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Naturally occurring 2′-hydroxyl-substituted flavonoids as high-affinity benzodiazepine site ligands

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Abstract

Screening of traditional medicines has proven invaluable to drug development and discovery. Utilizing activity-guided purification, we previously reported the isolation of a list of flavonoids from the medicinal herb *Scutellaria baicalensis* Georgi, one of which manifested an affinity for the benzodiazepine receptor (BDZR) comparable to that of the synthetic anxiolytic diazepam ($K_i = 6.4$ nM). In the present study, this high-affinity, naturally occurring flavonoid derivative, 5,7,2′-trihydroxy-6,8-dimethoxyflavone (K36), was chosen for further functional and behavioral characterization. K36 inhibited [^{3}H]flunitrazepam binding to native BDZR with a K_i value of 6.05 nM. In electrophysiological experiments K36 potentiated currents mediated by rat recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in *Xenopus* oocytes. This potentiation was characterized by a threshold (1 nM) and half-maximal stimulation (24 nM) similar to diazepam. This enhancement was demonstrated to act via the BDZR, since co-application of 1 μM of the BDZR antagonist Ro15-1788 reversed the potentiation. Oral administration of K36 produced significant BDZR-mediated anxiolysis in the mice elevated plus-maze, which was abolished upon co-administration of Ro15-1788. Sedation, myorelaxation and motor incoordination were not observed in the chosen dosage regimen. Structure–activity relationships utilizing synthetic flavonoids with different 2′ substituents on the flavone backbone supported that 2′-hydroxyl-substitution is a critical moiety on flavonoids with regard to BDZR affinities. These results further underlined the potential of flavonoids as therapeutics for the treatment of BDZR-associated syndromes.

Keywords: Benzodiazepines; Benzodiazepine receptor; Flavonoids; Structure–activity relationship; *Scutellaria baicalensis* Georgi; Anxiolytics

1. Introduction

GABA, the major inhibitory neurotransmitter in the central nervous system (CNS), is essential for the overall balance between neuronal excitation and inhibition through interaction with specific membrane receptors. Being a member of the fast-acting transmitter-gated ion channel superfamily, $GABA_A$ receptors share structural and functional similarities with the nicotinic acetylcholine receptor, glycine receptor and 5-HT_3 receptor, which include a pentameric pseudosymmetrical *trans*-membrane structure with a central ion pore. $GABA_A$ receptors, mainly located postsynaptically, mediate most of the inhibitory synaptic transmission in the CNS and serve as the target for many important neuroactive drugs including BDZs, barbiturates, steroids, general anesthetics and possibly alcohol [1].

The allosteric BDZR has been proposed to reside at the interface between α and γ subunits [2–4]. Upon ligand recognition, the affinity of the GABA binding site for its substrate is modified to result in the differential regulation of chloride flux through the ion channel situated at the center of the assembly. Pharmacologically, BDZs are potent anxiolytic, sedative, muscle relaxant and anticonvulsant drugs. However, untoward effects, including ethanol potentiation and amnesia, that accompany treatment with BDZs, have stimulated research into alternatives to conventional BDZs.

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Abbreviations: GABA, γ-aminobutyric acid; BDZ, benzodiazepine; BDZR, benzodiazepine receptor; K36, 5,7,2′-trihydroxy-6,8-dimethoxyflavone; SAR, structure–activity relationship.

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Apart from the putative differential pharmacology mediated by different BDZR subtypes, BDZR ligands are also known to modulate the $GABA_A$ receptor function in both directions and in different amplitudes. In this aspect, BDZR agonists and inverse agonists have been shown to increase and decrease, respectively, the affinity of GABA for its receptor, while an antagonist at the recognition site exerts negligible effect. That these ligands possess the capacity to regulate the physiological response in a spectrum of efficacies has raised hope for the development of partial allosteric modulators at the recognition site as potential therapeutics devoid of side-effects [5,6]. In particular, BDZR partial agonists are postulated to exert potent anxiolysis without sedative and myorelaxant effects, both of which are considered to be elicited when a majority of receptor–drug complexes are activated [7,8]. Several lines of neuropharmacological evidences are supportive of this hypothesis, showing that BDZR partial agonists potentiate the GABA-activated current in a sub-maximal manner in comparison to full agonists, and that the therapeutic window separating anxiolysis and other pharmacological effects of these ligands are much wider than conventional BDZs [9].

In recent years, drug screening from traditional medicinal herbs has attracted much attention in the hope to identify novel therapeutics for the treatment of various diseases. The discovery of chrysin, one of the first flavonoids shown to possess *in vivo* activity through interaction with the BDZR [10], marked the search for such natural anxiolytics. A number of flavonoids have been found to possess partial allosteric modulatory action at the $GABA_A$ receptor complex, and play a role in the modulation of anxiety [11–13]. They therefore constitute a promising class of naturally occurring compounds for the treatment of anxiety.

Naturally occurring flavonoids often bind to the BDZR with only moderate affinities. However, through synthesis of chemical libraries and molecular modeling of the flavonoid binding to the BDZR pharmacophore, several groups have been able to generate synthetic derivatives with higher affinities for the BDZR [14–18].

As part of our effort in identifying potent BDZR ligands from natural resources, a range of flavonoids was isolated from the medicinal herb *Scutellaria baicalensis* Georgi guided by radioreceptor binding assay for the BDZR [19,20]. One of these naturally occurring flavonoids, K36, exhibited the highest affinity for the BDZR, comparable to that of diazepam. In the present study, K36 was further investigated with respect to its functional and behavioral properties. Using electrophysiological techniques, its role as a $GABA_A$ receptor function modulator was examined. Neuropharmacological studies employing animal models routinely adopted for BDZ evaluations were carried out. In addition, affinities of a series of flavonoid derivatives for the BDZR were examined in order to better understand the structural basis of the high-affinity of K36 for the BDZR.

2. Methods

2.1. Chemicals

Radioactive [^{3}H]flunitrazepam (*N*-methyl-[^{3}H], 88.0 Ci $mmol^{-1}$) was purchased from Amersham. [^{3}H]Ro15-1788 (*N*-methyl-[^{3}H], 78.6 Ci $mmol^{-1}$) was from NEN Life Science Products. Diazepam was from Sigma Chemical Co. Anexate (Ro15-1788, 0.1 mg mL^{-1} ampoules) was purchased from Hoffmann-La Roche Ltd. 5,7,2′-Trihydroxy-6,8-dimethoxyflavone, 5,7,2′-trihydroxy-6-methoxyflavone, 5,7-dihydroxy-6-methoxyflavone, 5,7-dihydroxy-6,8-dimethoxyflavone, 5,7-dihydroxy-8-methoxyflavone and 5,6,7-trihydroxyflavone were purified from the Chinese herb *Scutellaria baicalensis* Georgi as described previously [19,20]. Other flavonoids were from Indofine Chemical Company. All other materials were of analytical grade from standard commercial sources.

2.2. Radioreceptor binding studies

2.2.1. [^{3}H]Flunitrazepam binding assay

Synaptosomal membranes were prepared from rat forebrains [21] and incubation assay conditions were as previously described [22], employing 1 nM [^{3}H]flunitra- 5 min at 4°. Non-specific binding was determined in the presence of 10 μM diazepam constituting <10% of total binding. Saturation experiments were performed using 0.1–150 nM [^{3}H]flunitrazepam.

2.2.2. GABA shift experiment

The GABA shift experiment was identical to the [^{3}H]-flunitrazepam binding assay except that synaptosomal membrane preparation was washed for three additional times in ice-cold double-distilled water, and incubated in 1 nM [^{3}H]Ro15-1788. For calculation of GABA ratios, IC_{50} of test compound in the absence of added GABA was divided by IC_{50} of the compound in the presence of 10 μM GABA.

Results were determined by non-linear regression analysis (sigmoidal curve fitting) of specifically bound radioligand, % of control, vs. semi-log concentration (M). K_i values were calculated using the equation $K_i = IC_{50}/[1 + ([^3H]/K_d)]$, where K_d is the dissociation constant of [^{3}H]flunitrazepam at the high-affinity site. K_d and B_{max} from the saturation experiment were determined using Prism 3.0 (GraphPad Software).

2.3. Electrophysiological studies

Xenopus laevis oocytes were prepared, injected, defolliculated and currents recorded as described [23,24]. Briefly, oocytes were injected with 50 nL of capped, polyadenylated cRNA dissolved in 5 mM KCl–HEPES (pH 6.8). This solution contained the transcripts coding for the different rat $GABA_A$ subunits at concentrations of 40, 40 and 200 nM

for α_1, β_2 and γ_2, respectively. This combination was chosen for initial experimentation as they represent the major subtype in the mammalian brain. 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 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 and where indicated K36 were applied for 30–45 s 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 a xy-recorder or digitized using a MacLab/200 (AD Instruments).

2.4. Behavioral characterization

2.4.1. Animals

Male ICR mice (16–20 g; Animal Care Centre, HKUST) were housed in groups of four to five with food and water *ad libitum* and kept on a 08:00–20:00 hr light cycle. Experiments were conducted between 08:30 and 12:00 hr. Time between dosing and experimental sessions was based on pilot studies. The animals were test-naïve and was scored only once in each test.

2.4.2. Drug solutions

K36 and diazepam were dissolved into deionized-distilled water by ultrasonication with the addition of a drop of Tween 80 to give an injection volume of 10 mL kg^{-1}. Anexate (Ro15-1788, 0.1 mg mL^{-1} ampoules) was diluted and administered i.p. 15 min prior to testing.

2.4.3. Locomotor activity test

The ZIL-2 apparatus (Beijing Institute of Materia Medica) was employed, with dimensions of 60 cm × 60 cm × 12 cm, consisted of four circular plastic boxes (diameter: 25 cm) each with six evenly spaced infrared photocells. The number of transitions across the light beams detected by the photocells was counted automatically during a 5-min period.

2.4.4. Hole-board test

The hole-board apparatus is a wooden box (60 cm × 60 cm × 30 cm) with four holes (3 cm in diameter) equally spaced in the floor. The entire apparatus was painted white. Mice were placed individually at the center of the floor facing away from the observer, and the number of head-dips, the time spent head-dipping and the number of rears were counted in a 5-min period [25]. An increase in the number and time spent headdipping, and the number of rears reflect a greater exploratory activity. A decrease of these three parameters as compared to control reveals a sedative behavior [26,27].

2.4.5. Elevated plus-maze test

The maze had two opposite arms, 25 cm × 10 cm, crossed with two enclosed arms of the same dimension but having 20 cm high walls. The arms were connected with a central platform, 5 cm × 5 cm, giving the apparatus shape of a plus sign. The maze was kept in a dimly-lit room and elevated 40 cm above ground. At the start of the experiment, each mouse was 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. Total number of closed arm entries provided a measure of general activity and a selective increase in the parameters corresponding to open arms reveals an anxiolytic effect [28,29]. In a separate experiment where mice were subjected to the co-administration of K36 and Ro15-1788, mice were orally administered K36 (4 mg kg^{-1}) and received i.p. injection of Ro15-1788 (1.25 mg kg^{-1}) 20 and 15 min prior to testing, respectively.

2.4.6. 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 ground) with their forepaws, and released [30]. Each mouse was tested prior to drug administration. Normal animals would actively grasp the wire with their hind limbs. Only mice successful in grasping the wire with their hind limbs are tested and scored. A myorelaxant drug impairs the mice to grasp the wire and muscle relaxation is commonly associated with sedation.

2.4.7. Rotarod test

The rotarod test utilized a custom-built apparatus consisting of an elevated cylinder (diameter: 2.5 cm) placed 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 period of 1 min. Mice that failed to stay on the rotarod during the test interval was scored as an index of motor incoordination.

2.4.8. Statistical analysis

Behavioral data obtained from each response measures were subjected to one-way ANOVA, and multiple group

comparisons were made by Dunnett's *t*-test for those responses that yielded significant treatment effects in the ANOVA test. Chi's square test was used when necessary.

3. Results

3.1. Radioreceptor binding assays

3.1.1. Determination of K_d and B_{max} of [^{3}H]flunitrazepam binding to the BDZR

From representative Rosenthal (Scatchard) plot analysis of [^{3}H]flunitrazepam saturation binding experiments, the dissociation constant (K_d) and the maximal binding density (B_{max}) of the high and low affinity binding sites were determined to be 1.23 ± 0.08 nM and 2.17 ± 0.17 pmol mg^{-1}, and 18.90 ± 1.63 nM and 2.30 ± 0.16 pmol mg^{-1} membrane preparation, respectively. From competitive binding experiments carried out in the presence of 1 nM of [^{3}H]flunitrazepam, K_i values were calculated from IC_{50} of test compounds using the high-affinity K_d value 1.23 nM.

3.1.2. BDZR affinities and GABA ratios of flavonoids

BDZR affinities of six flavonoids isolated from *Scutellaria baicalensis* Georgi, and together with that of the commercially available derivatives are shown in Table 1, ranging from low nanomolar to high micromolar concentrations. GABA ratios were estimated for these flavonoids and the values represented a spectrum of efficacies, from that of a antagonist to that of a partial agonist. Of the different flavonoids obtained from this medicinal herb, the monoflavonoid, K36 (Fig. 1), was most effective in inhibiting [^{3}H]flunitrazepam binding to the rat cerebral cortex, with a K_i of 6.05 ± 0.63 nM (Fig. 2). A GABA ratio of 1.20 ± 0.07 suggests that this flavonoid interacts with the BDZ in the mode of a partial agonist when compared to the full agonist diazepam (GABA ratio of 2.24 ± 0.24). K36, being a naturally occurring monoflavonoid with the highest affinity for the BDZ reported to date, was subjected to

Table 1
K_i and GABA ratios of flavonoids used in this study

Compound	Name	Inhibition of [^{3}H]flunitrazepam (μM)	
		K_i	GABA ratio
1	5,7,2′-Trihydroxy-6,8-dimethoxyflavone (K36)	0.0061 ± 0.0001	1.20 ± 0.07
2	2′-Hydroxy-β-naphthoflavone	0.027 ± 0.003	1.06 ± 0.05
3	6,2′-Dihydroxyflavone	0.034 ± 0.001	0.89 ± 0.13
4	5,7,2′-Trihydroxy-6-methoxyflavone	0.038 ± 0.005	1.32 ± 0.05
5	5,7,2′-Trihydroxyflavone	0.075 ± 0.004	0.90 ± 0.10
6	2′-Hydroxyflavone	0.21 ± 0.10	1.17 ± 0.10
7	5,7-Dihydroxy-6,8-dimethoxyflavone	0.20 ± 0.05	ND
8	7,2′-Dihydroxyflavone	0.56 ± 0.07	0.99 ± 0.09
9	5,7-Dihydroxy-6-methoxyflavone (Oroxylin A)	0.89 ± 0.06	1.09 ± 0.04
10	5,7-Dihydroxyflavone	0.64 ± 0.26	0.90 ± 0.11[a]
11	5,7-Dihydroxy-8-methoxyflavone (Wogonin)	1.52 ±0.13	1.03 ± 0.04
12	6-Hydroxyflavone	2.64 ± 0.36	0.90 ± 0.11
13	7-Hydroxyflavone	4.20 ± 0.27	1.14 ± 0.08[a]
14	5,6,7-Trihydroxyflavone (Baicalein)	5.58 ± 0.02	ND
15	Flavone	7.81 ± 1.81	1.05 ± 0.03[a]
16	6-Hydroxy-2′-methoxyflavone	9.46 ± 1.45	ND
17	2′-Methoxyflavone	32.24 ± 1.96	ND
18	Diazepam	0.0064 ± 0.00021	2.24 ± 0.24
19	Flumazenil	0.005 ± 0.0003	0.91 ± 0.01
20	FG7142	0.43 ± 0.05	0.82 ± 0.04
21	2′-Chloroflavone	>100	ND
22	8-Bromo-2′,6-dichloroflavone	>100	ND
23	2′,6-Dichloro-7-methoxyflavone	>100	ND
24	2′,6-Methoxyflavone	>100	ND
25	6-Bromo-2′-chloroflavone	>100	ND
26	2′,6-Dichloroflavone	>100	ND
27	2′-Nitroflavone	>100	ND
28	2′-Amino-6-methoxyflavone	3.50 ± 0.5	ND
29	2′-Chloro-6-methoxyflavone	>100	ND
30	2′-Chloro-6-hydroxyflavone	>100	ND

Dose–inhibition curves were generated with 8–10 drug concentrations. K_i values for various compounds were estimated by displacement of [^{3}H]flunitrazepam binding to synaptosomal membranes from cerebral cortex of Sprague–Dawley rat (approximately 250 g). K_i values were calculated according to the equation: $K_i = IC_{50}/[1 + ([^3H]/K_d)]$, where [^{3}H] is the concentration of [^{3}H]flunitrazepam (1 nM), and K_d is the dissociation constant of [^{3}H]flunitrazepam from the high-affinity binding site (1.23 ± 0.08 nM). GABA ratios are determined in the presence of 10^{-5} M GABA with synaptosomal membrane preparation from rat cerebral cortex employing 1 nM [^{3}H]Ro15-1788.

[a] GABA ratios referred from Dekermendjian *et al.* [15].

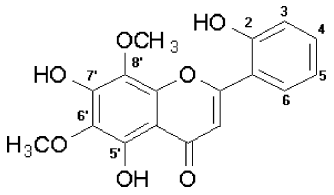

Fig. 1. The chemical structure and molecular weight of K36 (MW: 330).

further characterization by means of electrophysiological studies and animal models typical for BDZ evaluations.

3.2. Partial positive allosteric modulation of recombinant BDZR by K36

Rat recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were functionally expressed in *Xenopus* oocytes. GABA elicited μA sized currents. At 3 μM, K36 itself did not activate any current. A GABA concentration eliciting approximately 1% of the maximal current amplitude was applied alone and subsequently in combination with increasing concentrations of K36 (Fig. 3a). At 1 nM K36 started to allosterically stimulate the GABA-induced currents. Figure 3b shows the mean stimulation by different concentrations of K36 observed in four experiments. The concentration response curve was best-fitted by assuming a half-maximal stimulation at 24 nM K36 and a Hill coefficient of 0.8. In four experiments, maximal stimulation by K36 amounted to about 54 ± 8% of that by 0.3 μM diazepam (two batches of oocytes). The BDZ antagonist Ro15-1788, at 1 μM, abolished most of the stimulation by 0.3 μM K36 (Fig. 3c). In four individual experiments, 0.3 μM K36 stimulated the current elicited by GABA as determined 45 s after application of the drugs by 66 ± 10% (N = 4). Subsequently, 0.3 μM K36 was co-applied with 1 μM Ro15-1788 and this stimulation was decreased to 4 ± 1% (N = 4). Ligands of the BDZR are known to depend on the presence of a γ subunit [2,24,31]. We therefore tested the stimulation by 3 μM K36 at the dual subunit combination $\alpha_1\beta_2$ and found that current stimulation was negligible (2 ± 5%, N = 3).

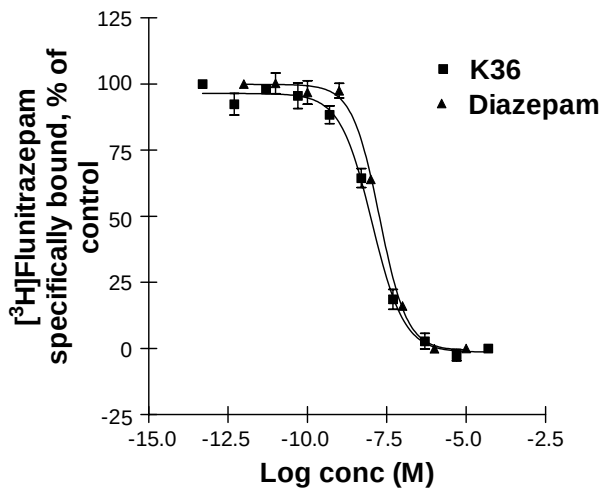

Fig. 2. Dose–inhibition of [^{3}H]flunitrazepam (1 nM) binding to the rat cerebral cortical membrane by K36 and diazepam. K_i value of K36 and diazepam is 6.1 ± 0.7 nM and 6.4 ± 0.2 nM, respectively, obtained from two individual experiments each performed in triplicates.

In accord with the ligand binding results, these electrophysiological measurements demonstrated a partial positive allosteric modulatory role of K36 via interaction with the BDZR *in vitro*. To correlate between *in vitro* and *in vivo* effects, the locomotor activity test, the elevated plus-maze, the hole-board, the horizontal wire, and the rotarod tests were also performed.

3.3. Pharmacological effects of K36

3.3.1. Effects of K36 in the locomotor activity test

To differentiate between possible stimulant effects of tested drugs from their modulation with exploratory behavior, the locomotor activity test was performed. K36 produced no significant changes in the locomotor activity of mice administered with doses 1–8 mg kg^{-1}. Diazepam, at 1 mg kg^{-1}, likewise did not alter the locomotor activity in comparison with vehicle-treated controls. However, mice administered 3 mg kg^{-1} diazepam exhibited significantly lower locomotor activities than control (Fig. 4a).

3.3.2. Effects of K36 in the hole-board test

In the hole-board test where exploratory activity was assayed, although the mice displayed a general increase in the number and time spent head-dipping, acute administrations of K36 (1–8 mg kg^{-1}) or diazepam (1 mg kg^{-1}) did not result in significant changes in any of the parameters indicative of potential sedative effects when compared to control (data not shown). Conversely, diazepam, at the higher dosage (3 mg kg^{-1}), resulted in significant decrease in the number of rears made in mice with no changes in the parameters corresponding to head-dips, demonstrating the potential sedative effect of diazepam at its anxiolytic dosage. These results therefore demonstrated the lack of sedation by K36 over its anxiolytic dose range.

3.3.3. Effects of K36 in the elevated plus-maze

K36, at 4 and 8 mg kg^{-1}, 20 min after dosing, gave rise to significant increases in both the percentage of entries and time spent in open arms while other dosage regimen did not alter these parameters as compared to control. At 4 mg kg^{-1}, diazepam (1 and 3 mg kg^{-1}) also showed significant anxiolysis as observed in the dose-dependent increase in the parameters corresponding to the open arms in comparison to control mice (Fig. 4b). When K36 (4 mg kg^{-1}) was co-administered with the BDZR antagonist Ro15-1788 (1.25 mg kg^{-1}), both the K36-induced increase in the percentage of entries and time spent in open arms were reversed back to basal levels, demonstrating that K36 elicited its anxiolytic effect specifically through interaction with the BDZR (Fig. 5).

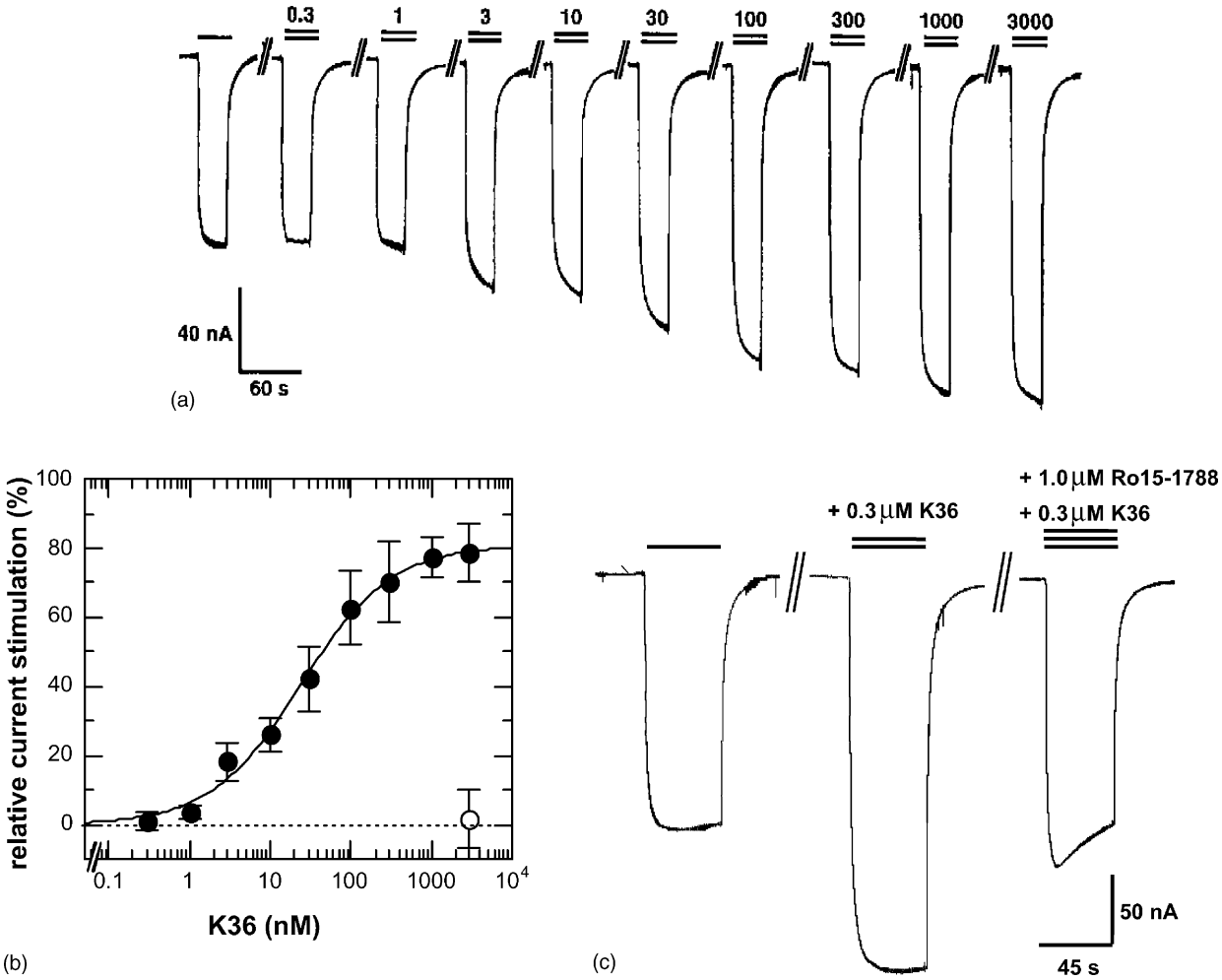

Fig. 3. (a) Stimulation of currents elicited by GABA in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Recombinant rat $GABA_A$ receptors were expressed in *Xenopus laevis* oocytes. Application of 1.5 μM GABA (lower bar) alone resulted in approximately 1% of the maximal current amplitude. Increasing concentrations of K36 was co-applied with GABA. The bars indicate duration of drug applications. Numbers above the upper bars indicated the drug concentration in nM. (b) Allosteric stimulation of currents elicited by GABA in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by K36. Conditions were as showed in Fig. 4. This figure shows cumulative concentration response curve (●). Stimulation was absent in the subunit combination $\alpha_1\beta_2$ (○). Values are showed as mean ± SEM for four and three oocytes, respectively. (c) Stimulation by K36 is inhibited by a BDZR antagonist Ro15-1788. The concentration of GABA eliciting approximately 2% of the maximal current amplitude at recombinant rat $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors was determined first; 0.3 μM K36 markedly stimulated these currents. When 1 μM Ro15-1788 was co-applied with 0.3 μM K36, the stimulation was inhibited completely. The bars show the periods of drug application. The lowest bar indicates application of 1.5 μM GABA.

3.3.4. Effects of K36 in the horizontal wire test

K36-treated mice did not display signs of myorelaxation at any of the dosages tested. Diazepam, at 1 mg kg^{-1}, did not result in significant changes in the wire-grasping performance of the mice. However, mice treated with 3 mg kg^{-1} diazepam showed significant impairment in their performance to grasp the wire as compared to control (Fig. 4c), once again illustrating the compromising side-effect of diazepam at its effective dose range for anxiolysis.

3.3.5. Effects of K36 in the rotarod test

Mice administered with either K36 (1–8 mg kg^{-1}) or diazepam (1 mg kg^{-1}) did not manifest motor incoordination as monitored by their ability to stay on the rotarod after treatment (Fig. 4d). When mice were subjected to the higher dose of diazepam (3 mg kg^{-1}), they exhibited significant motor incoordination in comparison to controls.

4. Discussion

Flavone and its derivatives have been shown to possess affinity for the BDZR [19,32,33] and a number of them have been demonstrated to be active *in vivo* [34,35]. However, these naturally occurring flavonoids characterized so far exhibited affinities for the BDZR only in the micromolar range, substantially lower than that of conventional BDZs. Until the protein structure of the BDZR is elucidated, facilitating rational drug design to generate potent ligands for the recognition site, there are essentially two approaches for identifying high-affinity BDZR

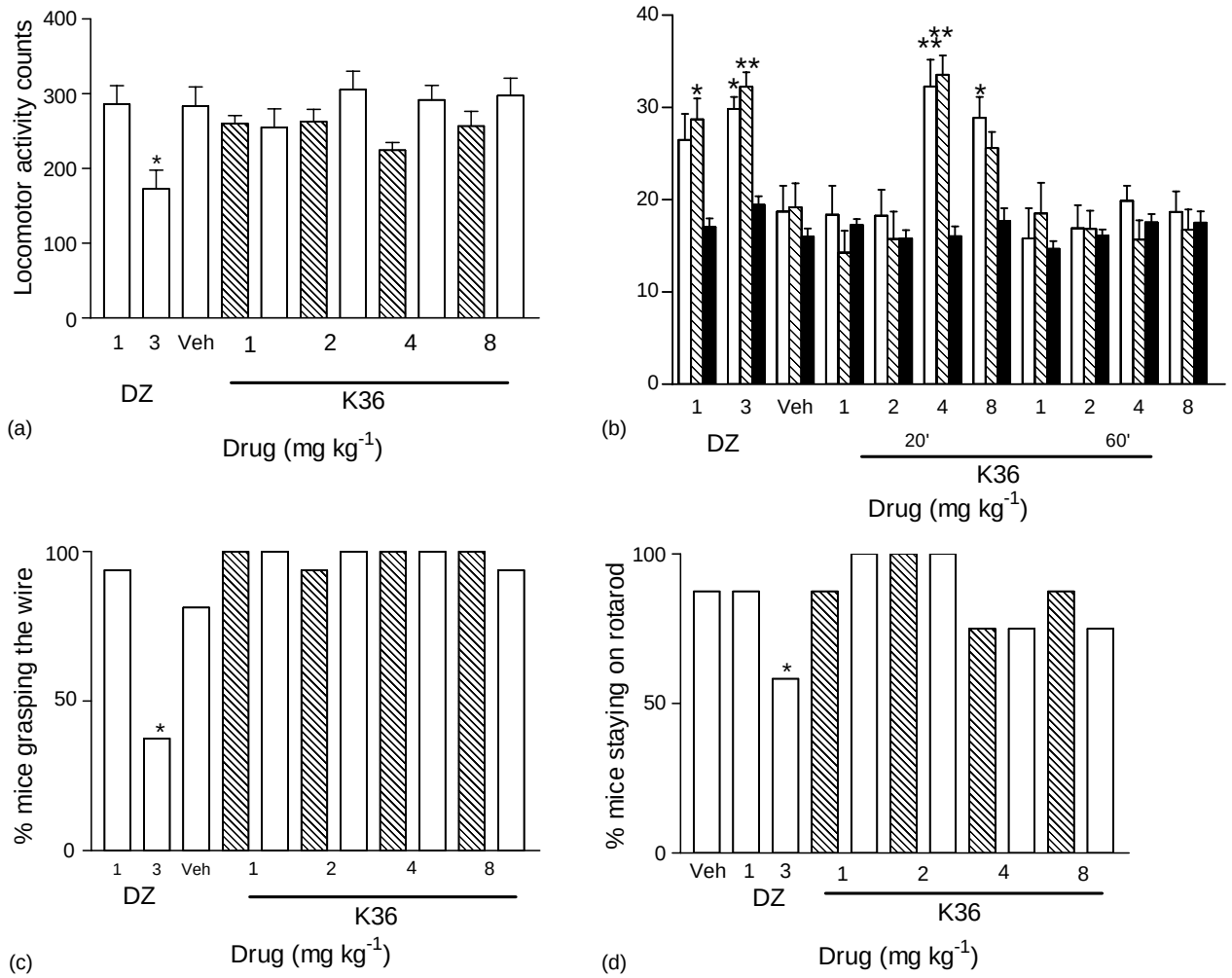

Fig. 4. (a) Locomotor activity assessment in mice after K36 treatment. Locomotor activity (mean ± SEM) counts during a 5-min test session in a ZIL-2 apparatus after oral administration with vehicle (Veh), diazepam (DZ; 1 and 3 mg kg^{-1}) or K36, $^{*}P < 0.01$, significantly different from control, Dunnett's *t*-test after one-way ANOVA, N = 16 mice per group. Open and hatched bars represents 1 hr and 20 min after p.o., respectively. (b) Assessment of anxiolytic effects of K36 in the mice elevated plus-maze. Data represents mean ± SEM of the percentage of entries (open bar) and time spent (hatched bar) in open arms, and the total number of closed arm entries (solid bar) made in mice after oral administration of diazepam (DZ; 1 and 3 mg kg^{-1}), vehicle (ddH_2O, pH 7.0) or K36 (1–8 mg kg^{-1}) in the mice elevated plus-maze during a 5-min test, N = 16 mice per group, $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control, Dunnett's *t*-test after one-way ANOVA. (c) Assessment of myorelaxant effects of diazepam, vehicle or K36 in the horizontal wire test. Data represents the percentage of mice grasping the wire after oral administration of diazepam (DZ; 1 and 3 mg kg^{-1}), vehicle (Veh, ddH_2O) or K36 (1–8 mg kg^{-1}) $^{*}P < 0.01$, significantly different from control, Chi's *t*-test, N = 16 mice per group. Open and hatched bars represents 1 hr and 20 min after p.o., respectively. (d) Assessment of motor coordination in diazepam, vehicle or K36-treated mice in the rotarod test. Data represent the percentage of mice that stayed on the rotarod for a period of 1 min given two opportunities after oral administration of diazepam (DZ; 1 and 3 mg kg^{-1}), vehicle (Veh, ddH_2O) or K36 (1–8 mg kg^{-1}), $^{*}P < 0.05$, significantly different from control, Chi's square test, N = 16 mice per group. Open and hatched bars represents 1 hr and 20 min after p.o., respectively.

ligands. Apart from synthesizing chemical libraries from a drug lead, the search for potent ligands continues from that of natural origins. It is evident that a vast number of potential therapeutics lies in traditional medicines and clinically, the opiates are probably one of the most recognized class of compounds that has evolved from natural sources. Adopting the latter approach, we previously reported the isolation and identification of a list of BDZR ligands from the traditional medicinal herb *Scutellaria baicalensis* Georgi guided by the [^{3}H]flunitrazepam displacement assay. Notably, amongst the list of naturally occurring flavonoid derivatives that manifested significant affinities for the BDZR, K36, possessing the highest affinity for the receptor, was chosen for more in-depth evaluation into its functional and behavioral role. In addition, these flavonoids were also used in a SAR study, thus having additional significance apart from their possible value as therapeutics.

K36 displaced [^{3}H]flunitrazepam binding in a very similar manner as diazepam (Fig. 2). Consistent with the GABA ratio of 1.2 (Table 2), electrophysiological experiments in *Xenopus* oocytes showed that K36 acts as a partial positive allosteric modulator of the $GABA_A$ receptor via the BDZR. At 3 μM K36, stimulation of the GABA-induced current was nearly saturated. At this concentration, K36 still did not activate any current alone.

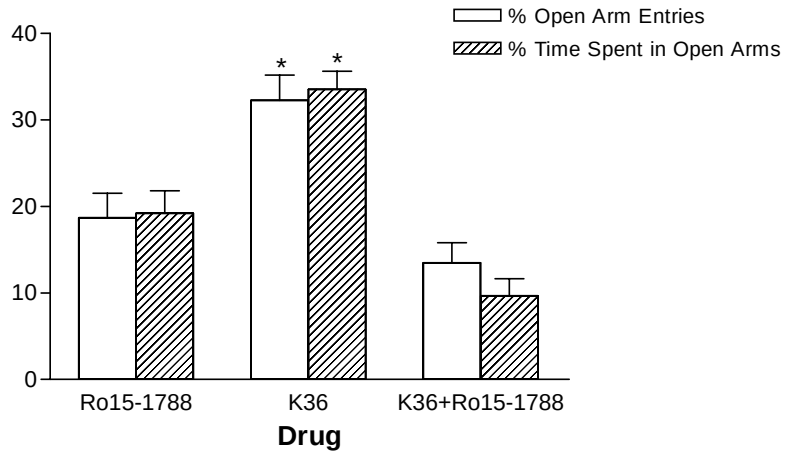

Fig. 5. Anxiolytic effect of K36 mediated by the BDZR. Data represents mean ± SEM of the percentage of entries (open bar) and time spent (hatched bar) in open arms in the mice elevated plus-maze after administration of K36 (4 mg kg^{-1}, p.o.) alone or with Ro15-1788 (1.25 mg kg^{-1}, i.p.), $^{*}P < 0.01$, significantly different from control (Ro15-1788, i.p.), Dunnett's *t*-test after one-way ANOVA, N = 16 mice per group.

In three batches of oocytes, maximal stimulation by K36 amounted to approximately 54 ± 8% of that by 0.3 μM diazepam. Threshold of stimulation is at approximately 1 nM and half-maximal stimulation at 24 nM (Fig. 3a and b). These properties are comparable to those of diazepam. In order to investigate a possible subunit specificity of K36, we measured allosteric stimulation by 3 μM K36 in $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_3\gamma_2$ and $\alpha_1\beta_2\gamma_3$ $GABA_A$ receptors (data not shown). There was a slight but not significant preference for $\alpha_3\beta_2\gamma_2$ over the other subunit combinations. In contrast to classical BDZs, K36 also acts at $\alpha_6\beta_2\gamma_2$. In addition, unlike loreclezole, K36 acts also at β_1 containing receptor (data not shown).

Subsequent to the first identification of flavonoid as ligands of the BDZR, numerous naturally occurring flavonoids have been found active in this regard [36]. However, apart from amentoflavone, a biflavone which exhibited low nanomolar affinity for the recognition site [37], all other have been shown to possess affinities within the micromolar range only. Thus K36 represents the first instance of a naturally occurring monoflavonoid with affinity and potency for the BDZR comparable to that of diazepam *in vitro*. In addition, the sub-maximal potentiation of the GABA-activated current by K36 in comparison to the potentiation of diazepam establishes that K36 is a partial agonist at the recombinant $GABA_A$ recptor in contrast to the full agonistic nature of diazepam.

In the murine models useful for BDZ evaluations, K36 elicited significant anxiolysis in the elevated plus-maze, evidenced by the selective increases in the parameters corresponding to the open arms, with no changes in locomotor activities and the number of closed arm entries. In the hole-board, horizontal wire and rotarod tests, K36 did not elicit significant sedation, myorelaxation and motor incoordination at the anxiolytic dosages. More importantly, the K36-elicited anxiolytic effect was shown to

Table 2
Branch chains on the flavonoids used in this study

Compound	Name	Side chain				
		2′	5	6	7	8
1	5,7,2′-Trihydroxy-6,8-dimethoxyflavone (K36)	OH	OH	OCH_3	OH	OCH_3
2	2′-Hydroxy-β-naphthoflavone	OH	H	C_4H_4	H	H
3	5,7,2′-Trihydroxy-6-methoxyflavone	OH	OH	OCH_3	OH	H
4	6,2′-Dihydroxyflavone	OH	H	OH	H	H
5	5,7,2′-Trihydroxyflavone	OH	OH	H	OH	H
6	2′-Hydroxyflavone	OH	H	H	H	H
7	5,7-Dihydroxy-6,8-dimethoxyflavone	H	OH	OCH_3	OH	OCH_3
8	7,2′-Dihydroxyflavone	OH	H	H	OH	H
9	5,7-Dihydroxy-6-methoxyflavone (Oroxylin A)	H	OH	OCH_3	OH	H
10	5,7-Dihydroxyflavone	H	OH	H	OH	H
11	5,7-Dihydroxy-8-methoxyflavone (Wogonin)	H	OH	H	OH	OCH_3
12	6-Hydroxyflavone	H	H	OH	H	H
13	7-Hydroxyflavone	H	H	H	OH	H
14	5,6,7-Trihydroxyflavone (Baicalein)	H	OH	OH	OH	H
15	Flavone	H	H	H	H	H
16	6-Hydroxy-2′-methoxyflavone	OCH_3	H	OH	H	H
17	2′-Methoxyflavone	OCH_3	H	H	H	H

be specifically mediated by the BDZR, insofar that it was completely abolished by Ro15-1788.

Although electrophysiological measurements provided evidence for a partial agonistic modulatory action on the $GABA_A$ receptor function by K36, the extent of its contribution to the anxioselective effect of K36 in mice remains to be determined. Transgenic studies have revealed that α_2-containing receptor assembly-mediated anxiety in mice [38] and that the α_1 subunit is involved in the diazepam-induced sedation and anticonvulsant effect [39,40]. Thus partial agonistic properties and receptor selectivity are plausible contribution to the anxioselective effect of K36.

It is interesting that the K36-induced anxiolysis was observed 20 min, but not 1 hr after oral administration. One possible explanation for its fast-acting but short-lasting effect may be due to the rapid metabolism and excretion of this hydroxyl-rich flavonoid, as pharmacokinetic experiments have shown that metabolism of flavonoids proceed through the attachment of hydroxyl groups which render them more hydrophilic [41,42]. Since K36 possesses multiple hydroxyl substitutions, it is likely to be excreted rapidly. Obviously, whether the anxiolytic effect was as a result of interaction with the BDZR by K36 itself, its active metabolites, or both, remains to be identified, and the exact mechanism behind such atypical pharmacological profile remains to be unraveled with further studies. Despite the vast possibilities raised, unlike amentoflavone which was suggested that it does not cross the blood–brain barrier based on its failure to displace [^{3}H]diazepam binding *in vivo* [37], K36, with high-affinity for the BDZR, is demonstrated to be centrally active. Although further neuropharmacological experiments are needed to elaborate its therapeutic potentials, this represents the first instance where a high-affinity naturally occurring flavonoid derivative modulates BDZR-mediated anxiolytic effects *in vivo*.

4.1. 2′-Substitution of flavonoids as a determinant of BDZR affinity

In the present study, hydroxyl substitution at the 2′ carbon onto compounds **1**, **3**, **4**, **5**, **6** and **8**, compared to their unsubstituted counterparts (compounds **7**, **12**, **9**, **10** and **6**, respectively), all displayed enhanced binding affinities for the BDZR on the $GABA_A$ receptor (Tables 1 and 2). This further illustrates that the 2′-hydroxyl group on flavonoid molecules is an important enhancing factor that is responsible for the interaction between the ligand and the receptor. In general, the substitution of a hydroxyl group for a hydrogen at the 2′ carbon resulted in 7-fold or higher affinity for the BDZR.

To investigate the role of 2′ substituents in the determination of binding affinities for the BDZR, flavonoids with various substituents, i.e. –Cl, $–NO_2$ and $–NH_2$, at the 2′ carbon were organically synthesized for further investigation. It was found that only the flavonoid with an $–NH_2$ substitution displayed significant affinity for the BDZR (Table 1). This suggests that the electron donation effect of the –OH and $–NH_2$ substitution, as opposed to the electron withdrawal effects of the nitro and halogen moieties, brings about tighter binding of the flavonoids to its receptor. Recently, Kahnberg *et al.* [14] have also reported independently the importance of 2′-hydroxyl substitution of flavonoids in determining their interaction with the BDZR. Thus our results complement such findings and provide further evidence to support the importance of a 2′-hydroxyl group plays in the flavonoid–BDZR interaction.

Incorporation of electronegative groups to the 3′ and 6 carbon on the flavonoid backbone yielded significant increases in the binding affinities of the class of compounds for the BDZR [18]. 6,3′-Dinitroflavone and 6-chloro-3′-nitroflavone are examples of these high-affinity synthetic flavonoids [13,43]. However, these electronegative moieties when substituted onto other carbons on the flavone backbone did not give rise to enhanced affinities for the BDZR, which is consistent with our results.

The present findings highlight the potential of drug discovery from traditional medicines. Notably, we have isolated a list of BDZR ligands from *Scutellaria baicalensis* Georgi, a commonly prescribed Chinese medicinal herb, one of which possessed the highest affinity for the BDZR amongst naturally occurring flavonoids reported to date. In this paper, K36, manifesting affinity and potency for the BDZR comparable to that of diazepam *in vitro*, was demonstrated to elicit significant anxiolysis without sedative, myorelaxant and motor incoordination effects in the chosen dosage regimen. As mentioned earlier, the potential of K36 as a therapeutics awaits for more in-depth studies, nevertheless, not only does it represent the first instance in which such high-affinity naturally occurring flavonoid derivative exerted CNS modulation at the BDZR level, it also serves as a lead for drug developments and for further QSAR studies leading to a refined pharmacophore model of the receptor.

Furthermore, the SAR study of flavonoid binding at the BDZR generated by the list of derivatives tested in this study are consistent with previous reports and provides the basis for enhancing the affinities of this class of compounds for the BDZR.

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