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Confirmation of the anxiolytic-like effect of dihydrohonokiol following behavioural and biochemical assessments

Yuji Maruyama, Hisashi Kuribara, Eiko Kishi, Susan T. Weintraub and Yoshihisa Ito

Abstract

Previous studies in this laboratory revealed that dihydrohonokiol-B (DHH-B; 3′-(2 propenyl)-5-propyl-(1,1′-biphenyl)-2,4′-diol), a partially reduced derivative of honokiol, was an effective anxiolytic-like agent in mice at an oral dose of 0.04 mg kg^{-1}, and at higher doses, when evaluated by the elevated plus-maze test. The aim of this study was to further confirm the anxiolytic-like effect of DHH-B using an additional behavioural procedure (Vogel's conflict test in mice) and a biochemical assessment (in-vitro determination of muscimol-stimulated $^{36}Cl^-$ uptake into mouse cortical synaptoneurosomes). As in earlier experiments, DHH-B (0.04–1 mg kg^{-1}, p.o.) was shown to prolong the time spent in the open-sided arms of the elevated plus-maze in a dose-dependent manner. Moreover, in the Vogel's conflict test, DHH-B (5 mg kg^{-1}, p.o.) significantly increased punished water intake. In tests with mouse cerebral cortical synaptoneurosomes, 10 and 30 μM of DHH-B significantly increased $^{36}Cl^-$ influx in the absence of muscimol. In the presence of 25 μM muscimol, the addition of 1 μM DHH-B led to significant enhancement of $^{36}Cl^-$ uptake, while 30 μM DHH-B was required to further stimulate the $^{36}Cl^-$ uptake induced by 250 μM muscimol. The results of these studies confirm that DHH-B is a potent anxiolytic-like agent and that $GABA_A$ receptor-gated Cl^--channel complex is involved in the anxiolytic-like efficacy of DHH-B.

Department of Neuropsychopharmacology (Tsumura), Gunma University School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan

Yuji Maruyama, Hisashi Kuribara, Eiko Kishi

Department of Pharmacology, College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan

Yoshihisa Ito

Department of Biochemistry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7760, USA

Susan T. Weintraub

Correspondence: Y. Maruyama, Department of Neuropsychopharmacology (Tsumura), Gunma University School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. E-mail: yuji@med.gunma-u.ac.jp

Introduction

Honokiol and magnolol, neolignan isomers, are major components of the bark of *Magnolia obovata* Thunb (Fujita et al 1972). The anxiolytic-like effect of honokiol was first reported by Kuribara et al (1998, 1999) and Maruyama et al (1998, 2000). In those initial studies using male mice, it was shown that oral administration of 0.2–2 mg kg^{-1} of honokiol for 7 days produced a significant anxiolytic-like response as assessed on an elevated plus-maze (Pellow et al 1985; Dawson & Trickebank 1995; Kulkarni & Reddy 1996), as modified by Kuribara and co-workers (Kuribara et al 1998). However, 20 mg kg^{-1} honokiol was required for anxiolytic-like activity following a single oral administration in mice. These results suggested that a metabolite(s) of honokiol, rather than the parent compound, might be responsible for the anxiolytic-like effect. To obviate the need for subchronic treatment with honokiol for development of a clear anxiolytic response, the authors evaluated analogues of honokiol which might be effective at an acceptably low dose following acute oral administration.

It has previously been shown through pharmacokinetic studies that hydrogenation of the propenyl side chains is the main metabolic pathway of magnolol (Tsai et al 1995). It was, therefore, expected that metabolism of honokiol would also generate reduced derivatives. As such, we assessed the anxiolytic-like potential of various hydrogenated, methylated or hydroxylated derivatives of honokiol (8 compounds in total) and found that dihydrohonokiol-B (DHH-B; (3′-(2-propenyl)-5-propyl-(1,1′-biphenyl)-2,4′-diol) exhibited significant anxiolytic-like activity (Kuribara et al 2000).

In this study, we compared results obtained on the elevated plus-maze with a behavioural assessement using Vogel's conflict test (Vogel et al 1971) as modified for mice (Kuribara et al 1989; Umezu 1999). The Vogel's test was selected because it is a standard measure of the anxiety level of animals (Commissaris 1993). We also conducted a biochemical in-vitro investigation to determine whether the potent anxiolytic-like effect of DHH-B was mediated by the $GABA_A$-receptor chloride-ionophore complex (Olsen & Tobin 1990; Ito et al 1992; Sieghart 1992). In these in-vitro experiments, the effect of DHH-B on $^{36}Cl^-$ uptake into cortical synaptoneurosomes (Hollingsworth et al 1985; Schwartz et al 1986; Suzudak et al 1986) was determined in the absence and presence of muscimol, a $GABA_A$ agonist.

Materials and Methods

Animals

Male ddY mice, 7 weeks old, weighing 30–32 g, were purchased from SLC Japan Co. Ltd (Hamamatsu, Japan). Ten animals were housed in each Plexiglas cage which had roll paper bedding (Paper Clean; SLC, Japan). The mice were allowed free access to water and standard laboratory food (MF: Oriental Yeast Co. Ltd, Tokyo, Japan) and were maintained in a facility at a temperature of 24 ± 1°C and relative humidity of 55 ± 5%, with lights on at 0700–1900 h daily. All experimental animal protocols met the Guidelines for Animal Experimentation approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society.

Drugs

DHH-B was synthesized at Tsumura & Co. Ltd (Tokyo, Japan) as previously described (Kuribara et al 2000). Diazepam (Cercine Inj.) was purchased from Takeda Chemical Industries Ltd (Osaka, Japan) and ^{36}chlorine radionuclide (sodium chloride solution, 3 mCi g^{-1}) was obtained from Amersham Life Science (Tokyo, Japan).

For assessment by the plus-maze and Vogel's conflict tests, DHH-B was first dissolved in 50 μL of ethanol, and the solution was diluted with distilled water containing Tween-80 (0.1%) so that the final ethanol concentration was 0.5%. The injectable preparation of diazepam was diluted with physiological saline. The concentration of each drug solution was adjusted so that the volume administered was kept constant at 0.1 mL/10 g mouse body weight.

The elevated plus-maze test

The elevated plus-maze used in this study was the same as described in our previous reports (Kuribara et al 1988, 2000; Maruyama et al 1998). The apparatus was a slightly modified version of the original plus-maze used for rats (Pellow et al 1985) and mice (Lister 1987). The plus-maze, which was elevated 40 cm above a base, had four arms (6 cm wide, 30 cm long) extending from a central platform (8×8 cm). Two of the arms had side-walls (10 cm high), and they were non-transparent grey in colour. The other two arms had no side-walls and were constructed with a transparent floor. The central platform was non-transparent grey.

For testing, a mouse was placed on the central platform facing one of the closed-sided arms; the total time spent in the open-sided arms (all four paws entering) during the ensuing 5 min observation period was recorded. Groups of 10 drug-naive mice were used for each test: DHH-B (0.008, 0.04, 0.2 and 1 mg kg^{-1}) and diazepam (1 mg kg^{-1}). On the basis of time-course experiments for DHH-B and diazepam reported previously (Kuribara et al 2000), DHH-B was given orally 3 h before the plus-maze test, and diazepam given orally 10 min before testing. Mice in control groups received the appropriate vehicle for each drug.

Modified Vogel's conflict test

The Vogel's conflict test was conducted in a Plexiglas chamber (13 cm long, 9 cm wide, 9 cm high) equipped with a recorder (VC-3002-L) and control unit (VC-2050-L; O'Hara & Co. Ltd, Tokyo, Japan). In keeping with our standard protocol (Kuribara et al 1989), a mouse deprived of water for 24 h was placed in a test chamber and allowed free access to water from the spout for 30 min. The amount of water taken in was

measured in 0.05-mL increments. The mouse was then returned to its home cage and deprived of water. The following day, the mouse was brought to the same chamber and again allowed to drink for 30 min; however, on this day, the mouse received an electric foot-shock (60 V, 0.2 mA, AC, for 0.3 s) delivered through the stainless floor grid of the chamber with every water intake of 0.05 mL. Groups of 10–30 drug-naive mice were used for these tests: DHH-B (vehicle, 1, 2 and 5 mg kg^{-1}) and diazepam (vehicle and 1 mg kg^{-1}). In the same way as described above, DHH-B and diazepam were given orally 3 h and 10 min, respectively, before the conflict test.

$^{36}Cl^-$ uptake into cortical synaptoneurosomes

Cortical synaptoneurosomes were prepared in the same way as previously reported (Hollingsworth et al 1985; Suzudak et al 1986; Ito et al 1992, 1995, 1996). Briefly, mouse cerebral cortices were homogenized in 5 volumes of ice-cold Krebs-Henseleit buffer (pH 7.4) using a glass–glass homogenizer. The homogenates were diluted with 20 volumes of buffer and then filtered through three layers of nylon cloth and a 10-μm Millipore filter (LCWP 047: Millipore Corp., Bedford, USA). The filtrates were centrifuged at 1000 *g* for 15 min, and the resulting pellets were suspended in ice-cold solution containing (in mM): 118 NaCl, 4.7 KCl, 1.18 $MgSO_4$, 2.5 $CaCl_2$ and 20 HEPES-Tris (pH 7.4, buffer-A). Samples of the synaptoneurosomal fraction (1.5–1.8 mg protein) were pre-incubated at 30°C for 3 min and then a test solution of DHH-B was added. Based on time-course experiments of muscimol-stimulated $^{36}Cl^-$ uptake (Ito et al 1996), $^{36}Cl^-$ uptake was measured after a 3-s incubation period at 30°C. Uptake of $^{36}Cl^-$ was terminated by addition of 5 mL of ice-cold buffer-A containing 0.1 mM picrotoxin, followed by rapid filtration under vacuum through Whatman GF/C glass-fibre filters pretreated with 0.05% polyethyleneimine. The filters were then washed with two 5-mL portions of the same buffer. Radioactivity trapped on the filters was determined using a scintillation counter. Protein concentrations were measured by the method of Lowry et al (1951).

Statistical analysis

A one-way analysis of variance followed by a Student-Newman-Keuls test was used to assess statistical significance for the results of the plus-maze test (time spent in the open-sided arms), the activity test (activity counts) and Vogel's conflict test (the amount of water intake). For measurements of the level of $^{36}Cl^-$ uptake into cortical synaptoneurosomes, a one-way analysis of variance was employed with subsequent comparisons between treatment groups and corresponding controls carried out using Duncan's Multiple Range Test. Values of $P < 0.05$ were considered significant for all statistical measures.

Results

Elevated plus-maze test

As shown in Table 1, both diazepam (1 mg kg^{-1}) and DHH-B (0.04–1 mg kg^{-1}) significantly prolonged the

Table 1 Assesment of the anxiolytic-like effect of dihydrohonokiol-B (DHH-B) and diazepam by the elevated plus-maze test in mice.

Treatment[a]		n	Time in open-arms (s/5 min)[b]
Vehicle		20	5.8 ± 2.0
DHH-B	0.008 mg kg^{-1}	10	6.8 ± 2
	0.04 mg kg^{-1}	10	12.3 ± 3.4*
	0.2 mg kg^{-1}	10	18.1 ± 5.6*
	1.0 mg kg^{-1}	10	24.9 ± 5.0*
Vehicle		10	3.2 ± 1.3
Diazepam	1.0 mg kg^{-1}	10	24.3 ± 5.4*

[a]DHH-B and diazepam were orally administered 3 h and 10 min, respectively, before the plus-maze test. [b]Values represent the mean ± s.e. for evaluation of n drug-naïve mice. *$P < 0.05$ compared with the corresponding vehicle-treated control.

Table 2 Assessment of anxiolytic-like effect of dihydrohonokiol (DHH-B), and diazepam by the Vogel's conflict test in mice.

Treatment[a]		n	Punished drinking/ 30 min[b]
Vehicle		30	4.3 ± 1.5
DHH-B	1.0 mg kg^{-1}	10	7.1 ± 3.7
	2.0 mg kg^{-1}	10	6.0 ± 2.8
	5.0 mg kg^{-1}	10	17.9 ± 5.6**
Vehicle		30	4.3 ± 1.5
Diazepam	1.0 mg kg^{-1}	30	12.8 ± 2.7*

[a]DHH-B and diazepam were administered 3 h and 10 min, respectively, before the conflict test. [b]Values represent the mean ± s.e. for n drug-naive mice. *$P < 0.05$, **$P < 0.01$ compared with the corresponding vehicle-treated control.

time spent by mice in the open-sided arms, strongly suggesting efficacy as an anxiolytic-like agent.

Vogel's conflict test

The anxiolytic-like effect of DHH-B was also confirmed with the modified Vogel's conflict test. From Table 2, it can be seen that oral administration of DHH-B increased punished drinking, although statistical significance was only achieved at 5 mg kg^{-1}, approximately 100 times higher than the 0.04-mg-kg^{-1} dose shown to be effective in the elevated plus-maze test. Diazepam significantly increased punished drinking at the same 1-mg-kg^{-1} dose that was effective in the plus-maze test.

$^{36}Cl^-$ uptake into mouse cerebral cortical synaptoneurosomes

Uptake of $^{36}Cl^-$ into mouse cortical synaptoneurosomes was assessed using three different concentrations of DHH-B (1, 10 and 30 μM), in the absence and presence of muscimol (25 and 250 μM). The results of these experiments are shown in Table 3. It is noteworthy that in the absence of muscimol, 10 and 30 μM DHH-B significantly increased $^{36}Cl^-$ uptake into synaptoneurosomes as compared with control levels. Moreover, increases in $^{36}Cl^-$ uptake induced by 25 and 250 μM of muscimol were significantly enhanced by 1 and 30 μM of DHH-B, respectively.

Table 3 Effects of dihydrohonokiol (DHH-B) on $^{36}Cl^-$ uptake into mouse cortical synaptoneurosomes.

Treatments[a]	$^{36}Cl^-$ uptake[b] (% of control)
Vehicle (muscimol, 0 mol)	100
DHH-B 1 μM	106.4 ± 2.5
DHH-B 10 μM	111.6 ± 2.6*
DHH-B30 μM	113.9 ± 3.8*
Muscimol 25 μM	158.6 ± 5.3#
Muscimol + DHH-B (1 μM)	190.9 ± 13.1*
Muscimol + DHH-B (10 μM)	194.1 ± 8.9*
Muscimol + DHH-B (30 μM)	199.8 ± 11.8*
Muscimol 250 μM	186.7 ± 4.6#
Muscimol + DHH-B (1 μM)	189.0 ± 7.8
Muscimol + DHH-B (10 μM)	207.9 ± 7.3
Muscimol + DHH-B (30 μM)	227.1 ± 13.9*

[a]Incubation of mouse cortical synaptoneurosomes with muscimol or DHH-B, or both, as indicated. [b]The values represent mean ± s.e. for six samples obtained in triplicate, expressed as a percentage of the vehicle control (21.5 ± 0.9 nmol (mg protein)$^{-1}$ per 3 seconds). *$P <$ 0.05 compared with the corresponding vehicle or muscimol-alone value. #$P < 0.05$ compared with the vehicle (muscimol 0 μM) value.

Discussion

In agreement with our previous observations (Kuribara et al 2000), DHH-B was shown to significantly increase the time spent by mice in the open-sided arms of the plus-maze at oral doses of 0.04 mg kg^{-1} and higher. Furthermore, DHH-B increased punished drinking in Vogel's conflict test at an oral dose of 5 mg kg^{-1}. These behavioural studies clearly indicate an anxiolytic-like effect of DHH-B. Diazepam was also shown to be an effective agent in both tests, but, in contrast to DHH-B, statistical significance for diazepam was achieved at the same dose (1 mg kg^{-1}) in each. It is not known at this time why a 100-fold higher dose of DHH-B was required for anxiolytic-like activity in Vogel's conflict test as compared with the elevated plus-maze. However, it has been reported that the effective doses of non-benzodiazepine anxiolytics, such as 5-HT_{1A} agonists and 5-HT_3 antagonists, were lower in the plus-maze test than in the conflict test (Hogg 1996).

Several drugs such as benzodiazepines, barbiturates, neurosteroids, ethanol, some of the anticonvulsants and general anaesthetics interact with $GABA_A$ receptors in the process of eliciting their pharmacological effects (Gee et al 1987; Luu et al 1987; Morrow & Paul 1988; Mehta & Ticku 1999). In particular, the traditional benzodiazepines develop pharmacological effects through benzodiazepine receptors that are modulatory sites coupled to the $GABA_A$-receptor chloride channel (Ketelaars et al 1988; Haefely 1990; Sannerud et al 1993). A variety of benzodiazepine-receptor agonists have been shown to increase $^{36}Cl^-$ uptake into synaptoneurosomes (Miller et al 1988a, b).

In these biochemical experiments, the effect of DHH-B on $^{36}Cl^-$ uptake in the mouse cortical synaptoneurosomes was determined in the absence and presence of muscimol. The method developed by Ito et al (1996) was employed so that it would be possible to determine whether DHH-B was acting through the $GABA_A$-receptor chloride-ionophore complex (Ito et al 1992; Sieghart 1992). The results of the $^{36}Cl^-$ uptake studies were consistent with the behavioural tests because DHH-B not only stimulated $^{36}Cl^-$ uptake into synaptoneurosomes following a single treatment but also enhanced muscimol-stimulated $^{36}Cl^-$ uptake. It is, therefore, highly likely that a $GABA_A$ receptor-gated Cl^- channel complex is involved, at least in part, in the anxiolytic-like activity of DHH-B. However, further

investigations are required to more fully elucidate the mechanisms underlying the pharmacological actions of DHH-B.

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