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Anxiolytic effect of wogonin, a benzodiazepine receptor ligand isolated from *Scutellaria baicalensis* Georgi

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Abstract

The search for novel anxiolytics devoid of undesirable side-effects typical of classical benzodiazepines (BDZs) has been intense, and flavonoids, as a relative new class of ligands, have been shown to possess anxiolytic effects *in vivo*. The present study evaluated the pharmacological properties of a naturally occurring monoflavonoid, 5,7-dihydroxy-8-methoxyflavone or wogonin. The affinity (K_i) of wogonin for the benzodiazepine site (BZD-S) on the γ-aminobutyric acid$_A$ ($GABA_A$) receptor complex was 0.92 μM. Using electrophysiological techniques, we showed that wogonin enhanced the GABA-activated current in rat dorsal root ganglion neurons, and in *Xenopus laevis* oocytes expressing recombinant rat $GABA_A$ receptors, the enhancement was partially reversed by the co-application of a 1 μM concentration of the BZD-S antagonist anexate (Ro15-1788). Acute toxicity and behavioral effects were examined in mice. Acute lethal activity was low, with an LD_{50} of 3.9 g/kg. Oral administration of wogonin (7.5 to 30 mg/kg) elicited an anxiolytic response that was similar to that elicited by diazepam in the elevated plus-maze; a dose-dependent increase in open arm entries and time spent in open arms was observed. More importantly, its anxiolytic effect was blocked by the co-administration of Ro15-1788. In the holeboard test, not only did wogonin-treated mice experience an increased number of head-dips but they also spent more time at it, showing no signs of sedation. Furthermore, wogonin did not cause myorelaxant effects in the horizontal wire test. Taken together, these data suggest that wogonin exerts its anxiolytic effect through positive allosteric modulation of the $GABA_A$ receptor complex via interaction at the BZD-S. Its anxiolytic effect was not accompanied by sedative and myorelaxant side-effects typical of BDZs.

Keywords: $GABA_A$ receptor; Benzodiazepine; Wogonin; Anxiolytic; *Scutellaria baicalensis* Georgi; Flavonoid

1. Introduction

The $GABA_A$ receptor is a member of the ligand-gated ion channel superfamily, GABA being the major inhibitory transmitter in the CNS. Binding of GABA to the $GABA_A$ receptor activates a chloride ion flux through the channel, and ligands for the BDZ-S modulate the inhibitory effects of GABA [1]. Such BDZ-S ligands are classified as positive allosteric modulators, antagonists, or negative allosteric modulators according to their spectrum of intrinsic efficacy towards the $GABA_A$ receptor [2]. Positive allosteric modulators increase the frequency of chloride channel openings without altering the channel conductance or duration of opening. Therapeutically, they are used as anxiolytic, anticonvulsant, sedative-hypnotic, and muscle relaxant drugs.

Scutellaria baicalensis Georgi is one of the most important medicinal herbs in traditional Chinese medicine. It possesses anti-bacterial activity and sedative effects [3], and finds application in the treatment of a range of conditions including diarrhea [4] and hepatitis [5]. Wogonin, the major chemical constituent of this herb, is a flavone derivative containing a phenylbenzopyrone nucleus. Flavonoids, a relatively new class of ligands for the BDZ-S, can be isolated from a wide variety of fruits, vegetables, nuts, and flowers, as well as tea, which are important components of the human diet [6]. The biological and

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Abbreviations: BDZ, benzodiazepine; BDZ-S, benzodiazepine site(s); B_{max}, maximal binding density; DRG, dorsal root ganglion; and GABA, γ-aminobutyric acid.

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pharmacological properties of flavonoids are broad and include anti-viral and anti-inflammatory actions [7,8], the reduction of neuronal oxidative metabolism [9], estrogenic effects [10], and the inhibition of enzymes including protein kinase C and tyrosine kinase [11,12]. Until recently, little was known regarding their effects on the CNS; however, several groups have now reported on natural and synthetic flavonoids that exert anxiolytic effects [13–15]. Even though BDZs are extremely effective anxiolytics and constitute the most widely prescribed class of psychoactive drugs in current therapeutic use, the search continues for novel anxiolytic compounds devoid of the undesirable effects of BDZ, the most common and important of which are sedative and myorelaxant effects.

The purpose of the present study was to characterize the functional and pharmacological properties of wogonin isolated from *S. baicalensis* Georgi. Electrophysiological studies were carried out to examine its efficacies for the $GABA_A$ receptor and the acute toxicity of wogonin was assessed. Additionally, the anxiolytic, sedative, and myorelaxant effects typical of BDZ were determined by the elevated plus-maze, holeboard, and horizontal wire tests, respectively.

2. Materials and methods

2.1. Chemicals

S. baicalensis Georgi was obtained from herbal suppliers in Hong Kong, and voucher specimens (USTHQ0002) were maintained. Radioactive [^{3}H]flunitrazepam (*N*-methyl-[^{3}H], 88 Ci/mmol) was purchased from Amersham. Diazepam was obtained from the Sigma Chemical Co. Anexate (Ro15-1788, 0.1 mg/mL ampoules) was purchased from Hoffmann-La Roche Ltd. All other materials were of the highest grade from standard commercial sources.

2.2. Sample preparation

The roots of *S. baicalensis* Georgi were ground into powder, and 500 g of the resulting powder was extracted three times, each time with 2 L of dichloromethane, at room temperature. The extract was filtered through Whatman No. 1 filter paper and concentrated to 200 mL in a rotary vacuum evaporator at 60°. The yellow precipitate formed was filtered with a Whatman No. 1 filter and dried. The precipitate (1.5 g) was dissolved in 40 mL ethanol under reflux. This solution was filtered and kept at room temperature for crystal formation. The crystals, consisting of wogonin (purity >95%), were filtered and washed with ethanol.

2.3. Radioreceptor binding assay

Wogonin was dissolved in DMSO (Acros) and assayed at less than a 0.2% final DMSO concentration. DMSO itself at a concentration of up to 0.5% showed no significant effects on the BDZ-S assay. The radioreceptor binding assay was performed with synaptosomal membranes isolated from rat forebrains [16,17] and was carried out as previously described [18], employing 1 nM [^{3}H]flunitrazepam as radioligand and duplicate 30-min incubations at 4°. Non-specific binding was determined by the addition of 10 μM diazepam. In the saturation assays, nine concentrations (0.2 to 25 nM) of [^{3}H]flunitrazepam were used.

2.4. Electrophysiological studies

2.4.1. DRG neurons

Freshly isolated DRG neurons from adult rats (200–250 g Sprague–Dawley rats, of either sex) were prepared as described previously [19,20]. Whole-cell patch-clamp recordings were performed at room temperature (22–25°) using a CEZ-2400 patch/whole-cell patch amplifier (Nihon Kohden). Gigaohm seals were made using borosilicate glass microelectrodes with a tip resistance of 2–4 MΩ. The membrane potential was held at −60 mV. Neurons were placed in an extracellular medium containing: 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose; pH was adjusted to 7.3 with NaOH, and osmolarity was adjusted to 340 mOsmol/L with sucrose. The patch pipettes were filled with an intracellular solution containing: 140 mM CsCl, 2.5 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, and 5 mM Mg-ATP; pH was adjusted to 7.3 with CsOH, and osmolarity was adjusted to 310 mOsmol/L with sucrose. Membrane currents were filtered at 1 kHz (−3 dB), and data were stored and analyzed on a laboratory computer using data acquisition software and hardware (Huazhong University of Science and Technology).

GABA and diazepam were dissolved in the extracellular solution. Wogonin was dissolved in DMSO and further diluted with extracellular solution. Drug solutions were applied by gravity flow using a linear barrel array made of fused silica tubes (external diameter/internal diameter = 500/200 μm) connected to a series of independent reservoirs. The tubes were placed within 100 μm of the neurons. Neurons were bathed constantly in extracellular medium flowing from one barrel, and drug solutions were applied by opening the appropriate valve and rapidly shifting the pipette array horizontally using a micromanipulator. To ensure full recovery of $GABA_A$ receptor from desensitization after each application, drugs were applied at 4-min intervals.

2.4.2. Xenopus laevis oocytes

X. laevis oocytes were prepared, injected, and defolliculated, and currents were recorded, as described [21,22]. Briefly, oocytes were injected with 50 nL of capped, polyadenylated cRNA dissolved in 5 mM K-HEPES (pH 6.8). For dual subunit combinations, a concentration of 75 nM

was used for each transcript. For the triple subunit combinations, a 10 nM concentration of the cRNA coding for the different α subunits (α1, α2, α3, α5, or α6) and the β2 subunit was used, whereas a 50 nM concentration of the γ2 subunit cRNA was used. RNA transcripts were synthesized from linearized plasmids encoding the desired protein using the mMESSAGE mMACHINE™ kit (Ambion) according to the recommendations of the manufacturer. A poly(A) tail of ~300 residues was added to the transcripts by using yeast poly(A) polymerase (Amersham). The cRNA combinations were coprecipitated in ethanol and stored at −20°. Transcripts were quantified on agarose gels after staining with Radiant Red Fluorescent RNA Stain (Bio-Rad) by comparing staining intensities with various amounts of molecular weight markers (RNA Ladder, Gibco BRL). Electrophysiological experiments were performed by the two-electrode voltage clamp method at a holding potential of −80 mV. The medium contained 90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES-NaOH, pH 7.4. GABA, diazepam, and test drugs were applied for 20 sec, and a washout period of 3–15 min was allowed to ensure full recovery from desensitization. Current responses have been fitted to the Hill equation: $I = I_{max}/(1 + (EC_{50}/[A])^n)$, where I is the peak current at a given concentration of $GABA_A$; I_{max} is the maximum current; EC_{50} is the concentration of agonist eliciting half maximal current; and n is the Hill coefficient. Currents were measured using a modified OC-725 amplifier (Warner Instruments Corp.) in combination with an xy-recorder or digitized using a MacLab/200 (AD Instruments).

2.5. Behavioral and toxicity tests

2.5.1. Animals

Male ICR mice weighing 18–23 g were used. They were housed in groups of four or five with free access to food and water and maintained on an 11 hr light:13 hr dark cycle. All of the experimental groups except for the one used for the acute lethal activity test (10 mice of either sex) consisted of 16 animals per group.

2.5.2. Drugs

Diazepam and wogonin were dissolved in water (adjusted to pH 10 with NaOH) and administered orally 1 hr before testing. Their concentrations were adjusted to give a delivery volume of 10 mL/kg. Anexate (Ro15-1788, 0.1 mg/mL ampoules) was injected i.p. to give a dose of 1.25 mg/kg 15 min prior to testing. The dose range of wogonin was selected based on pilot experiments.

2.5.3. Acute lethal activity

The average body weight was 20.03 ± 0.17 g (means ± SEM) for male mice (30) and 21.80 ± 0.22 g (means ± SEM) for female mice (30). After the pilot test, six doses of wogonin were chosen (see Table 1). For each dose, 10 mice of either sex were randomly selected and grouped. Food was withheld for 12 hr prior to dosing. Wogonin was given orally as a suspension in olive oil. Three doses were administered at a constant volume of 15 mL/kg. The LD_{50} value was determined 10 days after oral administration, and its 95% confidence limits were calculated by use of a probit analysis.

Table 1
Acute lethality of wogonin

Dose (g/kg)	Number of deaths		Mortality (%)
	Male	Female	
2.19	2	1	30
2.74	2	1	30
3.43	2	2	40
4.29	3	2	50
5.36	3	3	60
6.70	4	4	80

The LD_{50} and its 95% confidence limits were calculated to be 3919 (3262–4576) mg/kg.

2.5.4. Locomotor activity test

A ZIL-2 apparatus (Beijing Institute of Materia Medica), with dimensions of 60 × 60 × 12 cm and consisting of four circular plastic boxes (diameter 25 cm) each with 6 evenly spaced infrared photocells, was employed. Locomotor activity was assessed automatically during a 5-min test period and expressed as the number of transitions across the photocells.

2.5.5. Holeboard test

The holeboard was a wooden box, 60 × 60 × 30 cm, with four holes (diameter 3 cm) evenly spaced in the floor. Mice were placed at the center of the holeboard, and the number of head-dips into the holes, the time spent head-dipping, and the number of rears were evaluated over a 5-min period [23]. Mice were tested in a randomized order with respect to drug treatment between 8:00 a.m. and 12:00 p.m. in a room lit by dim light. They were randomly allocated to the following groups: vehicle control, wogonin (3.75, 7.5, 15, and 30 mg/kg), and diazepam (1 mg/kg), N = 16 per group. After each trial, the floor of the apparatus was wiped with 70% ethanol and dried thoroughly with tissue to remove traces of previous paths. An increase in the number of head dips, the time spent head-dipping, and the number of rears reflects a greater exploratory activity. A decrease in these three parameters relative to controls reveals a sedative behavior [24–26].

2.5.6. Elevated plus-maze

The wooden elevated plus-maze consisted of two opposite open arms (25 × 5 cm) and two opposite closed arms enclosed by 20-cm high walls. The arms extended from a central platform (5 × 5 cm). The plus-maze was elevated to a height of 40 cm. The maze was put inside a box (30 × 30 × 50 cm). After a 5-min holeboard test, each mouse was immediately placed in the central square facing

a closed arm, and its behavior was observed for 5 min. This method allows the separation of exploratory (head-dipping) from locomotor activity and rearing [25], and increases the overall activity of mice in the plus-maze [27,28]. The number of entries into and the time spent on the open and closed arms were scored. The total number of arm entries provided a measure of general activity. The number of entries into open arms was expressed as a percentage of the total number of arm entries. Time spent on open arms was also expressed as a percentage of time spent on both open and closed arms. Testing took place between 8:00 a.m. and 12:00 p.m. in a randomized order with respect to drug treatment in a room lit by dim light. Mice were randomly allocated to the following test groups: vehicle control, wogonin (3.75, 7.5, 15, and 30 mg/kg), and diazepam (1 mg/kg), N = 16 per group. In a separate experiment where mice were subjected to the co-administration of wogonin and Ro15-1788, mice were administered wogonin (15 mg/kg) orally and received an i.p. injection of Ro15-1788 (1.25 mg/kg), 1 hr and 15 min prior to testing, respectively. A selective increase in the parameters corresponding to open arms reveals an anxiolytic effect [27,28].

2.5.7. Horizontal wire test

Mice were lifted by the tail and allowed to grasp a horizontally strung wire (1 mm diameter, 15 cm long, and placed 20 cm above the table) with their forepaws, and then were released [29]. Testing took place after two trials performed at 5-min intervals. The number of mice from each treatment group that did not grasp the wire with the forepaws or actively grasped the wire with at least one hind paw within 3 sec was recorded. A myorelaxant drug would impair the ability of the mice to grasp the wire, and muscle relaxation is commonly associated with sedation.

Table 2
[^{3}H]Flunitrazepam displacement studies of wogonin

Compound	Inhibition of [^{3}H]flunitrazepam binding[a] (μM)	
	IC_{50}	K_i
Diazepam	0.012 ± 0.0011	0.0089 ± 0.00008
Wogonin	1.26 ± 0.20	0.92 ± 0.14

[a] Estimated IC_{50} and K_i values are means ± SEM for three separate experiments, each with duplicate determinations.

2.5.8. Statistics

All data were subjected to ANOVA. Post hoc comparisons between individual treatment groups and controls were made with Dunnett's *t*-test.

3. Results

3.1. Radioreceptor binding assay

The IC_{50} and K_i values of wogonin and diazepam for the displacement of [^{3}H]flunitrazepam from synaptosomal membrane fractions are shown in Table 2. Diazepam served as a standard compound to assess the relative potency of wogonin. Based on the data, wogonin (IC_{50} = 1.26 μM) was approximately 100-fold less potent than diazepam (IC_{50} = 0.012 μM). From Scatchard plot analysis of [^{3}H]flunitrazepam saturation binding (Fig. 1), the dissociation constant (K_d) of the high-affinity binding site for [^{3}H]flunitrazepam was 2.73 ± 0.65 nM and the B_{max} 0.73 ± 0.012 pmol/mg protein. Scatchard plot analysis showed that wogonin at 1 and 5 μM caused a decrease in the K_d without changing the B_{max}, suggesting a competitive inhibition of [^{3}H]flunitrazepam binding. However, at 25 μM

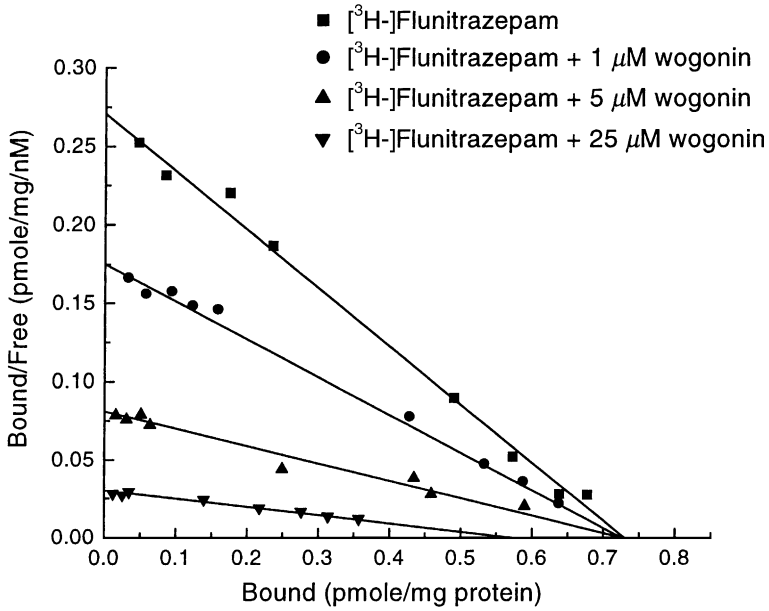

Fig. 1. Representative Scatchard plot of [^{3}H]flunitrazepam (0.2 to 25 nM) binding to the BDZ-S *in vitro* in the absence and presence of different concentrations of wogonin.

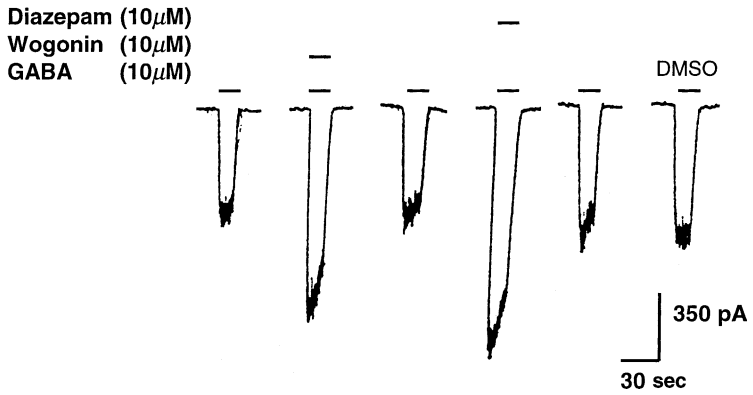

Fig. 2. Representative current trace (N = 6) obtained from DRG neurons using the whole-cell patch-clamp technique. Both wogonin and diazepam enhanced the GABA-activated current.

wogonin, non-competitive inhibition was observed with alterations of both K_d and B_{max}.

3.2. Electrophysiological studies

The functional effects of wogonin on DRG neurons were investigated by electrophysiological techniques. The GABA-activated current was enhanced by the co-application with GABA of either wogonin (10 μM) or diazepam. Application of wogonin alone up to 30 μM induced no detectable changes in the membrane conductance (data not shown). The current trace shown in Fig. 2 was obtained from the same DRG neuron voltage-clamped at −60 mV (N = 6). DMSO alone also had no effect.

GABA elicited μA sized currents on *Xenopus* oocytes expressing recombinant rat α1β2γ2 $GABA_A$ receptors. At 30 μM, its limit of solubility, wogonin alone did not activate any current. In Fig. 3, a GABA concentration eliciting about 1% of the maximal current amplitude was applied alone, and subsequently in combination with increasing concentrations of wogonin. From approximately 1 μM, wogonin started to allosterically stimulate these currents. Fig. 4 shows the mean stimulation by different concentrations of wogonin in three experiments. Half-maximal stimulation was observed at 3 μM wogonin, which was comparable in order of magnitude to the radio-receptor binding assay. At 30 μM wogonin, current stimulation by GABA approached saturation. In six individually prepared batches of oocytes, wogonin potentiation averaged 37 ± 7% (SEM) at 0.3 μM when diazepam at this concentration elicited maximal potentiation (data not shown).

The benzodiazepine antagonist Ro15-1788 at 1 μM partially inhibited the potentiation by 30 μM wogonin (Fig. 5). In seven separate experiments, 30 μM wogonin potentiated the current elicited by GABA by 57 ± 6%, but the potentiation was decreased to 11 ± 6% when 30 μM wogonin was subsequently co-applied with 1 μM Ro15-1788. Ro15-1788 at 1 μM by itself did not affect the current response to GABA (data not shown). Inhibition of wogonin potentiation by Ro15-1788, therefore, amounted to 46 ± 9% (N = 7). It is of interest to compare the partial inhibition of wogonin potentiation by Ro15-1788 to the findings in radioreceptor binding assays where

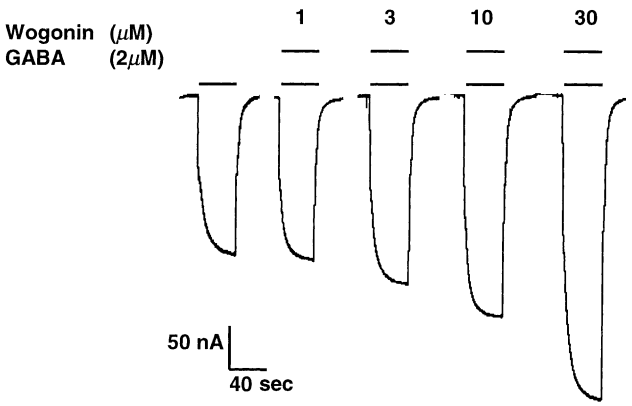

Fig. 3. Stimulation of currents elicited by GABA in α1β2γ2 $GABA_A$ receptors. Recombinant rat $GABA_A$ receptors were expressed in *X. laevis* oocytes. Application of 2 μM GABA (lower bar) alone resulted in approximately 1% of the maximal current amplitude. The bars indicate duration of drug applications. Numbers above the upper bars indicate wogonin concentration in micromolar co-applied with GABA (lower bars). N = 7.

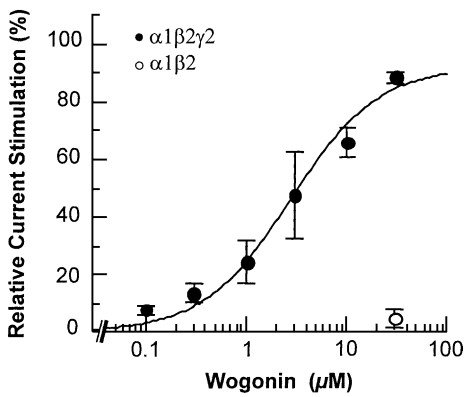

Fig. 4. Allosteric stimulation by wogonin of currents elicited by GABA in α1β2γ2 $GABA_A$ receptors. Conditions were the same as in Fig. 2. In the cumulative concentration response curves shown, the points indicate the means ± SEM of 6 oocytes from three different batches. Stimulation by 30 μM wogonin obtained with the dual subunit combination α1β2 is also shown (○; means ± SEM, N = 3).

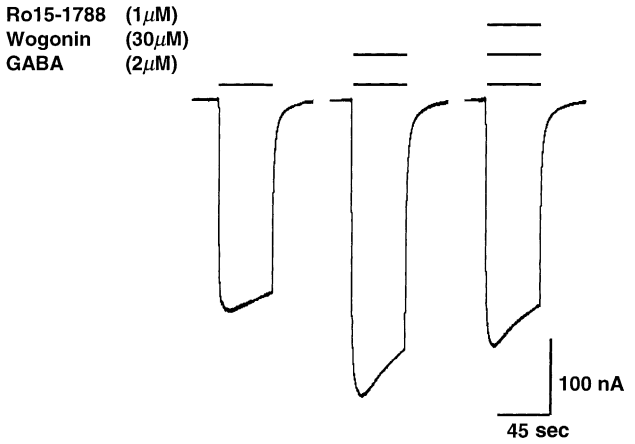

Fig. 5. Stimulation by wogonin inhibited by the BDZ-S antagonist Ro15-1788. The concentration of GABA (2 μM, lower bar) eliciting approximately 2% of the maximal current amplitude with recombinant rat α1β2γ2 $GABA_A$ receptors was first determined. Wogonin at 30 μM markedly stimulated these currents. When 1 μM Ro15-1788 (upper bar) was co-applied with 30 μM wogonin (middle bar), the stimulation exerted by wogonin was inhibited partially. The bars indicate the periods of drug application. N = 7.

non-competitive binding was observed at the highest concentration (Fig. 1), which suggests an alternative modulating effect of wogonin independent of the BDZ-S. Since ligands of the BDZ-S are known to depend upon the presence of a γ subunit [22,30,31], potentiation by 30 μM wogonin was also measured using the dual subunit combination of α1β2. Current stimulation was only 5 ± 3% (N = 3), i.e. nearly abolished, in the absence of the γ subunit.

3.3. Acute toxicity

After oral administration and a 10-day observation period, the percentage of mortality, LD_{50}, and 95% confidence limits of wogonin were documented in Table 1. The acute toxicity of wogonin was moderately low.

3.4. Behavioral test

3.4.1. Locomotor activity test

To differentiate between possible stimulant effects of tested drugs from their modulation with exploratory

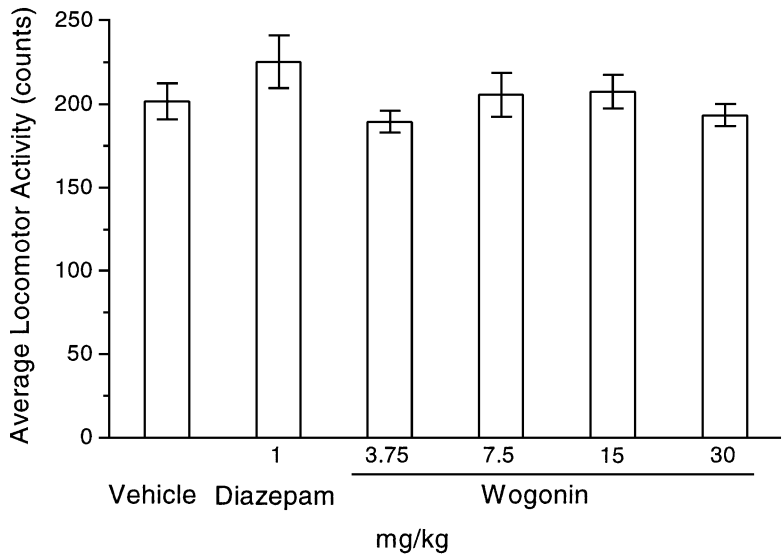

Fig. 6. Locomotor activity (means ± SEM) counts during a 5-min test session in a ZIL-2 apparatus 1 hr after the oral administration of vehicle, diazepam, or wogonin. N = 16 mice per group.

Table 3
Effects of vehicle, wogonin, and diazepam in the holeboard test in mice

Drug	Dose (mg/kg)	Number	Time	Rears
Vehicle		23.13 ± 1.84	26.88 ± 2.01	22.06 ± 1.67
Wogonin	3.75	23.88 ± 1.08	27.00 ± 1.10	18.13 ± 0.86
	7.5	31.06 ± 1.88*	36.38 ± 1.93*	19.25 ± 2.72
	15	37.00 ± 1.94**	42.25 ± 2.54**	25.38 ± 2.30
	30	42.75 ± 3.58**	48.19 ± 3.92**	23.50 ± 1.94
Diazepam	1	28.88 ± 2.16	37.00 ± 0.79*	24.56 ± 2.56

Data are expressed as means ± SEM, N = 16 per group.
* $P < 0.05$, significantly different from control (Dunnett's *t*-test after ANOVA).
** $P < 0.01$, significantly different from control (Dunnett's *t*-test after ANOVA).

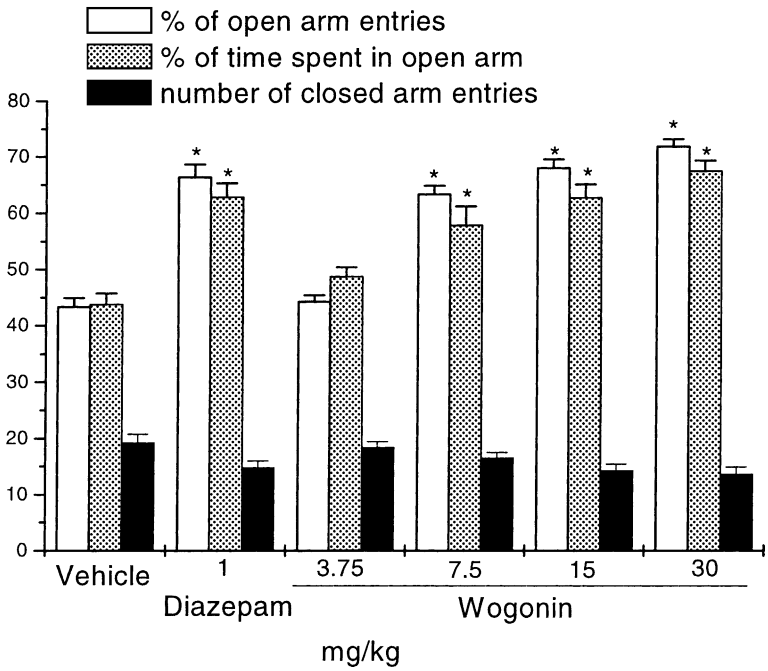

Fig. 7. Effects of vehicle, diazepam, or wogonin on the behavior of mice in the elevated plus-maze. Data are expressed as the means (±SEM) percentage of open arm entries or of time spent in open arms, and the number of closed arm entries in mice given a 5-min test, 1 hr after the oral administration of the compounds. N = 16 mice per group. Key: (∗) $P < 0.01$, significantly different from controls (Dunnett's *t*-test after ANOVA).

behavior, the locomotor activity test was performed. Wogonin produced no significant changes in the locomotor activity of mice administered doses of 3.75 to 30 mg/kg [$F_{(4,75)} = 0.64$] (Fig. 6). Diazepam (1 mg/kg) likewise did not alter the locomotor activity [$F_{(1,30)} = 1.35$].

3.4.2. Holeboard test

The holeboard test is well-established as a means to assay potential sedative effects [32]. Acute administration of diazepam significantly increased the time mice spent head-dipping [$F_{(1,30)} = 6.97$, $P < 0.05$] (Table 3). Neither wogonin nor diazepam had a significant effect on the number of rears [$F_{(4,75)} = 2.23$ and $F_{(1,30)} = 0.67$, respectively] (Table 3). Like the diazepam-treated group, wogonin-treated mice experienced a significant increase in the number of head-dips and time spent head-dipping [$F_{(4,75)} = 14.45$, $P < 0.0001$ and $F_{(4,75)} = 14.29$, $P < 0.0001$, respectively]. The lowest dose of wogonin (3.75 mg/kg) tested was without effect (Table 3).

Table 4
Effects of vehicle, wogonin, and diazepam in the elevated plus-maze in mice

Drug	Dose (mg/kg)	Total arm entries
Vehicle		34.31 ± 1.78
Wogonin	3.75	34.56 ± 0.97
	7.5	45.56 ± 1.71*
	15	43.50 ± 1.90*
	30	47.00 ± 1.93*
Diazepam	1	45.13 ± 2.31*

Data are expressed as means ± SEM, N = 16 per group.
* $P < 0.01$, significantly different from control (Dunnett's *t*-test after ANOVA).

3.4.3. Elevated plus-maze test

Both diazepam and wogonin significantly altered the total number of arm entries [$F_{(1,30)} = 13.73$, $P < 0.001$ and $F_{(4,75)} = 12.99$, $P < 0.0001$, respectively] with little effect in the number of closed arm entries (Fig. 7). Analysis showed that diazepam and wogonin (7.5, 15, and 30 mg/kg)

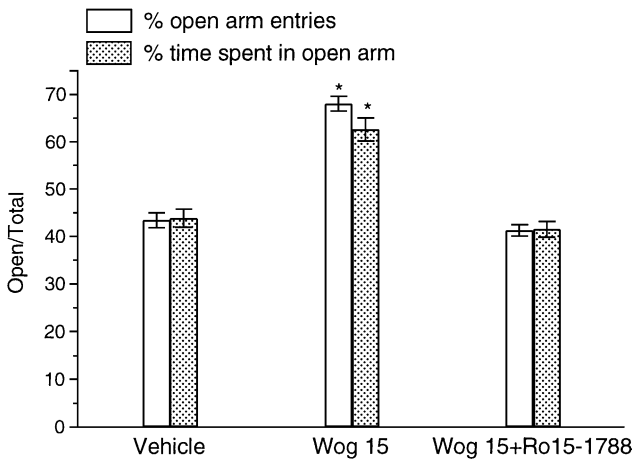

Fig. 8. Anxiolytic effect of wogonin blocked by Ro15-1788. Data are expressed as the means (±SEM) percentage of open arm entries or of time spent in open arms in mice given a 5-min test, 1 hr after the oral administration of wogonin (15 mg/kg), wogonin (15 mg/kg) + Ro15-1788 (1.25 mg/kg, i.p. administration 15 min prior testing), or vehicle; N = 16 mice per group. Key: (∗) $P < 0.01$, significantly different from control (Dunnett's *t*-test after ANOVA).

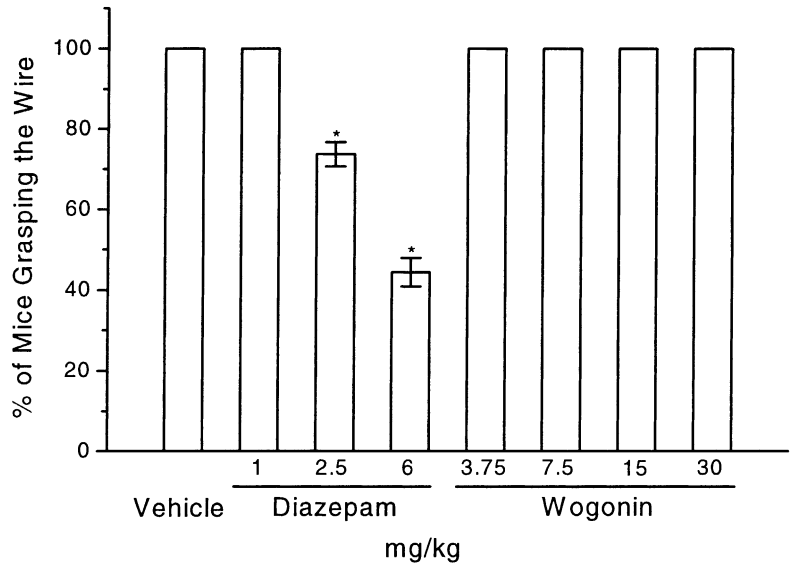

Fig. 9. Performance of mice in the horizontal wire test 1 hr after oral administration of vehicle, diazepam, or wogonin. N = 16 mice per group. For the 2.5 and 6 mg/kg diazepam-treated groups, the error bars represent the variation (SEM) among three groups, consisting of a total of 16 mice, in the percent of mice grasping the wire. Key: (∗) $P < 0.01$, significantly different from controls (Dunnett's *t*-test after ANOVA).

significantly increased the total number of arm entries (Table 4), and significantly elevated the percentage of open arm entries [$F_{(1,30)} = 70.35$, $P < 0.0001$ and $F_{(4,75)} = 89.90$, $P < 0.0001$, respectively]. Dunnett's *t*-tests showed that diazepam and wogonin (7.5, 15, and 30 mg/kg) significantly differed from control (Fig. 7). The increment was most pronounced between 3.75 and 7.5 mg/kg. It was more gradual beyond 7.5 mg/kg, although a definite increment was observed with respect to both open arm entries and time spent in open arms when the dosage was increased from 7.5 to 15 mg/kg, and finally to 30 mg/kg. To test whether the anxiolytic effect of wogonin was exerted via the BDZ-S, mice were subjected to the co-administration of the BDZ-S antagonist Ro15-1788 with wogonin (15 mg/kg). Results showed that Ro15-1788 injection reduced the percentage of open arm entries and the percentage of time spent in open arm to basal levels (Fig. 8).

3.4.4. Horizontal wire test

At 2.5 and 6 mg/kg, diazepam significantly decreased the percentage of mice grasping the wire (Fig. 9). In contrast, wogonin, up to a dose of 30 mg/kg, did not elicit a myorelaxant effect.

4. Discussion

Flavonoids, as a class of naturally occurring compounds found in most vascular plants, have been demonstrated by a number of groups to be centrally active, possessing efficacies for a number of receptor systems in the CNS. Ligands upon binding at the BDZ-S on the $GABA_A$ receptor complex exert pharmacologically and clinically important profiles including anxiolysis, anti-convulsion, muscle-relaxation, and sedation. With our effort in the search for BDZ-S ligands from traditional herbal medicines, we have isolated flavonoids with BDZ-S binding activity from the herb *S. baicalensis* Georgi [18]. In the present study, we show that one such flavonoid, wogonin, is an orally active anxiolytic.

From the radioreceptor binding assay, wogonin displayed moderate affinity for the BDZ-S. Scatchard plot analysis demonstrated that wogonin interacted competitively at 1 and 5 μM. However, at 25 μM, both competitive and non-competitive interactions were illustrated. This is in agreement with the partial inhibition of the GABA-activated current as seen in electrophysiological experiments employing DRG neurons when the BDZ-S antagonist Ro15-1788 was co-applied with 30 μM wogonin.

In the electrophysiological studies, wogonin demonstrated a positive allosteric modulatory effect by enhancing the GABA-stimulated current. The potentiation of 30 μM wogonin in *X. laevis* oocytes expressing recombinant rat $GABA_A$ receptor was partially blocked by the co-application of the BDZ-S antagonist Ro15-1788, suggesting that wogonin acts at least partly through the BZD-S. The positive allosteric modulatory effect of wogonin was abolished in the dual subunit combination of α1β2, demonstrating that wogonin potentiates the GABA-activated current in a γ-subunit-dependent manner (Fig. 4), characteristic of BDZ-S ligands.

Notably, in the saturation experiment, a non-competitive interaction of wogonin with flunitrazepam was observed at the highest concentration employed (Fig. 1). Moreover, in the electrophysiological studies, the wogonin-potentiated current was inhibited only partially by Ro15-1788 (Fig. 5). Both observations suggested that wogonin might have

interacted with the receptor in a way not identical to that of conventional BDZs. Nevertheless, the finding that the wogonin-potentiated current was γ-subunit dependent (Fig. 4), and that wogonin in its own right did not induce any current, along with the fact that wogonin competed specifically with BDZs in binding assays, all support the hypothesis that the interaction of wogonin with the receptor resembles, at least in part, the interaction with the conventional BDZ-S. However, it would be of interest for further investigations to unravel any possible differences between wogonin and BDZs in the mechanism(s) of interaction with the receptor complex, and how these differences may associate with their respective pharmacological profiles.

At present, these *in vitro* data indicate positive modulatory efficacies of wogonin for the $GABA_A$ receptor via interaction with the BDZ-S. This compound was studied further for its pharmacological profiles employing the locomotor activity test, the elevated-plus maze, the holeboard, and the horizontal wire tests, all typical of *in vivo* evaluation of BDZs.

In the behavioral experiments, wogonin did not alter the locomotor activity while it significantly increased the exploratory (head-dipping) activity and exerted no sedative effect at the chosen dosage regimen as shown in the holeboard test. As for the diazepam control group, locomotor activity was not altered in diazepam-treated mice, although a low dose of diazepam was reported previously to increase locomotor activity [13]. One possible cause of such a discrepancy is the difference in the mouse strain used in the present and previous studies, which employed ICR and CF1 mice, respectively [13]. Earlier studies reported the absence of an increase in exploratory behavior in the BALB/c strain at any dose level of diazepam [33] and that mouse strains different in spontaneous exploratory behavior also display different responsiveness to diazepam in an anxiety-related exploratory model [34].

The elevated plus-maze is considered to be an etiologically valid animal model of anxiety because it uses natural stimuli (fear of a novel, brightly-lit open space and fear of balancing on a relatively narrow, raised platform) that can induce anxiety in humans [35]. Here, wogonin elicited anxiolysis as observed in the selective increase in the number of entries and time spent in open arms. The fact that Ro15-1788 completely abolished the anxiolytic effect of wogonin clearly indicated that wogonin exerted its effect via the BDZ-S (Fig. 8). That the increase in total arm entries was due to an increase in open arm entries rather than closed arm entries correlates well with the previous finding [36] that closed arm entries were correlated with locomotor activity, which also indicated the exclusive stress-alleviating effect of wogonin. It is shown that wogonin at 3.75–30 mg/kg did not alter locomotor activity (Fig. 6).

In the horizontal wire test, no significant myorelaxant effect was observed after oral administration of wogonin (3.75 to 30 mg/kg). In contrast, diazepam (2.5 and 6 mg/kg) produced a clear-cut myorelaxant effect (Fig. 9).

In summary, wogonin has been shown to be centrally active via oral administration. Its anxiolytic effect is exerted through interaction at the BDZ-S. A dose producing such an effect was not accompanied by sedative and myorelaxant actions. The non-competitive interaction at the $GABA_A$ receptor complex observed in the binding assay with a high concentration of wogonin (25 μM) should have a minimal effect in the *in vivo* pharmacological tests, as this concentration of wogonin, required for the non-competitive interaction, is unlikely to be achieved in animals and is rarely obtained *in vivo* in the brain.

Modification of side chains on the flavone molecule has been shown to be selective for a number of receptor systems including the opiate receptor [37] and the BDZ-S. The present study adds wogonin to the list of CNS active flavone derivatives and is of great value to the construction of structure–activity relationships among the class of compounds with affinity for the BDZ-S. An investigation into the other *in vivo* efficacies of wogonin, including ethanol potentiation and tolerance, will be the subject of further studies.

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References

[1] Wang Q, Han YF, Xue H. Central benzodiazepine binding site ligands. CNS Drug Rev 1999;5:125–44.
[2] Gardner CR, Tully WR, Hedgecock CJR. The rapidly expanding range of neuronal benzodiazepine receptor ligands. Prog Neurobiol 1993; 40:1–61.
[3] Zhu YP. Chinese Materia Medica: chemistry, pharmacology, and applications. The Netherlands: Harward Academic, 1998.
[4] Kubo M, Kimura Y, Odani T, Tani T, Namba K. Studies on Scutellariae radix. Part II: the antibacterial substance. Planta Med 1981;43: 194–201.
[5] Kimuya Y, Kubo M, Tani T, Arichi S, Okuda H. Studies on Scutellariae Radix. IV. Effects on lipid peroxidation in rat liver. Chem Pharm Bull (Tokyo) 1981;29:2610–7.
[6] Harborne JB. The flavonoids, advances in research since 1986. London: Chapman & Hall, 1994.
[7] Lin CC, Shieh DE. The anti-inflammatory activity of *Scutellaria rivularis* extracts and its active components, baicalin, baicalein and wogonin. Am J Chin Med 1996;24:31–6.
[8] Barnard L, Smee F, Huffman JH, Meyerson LH, Sidwell RW. Antiherpes virus activity of 59-303, a novel plant flavonoid. Chemotherapy 1993;39:203–11.
[9] Oyama Y, Fuchs PA, Katayama N, Noda K. Myricetin and quercetin, the flavonoid constituents of *Ginkgo biloba* extract, greatly reduce

oxidative metabolism in both resting and Ca^{2+}-loaded brain neurons. Brain Res 1994;635:125–9.
[10] Miksicek RJ. Commonly occurring plant flavonoids have estrogenic activity. Mol Pharmacol 1993;44:37–43.
[11] Ferriola PC, Coy V, Middleton E. Protein kinase C inhibition by flavonoids: kinetic mechanisms and structure–activity relationships. Biochem Pharmacol 1989;38:345–54.
[12] Cushman M, Nagarathnam D, Burg DL, Geahlen RL. Synthesis and protein-tyrosine kinase inhibitory activities of flavonoid analogues. J Med Chem 1991;34:798–806.
[13] Wolfman C, Viola H, Paladini A, Dajas F, Medina JH. Possible anxiolytic effects of chrysin, a central benzodiazepine receptor ligand isolated from *Passiflora coerulea*. Pharmacol Biochem Behav 1994; 47:1–4.
[14] Salgueiro JB, Ardenghi P, Dias M, Ferreira MBC, Izquierdo I, Medina JH. Anxiolytic natural and synthetic flavonoid ligands of the central benzodiazepine receptor have no effect on memory tasks in rat. Pharmacol Biochem Behav 1997;58:887–91.
[15] Paladini AC, Marder M, Viola H, Wolfman C, Wasowski C, Medina JH. Flavonoids and central nervous system: from forgotten factors to potent anxiolytic compounds. J Pharm Pharmacol 1999;51:519–26.
[16] Schacht U, Baecker G. Effects of clobazam in benzodiazepine-receptor binding assay. Drug Dev Res Suppl 1982;1:83–93.
[17] Vogel HG, Vogel WH. Drug discovery and evaluation: pharmacology assay. New York: Springer, 1997.
[18] Hui KM, Wang XH, Xue H. Interaction of flavones from the roots of *Scutellaria baicalensis* with the benzodiazepine site. Planta Med 2000;66:91–3.
[19] Hu HZ, Li ZW. Modulation by adenosine of GABA-activated current in rat dorsal root ganglion neurons. J Physiol (Lond) 1997;501:67–75.
[20] Hu HZ, Li ZW, Si JQ. Evidence for the existence of substance P autoreceptor in the membrane of rat dorsal root ganglion neurons. Neuroscience 1997;77:535–41.
[21] Sigel E. Properties of single sodium channels translated by *Xenopus* oocytes after injection with messenger ribonucleic acid. J Physiol (Lond) 1987;386:73–90.
[22] Sigel E, Baur R, Trube G, Möhler H, Malherbe P. The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. Neuron 1990;5:703–11.
[23] File SE, Pellow S. The effects of triazolobenzodiazepines in two animal tests of anxiety and in the holeboard. Br J Pharmacol 1985; 86:729–35.
[24] Nolan NA, Parkes MW. The effects of benzodiazepines on the behavior of mice on a hole-board. Psychopharmacologia 1973;29: 277–86.
[25] File SE, Wardill AG. Validity of head-dipping as a measure of exploration in a modified hole-board. Psychopharmacologia 1975; 44:53–9.
[26] File SE, Pellow S. Intrinsic actions of the benzodiazepine receptor antagonist Ro 15-1788. Psychopharmacology (Berl) 1986;88:1–11.
[27] Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 1985;14:149–67.
[28] Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 1987;92:180–5.
[29] Bonetti EP, Pieri L, Cumin R, Schaffner R, Pieri M, Gamzu ER, Muller RKM, Haefely W. Benzodiazepine antagonist Ro 15-1788: neurological and behavioral effects. Psychopharmacology (Berl) 1982;78:8–18.
[30] Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenman H, Schoefield PR, Seeburg PH. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 1989;338:582–5.
[31] Günther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, Crestani F, Aguzzi A, Arigoni M, Lang Y, Bluethmann H, Mohler H, Lüscher B. Benzodiazepine insensitive mice generated by targeted disruption of the γ_2 subunit gene of γ-aminobutyric acid type A receptors. Proc Natl Acad Sci USA 1995;92:7749–53.
[32] File SE. What can be learned from the effects of benzodiazepines on exploratory behavior? Neurosci Biobehav Rev 1985;9:45–54.
[33] Robertson HA. Benzodiazepine receptors in ''emotional'' and ''non-emotional'' mice: comparison of four strains. Eur J Pharmacol 1980;56:163–252.
[34] Crawley JN, Davis LG. Baseline exploratory activity predicts anxiolytic responsiveness to diazepam in five mouse strains. Brain Res Bull 1982;8:609–12.
[35] Dawson GR, Tricklebank MD. Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol Sci 1995;16:33–6.
[36] Jardim MC, Nogueira RL, Graeff FG, Nunes-de-Souza RL. Evaluation of the elevated T-maze as an animal model of anxiety in the mouse. Brain Res Bull 1999;48:407–11.
[37] Thirugnanasambantham P, Viswanathan S, Ramaswamy S, Krishnamurty V, Mythirayee C, Kameswaran L. Analgesic activity of certain flavone derivatives: a structure–activity study. Clin Exp Pharmacol Physiol 1992;20:59–63.