J. Pharm. Pharmacol. 2000, 52: 1017–1022
Received January 11, 2000
Accepted March 22, 2000

Does Dihydrohonokiol, a Potent Anxiolytic Compound, Result in the Development of Benzodiazepine-like Side Effects?

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Abstract

The aims of this study were to assess whether dihydrohonokiol, 3′-(2-propenyl)-5-propyl-(1,1′-biphenyl)-2,4′-diol (DHH-B), a potent anxiolytic compound, developed benzodiazepine-like side effects.

A 1 mg kg^{-1} dose of diazepam, almost equivalent to the minimum dose for the anxiolytic effect, disrupted the traction performance, potentiated hexobarbital-induced sleeping and impaired learning and memory performance. DHH-B, even at a dose of 1 mg kg^{-1} (i.e. five times higher than the minimum dose for significant anxiolytic effect) neither developed diazepam-like side effects nor enhanced the side effects of diazepam. Rather, the potentiation by diazepam of hexobarbital-induced sleeping was reduced by 1 mg kg^{-1} DHH-B. Furthermore, mice treated with 10 daily administrations of 1 and 5 mg kg^{-1} diazepam, but not 0·2–5 mg kg^{-1} DHH-B, showed precipitated withdrawal symptoms characterized by hyper-reactivity, tremor and tail-flick reaction when they were challenged with flumazenil (10 mg kg^{-1} i.p.).

These results suggest that, unlike the benzodiazepine anxiolytic diazepam, DHH-B is less likely to induce motor dysfunction, central depression, amnesia or physical dependence at the effective dose required for the anxiolytic effect.

Benzodiazepine anxiolytics frequently cause acute central depressant symptoms such as ataxia, oversedation, disinhibition, amnesia, ethanol and barbiturate potentiation (Shader & Greenblatt 1995), and tolerance and dependence characterized by withdrawal symptoms such as anxiety, insomnia, hyper-reactivity and convulsion following the termination of long-term use (Woods et al 1992, 1995; Schweizer et al 1995; Woods & Winger 1995). Such unwanted side effects limit the clinical efficacy and use of benzodiazepine anxiolytics. To minimize the side effects, new drugs capable of partial allostic modification of $GABA_A$ receptors have been proposed (Haefely et al 1990; Doble & Martin 1992; Gardner et al 1993).

Recently, Kuribara et al (2000) found that dihydrohonokiol, 3′-(2-propenyl)-5-propyl-(1,1′-biphenyl)-2,4′-diol (DHH-B), revealed a potent anxiolytic effect following single oral administration in the elevated plus-maze test in mice. Honokiol, the original compound of DHH-B, prolonged the time spent in the open arms of the maze after seven daily, but not single, oral administrations of 0·2–5 mg kg^{-1} (Kuribara et al 1998; Maruyama et al 1998), whereas a single dose of 0·2 mg kg^{-1} DHH-B was sufficient for producing almost the same prolongation (Kuribara et al 1999). Honokiol was less likely than benzodiazepine anxiolytic diazepam to elicit side effects such as disruption of motor function and learning and memory, potentiation of hexobarbital-induced sleeping or induction of physical dependence (Kuribara et al 1998, 1999). However, concern about the possibility of benzodiazepine-like side effects remains with DHH-B use because the combined administration of DHH-B with diazepam enhances the anxiolytic effect and the anxiolytic effect of DHH-B is inhibited by a benzodiazepine receptor antagonist, flumazenil (Kuribara et al 2000), indicating the strong involvement of benzodiazepine receptors in the anxiolytic effect of DHH-B.

The aim of this study was to assess whether or not DHH-B produced benzodiazepine-like side effects such as motor dysfunction, central

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depression, amnesia or physical dependence at doses of 0·2–5 mg kg^{-1}, equivalent to 25 times the minimum dose required for a significant anxiolytic effect (H. Kuribara et al, unpublished data).

Materials and Methods

Mice

Male mice of the ddY strain (Japan SLC, Hamamatsu, Japan) were purchased at 6 weeks, and bred for 1 week in polycarbonate cages (20 × 25 × 15 cm) in groups of 10, each with free access to a solid diet (MF, Oriental Yeast, Tokyo) and tap water. The conditions of the breeding room were controlled as follows: temperature 23 ± 1°C, relative humidity 55 ± 3% and a 12-h light–dark cycle.

All the experimental protocols were approved by the Committee of Animal Experiments in Gunma University School of Medicine and met the Guidelines for Animal Experimentation of the Japan Association of Laboratory Animal Science. The mice were used only once at 7 weeks of age and 34–37 g.

Drugs

The drugs used were DHH-B (synthesised by the authors), diazepam (Hoffmann-La Roche, Nutley, NJ), flumazenil (Hoffmann-La Roche) and hexobarbital-Na (Sigma, St Louis, MO). DHH-B was dissolved in a very small amount of ethanol, and the resultant solution was diluted with Tween-80 (0·1%)/physiological saline solution. The final ethanol concentration was 0·5%. Diazepam and flumazenil were suspended in the Tween-80/physiological saline solution, and hexobarbital-Na was dissolved in physiological saline. The concentration of each drug solution or suspension was adjusted to ensure a constant administration volume at 0·1 mL/10 g body weight of the mouse.

Experimental procedures

Traction test. The traction test was carried out according to the method previously described (Kuribara et al 1977). Briefly, a wire (diameter 1·6 mm and length 30 cm) was set horizontally 30 cm above the floor. The mouse was forced to grasp the wire with its four paws and the clinging time was recorded up to 60 s. Once the mouse had clung on for 60 s, it was released from the wire and the clinging time was recorded as 60 s. The effects of oral doses of DHH-B (0·2, 1·0 and 2·0 mg kg^{-1}, 3 h before), diazepam (0·5, 1 and 2 mg kg^{-1}, 10 min before) and DHH-B (1 mg kg^{-1}) + diazepam (1 mg kg^{-1}) were evaluated.

Hexobarbital-induced sleeping test. Groups of 10 mice were orally treated with DHH-B (vehicle, 0·2 or 1 mg kg^{-1}, 3 h before), diazepam (vehicle, 1 mg kg^{-1}, 10 min before) or DHH-B (0·2 or 1 mg kg^{-1}) + diazepam (1 mg kg^{-1}). They were then given hexobarbital-Na (100 mg kg^{-1}, i.p.). The duration of the loss of the righting reflex was recorded as the sleeping time.

Learning and memory test. The experimental procedure was carried out with a minor modification to the original method by Itoh et al (1990, 1991). Briefly, the experimental apparatus was an elevated plus-maze (open arms 6 × 30 × 10 cm; closed arm 6 × 30 cm; centre platform 8 × 8 cm). All the floors of the open and closed arms and the centre platform, and the side walls of the closed arms were non-transparent. In the training trial (1st day), each mouse was placed at the end of a randomly selected open arm facing away from the centre platform. The time of movement from the open arm to one of the closed arms was recorded as the transfer latency. The criterion of the mouse's entry into the closed arm was the crossing with all four paws of the borderline separating the centre platform and the closed arm. After measurement of the transfer latency, the mouse was allowed to move freely in the plus-maze for 2 min. Subsequently, the mouse was gently returned to its home cage. The next day the retention trial was carried out in the same way as in the training trial. Thus, the mouse was placed in the same position as in the training trial, and the transfer latency was recorded.

Groups of 10 mice were orally treated with either DHH-B (vehicle, 1 or 5 mg kg^{-1}, 3 h before) or diazepam (vehicle, 1 or 2 mg kg^{-1}, 10 min before) in either the training or the retention trial.

Physical dependence test. The drug administration schedules were similar to our previous study (Kuribara et al 1999). Briefly, groups of 10 mice were orally given either vehicle (Tween-80/ethanol solution), DHH-B (0·2, 1 or 5 mg kg^{-1}) or diazepam (1 or 5 mg kg^{-1} p.o.) daily for 10 days. Twenty-four hours after the last drug administration, all mice were challenged with flumazenil (10 mg kg^{-1} i.p.), and occurrence of the following abstinence symptoms was observed for 30 min: 1) hyper-reactivity (vocalization induced by a light pushing of the back), 2) tremor, 3) clonic convulsion, 4) tonic convulsion, 5) tail-flick reaction, 6)

running fit (wild running evoked by keyring sound). During the measurement of tremor, clonic and tonic convulsion, and tail-flick reaction, presentation of any external stimuli to the mouse was avoided.

Statistical analysis

The clinging time in the traction test, the sleeping time in the hexobarbital-induced sleeping test and the transfer latency in the learning and memory test were analysed by one-way analysis of variance, followed by Students *t*-test. In the physical dependence test, the numbers of mice exhibiting the symptoms were analysed by the chi-square test. $P < 0.05$ was considered significant.

Results

Traction test

As shown in Table 1, 0·2–2·0 mg kg^{-1} DHH-B did not disrupt the traction performance, whereas 0·5–2 mg kg^{-1} diazepam shortened the clinging time in a dose-dependent manner.

Hexobarbital-induced sleeping test

As shown in Table 2, 0·2 and 1 mg kg^{-1} DHH-B did not change the hexobarbital-induced sleeping time, but 1 mg kg^{-1} diazepam significantly prolonged the sleeping time. DHH-B (1 mg kg^{-1}) significantly reversed the diazepam-induced sleeping prolongation.

Learning and memory test

Table 3 represents the transfer latencies in the training (1st day) and retention (2nd day) trials in mice treated with DHH-B or diazepam. The treatment with 1 or 5 mg kg^{-1} DHH-B prior to the training trial did not change the transfer latencies in either the training or the retention trial. The pre-training treatment with 1 and 2 mg kg^{-1} diazepam shortened the latency time in the training trial, but treatment with 2 mg kg^{-1} diazepam prolonged the latency time in the retention trial. The pre-retention treatment with 5 mg kg^{-1} DHH-B significantly prolonged the latency time in the retention trial whereas no significant change in the latency time was produced when diazepam was administered prior to the retention trial.

Physical dependence test

As shown in Table 4, the challenge with flumazenil to the mice treated with 0·2–5 mg kg^{-1} DHH-B or

Table 1. Effects of DHH-B and diazepam on traction performance in mice.

Drug		Clinging time (s)
Vehicle		60·0
DHH-B	0·2 mg kg^{-1}	60·0
	1·0 mg kg^{-1}	60·0
	2·0 mg kg^{-1}	60·0
Vehicle		60·0
Diazepam	0·5 mg kg^{-1}	57·9 ± 1·4
	1·0 mg kg^{-1}	46·9 ± 4·9*
	2·0 mg kg^{-1}	38·8 ± 5·8**
DHH-B 1 mg kg^{-1} + diazepam 1 mg kg^{-1}		44·0 ± 4·8*

DHH-B and diazepam (p.o.) were administered 3 h and 10 min, respectively, before the traction test. Values are mean ± s.e.m. of 10 mice. *$P < 0.05$, **$P < 0.01$ the vehicle-treated group.

Table 2. Effects of oral DHH-B and diazepam on hexobarbital-induced sleeping.

Drug		Sleeping time (s)
Vehicle (DHH-B)		2335 ± 193
DHH-B	0·2 mg kg^{-1}	2385 ± 116
	1·0 mg kg^{-1}	2422 ± 211
Vehicle (DHH-B) + vehicle (diazepam)		2255 ± 248
Vehicle (DHH-B) + diazepam 1·0 mg kg^{-1}		4614 ± 202**
DHH-B 0·2 mg kg^{-1} + diazepam 1 mg kg^{-1}		4166 ± 470**
DHH-B 1·0 mg kg^{-1} + diazepam 1 mg kg^{-1}		3240 ± 213*,‡

DHH-B and diazepam were administered 3 h and 10 min, respectively, before the challenge with hexobarbital-Na (100 mg kg^{-1}, i.p.). Values are mean ± s.e.m. of 10 mice. *$P < 0.05$, **$P < 0.01$ vs the vehicle (DHH-B) + vehicle (diazepam)-treated group; ‡$P < 0.01$ vs vehicle (DHH-B) + diazepam-treated group.

Table 3. Effects of oral DHH-B and diazepam on transfer latency in training and retention trials.

Treatment		Training (s)	Retention (s)
Administration before the training trial			
Vehicle		34·7 ± 7·2	16·3 ± 6·6
DHH-B	1·0 mg kg^{-1}	36·3 ± 4·7	16·7 ± 3·3
	5·0 mg kg^{-1}	41·1 ± 6·1	8·6 ± 0·9
Vehicle		37·6 ± 3·9	15·2 ± 3·4
Diazepam	1·0 mg kg^{-1}	20·5 ± 3·2**	18·7 ± 4·9
	2·0 mg kg^{-1}	20·5 ± 3·1**	30·3 ± 2·8*
Administration before the retention trial			
Vehicle		36·0 ± 6·9	13·2 ± 3·8
DHH-B	1·0 mg kg^{-1}	27·0 ± 3·0	12·1 ± 1·4
	5·0 mg kg^{-1}	40·6 ± 8·0	23·5 ± 4·2*
Vehicle		32·2 ± 3·8	15·2 ± 3·4
Diazepam	1·0 mg kg^{-1}	38·9 ± 4·9	16·0 ± 1·7
	2·0 mg kg^{-1}	35·1 ± 6·9	20·0 ± 3·8

DHH-B and diazepam were administered 3 h and 10 min, respectively, before either the training or retention trial. Values are mean ± s.e.m. of 10 mice. *$P < 0.05$, **$P < 0.01$ vs the vehicle-treated group.

its vehicle was followed by very mild hyper-reactivity in 1–4 out of 10 mice. However, there was no significant difference in the occurrence among the treatments, whereas in the mice treated with diazepam, the challenge with flumazenil resulted in precipitated withdrawal symptoms in a dose-dependent manner; the occurrences of hyper-reactivity and tail-flick reaction were significantly higher in the groups treated with diazepam at 1 and 5 mg kg^{-1}, respectively. The level of hyper-reactivity was much more severe in the diazepam-treated mice than in the mice treated with DHH-B or its vehicle. Some mice treated with diazepam showed tremors, clonic convulsions or running fits following the challenge with flumazenil, although the occurrences did not reach the significant level.

Discussion

This study revealed that, unlike diazepam, DHH-B did not disrupt the traction performance. This result suggests that DHH-B use has a low risk of motor dysfunction and/or ataxia, which are frequently induced by benzodiazepine anxiolytics (Kuribara et al 1977).

Generally, benzodiazepine anxiolytics depress the central nervous system, and this effect is easily detectable as an enhancement of barbiturate- or ethanol-induced sleeping. Thus, the combined use (or abuse) of benzodiazepine anxiolytics with ethanol or barbiturate increases the risk of the induction of deep (sometimes fatal) central depression (Schweizer et al 1995). In this study, 1 mg kg^{-1} diazepam potentiated hexobarbital-induced sleeping whereas 0·2 and 1 mg kg^{-1} DHH-B scarcely affected hexobarbital-induced sleeping, suggesting that DHH-B has no (or a very weak) central depressant effect. Interestingly, 1 mg kg^{-1} DHH-B significantly reduced the potentiation by diazepam of hexobarbital-induced sleeping.

We have reported that the combined administration of 1 mg kg^{-1} DHH-B and 1 mg kg^{-1} diazepam enhanced the anxiolytic effect, and that the anxiolytic effect of DHH-B was inhibited by the benzodiazepine receptor antagonist flumazenil (Kuribara et al 2000), clearly indicating that the $GABA_A$–benzodiazepine receptor complex is responsible for the development of the anxiolytic effect of DHH-B. Currently, $GABA_A$–benzodiazepine receptor heterogeneity is considered to be involved in the individual pharmacological and side effects of benzodiazepine anxiolytics (Gardner et al 1993; Lüddens et al 1995). Taken together, there is a possibility that the characteristics of action of DHH-B on the $GABA_A$–benzodiazepine receptor complexes are different from those of benzodiazepine anxiolytics. This consideration may be supported by the other behavioural evidence.

Benzodiazepine anxiolytics sometimes induce amnesia through disruption of the input of information into the store site rather than through memory recall (Haefely et al 1990; Doble & Martin 1992; Schweizer et al 1995). In agreement with such considerations, the pre-training, but not pre-retention, treatment with 2 mg kg^{-1} diazepam caused significant prolongation in the transfer latency in the retention trial, indicating an induction of amnesia. The pre-training treatment with diazepam shortened the transfer latency in the training trial. This behavioural change was almost the same as that previously reported (Kuribara et al 1999), probably partially reflecting a behavioural excitation induced by suppression of the prefrontal cortex function, the disinhibition by diazepam. Different from the effects of diazepam, the pre-retention treatment with 5 mg kg^{-1} DHH-B significantly prolonged the transfer latency in the

Table 4. Number of mice showing symptoms after the challenge with flumazenil.

Treatment (10 days)		HR	TR	CC	TC	TF	RF
Vehicle		4/10	0/10	0/10	0/10	0/10	0/10
DHH-B	0·2 mg kg^{-1}	1/10	0/10	0/10	0/10	0/10	0/10
	1·0 mg kg^{-1}	3/10	0/10	0/10	0/10	0/10	0/10
	5·0 mg kg^{-1}	3/10	0/10	0/10	0/10	0/10	0/10
Vehicle		0/10	0/10	0/10	0/10	1/10	0/10
Diazepam	1·0 mg kg^{-1}	5/10**	1/10	0/10	0/10	0/10	3/10
	5·0 mg kg^{-1}	8/10**	4/10	1/10	0/10	8/10**	1/10

Vehicle, DHH-B and diazepam were administered p.o. once a day for 10 days, and the challenge with flumazenil (10 mg kg^{-1} i.p.) was performed 24 h after the last administration. The figures presented are the numbers of mice that showed the symptoms during the observation period for 30 min. HR = Hyper-reactivity (vocalization induced by light pushing of the back); TR = tremor; CC = clonic convulsion; TC = tonic convulsion; TF = tail-flick or tail reaction; RF = running fit (wild running evoked by keyring sound). *$P < 0·05$, **$P < 0·01$ vs the vehicle-treated group.

retention trial. It is probable that the anxiolytic effect of DHH-B was responsible for this. However, this group of mice demonstrated the longest latency time among the groups in the training trial, suggesting another possibility: the change after the pre-retention treatment with $5\,mg\,kg^{-1}$ DHH-B occurred accidentally. On the other hand, the pre-training treatment with DHH-B caused no significant change in the transfer latencies in both trials, indicating that DHH-B has much less risk of induction of amnesia or disinhibition compared with benzodiazepine anxiolytics during the treatment of anxiety disorders.

Flumazenil can rapidly inhibit almost all the acute pharmacological effects of benzodiazepine anxiolytics. When mice are physically dependent on benzodiazepine anxiolytics, the challenge with flumazenil is followed by precipitated withdrawal symptoms characterized by behaviours that are opposite to those reflecting the acute pharmacological effects of benzodiazepine anxiolytics (Cumin et al 1982; McNicholas & Martin 1982; Wilson & Gallager 1988; Martinez et al 1992; Martin et al 1993; Jing et al 1995). In this study, similar to the previous reports in rats (Jing et al 1995) and mice (Kuribara et al 1999), 4 out of 10 mice treated with a vehicle of DHH-B, but not diazepam, exhibited very mild excitation, showing hyper-reactivity, after the challenge with flumazenil. Such behavioural excitation may be caused by an anxiogenic effect of a comparatively higher dose of flumazenil (Lee & Rodgers 1991). The 10 daily treatments with 1 and $5\,mg\,kg^{-1}$ diazepam were responsible for the production of flumazenil-induced precipitated withdrawal symptoms characterized by hyper-reactivity and tail-flick/tremor. The level of hyper-reactivity in the diazepam-treated mice was much more severe than in the mice treated with DHH-B or its vehicle, indicating that this behavioural change reflected the abstinence symptom of diazepam rather than the anxiogenic effect of flumazenil. Tremors, running fits and clonic convulsions were also observed in small numbers of mice treated with diazepam. These results indicate that physical dependence on diazepam is induced after long-term use of diazepam even at near-therapeutic doses for anxiety disorder whereas the mice treated with DHH-B, even at $2\,mg\,kg^{-1}$, 10 times as high as the minimum dose for anxiolytic effect, did not show any withdrawal symptoms after the challenge with flumazenil, indicating that DHH-B has a low risk for induction of benzodiazepine-like physical dependence.

In conclusion, it is thought that DHH-B, a potent anxiolytic compound, has a lower risk of production of the unwanted side effects that are frequently produced by benzodiazepine anxiolytics. The mechanism of action of DHH-B has not been clearly determined. However, there is a possibility that DHH-B has a unique characteristic action on the $GABA_A$–benzodiazepine receptor subtype(s) that are responsible for the side effects of benzodiazepine anxiolytics.

Acknowledgement

We thank Hoffmann-La Roche Inc. (Nutley, NJ) for the generous gift of flumazenil.

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