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Pharmacological Characterization of 6-Bromo-3′-nitroflavone, a Synthetic Flavonoid with High Affinity for the Benzodiazepine Receptors

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WOLFMAN, C., H. VIOLA, M. MARDER, P. ARDENGHI, C. WASOWSKI, N. SCHRÖDER, I. IZQUIERDO, E. RUVEDA, A. PALADINI AND J. H. MEDINA. *Pharmacological characterization of 6-bromo-3′-nitroflavone, a synthetic flavonoid with high affinity for the benzodiazepine receptors.* PHARMACOL BIOCHEM BEHAV **61**(3) 239–246, 1998.—6-Bromo-3′-nitroflavone is a synthetic flavone derivative that selectively recognizes benzodiazepine receptors and has potent anxiolytic-like effects. Here, we describe in detail its pharmacological characterization. When IP injected in mice, 6-bromo-3′-nitroflavone (0.01–0.3 mg/kg) had an anxiolytic-like effect in the elevated plus-maze test. This effect was blocked by the specific benzodiazepine receptor antagonist, flumazenil. In addition, it exhibited anxiolytic-like actions when given orally (1 mg/kg). 6-Bromo-3′-nitroflavone did not exhibit myorelaxant effects (up to 30 mg/kg, IP). Unlike diazepam, this flavonoid produced no anterograde amnesia in a one-trial inhibitory avoidance learning. On the other hand, 6-bromo-3′-nitroflavone possessed mild anticonvulsant activity (0.1 mg/kg, IP) and provoked sedative-depressant actions only at doses 100–1000 times higher than those producing anxiolytic-like effects. 6-Bromo-3′-nitroflavone (0.1–1 mM) produced a lower potentiation of γ-amino-butyric acid (GABA)-stimulated $^{36}Cl^-$ influx (126–138%) in comparison to diazepam (0.1 mM: 166%) in cerebral cortical membrane vesicles. Taken together, these findings suggest that 6-bromo-3′-nitroflavone has anxiolytic-like action possibly behaving as a partial agonist of the benzodiazepine receptors. © 1998 Elsevier Science Inc.

Flavonoids Benzodiazepine receptors Anxiety Sedation Agonist

RESEARCH in our laboratories during the past few years was focused on the detection of natural ligands for the central benzodiazepine receptor (20).

These efforts were rewarded with the isolation of chrysin (5,7-dihydroxyflavone) from *Passiflora coerulea* (19,35), and apigenin (5,7,4′-trihydroxyflavone) from *Matricaria recutita* (32), plants both widely used as "tranquilizers" in folklore medicine. Chrysin and apigenin are competitive ligands for the central benzodiazepine receptors and possess anxiolytic-like but not depressant actions in mice. These findings led us to postulate the existence of a new family of benzodiazepine receptor ligands with a flavonoid structure (18,20). In support of this assumption, some naturally occurring flavonoids have been shown to possess benzodiazepine-like actions (12, 22,28).

The next step attempted was to increase the potency of the natural flavonoids, or of their phenyl benzopyrone nucleus (flavone), by introducing electronegative substituents in their molecules. This chemical modification is known to be very effective with the classical benzodiazepines (30).

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Nitration of flavone in positions 6 and 3′ produced a high-affinity ligand for the central benzodiazepine receptors with potent anxioselective properties in mice (15,36). More recently, it was found that the addition of a bromine atom in position 6 to the same nucleus produced a high-affinity compound with anxiolytic-like and sedative effects in mice, resembling a full agonist of the central benzodiazepine receptors (16).

These findings led us to synthesize 6-bromo-3′-nitroflavone, a compound shown in a preliminary study (33), to possess an anxiolytic-like action in mice and, most significantly, exhibiting a different affinity for central benzodiazepine receptors types I and II (33). The purpose of the present study was to characterize in detail the pharmacological profile of this high-affinity central benzodiazepine receptors ligand.

METHOD

Subjects

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. Adult male Wistar rats, weighing 250 g, were used for autoradiographic experiments. Animals were housed in a controlled environment, with free access to food and water and maintained on a 12 L:12 D cycle. Experiments were performed between 1100 and 1400 h.

Pharmacological Procedures

Locomotor activity test. An Opto-varimex® apparatus was used according to Viola et al. (31). 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 the following tests in mice, animals were IP injected with the vehicle, or with 6-bromo-3′-nitroflavone 15 min before the beginning of the test. In oral administration experiments, vehicle or 6-bromo-3′-nitroflavone (0.3–3 mg/kg) was administered 60 min before the beginning of the test. In each session, control mice were tested in paralell with those animals receiving drug treatment.

Elevated plus-maze test. The test was performed in the same session inmediately after the locomotor activity measurement (31,35,36). This test is widely validated for rats (23–25) and mice (5,14), and possesses several advantages over other tests for measuring anxiety (6,9). A selective increase in the number of entries in the open arms and/or the time spent in the open arms reveals an anxiolytic-like effect of the drug (23,25). In oral administration experiments, vehicle or 6-bromo-3′-nitroflavone (0.3–3 mg/kg) were administered 60 min before the beginning of the test.

Holeboard test. The test was performed according to Viola et al. (31) and Wolfman et al. (36). The number of head dips and the time spent head dipping were counted for 5 min. A decrease in these parameters reveals a sedative behavior (8,10).

Horizontal wire test. This test was carried out as previously described (31,32,35). The test took place after two trials, performed at 5-min intervals. A myorelaxant drug will impair mice to grasp the wire (3). The effect of increasing doses of 6-bromo-3′-nitroflavone (0.1–30 mg/kg) on diazepam-induced myorelaxation was also determined.

Sodium thiopental-induced sleeping time. Sodium thiopental (37 mg/kg) was IP injected 20 min after vehicle or 6-bromo-3′-nitroflavone (0.01–10 mg/kg). The disappearance and reappearance of the righting reflex were considered indications of latency and duration of sleep, respectively (1).

Sleep induction test. Different doses of 6-bromo-3′-nitroflavone (0.1 and 10 mg/kg) were administered IP 20 min before an infrahypnotic dose of sodium pentobarbital (20 mg/kg). The number of mice that have lost the righting reflex was registered during a 40-min test session (27).

Seizure testing. The effects of 6-bromo-3′-nitroflavone (0.1–10 mg/kg) on pentylenetetrazole-induced convulsions were evaluated according to Medina et al. (19) with slight modifications. Pentylenetetrazole (150 mg/kg) was administered IP to mice 15 min after injection of drug or vehicle. The number of mice presenting clonic and tonic–clonic convulsions was determined.

Inhibitory avoidance test. This test was performed according to Izquierdo et al. (13). The training apparatus was 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, 8-cm wide formica platform was placed on the left extreme 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 foot shock and were withdrawn from the box (training session). The test session was carried out 24 h later, and was similar to the training session in all respects except that the foot shock was omitted. Test step down latency (to a ceiling of 180 s) was taken as a measure of retention of inhibitory avoidance (13). Vehicle, diazepam, or 6-bromo-3′-nitroflavone were administered IP 1 h before the training session.

Tail-flick test. This test was performed according to Wolfman et al. (36). Analgesia was assessed with a tail-flick apparatus. Rats were wrapped in a towel and placed in 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 cutoff time of 10 s was used to prevent tissue damage. Briefly, the general procedure was as follows: the rats were wrapped in a towel and placed in the apparatus to determine the baseline tail-flick latency value for each animal. Following this, the rats were placed alone in a waiting cage and received an IP injection of vehicle or 6-bromo-3′-nitroflavone; 1 h later they were wrapped in a towel and placed in the apparatus to test the tail-flick latency value.

Drugs

6-Bromo-3′-nitroflavone (33) and diazepam were dissolved in 10% dimethylsulfoxide, 10% ethanol, in distilled water. The Ro 15-1788 (Hoffmann–La Roche) was administered together with the 6-bromo-3′-nitroflavone using the same vehicle. Oral administration of drugs was performed using small feeding curved needls (18 × 2″, Thomas Scientific, USA). The volume of administration was 0.1 ml/10 g in mice and 0.1 ml/100 g in rats.

Autoradiographic Experiments

Wistar rats were decapitated and 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-bromo-3′-nitroflavone displacement curves to [^{3}H]flunitrazepam binding (0.8 nM, NEN, specific activity 83 Ci/mmol), 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-bromo-3′-nitroflavone. For nonspecific binding we used 10 μM flunitrazepam. The

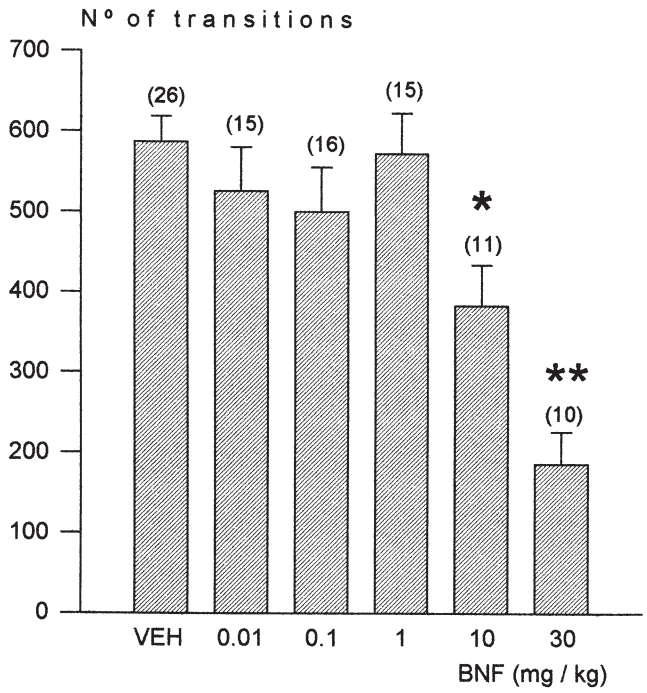

FIG. 1. Ambulatory locomotor activity counts during a 5-min test session in an Opto-varimex® apparatus 15 min after an IP injection of vehicle (VEH) or 6-bromo-3′-nitroflavone (BNF, 0.01–30 mg/kg). Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, significantly different from controls (Dunnett's multiple comparison test after ANOVA). Number of animals per group is shown in parentheses.

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 (21).

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) (2,4).

Potentiation of γ-amino-butyric acid (GABA)-stimulated $^{36}Cl^-$ influx in membrane vesicles. GABA-stimulated $^{36}Cl^-$ influx was determinated using the technique of Facklam et al. (7), with slight modifications. Briefly, male Wistar rats were killed by decapitation, their brains rapidly removed, and cerebral cortex dissected out on ice. The tissue was homogenized by hand (12 strokes) in a glass-glass homogenizer (1 g of fresh tissue in 30 ml of buffer), filtered through a 70 μm pore layer, and centrifuged for 15 min at 2800 rpm (937 × *g*). The pellet was washed twice (with 30 ml of buffer) and finally resuspended in 5 ml of buffer. Homogenization and resuspension buffers were prepared accordingly to Facklam et al. (7). A volume of 200 ml of this preparation was preincubated during 10 min at 30°C in a Dubnoff bath in the presence of different central benzodiazepine receptors ligands (diazepam, 6-bromo-3′-nitroflavone). Control samples were preincubated in the presence of vehicle solution. $^{36}Cl^-$ influx was initiated by addition and immediate vortexing of 200 μl of a solution of $^{36}Cl^-$ (1.25 μCi/ml; NEN, specific activity 13.37 mCi/g) containing 20 μM GABA. After 3 s, influx was stopped by the addition of 4 ml of ice-cold buffer containing bovine albumin (final concentration 0.01%) and picrotoxin (final concentration 100 μM), followed by rapid vacuum filtration through Whatman GF/C glass fiber filters soaked with 0.1% polyethylenimine. Filters were washed thrice with 4 ml of buffer. Basal influx was measured after incubation in the absence of central benzodiazepine receptors ligands and GABA. All experiments were performed in quadruplicate. Filters were put into vials, dried, and the radioactivity counted in a liquid scintillation counter. Potentiation was calculated as follows:

$$\frac{\text{GABA-induced flux (+ central benzodiazepine receptors ligand) − basal flux}}{\text{GABA-induced flux (− central benzodiazepine receptors ligand) − basal flux}} \times 100 - 100$$

Statistics

Analysis of variance (ANOVA) was used when several treatments in mice were compared. Post hoc comparisons between individual treatment and controls were made using Dunnett's multiple comparisons test. A chi-square frequency test was used when required. As nonparametric tests, Dunn's multiple comparison or Mann–Whitney *U*-tests were used.

RESULTS

Behavioral Results

Effect of 6-bromo-3′-nitroflavone on ambulatory locomotor activity. Figure 1 shows that the IP administration of 6-bromo-3′-nitroflavone (0.01–1 mg/kg) had no effect on spontaneous

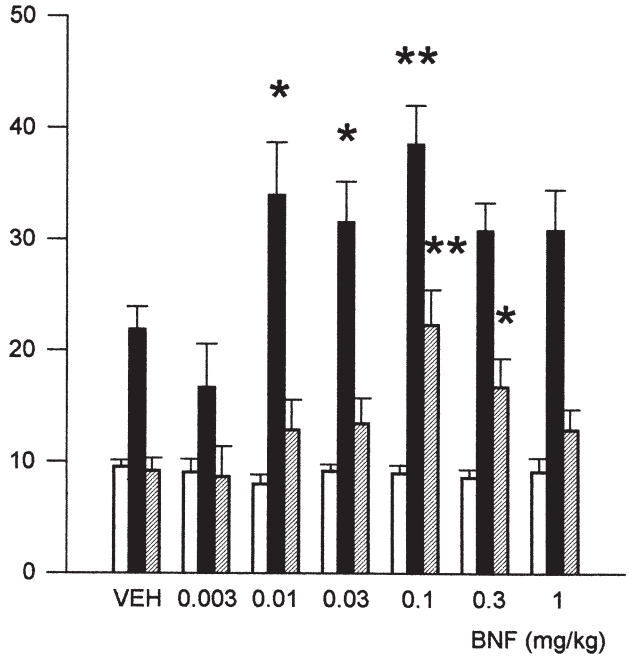

FIG. 2. Performance of mice during a 5-min test on the elevated plus-maze test, 20 min after IP injection with vehicle (VEH) or 6-bromo-3′-nitroflavone (0.003–1 mg/kg). Results are expressed as mean ± SEM of the number of closed-arm entries (open bars), percentage of open-arm entries (closed bars), and percentage of time spent in the open arms (hatched bars). *$p < 0.05$, **$p < 0.01$, significantly different from vehicle (Dunnett multiple comparison test after ANOVA). The number of experimental mice per group ranged between 13 and 28.

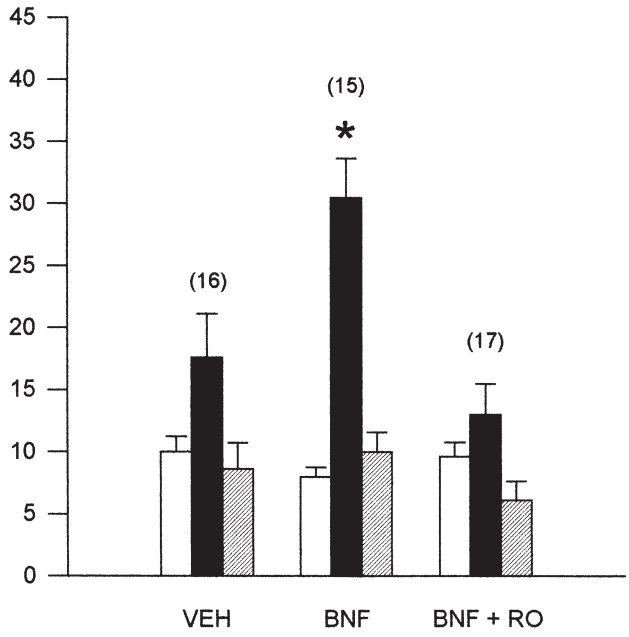

FIG. 3. Mean ± SEM of the number of closed-arm entries (open bars), percentage of open-arm entries (closed bars), and percentage of time spent in open arms (hatched bars) of mice given a 5-min session in the elevated plus-maze 20 min after IP injection of vehicle (VEH), 6-bromo-3′-nitroflavone (BNF, 0.1 mg/kg), or BNF (0.1 mg/kg) + Ro 15 1788 (RO, 3 mg/kg). *$p < 0.05$, significantly different from controls (Dunnett's t-test after ANOVA). Number of animals per group is shown in parentheses.

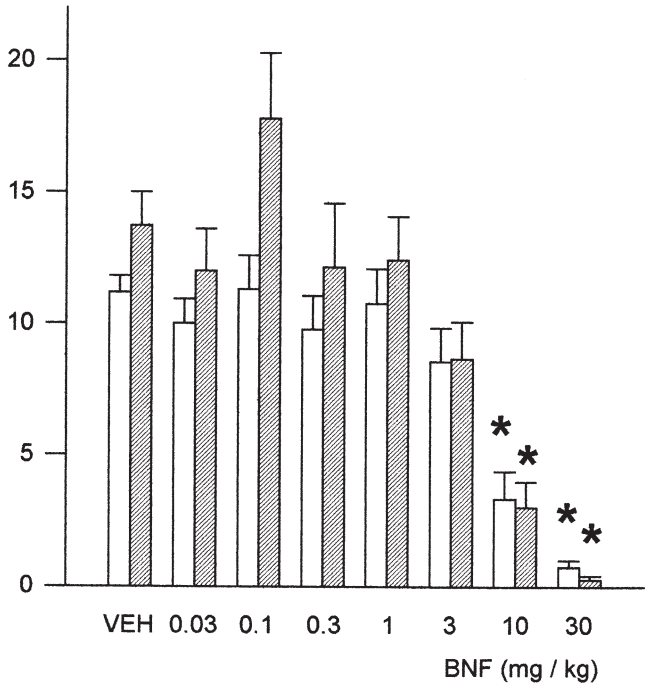

FIG. 4. Mean ± SEM number of head dips (open bars) and time (in seconds) spent head dipping (hatched bars) of mice given a 5-min session in the holeboard test 20 min after an IP injection of vehicle (VEH) or 6-bromo-3′-nitroflavone (BNF, 0.03–30 mg/kg). *$p < 0.01$, significantly different from controls (Dunnett's t-test after ANOVA). Number of animals in experimental groups ranged between 8 and 14.

ambulatory locomotion; at 10 and 30 mg/kg there was a 35 and 68% reduction in locomotion, respectively, $F(5, 87) = 8.143$, $p < 0.05$, and $p < 0.01$, respectively.

Effect of 6-bromo-3′-nitroflavone in the elevated plus-maze test. Confirming and extending preliminary findings (33), the IP injection of 0.01–0.3 mg/kg of 6-bromo-3′-nitroflavone in mice produced anxiolytic-like effects as determined by the increase in the percentage of open-arm entries (10–100 μg/kg), and the increase in time spent in the open arms (100 and 300 μg/kg) (Fig. 2). No changes were observed in the total arm entries, $F(6, 155) = 0.951$, $p > 0.05$ or in the number of closed-arm entries, $F(6, 155) = 0.356$, $p > 0.05$. The latter parameter is considered as the most valid measure of locomotor activity in this paradigm (5,14). Furthermore, the anxiolytic-like effect of 6-bromo-3′-nitroflavone (0.1 mg/kg) was blocked by the injection of 3 mg/kg Ro 15-1788, a specific central benzodiazepine receptor antagonist (11) (Fig. 3). Importantly, oral administration of 6-bromo-3′-nitroflavone (1 mg/kg, PO) produced a mild but significant anxiolytic-like effect (vehicle % open-arm entries: 20.8 ± 2.1, $n = 24$; 6-bromo-3′-nitroflavone % open-arm entries: 29.5 ± 2.5, $n = 27$, $p < 0.05$).

Effect of 6-bromo-3′-nitroflavone in the holeboard test. Performance of mice IP injected with vehicle or 6-bromo-3′-nitroflavone in the holeboard test is shown in Fig. 4. As can be seen, doses up to 3 mg/kg did not change the number of head dips and the time head dipping. Only at doses of 10 and 30 mg/kg (100–1000 times higher than the anxiolytic doses) a decrease in the number of head dips and the time spent head dipping was observed [head dips, $F(7, 99) = 11.97$, time, $F(7, 99) = 8.88$, $p < 0.01$; Dunnett comparison test after ANOVA]. This effect has been interpreted as indicative of sedation (24).

Effect of 6-bromo-3′-nitroflavone in the horizontal wire test. 6-Bromo-3′-nitroflavone (0.1–30 mg/kg) neither affected the percentage of mice grasping the wire nor antagonized the myorelaxant effect of 1 mg/kg of diazepam (data not shown).

Effect of 6-bromo-3′-nitroflavone on sodium thiopental-induced sleeping time. Figure 5 shows that IP administration of doses between 0.01 and 1 mg/kg of 6-bromo-3′-nitroflavone did not modify the latency or the sleeping time induced by 37 mg/kg of sodium thiopental. However, at 10 mg/kg, 6-bromo-3′-nitroflavone increased the sleeping time ($p < 0.001$, Dunn's comparison test after Kruskall–Wallis nonparametric ANOVA).

Effect of 6-bromo-3′-nitroflavone on pentobarbital sleep-induction test. A dose of 0.1 mg/kg of 6-bromo-3′-nitroflavone did not modify the number of mice that loss the righiting reflex after the administration of an infrahypnotic dose of sodium pentobarbital. In contrast, at 10 mg/kg, 6-bromo-3′-nitroflavone provoked sleep induction in 60% of the animals tested (Fig. 6) ($p < 0.025$, chi-square test).

Effect of 6-bromo-3′-nitroflavone on pentylenetetrazole-induced convulsions. The effect of the IP injection of 6-bromo-3′-nitroflavone on pentylenetetrazole-induced convulsions was tested in a wide range of doses (0.03–10 mg/kg). Only at 0.1 mg/kg, 6-bromo-3′-nitroflavone showed mild anticonvulsant activity, as determined by the number of mice without tonic–clonic seizures (7 of 15 animals, $p < 0.025$ chi-square test).

Effect of 6-bromo-3′-nitroflavone on inhibitory avoidance learning. In rats, the pretraining IP administration of 6-bromo-3′-nitroflavone, at doses that produce clear-cut anxiolytic-like effects in the elevated plus-maze (0.1–0.3 mg/kg), had no ef-

fect on test session performance of a one-trial step-down inhibitory avoidance training (Table 1). In addition, 6-bromo-3′-nitroflavone at the same doses, did not alter tail-flick latency [vehicle = 2.1 s (1.9/2.4, n = 7); 6-bromo-3′-nitroflavone 0.1 mg/kg = 2.1 s (1.8/2.3, n = 6); 6-bromo-3′-nitroflavone 0.3 mg/kg = 2.4 s (2.1/2.6, n = 7) median (interquartile range)]. As expected, diazepam (1 mg/kg, IP) hinders retention of the inhibitory avoidance learning (Table 1).

Biochemical Studies

Autoradiographic experiments. In a recent work we have demonstrated that 6-bromo-3′-nitroflavone is a high-affinity and competitive ligand for the central benzodiazepine receptors that differentially displaces [^{3}H]flunitrazepam binding to several regions of the rat brain (33). In fact, the K_i value was lower in cerebellum (3.6 nM) than in hippocampus (9.6 nM), striatum (9.8 nM), and spinal cord (12.7 nM), whereas in the cerebral cortex 6-bromo-3′-nitroflavone was able to recognize two different populations of binding sites (1.2 and 15.5 nM). In the present study, we have confirmed and extended these findings by using an autoradiographic method (Fig. 7). As can be seen, 6-bromo-3′-nitroflavone is more potent in displacing [^{3}H]flunitrazepam binding in the cerebellum than in the dentate gyrus of the hippocampus, superior colliculi, striatum, and in the frontal and parietal cortices.

GABA-stimulated $^{36}Cl^-$ influx in membrane vesicles. In rat cerebral cortex membrane vesicles, GABA (20 μM) stimulated $^{36}Cl^-$ influx (+30–40%). 6-Bromo-3′-nitroflavone (0.1 and 1 μM) potentiated the GABA-stimulated $^{36}Cl^-$ influx between 26–38% (Fig. 8). At a higher concentration (10 μM), it provoked a significant decrease in the GABA-stimulated $^{36}Cl^-$ influx. Experiments run in parallel with diazepam revealed that this classical benzodiazepine receptor full agonist produced maximal potentiation at 0.1 μM (+66%; $p < 0.05$ in comparison with the maximal effect induced by 6-bromo-3′-nitroflavone).

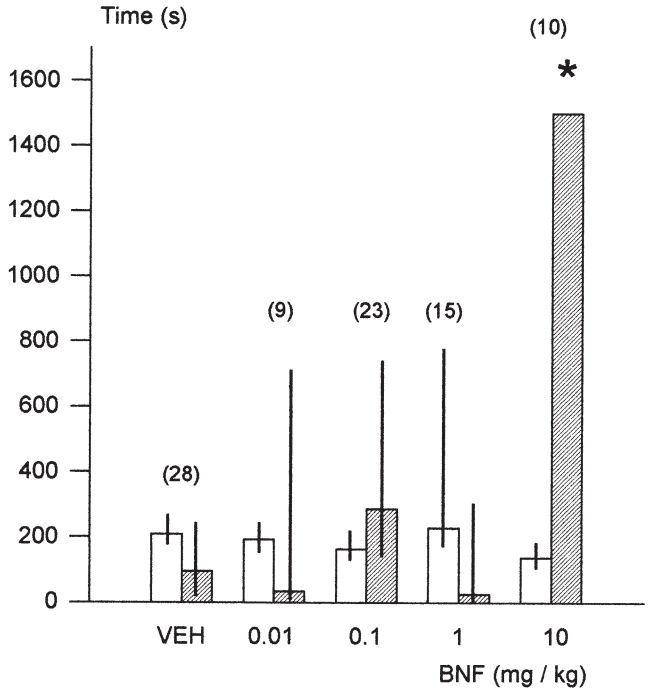

FIG. 5. Median (interquartile range) of latency (open bars) and sleeping time (hatched bars) of mice given a sodium thiopental-induced sleeping time test 20 min after an IP injection of vehicle (VEH) or 6-bromo-3′-nitroflavone (BNF, 0.01–10 mg/kg). *$p <$ 0.001, significantly different from controls (Dunn's multiple comparison test after Kruskall Wallis nonparametric ANOVA). Number of animals per group is shown in parentheses.

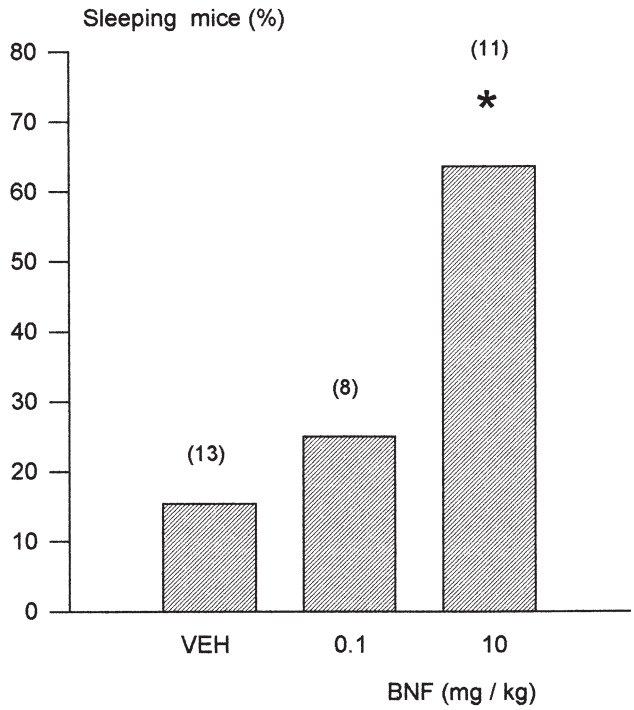

FIG. 6. Percentage of mice that have lost the righting reflex 20 min after an IP injection of vehicle (VEH) or 6-bromo-3′-nitroflavone (BNF, 0.1 and 10 mg/kg) in the sleep induction test. *$p < 0.025$, significantly different from controls (chi-square test). Number of animals per group is shown in parentheses.

DISCUSSION

The main finding of the present study is that 6-bromo-3′-nitroflavone has a pharmacological profile compatible with that of a benzodiazepine receptor agonist.

Preliminary work has demonstrated that 6-bromo-3′-nitroflavone is 10 times more potent as anxiolytic than diazepam in the elevated plus-maze test (33). In the present study, we confirmed and extended this data (Fig. 2), and found that the anxiolytic-like effect of this semisynthetic flavonoid was blocked by Ro 15-1788, a specific benzodiazepine receptor antagonist (Fig. 3). In addition, it is active when given orally.

On the other hand, 6-bromo-3′-nitroflavone possesses a mild depressant action, as shown by a reduction in spontaneous locomotion (Fig. 1), a decrease in both the number of head dips and the time spent head dipping in the holeboard (Fig. 4), and an increase in thiopental sleeping time and pentobarbital sleep induction (Figs. 5 and 6). However, it is important to stress that these depressant effects are produced with doses 100–1000 times higher than those required to induce the anxiolytic-like action. Therefore, in contrast to diazepam, which is a full agonist with a virtual overlapping of the doses causing therapeutic and unwanted side effects, 6-bromo-

3′-nitroflavone probably behaves as a partial agonist reducing anxiety at doses well below those causing sedation. In support of this contention, 6-bromo-3′-nitroflavone did not produce muscle relaxion in the wire test or anterograde amnesia in the inhibitory avoidance learning test. Thus, 6-bromo-3′-nitroflavone has a better separation index than diazepam because it is virtually devoid of unwanted side effects.

Alternatively, some of the pharmacological and biochemical data so far obtained with this flavonoid [bell-shaped dose–response curves (Fig. 2), inhibition of GABA-stimulated $^{36}Cl^-$ influx at a high concentration (Fig. 8)], could also be explained by the interaction of 6-bromo-3′-nitroflavone with another site on the GABA-A receptor that antagonises the anxiolytic-like effects mediated by the benzodiazepine site. It is important to stress that 6-bromo-3′-nitroflavone (10 μM) does not displace [^{3}H] muscimol binding to rat cerebral cortical synaptosomal membranes (33).

These behavioral data are similar to those obtained with another high-affinity benzodiazepine receptor ligand of flavonoid nature, 6, 3′-dinitroflavone. Based on its biochemical and pharmacological properties, we have also characterized 6, 3′-dinitroflavone as a partial agonist (36). However, several differences can be mentioned between these two compounds. First, the relative potency to produce anxiolytic-like effect is higher for 6, 3′-dinitroflavone than for 6-bromo-3′-nitroflavone; second, 6-bromo-3′-nitroflavone is able to partially prevent tonic–clonic seizures at a dose that also produces the anxiolytic-like effect, whereas 6, 3′-dinitroflavone exhibits no anticonvulsant activity (36); third, 6-bromo-3′-nitroflavone is able to better discriminate types I and II benzodiazepine receptors than 6, 3′-dinitroflavone (33); fourth, 6, 3′-dinitroflavone does not produce muscle relaxation but antagonizes the myorrelaxant effect of diazepam (36), whereas 6-bromo-3′-nitroflavone produces no muscle relaxation or abolition of diazepam-induced effects in the horizontal wire test. This difference might be due to a better selectivity of 6-bromo-3′-nitroflavone to discriminate between benzodiazepine receptor types I and II (33). In the present study, autoradiographic experiments revealed that 6-bromo-3′-nitroflavone differentially displaces the [^{3}H]flunitrazepam binding to various rat brain regions (Fig. 7), in accordance with the known distribution of type I benzodiazepine receptor (17,29,34). Alternatively, 6-bromo-3′-nitroflavone may not recognize those benzodiazepine receptors involved in muscle relaxation.

TABLE 1

EFFECT OF PRETRAINING IP ADMINISTRATION OF 6-BROMO-3′-NITROFLAVONE (BNF) AND DIAZEPAM (DZP) ON TRAINING AND TEST LATENCIES OF A STEP-DOWN INHIBITORY AVOIDANCE TASK IN RATS

Group (mg/kg)	—	Training Median (Interquartile range)*	Test Median (Interquartile Range)*
Vehicle	14	11 (4/20)	180 (35/180)
BNF, 0.1	13	9 (2/18)	180 (49/180)
BNF, 0.3	12	8 (4/11)	180 (31/180)
DZP, 1	12	13 (3/18)	12 (16/36)†

*In seconds.

†$p < 0.002$ MannWhitney *U*-test, with respect to the other three groups.

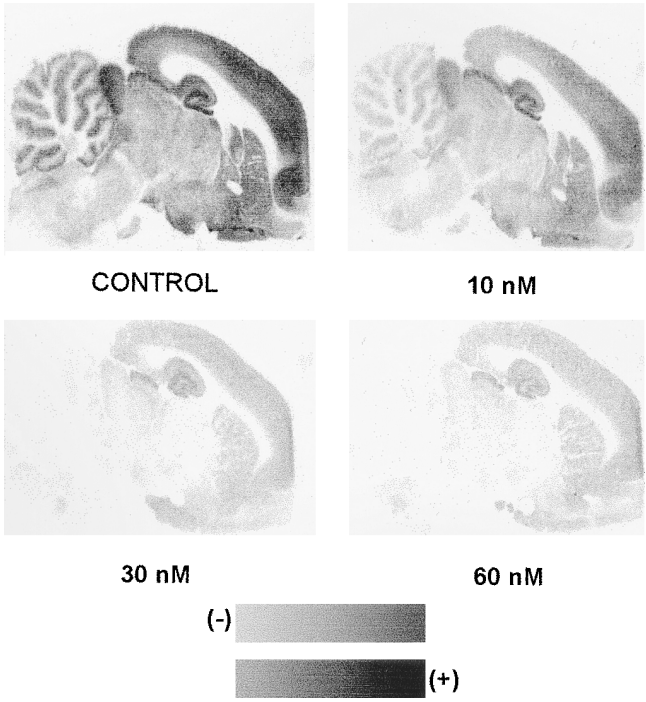

FIG. 7. Bright-field photographic images of autoradiograms generated by 0.65 nM [^{3}H]flunitrazepam exposed to [^{3}H]Hyperfilm for 14 days. Sagittal sections were incubated in the absence, or in the presence of 10, 30, or 60 nM 6-bromo-3′-nitroflavone. In these photographs, dark regions represent areas of high receptor densities. Note the dramatic decrease in cerebellar binding in comparison with that obtained in the dentate gyrus of the hippocampus, superior colliculus, and various regions of the cerebral cortex.

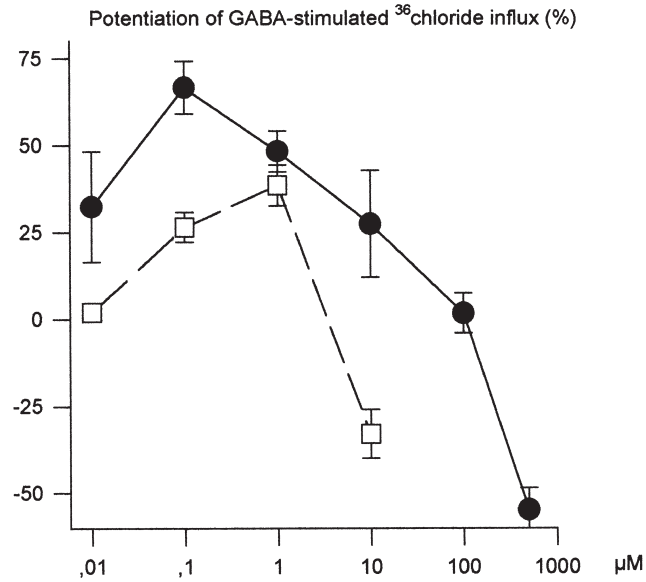

FIG. 8. Dose–response curves of diazepam (closed circles) and 6-bromo-3′-nitroflavone (open squares) on the potentiation of 20 μM GABA-stimulated $^{36}Cl^-$ influx (%) in rat cerebral cortex membrane vesicles (see the Method section). Data are means ± SEM of five to nine independent determinations done by quadruplicate.

Therefore, the pharmacologic profile of 6-bromo-3′-nitroflavone could be explained by partial agonism at the central benzodiazepine receptors that results in a reduced efficacy related to the maximal effect of the compound at receptor saturation (26). In support of this assumption, the GABA shift for 6-bromo-3′-nitroflavone and diazepam are 1.38 ± 0.1 and 2 ± 0.1, respectively (33). The GABA shift is a routinely used biochemical index that predicts the behavioral effects and efficacy of drugs acting on the benzodiazepine binding sites. Also, the potentiation of GABA-stimulated $^{36}Cl^-$ influx by diazepam is higher than that produced by 6-bromo-3′-nitroflavone (diazepam 0.1 μM: +66% vs. 6-bromo-3′-nitroflavone 1 μM: +38%; see Fig. 8).

In conclusion, 6-bromo-3′-nitroflavone is a high-affinity benzodiazepine receptor ligand that exhibits brain regional differences in displacing [^{3}H]flunitrazepam binding and possesses potent anxiolytic-like actions without evidencing muscle relaxant, amnestic, or severe sedative effects.

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