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Pharmacological studies on synthetic flavonoids: comparison with diazepam

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Abstract

The present experiments compared the central BZ-ω binding characteristics and pharmacological profiles of two synthetic flavonoids (6-bromoflavone and 6-bromo-3′-nitroflavone) with those of the benzodiazepine (BZ) diazepam. In vitro experiments showed that while diazepam displaced [^{3}H]flumazenil binding to the $GABA_A$ receptor in membranes from rat cerebellum and spinal cord, two brain areas enriched in the BZ-ω_1 and BZ-ω_2 receptor subtypes, with nearly equivalent half maximally effective concentrations, 6-bromo-3′-nitroflavone was somewhat more potent in displacing [^{3}H]flumazenil binding to membranes from rat cerebellum ($IC_{50} = 31$ nM) than from spinal cord ($IC_{50} = 120$ nM), indicating selectivity for the BZ-ω_1 receptor subtype. 6-Bromoflavone displayed weak ($IC_{50} = 970$ nM) affinity for the BZ-ω_1 and no affinity for the BZ-ω_2 ($IC_{50} > 1000$ nM) receptor subtypes. Diazepam, but not the synthetic flavonoids increased the latency to clonic seizures produced by isoniazid, thereby indicating that neither 6-bromoflavone nor 6-bromo-3′-nitroflavone display detectable intrinsic activity at $GABA_A$ receptors in vivo. Results from two conflict tests in rats showed that 6-bromoflavone (3–10 mg/kg) and 6-bromo-3′-nitroflavone (0.3–1 mg/kg) elicited anxiolytic-like activity in the punished drinking test, while both drugs were inactive in the punished lever pressing test. The positive effects displayed by the synthetic flavonoids in the punished drinking procedure were smaller than that of diazepam and were not antagonized by the BZ receptor antagonist flumazenil. In two models of exploratory activity, 6-bromoflavone (3–30 mg/kg) and 6-bromo-3′-nitroflavone (0.3–1 mg/kg) produced anxiolytic-like effects in the rat elevated plus-maze test, whereas both compounds failed to modify the behavior of mice in the light/dark test over a wide dose-range. The effects in the elevated plus-maze were antagonized by flumazenil. In the mouse defense test battery, where mice were confronted with a natural threat (a rat), 6-bromoflavone and 6-bromo-3′-nitroflavone failed to decrease flight reactions after the rat was introduced into the test area and risk assessment behavior displayed when subjects were constrained in a straight alley, and only weakly affected risk assessment of mice chased by the rat and defensive biting upon forced contact with the threat stimulus. In a drug discrimination experiment 6-bromoflavone and 6-bromo-3′-nitroflavone up to 30 and 3 mg/kg, respectively, did not substitute for the BZ chlordiazepoxide. Taken together, these results failed to demonstrate that the synthetic flavonoids 6-bromoflavone and 6-bromo-3′-nitroflavone possess anxiolytic-like properties similar or superior to that of diazepam, as was suggested previously. Furthermore, they question the contribution of BZ-ω receptors to the behavioral effects of 6-bromoflavone and 6-bromo-3′-nitroflavone.

Keywords: Anxiety; Benzodiazepines; Binding; Conflict tests; Convulsions; Defensive behaviors; Diazepam; Drug discrimination; Exploration models; Flavonoids; Flumazenil

1. Introduction

Benzodiazepines (BZs) have established efficacy in the treatment of anxiety, insomnia and epilepsy. They produce their pharmacological effects by positively modulating the action of GABA at $GABA_A$ receptors through allosteric binding sites called BZ_1 and BZ_2 (Squires et al., 1979; Sieghart and Schuster, 1984), also designated as ω_1 and ω_2, respectively (Langer and Arbilla, 1988). Even though BZs are relatively safe drugs when used in the treatment of pathological anxiety, they may produce untoward side effects such as sedation, muscle relaxation, memory impairment, tolerance and physical dependence (Lader, 1994). The search for

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positive modulators of BZ-ω receptors with more specific therapeutic actions without the concomitant unwanted effects has led to the development of drugs that either selectively bind to specific BZ-ω receptor subtypes (alpidem and abecarnil) (Stephens et al., 1990; Zivkovic et al., 1990, 1992) and/or modulate the action of GABA with low efficacy (partial agonists, e.g. bretazenil, imidazenil) (Martin et al., 1988; Giusti et al., 1993).

In the search for novel anxiolytic agents devoid of undesirable side effects, several BZ-ω receptor ligands, structurally unrelated to BZs, have recently been described. Based on the findings that some naturally occurring flavonoids possess selective affinity for BZ-ω receptors and exhibit anxiolytic-like activity in the absent of depressant effects (Wolfman et al., 1994), various synthetic derivatives of these compounds have been synthesized (Medina et al., 1997, 1998). Among these, much work has focused on 6-bromoflavone and 6-bromo-3′-nitroflavone which were found to display specific affinity for BZ-ω receptors (Medina et al., 1997). These two synthetic flavonoids also showed anxiolytic-like effects in the mouse elevated plus-maze (Marder et al., 1996; Viola et al., 1997; Wolfman et al., 1998). 6-Bromo-3′-nitroflavone produced positive effects over a wide dose-range, with minimum dose levels in the microgram range. The pharmacological profile of this compound is compatible with a partial agonist action as it antagonized the myorelaxant effects of diazepam and produced a lower potentiation of GABA-stimulated $^{36}Cl^-$ influx than diazepam in cerebral cortical membrane vesicles. In addition, 6-bromo-3′-nitroflavone did not impair motor activity or memory performance at doses 100–300-fold greater than those producing anxiolytic-like activity.

The present study was undertaken to investigate further the neurochemical and pharmacological properties of 6-bromoflavone and 6-bromo-3′-nitroflavone. Effects were directly compared to those of the prototypical anxiolytic diazepam, which was used throughout as a positive control. In a first series of experiments, the binding profile to native BZ-ω receptor subtypes, including BZ-ω_1, BZ-ω_2 and ω_5 (probably corresponding to $GABA_A$ receptors containing α_1, α_{2-3} and α_5 subunits, respectively, see Braestrup and Nielsen, 1980; Ruano et al., 1992; Tan and Schoemaker, 1994; Benavides et al., 1996), was established for each drug. The drugs' anticonvulsant action against isoniazid-induced seizures was then examined. This latter test was used in order to assess the intrinsic activity of the compounds at $GABA_A$ receptors (Mao et al., 1975). Anxiolytic-like properties were examined using several rodent tests sensitive to anxiolytic drugs including conflict procedures (rat punished lever pressing and Vogel drinking tests), exploratory models (rat elevated plus-maze and mouse light/dark test) and a mouse defense test battery (MDTB) which elicits and measures reactions to a natural threat stimulus (a rat) (Griebel et al., 1995). An additional experiment was undertaken to characterize further the behavioral actions of both synthetic flavonoids by examining their discriminative stimulus properties in rats trained to discriminate between a dose of the BZ chlordiazepoxide and saline.

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River France, Saint-Aubin-les-Elbeuf) were used in the punished lever pressing and drug discrimination procedures. They weighed 180–200 g at the beginning of training and 400–500 g at the time of drug testing. Male Sprague–Dawley rats (Iffa Credo, L'Arbresle and Charles River France) weighing 180–300 g at time of testing were used in the Vogel drinking procedure, the elevated plus-maze test and for radioligand binding studies. The latter animals were housed in groups of eight, whereas those used in the punished lever pressing and drug discrimination procedures were housed singly. Male Long Evans rats (400–500 g) (Iffa Credo) were used as threat stimulus in the MDTB. BALB/c (7 week-old), Swiss (10 week-old) (both supplied by Iffa Credo) and CD1 male mice (18–25 g) (Charles River France) were used in the light/dark test, the MDTB and the isoniazid-induced convulsions test, respectively. BALB/c mice were housed in groups of six, Swiss mice were isolated 1 week prior to testing and CD1 mice were housed in groups of 20. Different strains and housing conditions were used in the anxiety tests on the basis of preliminary findings. All animals were maintained under standard laboratory conditions (22–23°C) and kept on a 12:12 h light–dark cycle with light onset at 07:00 h. Rats used in the punished lever pressing and drug discrimination procedures were restricted to the food obtained during sessions and a daily ration of 15–20 g of standard laboratory chow given at the end of each weekday and over the weekend.

2.2. Drugs

The drugs used were diazepam, flumazenil, zolpidem, 6-bromoflavone, 6-bromo-3′-nitroflavone (synthesized by the Medicinal Chemistry Department, Synthélabo Recherche) and isoniazid (Sigma chemicals, St-Louis, USA). [^{3}H]Flumazenil was purchased from NEN Life Science Products (Paris, France). Diazepam, flumazenil, zolpidem and isoniazid were prepared as suspensions in physiological saline containing Tween 80 (0.1%), and the synthetic flavonoids were suspended in physiological saline containing Tween 80 (0.1%) and DMSO.

Vehicle controls contained Tween 80 (0.1%) in the case of diazepam, flumazenil, zolpidem and isoniazid, and Tween 80 (0.1%) and DMSO in the case of the synthetic flavonoids. The drugs were injected intraperitoneally in a volume of 2 ml/kg (rats) or 20 ml/kg (mice). All doses are expressed as the bases and were chosen on the basis of previous results with these compounds in behavioral studies (Marder et al., 1995; Griebel et al., 1996b; Wolfman et al., 1996; Medina et al., 1997; Viola et al., 1997).

2.3. In vitro binding to different BZ-ω receptor subtypes

Inhibition of [^{3}H]flumazenil binding to native BZ-ω receptor subtypes in vitro was studied as described by Schoemaker et al. (1997). Briefly, the cerebellum, spinal cord or hippocampus of rats were homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCl. The binding of [^{3}H]flumazenil (1 nM; specific activity 70–87 Ci/mmol) to the ω_1 receptor was studied in membranes from the rat cerebellum, a region enriched in this receptor subtype (Braestrup and Nielsen, 1980), using a 45 min incubation at 0–4°C and 1 μM diazepam to define nonspecific binding. [^{3}H]Flumazenil binding to the ω_2 receptor was studied using membranes from the rat spinal cord, where a majority of the expressed ω receptors appears to be of the ω_2 subtype (Ruano et al., 1992), under otherwise identical conditions. The native ω_5 receptor was studied using [^{3}H]flumazenil binding to membranes from the rat hippocampus in the presence of 5 μM zolpidem in order to mask the ω_1 and ω_2 receptor subtypes (Tan and Schoemaker, 1994), under otherwise identical conditions except for the use of 1 μM flunitrazepam to define nonspecific binding. Following incubation, membranes were recovered by vacuum filtration over Whatman GF/B filters, washed and the amount of radioactivity retained on the filter was quantified by liquid scintillation spectrometry. Data are presented as the compound concentration required to inhibit 50% of specific radioligand binding (IC_{50}).

2.4. Isoniazid-induced convulsions

Isoniazid (800 mg/kg, s.c.) was administered simultaneously with the test drugs. The anticonvulsant effect was assessed by measuring the latency to the appearance of the first clonic convulsion. Data were analyzed using one-way ANOVA followed by Dunnett's *t*-test.

2.5. Punished lever pressing

The procedure was a modification of that described previously (Sanger et al., 1985). Animals were tested in standard rat operant test chambers (MED Associates, Inc., Georgia) placed in sound-attenuated boxes with ventilation fans. Each chamber was fitted with a stainless steel grid floor. Electric shocks could be delivered to each grid by a shock generator and scrambler (MED Associates, Inc.). A total of 11 rats were trained initially to press a lever for food reward (45 mg precision food pellets, PJ Noyes, Inc., Lancaster). As training progressed, schedule parameters were gradually changed to a variable interval (VI 30 s) schedule of food reinforcement during daily 15 min sessions. After several sessions of VI 30 s responding, five 60 s periods of a visual stimulus were presented during a 25 min session. Each visual stimulus consisted of three stimulus lights situated above the food pellet dispenser and to the right of the response lever, which flashed at a rate of 1 s on, 1 s off. In this component, a footshock punishment schedule consisting of two independent VI schedules (VI 30 s for food, VI 10 s for shock) was in operation. Footshock was initially set at 0.1 mA. The first stimulus presentation started 5 min after the beginning of the session, and each following stimulus commenced 150 s after the end of the preceding stimulus. The magnitude of footshock was individually titrated for each rat (shock levels ranged from 0.3 to 0.65 mA) to obtain stable baselines of responding (i.e. an average lever pressing rate of 8 ± 2 presses in each 60 s punished responding period). To obtain stable levels of responding, an average of approximately 30 sessions after initiation of the punishment contingency was necessary. Once stable baselines of responding were obtained, drug studies were initiated.

Drug injections were given once or twice each week with at least two nondrug days intervening between two drug administrations. The vehicle was injected on all nondrug days. Drugs and doses were given in a mixed order. The effects of drugs were assessed on punished and unpunished responses rates. The former corresponds to those recorded during the presentation of the visual stimulus, whereas the latter were taken from the 60 s periods immediately preceding and immediately following each stimulus presentation. The mean values of punished and unpunished rates recorded during the nondrug session preceding the drug injection sessions were used as the control values. Thus, drug effects were analyzed statistically by comparing performances after drug administration with the mean values taken from appropriate control sessions using Friedman's ANOVA.

2.6. Punished drinking

The procedure was a modification of the technique described by Vogel et al. (1971). At the beginning of the experiment, rats, deprived of water for 48 h prior to testing, were placed individually in cages ($27 \times 22 \times 21$ cm) with a stainless steel grid floor. Each cage con-

tained a drinking tube connected to an external 50 ml buret filled with tap water. Trials were started only after the animal's tongue entered in contact with the drinking tube for the first time. An electric shock (0.06 mA) was delivered to the tongue after every twenty licks. The number of shocks was recorded automatically during a 3-min period. Data were analyzed with one-way ANOVA. Subsequent comparisons between treatment groups and control were carried out using Dunnett's *t*-test.

2.7. Elevated plus-maze

The test apparatus was based on that described by Pellow et al. (1985). All parts of the apparatus were made of dark polyvinylplastic with a black rubber floor. The maze was elevated to a height of 50 cm with two open (50×10 cm) and two enclosed arms ($50 \times 10 \times 50$ cm), arranged so that the arms of the same type were opposite each other, connected by an open central area (10×10 cm). To prevent rats falling off, a rim of Plexiglas (1 cm high) surrounded the perimeter of the open arms. The illumination in the experimental room consisted of one red neon tube fixed on the ceiling, so that experiments were performed under dim light conditions. The light intensity on the central platform was 10 lux. At the beginning of the experiment, a rat was placed in the center of the maze, facing one of the enclosed arms, and observed for 4 min. The apparatus was equipped with infrared beams and sensors capable of measuring: time spent in the open arms; the number of open-arm entries; and the number of closed-arm entries (defined as entry of all four limbs into an arm of the maze). In addition, rats were observed via video-link by an observer located in an adjacent room. This permitted the recording of a more ethologically-orientated measure, i.e. attempt at entry into open arms followed by avoidance responses. This includes stretched attend posture (the rat stretches forward and retracts to original position). The results were expressed as mean ratio of time spent in open arms to total time spent in both open and closed arms, mean ratio of entries into open arms to total entries into both open and closed arms, mean total number of both closed and open arm entries and mean total number of attempts. Data were analyzed by one-way ANOVA. Subsequent comparisons between treatment groups and control were carried out using Dunnett's *t*-test.

2.8. Light/dark test

This model of anxiety was based on that described by Misslin et al. (1989) and consists of two polyvinylchloride boxes ($20 \times 20 \times 14$ cm) covered with plexiglas. One of these boxes was darkened. A neon tube fixed on the ceiling provided the room illumination so that the light intensity in the center of the illuminated box was 150 lux. An opaque plastic tunnel ($5 \times 7 \times 10$ cm) separated the dark box from the illuminated one. Recording started immediately after the introduction of the animal in the apparatus. At the beginning of the experiment, a mouse was placed in the illuminated box, facing the tunnel. The apparatus was equipped with infrared beams and sensors capable of recording the following two parameters during a 4-min period: (a) time spent by mice in the lit box and; (b) total number of tunnel crossings. Data were analyzed by the nonparametric Kruskal–Wallis test. Subsequent comparisons between treatment groups and control were carried out using the Siegal and Castellan test.

2.9. Mouse defense test battery (MDTB)

The test was conducted in an oval runway, 0.40 m wide, 0.30 m high, and 4.4 m in total length, consisting of two 2 m straight segments joined by two 0.4 m curved segments and separated by a median wall ($2.0 \times 0.30 \times 0.06$). The apparatus was elevated to a height of 0.80 m from the floor to enable the experimenter to easily hold the rat, while minimizing the mouse's visual contact with him. All parts of the apparatus were made of black Plexiglas. The floor was marked every 20 cm to facilitate distance measurement. Activity was recorded with video cameras mounted above the apparatus. The room illumination was provided by one red neon tube fixed on the ceiling and two desk lamps with red bulbs placed on two tables (elevated to a height of 1 m) located 1 m away from the runway. The light intensity in the runway was 7 lux. Experiments were performed under red light between 09:30 h and 15:00 h. The experimenter was unaware of the drug treatment.

2.9.1. Effects on spontaneous locomotor activity: the pre-test

A subject was placed into the runway for a 3-min familiarization period during which line crossings were recorded.

2.9.2. Effects on flight responses: the rat avoidance test

Immediately after the 3-min familiarization period, a hand-held dead rat (killed by CO_2 inhalation) was introduced into the runway and brought up to the subject at a speed of approximately 0.5 m/s. Approach was terminated when contact with the subject was made or the subject ran away from the approaching rat. If the subject fled, avoidance distance (the distance from the rat to the subject at the point of flight) was recorded. This was repeated five times. Mean avoidance distance (cm) was calculated for each subject.

2.9.3. Effects on risk assessment: the chase and the straight alley tests

The hand-held rat was brought up to the subject at a speed of approximately 2.0 m/s. During the chase, the number of orientations (subject stops, then orients the head toward the rat) were recorded. After the chase was completed, the runway was then converted to a straight alley by closing a door at one end. During 30 s, the hand-held rat remained at a constant distance of 40 cm from the subject and the number of approaches/withdrawals (subject must move more than 0.2 m forward from the closed door, then return to it) were recorded. Both responses are described as risk assessment activities (Griebel et al., 1995).

2.9.4. Effects on defensive threat/attack responses: the forced contact test

Finally, the experimenter brought the rat up to contact the subject. For each such contact, bites by the subjects were noted. This was repeated three times. The results were expressed as mean number of bites. Data were analyzed by one-way ANOVA (Line crossings, avoidance distance, orientations, approaches/withdrawals) or the nonparametric Kruskal–Wallis test (bites). Subsequent comparisons between treatment groups and control were carried out using Dunnett's *t*-test or the nonparametric Siegal and Castellan test.

2.10. Discriminative stimulus properties

Rats were trained to discriminate between a dose of 5 mg/kg i.p. of chlordiazepoxide and saline using a standard, two-lever, fixed ratio 10 (FR10), food-rewarded operant procedure. Thus, rats obtained a food pellet (45 mg) each time they pressed 10 times on the appropriate lever in the 2-lever operant test chamber. Responses on one lever were rewarded in sessions which followed chlordiazepoxide injection and responses on the other lever were rewarded during sessions following saline injection (see Sanger and Zivkovic, 1986, for further details of the procedure). Daily sessions were 15 min in duration. When the animals had acquired the discrimination, they were given substitution tests with a range of doses of chlordiazepoxide followed by the drugs studied.

The results were recorded as the number of rats choosing the chlordiazepoxide-associated lever during the substitution tests and the number of lever presses emitted during these tests (expressed as a percentage of the number of lever presses following saline injection). Data were analyzed using the probit method of Litchfield and Wilcoxon in order to calculate the ED_{50} values. The ED_{50} discrimination is the dose at which 50% of the rats responded on the chlordiazepoxide-associated lever and the ED_{50} rate is the dose at which rates of lever pressing were decreased to 50% of those occurring after saline administration. In addition, the effects on lever pressing rates were analyzed statistically by comparing performances after drug administration with the mean values taken from appropriate control sessions using Friedman's ANOVA.

3. Results

3.1. In vitro binding to different BZ-ω receptor subtypes

Table 1 shows a comparison of the potency of diazepam and the synthetic flavonoids to displace the binding of [^{3}H]flumazenil from native BZ-ω_1, BZ-ω_2 and ω_5 receptors in cerebellar, spinal cord and hippocampal membranes, respectively. Unlike diazepam which binds to BZ-ω_1 and BZ-ω_2 receptors with similar half maximally effective concentrations, 6-bromo-3′-nitroflavone shows selectivity for the native BZ-ω_1 receptor. 6-Bromoflavone displays weak affinity for the native BZ-ω_1 receptor, whereas it has no detectable affinity for the BZ-ω_2 receptor. 6-Bromo-3'-nitroflavone, but not 6-bromoflavone displaces, albeit weakly, radioligand binding to the native ω_5 receptor.

3.2. Isoniazid-induced convulsions

Table 2 shows that diazepam [$F(5,53) = 83.02$, $P < 0.001$], but not 6-bromoflavone or 6-bromo-3′-nitroflavone significantly increased the latencies to isoniazid-induced convulsions. Post-hoc analysis revealed that the latencies to convulsions were significantly increased by diazepam from 1 mg/kg.

3.3. Punished lever pressing

Fig. 1 shows that the rates of lever pressing suppressed by punishment were significantly increased by diazepam ($\chi^2 = 20.75$, $P < 0.001$), but not by 6-bro-

Table 1
Effects of diazepam and two synthetic flavonoids on the binding of [^{3}H]flumazenil to the native BZ-ω_1 receptor in the cerebellum, the native BZ-ω_2 receptor in the spinal cord and the native ω_5 receptor in the hippocampus, studied in the presence of 5μM zolpidem, in order to mask the BZ-ω_1 and BZ-ω_2 receptors[a]

Drug	IC_{50} (nM)		
	BZ-ω_1	BZ-ω_2	ω_5
Diazepam	19 ± 2	12 ± 2	111 ± 5
6-Bromoflavone	970 ± 70	>1000	>1000
6-Bromo-3′-nitroflavone	31 ± 6	120 ± 23	770 ± 230

[a] Data are presented as the mean ± S.E.M. of three experiments.

Table 2
The anticonvulsant effects of diazepam and two synthetic flavonoids against clonic seizures produced in mice by 800 mg/kg, s.c. of isoniazid[a]

Drug	Dose (mg/kg, i.p.)	Latency to convulsions (min)
Diazepam	0	22.94 ± 1.3
	0.3	28.45 ± 1.1
	1	33.50 ± 2.1[b]
	3	46.75 ± 1.89[b]
	10	59.40 ± 2.5[b]
	30	68.15 ± 2.4[b]
6-Bromoflavone	0	21.25 ± 0.8
	1	21.90 ± 1.1
	3	17.95 ± 0.7
	10	20.20 ± 0.9
	30	19.85 ± 0.8
	100	19.70 ± 0.9
6-Bromo-3'-nitroflavone	0	20.10 ± 1.1
	1	18.25 ± 0.7
	3	19.45 ± 0.9
	10	18.35 ± 0.8
	30	17.60 ± 0.7
	100	18.95 ± 1.2

[a] Data represent means ± S.E.M. $n = 10$.
[b] $P < 0.05$ (Dunnett's t-test).

moflavone or by 6-bromo-3′-nitroflavone. Diazepam also significantly increased unpunished responding at 2.5 mg/kg ($\chi^2 = 9.3$, $P < 0.05$) (data not shown).

3.4. Punished drinking

Fig. 2 shows that all compounds significantly increased the number of shocks received [diazepam: $F(3,44) = 10.38$, $P < 0.001$; 6-bromoflavone: $F(4,95) =$

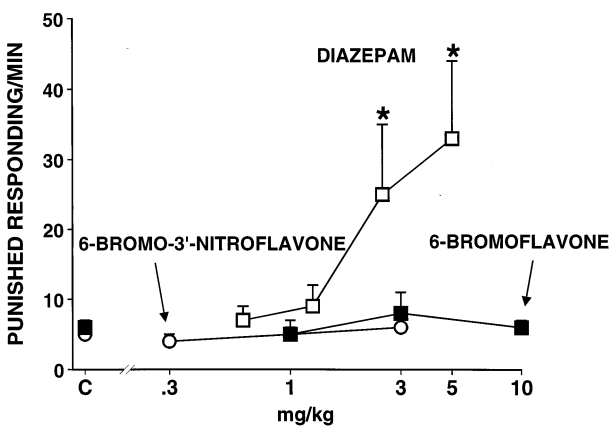

Fig. 1. Effects of diazepam and two synthetic flavonoids on rates of punished lever pressing in rats. The drugs were administered intraperitoneally 30 min (diazepam) or 20 min (6-bromoflavone and 6-bromo-3′-nitroflavone) before testing. Data represent mean ± S.E.M. $n = 8$, * $P < 0.05$ (Friedman test).

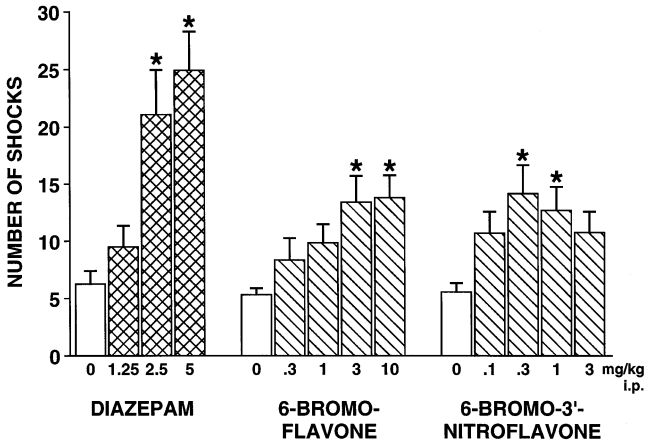

Fig. 2. Effects of diazepam and two synthetic flavonoids in the punished drinking conflict test in rats. The drugs were administered intraperitoneally 30 min (diazepam) or 20 min (6-bromoflavone and 6-bromo-3′-nitroflavone) before testing. Data represent mean ± S.E.M. $n = 12$–20, * $P < 0.05$ (Dunnett's t-test).

4.03, $P < 0.01$; 6-bromo-3′-nitroflavone: $F(4,95) = 2.98$, $P < 0.05$]. Post-hoc analysis indicated that diazepam (2.5 and 5 mg/kg), 6-bromoflavone (3 and 10 mg/kg) and 6-bromo-3′-nitroflavone (0.3 and 1 mg/kg) significantly increased punished responding. Fig. 3 shows that pretreatment with the BZ receptor antagonist flumazenil (5 mg/kg) had no effect on the anticonflict activity of 6-bromoflavone [$F(3,60) = 4.74$, $P < 0.01$] and 6-bromo-3′-nitroflavone [$F(3,52) = 4.51$, $P < 0.01$].

3.5. Elevated plus-maze

Fig. 4 shows that all drugs significantly modified both the percentage of time spent [diazepam: $F(3,30) =$ 2.87, $P < 0.05$; 6-bromoflavone: $F(5,64) = 2.87$, $P <$

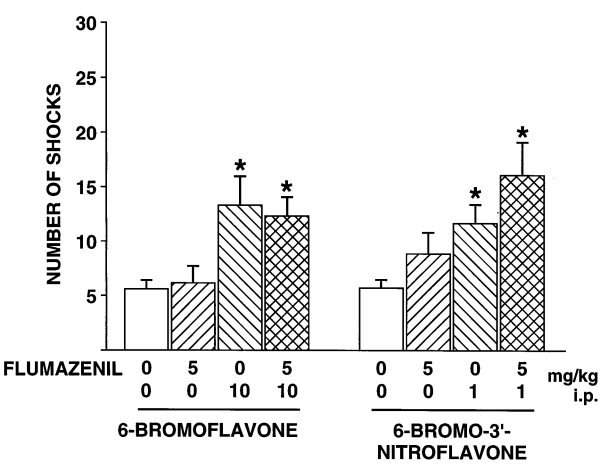

Fig. 3. Effects of 6-bromoflavone and 6-bromo-3′-nitroflavone alone or in combination with flumazenil in the punished drinking conflict test in rats. The drugs were administered intraperitoneally 20 min before testing. Data represent mean ± S.E.M. $n = 13$–17, * $P < 0.05$ (Dunnett's t-test).

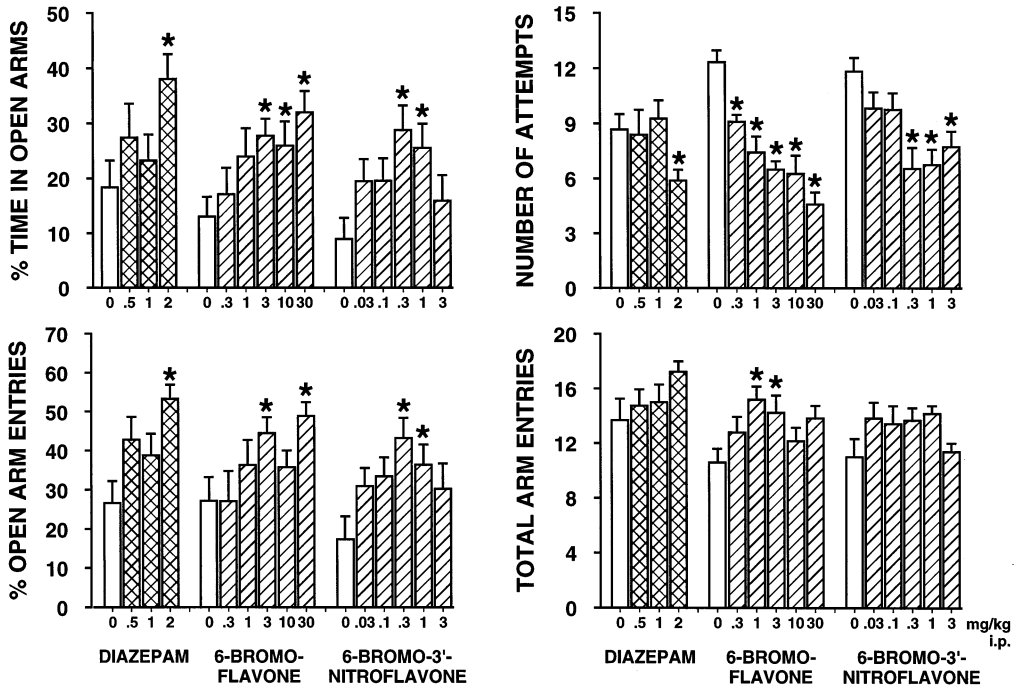

Fig. 4. Effects of diazepam and two synthetic flavonoids on the behavior of rats on the elevated plus-maze. The drugs were administered intraperitoneally 30 min (diazepam) or 20 min (6-bromoflavone and 6-bromo-3′-nitroflavone) before testing. Data represent mean ± S.E.M. $n = 8–12$, * $P < 0.05$ (Dunnett's t-test).

0.05; 6-bromo-3′-nitroflavone: $F(5,64) = 2.68$, $P < 0.05$] and the percentage of entries made [diazepam: $F(3,30) = 4.73$, $P < 0.01$; 6-bromoflavone: $F(5,64) = 2.63$, $P < 0.05$; 6-bromo-3′-nitroflavone: $F(5,64) = 2.94$, $P < 0.05$] into open arms. Post-hoc analysis indicated that diazepam (2 mg/kg), 6-bromoflavone (10–30 mg/kg) and 6-bromo-3′-nitroflavone (0.3 and 1 mg/kg) significantly increased activity in open arms. With respect to the ethologically-derivated measure, all compounds modified the number of attempts at entry into open arms followed by avoidance responses [diazepam: $F(3,30) = 2.5$, $P < 0.05$; 6-bromoflavone: $F(5,64) = 14.55$, $P < 0.001$; 6-bromo-3′-nitroflavone: $F(5,64) = 4.9$, $P < 0.001$]. Post-hoc analysis indicated that diazepam (2 mg/kg), 6-bromoflavone (0.3–30 mg/kg) and 6-bromo-3′-nitroflavone (0.3–3 mg/kg) significantly reduced attempts. 6-Bromoflavone [$F(5,64) = 2.54$, $P < 0.05$], but not the other drugs significantly increased the total number of arm entries at 1 and 3 mg/kg. In another experiment, the BZ receptor antagonist flumazenil (5 mg/kg) fully reversed the increase in open arm activity produced by 6-Bromoflavone (30 mg/kg) [open arm time: $F(3,32 = 2.72$, $P < 0.05$; open arm entries: $F(3,32) = 6.49$, $P < 0.01$] and by 6-bromo-3′-nitroflavone (0.3 mg/kg) [open arm time: $F(3,32) = 4.17$, $P < 0.05$; open arm entries: $F(3,32) = 3.59$, $P < 0.05$]. Flumazenil also blocked the decrease in attempts induced by 6-bromoflavone [$F(3,32) = 10.76$, $P < 0.001$], but failed to modify the effects of 6-bromo-3′-nitroflavone [$F(3,32) = 5.94$, $P < 0.01$] on this parameter (Fig. 5).

3.6. Light/dark test

In the diazepam experiment, statistical analysis indicated a significant increase in the time spent by mice in the bright area [$K = 48.76$, $P < 0.001$] and the total number of tunnel crossings [$K = 44.64$, $P < 0.001$] from 2 mg/kg. Neither synthetic flavonoids significantly affected these parameters (Fig. 6).

3.7. Mouse defense test battery

3.7.1. Effects on spontaneous locomotor activity: the pre-test

Table 3 shows that prior confrontation with the rat, none of the drugs significantly modified the number of line crossings.

3.7.2. Effects on flight responses: the rat avoidance test

The avoidance distance was significantly modified by diazepam [$F(3,27) = 16.1$, $P < 0.001$], but not by 6-bromoflavone or by 6-bromo-3′-nitroflavone. Post-hoc analysis indicated that diazepam (0.5 to 3 mg/kg) significantly reduced avoidance distance.

3.7.3. Effects on risk assessment

(a) Chase test: diazepam at 1 and 3 mg/kg

[$F(3,28) = 9.4$, $P < 0.001$], 6-bromoflavone at 0.3 and 3 mg/kg [$F(4,45) = 3.4$, $P < 0.05$] and 6-bromo-3′-nitroflavone at 0.0001, 0.001 and 1 mg/kg [$F(5,54) = 4.47$, $P < 0.01$] significantly decreased the number of orientations.

(b) Straight alley test: diazepam [$F(3,28) = 4.63$, $P < 0.05$], but not 6-bromoflavone or 6-bromo-3′-nitroflavone significantly increased the number of approaches followed by withdrawal responses.

3.7.4. Effects on defensive threat/attack responses the forced contact test

Diazepam at 1 and 3 mg/kg [$K = 21.83$, $P < 0.001$], 6-bromo-3′-nitroflavone at all doses except 0.01 mg/kg [$K = 11.4$, $P < 0.05$], but not 6-bromoflavone decreased significantly the number of bitings.

3.8. Discriminative stimulus properties

The effects of diazepam, 6-bromoflavone and 6-bromo-3′-nitroflavone in rats trained to discriminate a dose of 5 mg/kg of chlordiazepoxide from saline are shown in Table 4. The results are shown as the percentage of rats responding on the chlordiazepoxide-associated lever and the rates of lever pressing as a percentage of the rates obtained on saline days. Diazepam, but not the other drugs substituted for chlordiazepoxide. Complete substitution was reached by diazepam at 3 and 10 mg/kg ($ED_{50} = 2.2$ mg/kg). At the doses tested, none of the drugs significantly modified response rates.

4. Discussion

The aim of the present study was to compare the central BZ-ω binding characteristics and pharmacological profiles of 6-bromoflavone and 6-bromo-3′-nitroflavone, two synthetic flavonoids, with that of diazepam.

The in vitro binding assays showed that 6-bromoflavone displaced only weakly [^{3}H]flumazenil binding to $GABA_A$ receptor in membranes from rat cerebellum, a brain area enriched in the BZ-ω_1 receptor subtype, whereas it had no detectable affinity for $GABA_A$ sites endowed with the BZ-ω_2 and ω_5 receptor subtypes, respectively. In contrast, 6-bromo-3′-nitroflavone displayed high and moderate affinities for the BZ-ω_1 and BZ-ω_2 receptor subtypes, respectively, while it displaced [^{3}H]flumazenil that was only binding weakly to the ω_5 receptor subtype in membranes from rat hippocampus. Although previous studies using radioligand displacement of [^{3}H]flunitrazepam binding to synaptosomal membranes from different regions of the bovine or rat central nervous system indicated more potent displacement by both synthetic flavonoids, the present results confirm that these compounds show

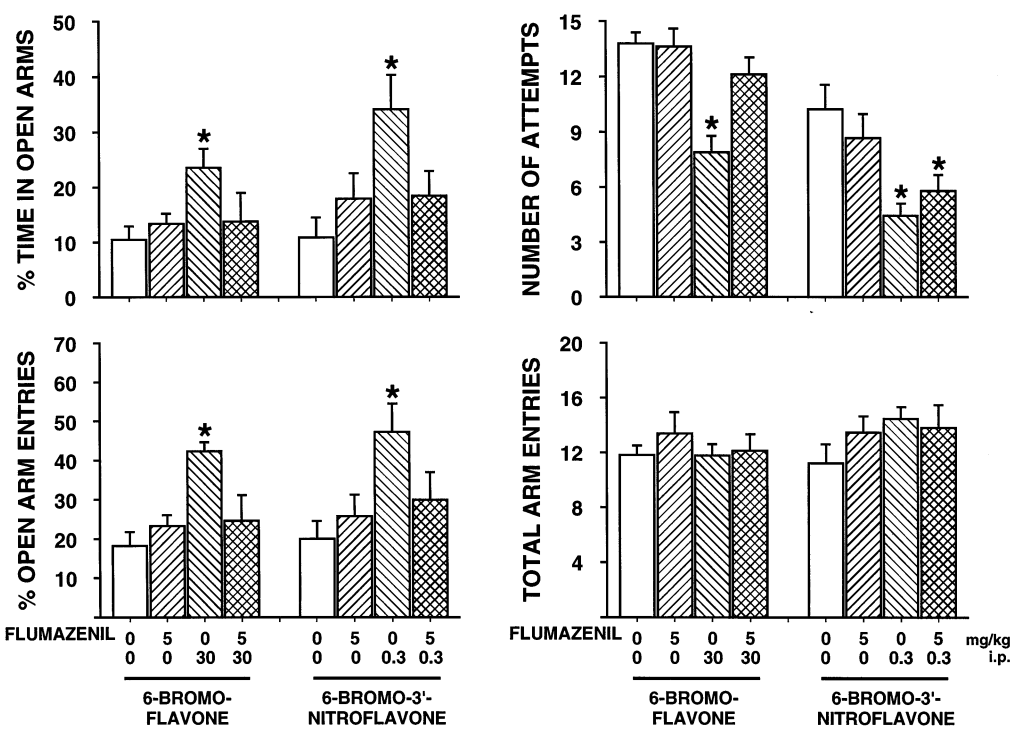

Fig. 5. Effects of 6-bromoflavone and 6-bromo-3′-nitroflavone alone or in combination with flumazenil on the behavior of rats on the elevated plus-maze. The drugs were administered intraperitoneally 20 min before testing. Data represent mean ± S.E.M. $n = 8–10$, * $P < 0.05$ (Dunnett's t-test).

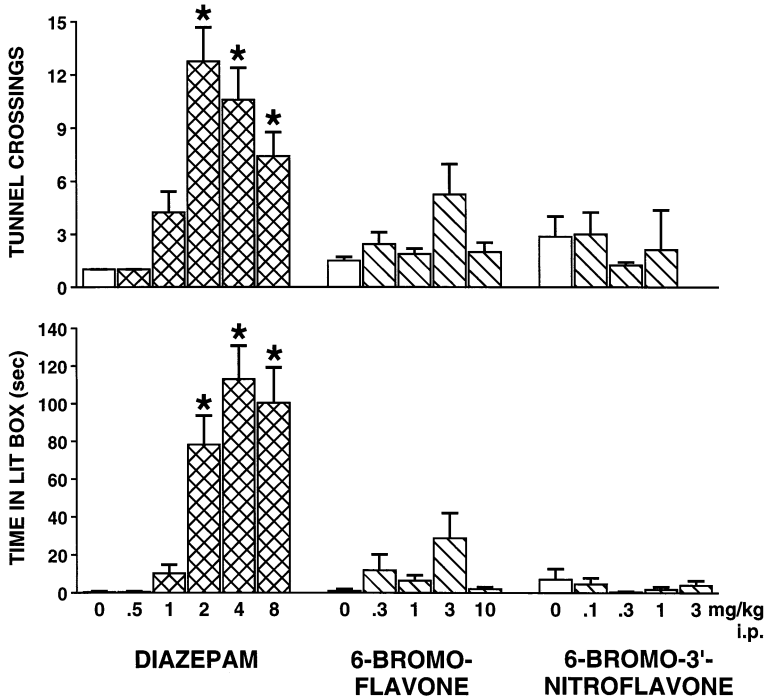

Fig. 6. Effects of diazepam and two synthetic flavonoids on the behavior of mice in the light/dark test. The drugs were administered intraperitoneally 30 min (diazepam) or 20 min (6-bromoflavone and 6-bromo-3′-nitroflavone) before testing. Data represent mean ± S.E.M. $n = 12–16$, * $P < 0.05$ (Siegal and Castellan test).

selectivity for the BZ-ω_1 relative to the BZ-ω_2 receptor subtype (Marder et al., 1996; Viola et al., 1997).

The in vivo experiments showed that diazepam, but not the synthetic flavonoids increased the latency to clonic seizures produced by isoniazid. Isoniazid inhibits glutamic acid decarboxylase, the enzyme that catalyzes the synthesis of GABA from glutamic acid, thereby reducing the neuronal stores of GABA available for nerve impulse-mediated release of this transmitter (Löscher and Frey, 1977). The maximal delay in onset of isoniazid-induced seizures produced by a test compound may therefore be taken as an index of increased GABAergic function. It has been proposed as an in vivo measure of the intrinsic activity of BZ-ω receptor ligands at $GABA_A$ receptors (Mao et al., 1975). The present results suggest that neither 6-bromoflavone nor 6-bromo-3′-nitroflavone displayed detectable intrinsic activity at $GABA_A$ receptors. Although no study has yet investigated the anticonvulsant properties of these two synthetic flavonoids, it is noteworthy that previous findings with 6,3'-dinitroflavone, a compound which is structurally related to the flavonoids tested in this study, showed that the drug had no anticonvulsant activity against pentylenetetrazole in mice (Wolfman et al., 1996).

In the punished lever pressing conflict test in rats neither 6-bromoflavone nor 6-bromo-3′-nitroflavone modified rates of punished responding, whereas in the punished drinking test both synthetic flavonoids produced anticonflict activity. However, it is important to note that the increase in punished responding with 6-bromoflavone and 6-bromo-3′-nitroflavone was smaller than that produced by diazepam, indicating a weaker anxiolytic-like activity. It is unlikely that the positive effects of 6-bromo-3′-nitroflavone in the punished drinking test are due to decreased sensitivity to electric shocks since the drug was reported to be inactive in the tail-flick analgesia test (Wolfman et al., 1998). The reason why the two conflict procedures yielded different behavioral profiles with these synthetic flavonoids is unclear, but it is noteworthy that previous findings with these tests showed that while non-selective BZ-ω receptor agonists are active in both situations, selective BZ-ω_1 receptor ligands generally produced anticonflict activity in the punished drinking model only (Sanger, 1995; Griebel et al., 1998a,b). Results from the present study thus confirm that BZ-ω_1-selective compounds have limited efficacy in conflict tests. Alternatively, the discrepancy between the two conflict tests may be explained by the fact that both models measure different facets or levels of anxiety. One can assume that the level of stress in the punished drinking test is higher than in the punished lever pressing procedure. In the former, the experimental situation was novel to the rats and they had never received electric shocks before testing. In contrast, in the lever pressing test, rats were trained for several months in the same cage and had already experienced electric shocks before drug testing.

Surprisingly, the non-selective BZ-ω receptor antagonist flumazenil failed to block the anticonflict activity of both synthetic flavonoids in the punished drinking test, thereby suggesting that these effects may not be mediated via an action at central BZ-ω receptors. However, these compounds have been described as selective BZ-ω receptor ligands since they did not displace the binding of specific radioligands to α_1 and β-adrenergic, muscarinic, cholinergic, $GABA_A$ or $5\text{-}HT_{1A}$ receptors (Medina et al., 1997). It must be noted that in the experiment with 6-bromo-3′-nitroflavone, a tendency to an increase in punished responding was observed when flumazenil was administered alone. Moreover, when coadministered with 6-bromo-3′-nitroflavone, flumazenil tended to potentiate the positive effects of the synthetic flavonoid. Although flumazenil is generally inactive in anxiety tests, there is some evidence that it may exert certain actions of its own (anxiogenic- or anxiolytic-like) (Lal and Harris, 1985; Pellow and File, 1985; Hodges and Green, 1987; Urbancic et al., 1990; Kapczinski et al., 1994; Griebel et al., 1995; Pokk and Zharkovsky, 1997), or may unmask other drug effects when given in combination with BZs (Barrett et al., 1985), depending on the level of stress and/or the model used. It is thus conceivable that flumazenil failed to counter the anticonflict effects of the synthetic flavonoids because it did not behave as a neutral ligand in this situation.

In the elevated plus-maze exploration test in rats, 6-bromoflavone and 6-bromo-3′-nitroflavone showed anxiolytic-like activity on all behavioral measures. Thus, on traditional behavioral indices, they increased percentage of time spent in open arms and percentage of open arm entries. Regarding the ethologically derived measure, they markedly decreased attempts. This latter effect indicates that animals treated with the synthetic flavonoids showed a reduced reluctance to leave relatively safe areas of the maze, a behavioral pattern that strengthens the conclusion of an anxiolytic-like action based upon the traditional index of anxiety. Furthermore, the positive effects produced by 6-bromoflavone and 6-bromo-3′-nitroflavone in the elevated plus-maze were of similar magnitude to those of diazepam, indicating a clear anxiolytic-like activity. The BZ-ω receptor antagonist flumazenil, which was inactive on its own, completely blocked the anxiolytic-like effects of 6-bromoflavone. While flumazenil antagonized the increase in open arm activity produced by 6-bromo-3′-nitroflavone, it failed to counter the effects on attempts.

Although 6-bromoflavone and 6-bromo-3′-nitroflavone were tested over a wide dose-range, no evidence for anxiolytic-like activity was observed in the mouse light/dark exploration test. In contrast, the positive control diazepam markedly increased the time spent in the lit box and the number of tunnel crossings, measures of anxiolytic-like action in this test (Misslin et al., 1989). The contrasting behavioral profiles displayed by the synthetic flavonoids in the two exploration models used in this study are somewhat unexpected, but may be explained by the use of different species. However, 6-bromoflavone and 6-bromo-3′-nitroflavone were

Table 3
Effects of diazepam and two synthetic flavonoids on several behavioral responses displayed by Swiss mice before (line crossings) and during (avoidance distance, approaches-withdrawals, orientations, bitings) exposure to a Long Evans rat in the mouse defense test battery[a]

Drug	Dose (mg/kg, i.p.)	Line crossings	Avoidance distance (cm)	Approaches –withdrawals	Orientations	Bitings
Diazepam	0	127.6 ± 9.6	160.6 ± 7.3	0.0 ± 0.0	9.6 ± 1.9	2.3 ± 0.3
	0.5	138.6 ± 16.1	126.4 ± 12.5[b]	0.3 ± 0.2	5.9 ± 1.5	1.8 ± 0.2
	1	139.5 ± 22.0	91.4 ± 8.9[b]	0.8 ± 0.3	2.3 ± 0.6[b]	0.8 ± 0.2[b]
	3	95.1 ± 13.7	76.0 ± 7.7[b]	1.6 ± 0.6[b]	1.3 ± 0.4[b]	0.1 ± 0.1[b]
6-Bromoflavone	0	133.0 ± 8.9	145.4 ± 11.9	0.2 ± 0.1	8.3 ± 0.6	2.1 ± 0.2
	0.1	114.4 ± 13.0	126.2 ± 10.8	0.5 ± 0.2	6.3 ± 1.2	1.1 ± 0.3
	0.3	126.5 ± 14.7	160.1 ± 6.7	0.8 ± 0.2	4.9 ± 0.6[b]	1.5 ± 0.2
	1	98.8 ± 9.6	92.0 ± 11.6	0.7 ± 0.4	5.6 ± 0.9	1.2 ± 0.3
	3	111.1 ± 10.8	127.2 ± 10.5	0.8 ± 0.3	4.1 ± 0.9[b]	1.3 ± 0.3
6-Bromo-3'-nitroflavone	0	105.7 ± 10.3	142.2 ± 8.2	0.2 ± 0.1	5.7 ± 1.0	2.3 ± 0.2
	0.0001	114.5 ± 6.3	126.8 ± 9.6	1.4 ± 0.4	3.9 ± 0.6[b]	0.9 ± 0.3[b]
	0.001	103.0 ± 9.2	137.3 ± 7.7	0.9 ± 0.3	2.8 ± 0.6[b]	1.4 ± 0.3[b]
	0.01	107.3 ± 10.0	134.6 ± 8.5	0.5 ± 0.2	1.9 ± 0.3[b]	1.7 ± 0.3
	0.1	92.4 ± 8.0	111.3 ± 11.9	1.1 ± 0.3	4.2 ± 0.8	1.1 ± 0.3[b]
	1	115.9 ± 9.2	129.2 ± 4.6	1.1 ± 0.3	2.4 ± 0.5[b]	1.6 ± 0.2[b]

[a] The drugs were administered intraperitoneally 30 (diazepam) or 20 (6-bromoflavone and 6-bromo-3′-nitroflavone) min before testing. Data represent mean ± S.E.M. $n = 8$–11.

[b] $P < 0.05$ (Dunnett's t-test).

Table 4
Discrimination stimulus properties of diazepam and two synthetic flavonoids[a]

Drug	Dose(mg/kg, i.p.)	Generalization (%)	Control rate (%)
Diazepam	1	25	118
	2	71	108
	3	100	109
	10	100	67
6-Bromoflavone	1	0	102
	3	0	101
	10	0	97
	30	0	96
6-Bromo-3'-nitroflavone	0.1	0	98
	0.3	0	95
	1	0	103
	3	0	92

[a] Drugs were tested in rats trained to discriminate between chlordiazepoxide and saline. The left column shows the percentage of rats choosing the drug-associated lever and the right column shows the total number of lever presses expressed as percentages of control. The drugs were administered intraperitoneally 30 min (diazepam) or 20 min (6-bromoflavone and 6-bromo-3'-nitroflavone) before testing. $n = 8$.

found to display anxiolytic-like activity in the murine elevated plus-maze (Marder et al., 1996; Viola et al., 1997), indicating that mice are suitable for investigating the behavioral effects of these flavonoids. Alternatively, these differences may be due to the fact that the two models assess different aspects of anxiety responses, some of which may not be modified by 6-bromoflavone and 6-bromo-3′-nitroflavone. Indeed, a previous factor analytic study performed on the variables from the elevated plus-maze and the light/dark tests showed that the former loaded on a factor related to exploration criteria, whereas the latter loaded on a factor related to neophobia (Belzung and Le Pape, 1994).

The MDTB is an experimental procedure designed for screening anxiety-modulating agents in mice. It elicits and measures reactions to a present threat (i.e. a rat). In this model, Swiss mice show an extremely precise delineation of defensive behaviors including flight, risk assessment and defensive attack, with each behavior controlled by specifiable characteristics of the threat stimulus and situation. Extensive pharmacological investigations have demonstrated that the MDTB is a useful tool for evaluating potential anxiolytics (Griebel et al., 1996a; Blanchard et al., 1997, 1998). The present results showed that unlike diazepam which markedly modified all defensive behaviors, 6-bromoflavone and 6-bromo-3′-nitroflavone either did not modify defense responses or produced weak and inconsistent effects, depending on the situation. Thus, while both synthetic flavonoids failed to produce significant effects on flight reactions (avoidance distance) after the rat was first introduced into the runway and risk assessment responses displayed when subjects were constrained in one part of the runway (approaches followed by withdrawals), they decreased, albeit non dose-dependently, risk assessment during the chase test (orientations). Furthermore, 6-bromo-3′-nitroflavone, but not 6-bromoflavone significantly decreased defensive attack behavior (bitings) upon forced contact with the rat at all but the 0.01 mg/kg dose. Taken together, these results indicate that the synthetic flavonoids studied produced only modest anxiolytic-like activity as compared to diazepam.

In the drug discrimination experiment, 6-bromoflavone and 6-bromo-3′-nitroflavone did not substitute for chlordiazepoxide up to 30 and 3 mg/kg, respectively. It is possible that this result may be related to the selectivity of these drugs for the BZ-ω_1 receptor subtype. However, previous findings showed that although the discriminative stimulus effects of selective BZ-ω_1 receptor ligands (e.g. zolpidem) differ from those produced by BZs, they produce at least some levels of responding on the chlordiazepoxide-associated level (Sanger, 1988; Sanger and Zivkovic, 1994).

In summary, the results of the present series of experiments failed to demonstrate that the synthetic flavonoids 6-bromoflavone and 6-bromo-3′-nitroflavone possess anxiolytic-like properties similar or superior to that of the BZ diazepam, as was suggested previously. Although flumazenil antagonized the anxiolytic-like effects of these flavonoids in the elevated plus-maze, the findings of a weak affinity of 6-bromoflavone for BZ-ω receptors, together with the lack of anticonvulsant activity, the failure of flumazenil to antagonize the anticonflict activity of both synthetic flavonoids and the complete lack of substitution of these compounds for the stimulus produced by chlordiazepoxide, question the contribution of BZ-ω receptors in the behavioral effects of 6-bromoflavone and 6-bromo-3′-nitroflavone.

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