

5,7-Dihydroxy-6-methoxyflavone, a benzodiazepine site ligand isolated from *Scutellaria baicalensis* Georgi, with selective antagonistic properties

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Received 23 December 2002; accepted 21 February 2003

Abstract

As part of an effort to identify naturally occurring GABA_A receptor benzodiazepine binding site (BDS) ligands from traditional medicinal herbs, we previously reported that flavonoid derivatives isolated from *Scutellaria baicalensis* (*S. baicalensis*) Georgi exhibited significant affinities for the BDS. The present study describes the characterization of 5,7-dihydroxy-6-methoxyflavone (oroxylin A), one of the major components of the herbal extract. Oroxylin A inhibited [³H]flunitrazepam binding to rat cerebral cortical membrane with a IC_{50} value of $1.09 \pm 0.07 \mu M$. A GABA ratio of 1.09 ± 0.04 suggests that oroxylin A interacts as an antagonist at the recognition site. In neuropharmacological studies, oral administration of oroxylin A (3.75 – 60 mg kg^{-1}) did not result in significant changes in animal models routinely employed for benzodiazepine (BD) evaluation. However, oroxylin A selectively abolished the anxiolytic, myorelaxant and motor incoordination, but not the sedative and anticonvulsant effects elicited by diazepam, a BDS agonist. These results add oroxylin A to the list of CNS active flavonoids, and as the first naturally occurring member endowed with selective antagonistic actions *via* the BDS. © 2003 Elsevier Science Inc. All rights reserved.

Keywords: Flavonoids; Benzodiazepine binding site; Anxiolytics; Pharmacology; Naturally-occurring; *Scutellaria baicalensis* Georgi

1. Introduction

BDs have widely been used as potent anxiolytics, sedative, hypnotics and anticonvulsants. Their recognition site, BDS, is one of the multiple binding sites on the GABA_A receptor, and serves as a regulatory element for the inhibitory GABA_A receptor function.

Despite the usefulness of conventional BD as potent anxiolytics, the accompanying compromising effects, including sedation, myorelaxation and memory loss following chronic administration, has initiated a search for alternatives devoid of these untoward actions. Since the discovery of amentoflavone, which manifested high-affinity for the BDS [1], much research has been devoted to the quest for alternative BDS ligands from natural sources. Chrysin was

amongst the first member of the class of compound to demonstrate CNS activities *via* the BDS [2]. To date, a list of both natural and synthetic flavonoids have been shown to exert BDS-mediated pharmacological actions with efficacies resembling to that of full agonists [3], partial agonists [4], and antagonists [5,6].

These ligands modulate in both directions and in varying amplitudes the binding of GABA to its receptor. It was observed that the presence of GABA enhanced the binding of a BDS agonist, reduced the binding of an inverse agonist, and exerted negligible effect on the binding of a BDS antagonist. The GABA shift experiment based on these allosteric modulatory properties provide a useful assay for correlating the *in vitro* behavior of a BDS ligand with its potential pharmacological profile [7].

Antagonists at the BDS are useful in the treatment of BD overdose and hepatic encephalopathy [8–10]. Ro15-1788, one of the most investigated synthetic antagonists, is the only prescribed drug amongst this class of compounds. However, due to the anxiogenic effects accompanying its administration, the development of novel antagonists as

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Abbreviations: GABA, γ -aminobutyric acid; BDS, benzodiazepine binding site; BD, benzodiazepine; Oroxylin A, 5,7-dihydroxy-6-methoxyflavone.

new pharmacological agents for research and therapeutic applications is much needed.

The root of *S. baicalensis* Georgi is widely employed in traditional Chinese prescriptions. Flavonoids from this medicinal herb have been shown to possess a broad spectrum of antiviral, antioxidant, anti-inflammatory, and antiallergic actions [11–14]. As part of the screening of traditional herbal extracts for BDS activity, we previously reported that several flavonoids isolated from this herb exhibited moderate affinities for the receptor [15]. Behavioral studies demonstrated that wogonin, one of these flavonoids, exerted potent anxiolysis in mice without sedative and myorelaxant actions [16].

The present study describes the characterization of another orally active flavonoid derivative, oroxylin A, isolated from *S. baicalensis* Georgi, with selective antagonistic properties at the BDS.

2. Materials and methods

2.1. Drugs

Radioactive ligands [^3H]flunitrazepam (*N*-methyl- ^3H], 88.0 Ci mmol) and [^3H]Ro15-1788 (*N*-methyl- ^3H], 78.6 Ci mmol $^{-1}$) were obtained from NEN Life Science Products. Diazepam, GABA, Ro15-1788 and picrotoxin were from Sigma. Valium (diazepam) ampoules were from Hoffmann-La Roche. All organic solvents were of analytical grade.

2.2. Isolation and identification of oroxylin A

The roots of *S. baicalensis* Georgi were purchased from traditional Chinese medicine suppliers in Hong Kong as a dried crude herb and was authenticated by Prof. Y.Z. Guo, Faculty of Traditional Chinese Medicine, Shenyang Pharmaceutical University, PR China. A voucher specimen (SB1) is deposited in the Department of Biochemistry, The Hong Kong University of Science and Technology.

Two hundred grams of the herb were extracted three times consecutively each with 4 L of dichloromethane. The resulting extract was dried (6 g) and was subjected to polyamide gel column chromatography. The sample was eluted from the column using successive mixtures of petroleum ether and chloroform in increasing polarity, up to a ratio of 7:1, to give oroxylin A (10 mg; Fig. 1). Identification of oroxylin A was carried out by ESI-MS, ^1H and ^{13}C NMR spectral analysis and spectroscopic data corresponds to that of previously described [17]. Purity of samples was >95%.

2.3. Synaptosomal membranes

Synaptosomal membranes were prepared as described previously [18], from male Sprague–Dawley rats. Briefly, the rats were decapitated and cerebral cortex dissected.

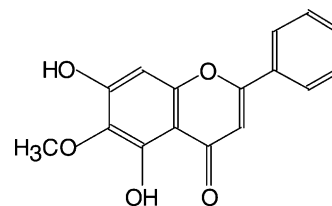


Fig. 1. The chemical structure of oroxylin A. 5,7-Dihydroxy-6-methoxyflavone (MW 284).

Tissues were stored frozen until used. The tissues were thawed and homogenized in 0.32 M sucrose (20 mL g $^{-1}$ of tissue) and centrifuged at 1000 g for 10 min at 4°. The supernatant was centrifuged at 140,000 g for 20 min at 4°. For [^3H]flunitrazepam binding studies, this 140,000 g pellet was resuspended in 2 vol. of 0.05 M Tris–Cl, pH 7.4 and stored frozen in aliquots of 100 μL until use. For [^3H]Ro15-1788 binding studies, the pellet was washed by resuspending in ice-cold distilled-deionized water and centrifuged again at 140,000 g for 20 min at 4°. Washing was repeated two more times in order to remove endogenous GABA from the synaptosomal membrane preparation. After the final centrifugation step, the pellets were resuspended in 0.05 M Tris–Cl (pH 7.4) and stored frozen (–80°) in 1 mL aliquots. On the day of the assay, they were thawed, washed one more time in distilled-deionized water as above and then resuspended for use in assays.

2.4. Radioreceptor binding studies

2.4.1. [^3H]Flunitrazepam binding assay

[^3H]Flunitrazepam binding was measured using filtration methods as described previously [18]. Briefly, aliquots (0.7–0.9 mg mL $^{-1}$) of synaptosomal membranes were resuspended in 0.05 M Tris–Cl, pH 7.4 and incubated with 1 nM [^3H]flunitrazepam at 4° for 60 min in the absence and presence of test drug. Nonspecific binding was measured in the presence of 10 μM diazepam, amounted to <10% of total binding. After incubation, the mixtures were filtered onto Whatman GF/B filters using a Brandel 24-well harvester and rapidly washed with ice-cold 0.05 M Tris–Cl, pH 7.4 buffer for 5 s. Individual filters were incubated overnight with 5 mL scintillation cocktail before measurement of radioactivity in a Wallac Reckbeta 1209 liquid scintillation counter. For saturation analysis, tissue aliquots were incubated with or without test drug and increasing concentrations of [^3H]flunitrazepam (0.1–10 nM). Results were determined by nonlinear regression analysis (sigmoidal curve fitting) of specifically bound radioligand, as % of control vs. log(*M*). K_d and B_{max} from the saturation experiments were determined using Prism 3.0 (Graphpad Software).

2.4.2. [^3H]Ro15-1788 binding assay

The [^3H]Ro15-1788 binding assay was identical to that of [^3H]flunitrazepam binding except extensively washed

membranes were incubated in 1 nM [^3H]Ro15-1788. For GABA shift experiments, test drug was incubated in the presence or absence of 10 μM GABA. GABA ratio was determined by dividing the IC_{50} of test drug in the absence of GABA by the IC_{50} of the test drug in the presence of GABA.

2.5. Behavioral studies

2.5.1. Drug solutions

Orally administered drugs were dissolved into distilled-deionized water by ultrasonication with the addition of one drop of Tween 80 to give an injection volume of 10 mL kg^{-1} . Diazepam (i.p.) and picrotoxin (s.c.) were dissolved in 10% DMSO and physiological saline, respectively.

2.5.2. Animals

Male ICR mice (16–20 g; Animal Care Centre, HKUST) were housed in groups of four to five with food and water *ad lib.* and kept on a 08:00 to 20:00 hr light cycle. Experiments were conducted between 08:30 and 12:00 hr.

2.5.3. Locomotor activity test

To differentiate between potential stimulant or depressant drug effects, the locomotor activity test was performed. The model ZIL-2 apparatus (Beijing Institute of Materia Medica, China) was employed, with dimensions of 60 cm \times 60 cm \times 12 cm, consisting of four circular plastic boxes (diameter 25 cm) each with six evenly spaced infrared photocells. Locomotor activity was counted automatically during a 5-min test period in terms of the number of transitions across the light beams detected by the photocells.

2.5.4. Holeboard test

The mouse holeboard was a wooden box with a 60 cm \times 60 cm square floor and 30 cm high walls. There were four equally spaced holes in the floor, each 3 cm in diameter. Each mouse was placed singly at the center of the board, facing away from the observer, and the number of head-dips and the number of rears made in a test period of 5 min were recorded. After each trial, the floor of the apparatus was wiped and dried to remove traces of the previous path. Due to wide variations in motor activity and head-dipping throughout the day [19], the mice were tested between 09:00 and 12:00 hr.

2.5.5. Elevated plus-maze

The maze had two opposite arms, 25 cm \times 10 cm, crossed with two enclosed arms of the same dimensions but having 20-cm high walls. The arms were connected to a central platform, 5 cm \times 5 cm, giving the apparatus the shape of a plus sign. The maze was kept in a dimly-lit room and elevated 40 cm above ground. Following the holeboard test, mice were placed individually at the center of the maze facing an enclosed arm. Number of entries and time spent in the open arms and closed arms were recorded in a

5-min period. An arm entry was defined by having all four paws inside the arm. At the end of the test, the number of entries and the time spent in the open arms were expressed as a percentage of the total number of entries in the arms and the total time spent in either arm, respectively. It is well established that the proportion of entries into the open arms in the elevated plus-maze is a good indicator of antianxiety action while the number of closed arm entries is related to drug effects on locomotor activities [20,21].

2.5.6. 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 25 cm above floor) with their forepaws, and then released [22]. Each mouse was tested prior to drug administration. Normal animals would actively grasp the wire with their hind limbs. Only these mice successful in grasping the wire with their hind limbs were scored. A myorelaxant drug impairs the ability of the mice to grasp the wire, and muscle relaxation is commonly associated with sedation.

2.5.7. Rotarod test

The rotarod test employed a custom-built apparatus consisting of an elevated cylinder (diameter 2.5 cm) 0.5 m above ground, with a textured surface that rotated at 10 rpm. After administration of the test substance, each mouse was tested for its ability to stay on the rotarod for a duration of 1 min.

2.5.8. Picrotoxin (PTX)-induced seizure test

Seizures were induced by the s.c. injection of picrotoxin (6 mg kg^{-1}). Oroxylin A and diazepam were administered p.o. 1 hr and i.p. 15 min prior to picrotoxin, respectively. The following parameters were recorded: (i) number of mice with convulsions (%), (ii) latency to the onset of the first jerk or clonus after injection, (iii) death rate, and (iv) latency to death. A clonic seizure was defined as forelimb clonus of >3 -s duration. Latencies represent the time, in minutes, between PTX injection and the observed parameters.

2.5.9. Data analysis

Behavioral data obtained from each response were subjected to one-way ANOVA, and multiple group comparisons were made by Dunnett's *t*-test for only those responses which yielded significant treatment effects in the ANOVA test. Fisher's exact test and Bonferroni's multiple comparison test were used when necessary. Significance was reported starting at the 0.05 level.

3. Results

3.1. Radioreceptor binding assays

Oroxylin A inhibited [^3H]flunitrazepam binding to the rat cerebral cortex membranes with a IC_{50} of $1.09 \pm 0.07 \mu\text{M}$

Table 1

Determination of IC_{50} and GABA ratio of oroxylin A by [3H]flunitrazepam binding assay and GABA shift experiment

Drug	Inhibition of [3H]flunitrazepam (μM) IC_{50}	GABA ratio
Oroxylin A	1.09 ± 0.07	1.09 ± 0.04
Diazepam	0.008 ± 0.0002	2.24 ± 0.24
Flumazenil	0.006 ± 0.0003	0.91 ± 0.01

IC_{50} values were estimated by displacement of [3H]flunitrazepam binding to synaptosomal membrane protein extracted from cerebral cortex of Sprague–Dawley rat (approximately 250 g). GABA ratios are determined in presence of 10 μM GABA with extensively washed synaptosomal membrane preparation from rat cerebral cortex employing 1 nM [3H]Ro15-1788. Data represent mean \pm SEM, $N = 3$.

and manifested a GABA shift of 1.09 ± 0.04 (Table 1), suggesting that this flavonoid might interact as an antagonist at the BDS. Scatchard plot analysis of [3H]flunitrazepam binding showed that oroxylin A at 1 and 5 μM decreased the binding affinity (K_d) with no change in the number of binding sites (B_{max}), indicative of competitive inhibition of [3H]flunitrazepam binding at the BDS (Fig. 2). In order to examine the *in vivo* effects of oroxylin A, animal models routinely employed for BDS ligand evaluations were performed.

3.2. Behavioral studies

3.2.1. Locomotor activity test

Oroxylin A-treated mice did not exhibit any changes in the number of transitions across photocells in comparison to vehicle. Likewise, diazepam at 1 mg kg^{-1} did not result in significant changes in mice in this regard (Table 2).

3.2.2. Holeboard test

No significant difference was observed in the number of head-dips made and the number of rears in oroxylin A (3.75–60 mg kg^{-1}) and diazepam-treated (1 mg kg^{-1}) mice in comparison to control, suggesting that no sedative

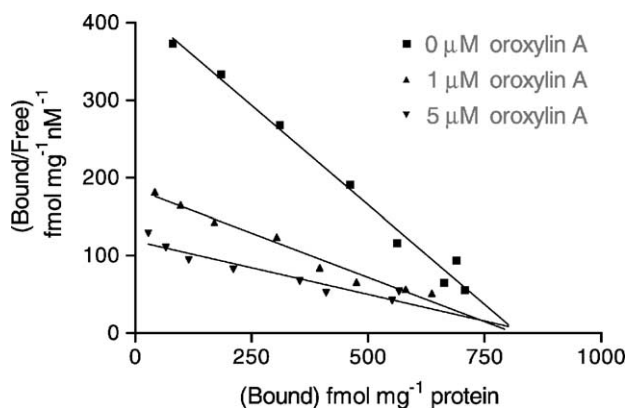


Fig. 2. A representative Scatchard plot of [3H]flunitrazepam (0.1–10 nM) binding to rat cerebral cortex benzodiazepine receptor *in vitro* in the absence and presence of oroxylin A (1 and 5 μM). Data represents mean of three individual experiments done in triplicates.

Table 2

Assessment of locomotor activity of oroxylin A-treated mice

Drug (mg kg^{-1})	Locomotor activity counts
Vehicle	238.6 ± 25.6
DZ (1)	286.3 ± 24.6
Oroxylin A (3.75)	292.6 ± 29.3
Oroxylin A (7.5)	281.1 ± 25.4
Oroxylin A (15)	286.8 ± 31.2
Oroxylin A (30)	266.9 ± 16.9
Oroxylin A (60)	285.0 ± 21.3

Locomotor activity counts (mean \pm SEM) in mice during a 5-min period 1 hr after oral administration of vehicle (Veh, ddH₂O), diazepam (DZ, 1 mg kg^{-1}) or oroxylin A (3.75–60 mg kg^{-1}), $N = 8$ –12 mice per group.

Table 3

Exploratory behavior of mice treated with oroxylin A in the holeboard test

Drug (mg kg^{-1})	Number of head-dips	Number of rears
Vehicle	20.23 ± 3.36	26.00 ± 2.99
DZ (1)	22.78 ± 1.77	24.22 ± 1.15
Oroxylin A (3.75)	22.58 ± 3.62	22.08 ± 4.45
Oroxylin A (7.5)	24.44 ± 5.14	22.11 ± 4.21
Oroxylin A (15)	19.50 ± 3.18	20.63 ± 4.24
Oroxylin A (30)	12.33 ± 4.09	23.00 ± 2.28
Oroxylin A (60)	19.30 ± 3.27	26.00 ± 3.37

Number of head-dips and rears made in mice orally administered oroxylin A (3.75, 7.5, 15, 30, 60 mg kg^{-1}), diazepam (DZ, 1 mg kg^{-1}) or vehicle (water). Data represent mean \pm SEM, $N = 16$.

effect was elicited at these dosages (Table 3). On the other hand, at 3 mg kg^{-1} i.p. diazepam, there were significantly decreased numbers of head-dips and rears. Pretreatment of oroxylin A (15–60 mg kg^{-1}) did not significantly affect the diazepam-induced sedation (3 mg kg^{-1} , i.p.) as measured by the number of head-dips and rears (Table 4).

3.2.3. Elevated plus-maze

Oroxylin A-treated mice (3.75–60 mg kg^{-1}) did not exhibit any significant differences with respect to the percentage of open arms entries, the percentage of time spent in open arms and the number of closed arm entries in comparison to control mice (Fig. 3a). In contrast, diazepam-treated mice (1 mg kg^{-1}) demonstrated significant

Table 4

Assessment of exploratory behavior of oroxylin A-treated mice in the holeboard test

Drug (mg kg^{-1})	Number of head-dips	Number of rears
Vehicle	17.94 ± 2.81	20.94 ± 3.78
DZ (3)	$4.23 \pm 1.33^{**}$	$7.00 \pm 1.38^{**}$
DZ (3) + oroxylin A (15)	$5.13 \pm 1.25^{**}$	$6.88 \pm 3.17^{**}$
DZ (3) + oroxylin A (30)	$10.00 \pm 0.53^{*}$	$5.08 \pm 3.46^{**}$
DZ (3) + oroxylin A (60)	$5.14 \pm 2.28^{**}$	$2.93 \pm 1.16^{**}$

Data represent (mean \pm SEM) the number of head-dips and rears made in mice pretreated with oroxylin A (0, 15, 30, 60 mg kg^{-1} , p.o.) 15 min prior to diazepam (3 mg kg^{-1} , i.p.), or vehicle (10% DMSO, i.p.), $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control, Bonferroni's multiple comparison test after one-way ANOVA, $N = 8$ –12 mice per group.

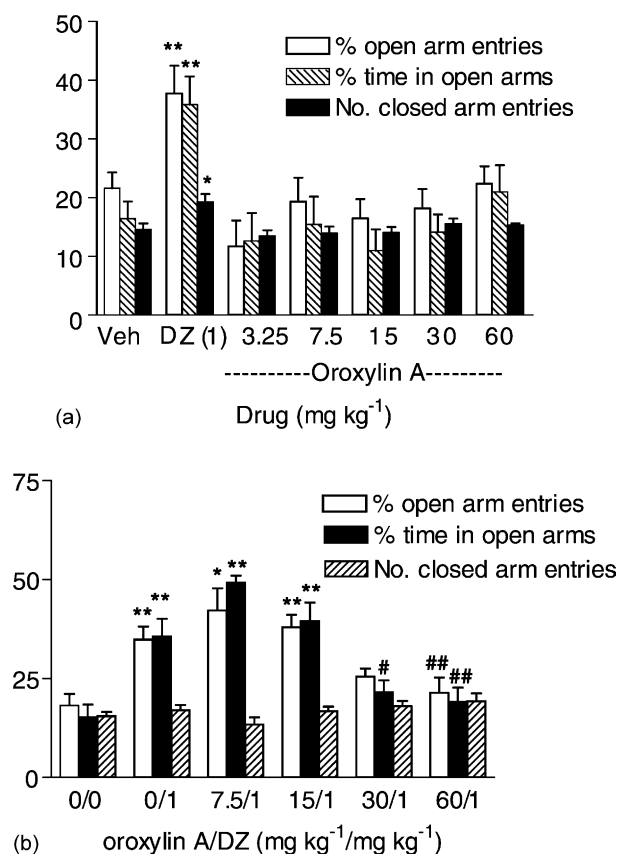


Fig. 3. (a) Assessment of the anxiolytic effect of oroxylin A in the mice elevated plus-maze. Data represent mean \pm SEM the percentage of entries and time spent in open arms, and the number of closed arm entries in mice 1 hr after oral administration of oroxylin A (3.75, 7.5, 15, 30, 60 mg kg⁻¹), diazepam (DZ, 1 mg kg⁻¹) or vehicle (ddH₂O, pH 7.4). * P < 0.05, ** P < 0.001 significantly different from control, Dunnett's t -test after one-way ANOVA, N = 8–12 mice per group. (b) Antagonistic effect of oroxylin A pretreatment in the mice elevated plus-maze. Data represent mean \pm SEM the percentage of entries and time spent in open arms, and the number of closed arm entries in mice co-administered oroxylin A (0, 7.5, 15, 30, 60 mg kg⁻¹, p.o.) and diazepam (DZ, 1 mg kg⁻¹, i.p.), or vehicle (10% DMSO, i.p.), * P < 0.05, ** P < 0.01 significantly different from Veh, # P < 0.05, ## P < 0.01 significantly different from DZ, Bonferroni's multiple comparison test after one-way ANOVA, N = 8–12 mice per group.

anxiolysis as observed in the selective increases in both open-arm parameters, with a slight increase in the total number of closed arm entries, thus indicating increased locomotor activity. Pretreatment of oroxylin A (30 and 60 mg kg⁻¹) reversed both diazepam-elicited (1 mg kg⁻¹, i.p.) open-arm parameters to basal levels, demonstrating that the anxiolytic effect of diazepam was abolished in the presence of oroxylin A. Such reversals were not observed at lower doses of oroxylin A (7.5 and 15 mg kg⁻¹; Fig. 3b).

3.2.4. Horizontal wire test

Both oroxylin A (3.75–60 mg kg⁻¹) and diazepam (1 mg kg⁻¹) did not compromise the grasping of wire by mice when compared to control, pointing to a lack of myorelaxation at these dosages (Fig. 4a). Diazepam at 3 mg kg⁻¹, i.p., in contrast, elicited significant myorelaxation in mice as signified by the decrease in the percentage

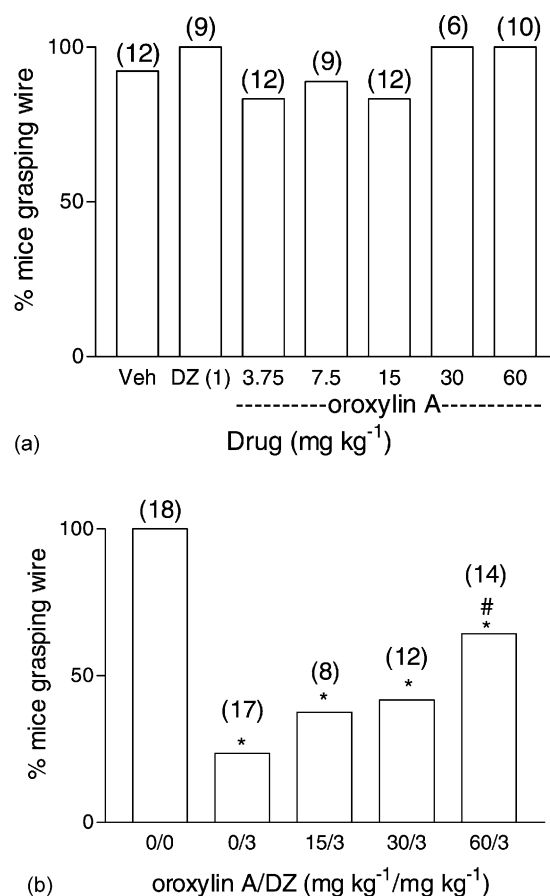


Fig. 4. (a) Performance of mice in the horizontal wire test. Percentage of mice grasping the wire after oral administration of oroxylin A (3.75, 7.5, 15, 30, 60 mg kg⁻¹), diazepam (DZ, 1 mg kg⁻¹) or vehicle (Veh, ddH₂O), number of mice per group in parentheses. (b) Reversal by oroxylin A pretreatment of the diazepam-induced myorelaxation in the horizontal wire test. Percentage of mice grasping the wire after co-administration of oroxylin A (0, 15, 30, 60 mg kg⁻¹, p.o.) and diazepam (DZ, 3 mg kg⁻¹, i.p.), or vehicle (10% DMSO, i.p.); number of mice per group in parentheses. * P < 0.01, significantly different from Veh, # P < 0.05 significantly different from DZ, Fisher's exact test.

of mice grasping the wire. This diazepam-induced myorelaxation was relieved by pretreatment of 60 mg kg⁻¹ of oroxylin A (Fig. 4b).

3.2.5. Rotarod test

Both oroxylin A (3.75–60 mg kg⁻¹) and diazepam (1 mg kg⁻¹) did not compromise the ability of mice to stay on the rotarod, revealing the absence of any motor incoordination effect (Fig. 5a). At 3 mg kg⁻¹ diazepam, the percentage of mice that stayed on the rotarod decreased significantly, but this decrease was reversed by pretreatment of oroxylin A (60 mg kg⁻¹; Fig. 5b).

3.2.6. PTX-induced seizure test

In the picrotoxin-induced seizure test, oroxylin A (3.75–60 mg kg⁻¹) did not alter the onset of the first seizure, death latency, the percentage of convulsant animals or the

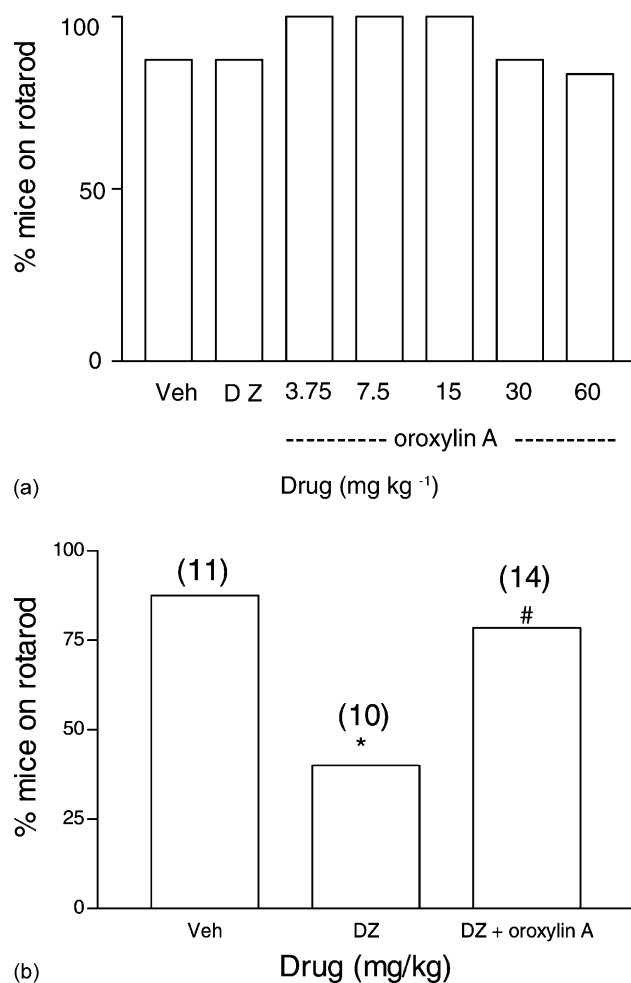


Fig. 5. (a) Performance of mice in the rotarod test. Percentage of mice that stayed on the rotarod 1 hr after oral administration of vehicle (Veh, ddH₂O), diazepam (DZ, 1 mg kg⁻¹) or oroxylin A (3.75–60 mg kg⁻¹), N = 8–12 mice per group. (b). Reversal by oroxylin A pretreatment of the diazepam-induced motor incoordination in the rotarod test. Percentage of mice that stayed on the rotarod after treatment of vehicle (10% DMSO, i.p.), diazepam (3 mg kg⁻¹, i.p.) alone or with oroxylin A (60 mg kg⁻¹, p.o.), **P* < 0.01 significantly different from Veh, #*P* < 0.05 significantly different from DZ, Fisher's exact test, number of mice per group in parentheses.

death rate. However, administration of 3 mg kg⁻¹ diazepam significantly altered the latency of the first seizure in PTX-treated mice, the percentage of convulsant animals, and the death rate. When oroxylin A was administered prior to diazepam treatment (3 mg kg⁻¹), these parameters did not change significantly in comparison to the diazepam-treated mice (Table 5).

4. Discussion

Flavonoids, an important class of naturally occurring compounds found in many vascular plants, have demonstrated CNS activities, interacting with a number of receptor systems in the CNS [23–25]. Ligand binding at the BDS on the GABA_A receptor complex are known to exert such pharmacological actions as anxiolysis, anticonvulsion, muscle relaxation and sedation [26]. Our search for BDS ligands from traditional herbal medicinal sources has led to the identification of several flavonoid derivatives from *S. baicalensis* Georgi with BDS activities [15]. In the present study, an additional flavonoid, oroxylin A, was found to be an orally active BDS ligand with some antagonistic properties.

In radioligand displacement studies employing [³H]flunitrazepam, oroxylin A exhibited moderate affinity for the BDS, and in the saturation experiments where oroxylin A (1 and 5 μM) was incubated with different concentrations of [³H]flunitrazepam, the ability of oroxylin A to modify the *K_d* without significant changes in the *B_{max}* suggests that this flavonoid derivative interacts competitively at the recognition site. A GABA ratio of 1.09 also suggests that oroxylin A behaves in a manner similar to that of a BDS antagonist (Table 1). To characterize the pharmacological profile of this flavonoid and to substantiate the extrapolation made utilizing these *in vitro* assays, a range of behavioral tests were conducted.

In the locomotor activity test, oroxylin A-treated mice did not exhibit any changes in comparison to control,

Table 5
Assessment of anticonvulsant properties of oroxylin A in the PTX-induced seizure model

Drug (mg kg ⁻¹)	Latency to first tonic-clonus seizure (min)	Death latency (min)	% Convulsant animals	Death rate
Vehicle	9.4 ± 1.1	16.1 ± 1.6	100	90
DZ (3)	22.8 ± 1.0*	–	18*	0*
Oroxylin A (3.75)	7.2 ± 0.9	17.8 ± 2.0	100	89
Oroxylin A (7.5)	8.7 ± 0.6	18.0 ± 1.6	100	83
Oroxylin A (15)	9.3 ± 0.5	19.1 ± 1.7	100	100
Oroxylin A (30)	11.1 ± 1.0	17.8 ± 1.2	100	100
Oroxylin A (60)	11.1 ± 1.4	18.6 ± 1.4	100	88
DZ (3) + oroxylin A (60)	22.9 ± 0.9*	–	25*	0*

Values represent (mean ± SEM) latency to the first tonic-clonic seizure, death latency, percentage of convulsant animals and death rate in mice after administration of vehicle, diazepam (DZ, 3 mg kg⁻¹, i.p.), oroxylin A (3.75–60 mg kg⁻¹, p.o.) or diazepam (DZ, 3 mg kg⁻¹, i.p.) and oroxylin A (60 mg kg⁻¹, p.o.). Diazepam and oroxylin A are administered 15 min and 1 hr prior to picrotoxin injections, respectively, **P* < 0.001 significantly different from control, Dunnett's *t*-test after one-way ANOVA or Fisher's exact test, N = 8–12 per group.

indicating that oroxylin A has no stimulant or depressant effects at the test doses (Table 2). In the holeboard test, an assay for potential sedative effects, oroxylin A, likewise, did not induce any change in the numbers of head-dips and rears made (Table 3). This flavonoid also did not give rise to any significant changes in the anxiolytic parameters as measured in the elevated plus-maze or any myorelaxant effects as observed in the horizontal wire test (Figs. 3a and 4a). Moreover, oroxylin A alone did not result in any motor incoordination as assessed in the rotarod test (Fig. 5a) and did not modify the parameters measured in the PTX-induced seizure test (Table 5). These results were entirely consistent with those expected of BDS antagonists.

To test if oroxylin A is capable of antagonizing the effects of the BDS agonist diazepam, it was administered to mice prior to the injection of two different dose levels of diazepam. Diazepam at 1 mg kg⁻¹ brought about evident anxiolytic effect as seen in the increases in the percentage of number of open-arm entries and time spent in open arms. However, these effects were abolished by oroxylin A, thereby revealing the antagonistic effect of this flavonoid *via* the BDS (Fig. 3b). Mice treated with 3 mg kg⁻¹ diazepam manifested sedative and myorelaxant effects in the holeboard test and the horizontal wire test, respectively. In contrast to abolishing the diazepam-induced anxiolysis, pretreatment of oroxylin A failed to block the sedation but partially reversed the myorelaxation caused by BDS agonist (Table 4 and Fig. 4b). The diazepam-elicited motor incoordination on the rotarod was also counteracted by 60 mg kg⁻¹ oroxylin A (Fig. 5b). In the PTX-induced seizure test, 3 mg kg⁻¹ diazepam demonstrated anticonvulsant action as measured in significant differences in the onset of the first seizure, the percentage of convulsant animals, death latency and death rate when compared to control. Oroxylin A (3.75–60 mg kg⁻¹), when administered alone, did not manifest any anticonvulsant properties. Likewise, oroxylin A pretreatment also failed to modify the diazepam-induced anticonvulsion in the chosen dosage regimen (Table 5).

In this study, the GABA shift assay, a useful means to characterize the pharmacological action of BDS ligands, was employed to characterize that of oroxylin A. Oroxylin A exhibited negligible changes in its binding affinity for the BDS in the presence or absence of GABA, a behavior resembling to that of an antagonist, which is in contrast to the expected enhancement by GABA of the binding affinity of a BDS agonist and reduction of that of an inverse agonist. Recent genetic knock-out and knock-in studies in mice, suggest that the sedative, amnesic and part of the anticonvulsant effects of GABA_A receptor ligands may be mediated by the α_1 subunit, and the anxiolytic, myorelaxant, motor impairment and ethanol potentiation actions mediated by the $\alpha_{2,3,5}$ subunits [27–31]. The pharmacological profile of oroxylin A as observed in the present study, with opposition to the diazepam-induced anxiolytic, myorelaxant and motor incoordination effects, but not the

sedative and anticonvulsant effects elicited by this BDS agonist, suggests that oroxylin A manifested antagonistic action mediated by the $\alpha_{2,3,5}$ -containing BDS. Further studies on the binding affinity of oroxylin A for different GABA_A receptor subunits will be needed to clarify its exact subtype selectivity.

In summary, our study demonstrated oroxylin A as the first known instance of a naturally occurring flavonoid possessing antagonistic properties at the BDS of the GABA_A receptors. The study also highlights the pharmacological selectivity of oroxylin A for antagonizing various physiological actions induced by diazepam. Together with earlier findings on both the selectivity and the wide spectrum of efficacies amid the class of compounds for the BDS-mediated pharmacology, our observation is consistent with the notion that flavonoids are capable of exerting a broad spectrum of intrinsic activities at the recognition site, and represents a promising class of compound for the treatment of BDS-associated syndromes. Since the initial search for alternative BDS ligands amongst the flavonoids, a list of successful semi-synthetic analogues has been generated with enhanced affinities for the BDS by the incorporation of different side chains onto the flavone backbone [4,32]. Molecular modeling of flavonoid binding to the BDS pharmacophore has also been carried out in attempts to further increase the affinity of these derivatives [33–35], however, much is awaited for the investigation of subunit selectivity manifested by these compounds. The structure–function relationships of flavonoids deserve to be thoroughly and systematically investigated.

Acknowledgments

This study was supported by the Hong Kong Government Innovation Technology Fund (Project No. UIM40). The authors thank Hongyan Wong for phytochemistry assistance.

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