An Anxiolytic-Like Effect of *Ginkgo biloba* Extract and Its Constituent, Ginkgolide-A, in Mice

Hisashi Kuribara,[†] Susan T. Weintraub,[‡] Tatsumi Yoshihama,[§] and Yuji Maruyama*[,†]

Center for Cooperative Research, Medical Division, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan, Department of Biochemistry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas, 78229-3900, Wako-kagaku Co., 3-2-3 Tonya-machi, Takasaki, Gunma 370-0006, Japan, and Research Division for Assessment of Complementary Medicine (NPO), 3-10-3 Ton-ya machi (6F-610), Takasaki, Gunma 370-0006, Japan

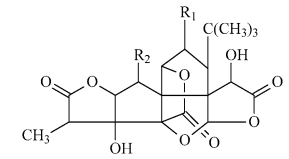
Received March 19, 2003

The anxiolytic-like effects of *Ginkgo biloba* extract (GBE) and its four terpenoid components (ginkgolide-A, ginkgolide-B, ginkgolide-C, and bilobalide) were assessed using the elevated plus-maze test in mice. Administration of GBE as a single oral dose (0.5 or 1 g/kg, po) caused a state of suppressed motor activity and, thus, shortened the time spent in the open-sided arms. However, when GBE (0.063−1 g/kg, po) was administered daily for 7 days and the plus-maze test was carried out 24 h after the final administration, the time spent in the open-sided arms was prolonged, with the peak anxiolytic-like effect at 0.125 g/kg. A combination of seven-day administration of GBE (0.125 g/kg) and a single dose of diazepam (1 mg/ kg, po, 10 min before testing) enhanced the anxiolytic-like effect. Flumazenil (0.3 mg/kg, ip, 10 min before testing) blocked the effect of diazepam, but not of GBE. Daily administration of ginkgolide-A (1 or 2 mg/kg, po) resulted in an anxiolytic-like effect by the third treatment, with the maximal effect observed after the fifth administration. Neither ginkgolide-B, ginkgolide-C, nor bilobalide produced any anxiolytic-like effects. At doses higher than 0.5 g/kg, GBE not only inhibited motor activity but also suppressed active avoidance behavior, reduced caffeine-induced stimulation, and enhanced pentobarbital-induced sleep, while ginkgolide-A (up to 20 mg/kg) did not exhibit these effects. Diazepam (1 mg/kg) is known to enhance pentobarbital-induced sleep. These results suggest that GBE produces a significant anxiolytic-like effect following repeated administration and that ginkgolide-A is most likely responsible for this effect. There are also indications that although GBE exerts a sedative effect at comparatively higher doses, ginkgolide-A has a relatively weak tendency to produce benzodiazepine-like side effects.

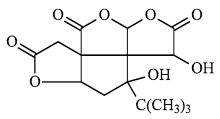
Ginkgo biloba extract (GBE), a plant extract obtained from *Ginkgo biloba* L. (Ginkgoaceae), is standardized to contain 24% ginkgo-flavoglycosides and 6% ginkgo-terpenoidlactones. This extract is now used as one of the leading herbal medicines in both Germany and France and is a widely utilized herbal supplement in Japan and the United States. Preclinical evaluations revealed that GBE exerts profound and widespread tissue effects, including membrane stabilization, and acts as an antioxidant and free radical scavenger.[1] These effects are achieved in a variety of ways, for example, through stimulation of endothelium-derived relaxing factor, prostacyclin inhibition of platelet aggregation and adhesion, and enhancement of degranulation.[1−3]

Since the 1970s there have been many human studies on GBE. Of interest, the extract has been found to be effective for the treatment of cerebral vascular insufficiency.[4−6] This condition is common in old age and is believed to be due to ischemia and a resultant decrease in oxygen levels in brain cells. Furthermore, the actions of GBE on the vascular systems have generally been considered to be effective for treatment of the signs, symptoms, and underlying pathophysiology of peripheral arterial insufficiency.[7−9] GBE is particularly effective for treatment of intermittent claudication,[10] a condition characterized by development of severe pain in the lower limbs associated with atherosclerosis and an increase in the production of toxic metabolites and cellular free radicals during exercise.

The central effects of GBE have also been investigated. Behavioral studies on herbal preparations containing GBE and GBE-related compounds have demonstrated improvement of memory,[11,12] an anxiolytic-like effect,[13−16] an antidepressant-like effect,[17] and a shortening of barbiturate-induced sleep.[18,15] However, because of the differences in experimental procedures as well as in the preparations of GBE, doses, schedules and routes of administration, and animals, it is difficult to arrive at a consensus about the central effects of GBE and its constituents.



	R_1	R_2
Ginkgolide-A	H	H
Ginkgolide-B	H	OH
Ginkgolide-C	OH	OH



Bilobalide

Figure 1. Structures of ginkgolide analogues and bilobalide.

In this study, we assessed the anxiolytic-like potentials of GBE and ginkgo-terpenoids (ginkgolide-A, ginkgolide-B, ginkgolide-C, and bilobalide; Figure 1). It was revealed that ginkgolide-A was responsible for the anxiolytic-like effect of GBE and that this compound did not have a pronounced tendency to produce benzodiazepine-like side effects.

* Corresponding author. Tel and Fax: 81-27-362-6004. E-mail: yuji_1@guitar.ocn.ne.jp.
[†] Gunma University.
[‡] The University of Texas Health Science Center at San Antonio.
[§] Wako-kagaku Co.

10.1021/np030122f CCC: $25.00
Published on Web 09/30/2003

Table 1. Effects of Single Administration of *Ginkgo biloba* Extract (GBE) and Diazepam Assessed by the Elevated Plus-Maze and Activity Tests in Mice

treatment[a]	time in open-sided arms (s)[b]	activity (counts/5 min)[b]
vehicle	5.7 ± 1.4	40.6 ± 4.8
GBE		
0.25 g/kg	5.4 ± 2.8	38.0 ± 4.9
0.5 g/kg	0.3 ± 0.2*	18.6 ± 2.0*
1.0 g/kg	0.8 ± 0.6*	17.8 ± 2.6*
vehicle	5.1 ± 1.5	44.7 ± 3.4
diazepam		
1.0 mg/kg	37.6 ± 9.6*	37.1 ± 2.3

[a] Drugs were administered orally at the following times before evaluation on the elevated plus-maze: GBE, 1 h; diazepam, 10 min. The activity test was conducted immediately after the plus-maze test. [b] Values represent the mean ± standard error of the mean for 10 mice per treatment group; statistical significance compared to the corresponding vehicle-treated group was assessed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test: *, $P < 0.01$ vs the vehicle-treated group.

Table 2. Effects of 7-Day Administration of *Ginkgo biloba* Extract (GBE) Assessed by the Elevated Plus-Maze and Activity Tests in Mice

treatment[a]		time in open-sided arms (s)[b]	activity (counts/5 min)[b]
drug	*N*		
vehicle	10	5.2 ± 2.2	32.3 ± 9.7
GBE			
0.063 g/kg	10	10.5 ± 2.8	24.4 ± 3.1
0.125 g/kg	10	12.5 ± 2.6**	30.2 ± 3.0
0.25 g/kg	10	9.0 ± 3.8	31.2 ± 3.9
0.5 g/kg	10	8.1 ± 3.0	25.0 ± 2.4
1.0 g/kg	5	6.0 ± 5.3	25.4 ± 4.8
vehicle	10	5.1 ± 1.5	44.7 ± 3.4
GBE (0.125 g/kg)	10	13.7 ± 4.8*	29.8 ± 3.3*
diazepam (1.0 mg/kg)	10	24.7 ± 6.0**	43.1 ± 4.6
flumazenil (0.3 mg/kg)	10	5.5 ± 2.5	31.5 ± 3.0
GBE + diazepam	10	55.1 ± 9.8$,#,**	52.3 ± 8.7
GBE + flumazenil	10	12.9 ± 3.9*	38.3 ± 5.1
diazepam + flumazenil	10	7.4 ± 3.0#	31.7 ± 5.0

[a] GBE or vehicle was administered orally for 7 days. The behavioral tests were carried out 24 h after the final (seventh) drug administration; *N*, number of mice per treatment group. The activity test was conducted immediately after the plus-maze test. [b] Values represent the mean ± standard error of the mean; statistical significance compared to the indicated treatment group was assessed by a one- or two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test: * and **, $P < 0.05$ and 0.01, respectively, vs the vehicle-treated control group; $, $P < 0.01$ vs the GBE-treated group (0.125 g/kg); #, $P < 0.01$ vs the diazepam-treated group.

Results and Discussion

Treatment with GBE. As shown in Table 1, a single administration of GBE (0.5 or 1 g/kg, but not 0.25 g/kg) suppressed motor activity and, as a consequence, shortened the time spent in the open-sided arms of the plus-maze. In contrast, it can be seen in Table 2 that administration of GBE for 7 days resulted in a significant increase in the time spent in the open-sided arms, with the peak anxiolytic-like effect observed at 0.125 g/kg. This drug treatment did not result in any significant changes in motor activity.

Combined Administration of GBE, Diazepam, and Flumazenil. The effects of combining a 7-day GBE treatment with a single administration of either diazepam or flumazenil are shown in Table 2. The combination of GBE and diazepam resulted in a significant enhancement of the anxiolytic-like effect. Although flumazenil caused no significant change in the time spent in the open-sided arms, it completely inhibited the anxiolytic-like effect of diazepam. In contrast, the anxiolytic-like effect of GBE was not modified by flumazenil.

Table 3. Effects of Four Terpenoids in *Ginkgo biloba* Extract Assessed by the Elevated Plus-Maze and Activity Tests in Mice

treatment[a]			time in open-sided arms (s)[b]	activity (counts/5 min)[b]
drug	days	*N*		
vehicle	1	10	5.7 ± 1.4	40.6 ± 4.8
ginkgolide-A				
1 mg/kg	1	10	6.1 ± 2.1	35.5 ± 3.1
2 mg/kg	1	10	5.9 ± 3.1	35.4 ± 3.3
vehicle	7	30	5.4 ± 1.1	31.0 ± 2.7
ginkgolide-A				
1 mg/kg	7	30	18.6 ± 3.0*	35.3 ± 2.6
2 mg/kg	7	20	17.4 ± 2.0*	29.5 ± 2.2
ginkgolide-B				
1 mg/kg	7	10	7.3 ± 2.6	31.0 ± 3.2
ginkgolide-C				
1 mg/kg	7	10	6.7 ± 2.2	33.7 ± 4.6
bilobalide				
1 mg/kg	7	20	6.1 ± 1.6	27.9 ± 2.5
2 mg/kg	7	10	7.9 ± 1.9	29.9 ± 4.5
vehicle	5	10	6.3 ± 2.5	38.4 ± 4.9
ginkgolide-A				
1 mg/kg	1	10	7.6 ± 2.4	39.1 ± 3.5
1 mg/kg	2	10	10.5 ± 2.5	31.6 ± 3.4
1 mg/kg	3	10	12.7 ± 2.9*	35.5 ± 3.5
1 mg/kg	5	10	17.8 ± 4.7*	40.1 ± 5.0
1 mg/kg	7	10	16.9 ± 2.2*	34.4 ± 3.3

[a] Test agents or vehicle was administered orally for the indicated number of days. Behavioral testing was conducted 1 h after drug administration for animals receiving a single dose and 24 h after the final drug administration for all others; *N*, number of mice per treatment group. The activity test was conducted immediately after the plus-maze test. [b] Values represent the mean ± standard error of the mean; statistical significance compared to the indicated treatment group was assessed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test: *, $P < 0.05$ vs the vehicle-treated group.

Terpenoids in GBE. As shown in Table 3, 7-day administration of ginkgolide-A produced an anxiolytic-like effect without affecting motor activity. However, no activity was detected after treatment with ginkgolide-B, ginkgolide-C, or bilobalide. The effect of ginkgolide-A was seen by the third administration and achieved the highest level by the fifth administration. However, acute treatment with ginkgolide-A was ineffective in producing an anxiolytic-like effect.

Pentobarbital-Induced-Sleep Test. As shown in Table 4, GBE (1 g/kg) and diazepam (1 mg/kg) each enhanced pentobarbital-induced sleep. However, ginkgolide-A did not change the sleep time, even at 20 mg/kg.

Discrete Shuttle Avoidance Test. As can be seen in Table 5, treatment with 1 and 2 g/kg GBE significantly inhibited avoidance behavior, resulting in a significant decrease in the number of shuttles and/or an increase in the number of shocks. However, at doses up to 5 mg/kg, ginkgolide-A did not influence avoidance behavior. As shown in Table 6, GBE (1 g/kg) completely abolished the caffeine-induced increase in avoidance. The inhibitory effect of GBE was not affected by diazepam. Conversely, ginkgolide-A exerted no interaction with either caffeine or diazepam.

General Observations. One to 3 h after the administration of 0.5−2 g/kg GBE, mice exhibited the characteristics of weak sedation. In contrast, ginkgolide-A, even at 20 mg/kg, caused neither sedation nor excitation. Diazepam, at 1 mg/kg, prompted light ataxia and muscle relaxation. The repeated administration of GBE or ginkgolide-A did not affect body weight gain.

The validity of the plus-maze test for evaluation of anxiolytic-like effects of drugs has been well docu-

Table 4. Effects of *Ginkgo biloba* Extract (GBE), Ginkgolide-A, and Diazepam on the Pentobarbital (50 mg/kg ip)-Induced Sleep in Mice

treatment[a]	sleep time (s)[b]
vehicle	3042 ± 312
GBE	3337 ± 306
0.063 g/kg	
0.125 g/kg	3504 ± 513
0.25 g/kg	3573 ± 440
0.5 g/kg	3783 ± 393
1.0 g/kg	4345 ± 395*
vehicle	3137 ± 596
ginkgolide-A	3272 ± 186
0.5 mg/kg	
1 mg/kg	2831 ± 355
2 mg/kg	3507 ± 309
5 mg/kg	3695 ± 550
10 mg/kg	3694 ± 467
20 mg/kg	3491 ± 363
vehicle	3213 ± 426
diazepam	
1 mg/kg	6358 ± 483 *

[a] GBE and ginkgolide-A were administered orally 30 min before the pentobarbital challenge, and diazepam was given 10 min before. [b] Interval between loss and recovery of righting reflex; values represent the mean ± standard error of the mean for 10 mice per treatment group; statistical significance compared to the corresponding vehicle-treated group was assessed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test: *, $P < 0.01$ vs the vehicle-treated group.

Table 5. Effects of *Ginkgo biloba* Extract (GBE) and Ginkgolide-A on the Discrete Shuttle Avoidance Test in Mice

treatment[a]	shuttles/h[b]	shocks/h[b]
vehicle	218.2 ± 20.8	1.90 ± 0.31
GBE		
0.125 g/kg	199.5 ± 20.5	0.60 ± 0.22
0.25 g/kg	182.9 ± 12.8	2.00 ± 0.33
0.5 g/kg	185.5 ± 15.8	2.20 ± 0.53
1 g/kg	156.4 ± 6.0**	2.20 ± 0.63
2 g/kg	149.7 ± 6.5**	6.10 ± 2.73*
vehicle	178.0 ± 10.4	0.90 ± 0.28
ginkgolide-A		
1 mg/kg	182.8 ± 14.8	1.60 ± 0.52
5 mg/kg	162.9 ± 8.5	1.10 ± 0.35

[a] GBE and ginkgolide-A were administered orally 30 min before the avoidance test. [b] Values represent the mean ± standard error of the mean for 10 mice per treatment group; statistical significance compared to the corresponding vehicle-treated group was assessed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test: *, $P < 0.05$; **, $P < 0.01$ vs the vehicle-treated group.

mented.[19−21] As expected, we found that treatment with diazepam, a benzodiazepine anxiolytic drug, led to a significant increase in the time a mouse spent in the open-sided arms of the plus-maze. It has been reported that acute administration of GBE combined with an extract of *Zingiber officinale*[14,15] or acute treatment with ginkgolic acid conjugates of Indian *G. biloba*[16] produces an anxiolytic-like effect in the elevated plus-maze test. However, in our experiments, acute administration of 0.25−1 g/kg GBE did not produce an anxiolytic-like effect but, rather, was sedating at doses above 0.5 g/kg. We conclude, therefore, that a single administration of GBE alone is not responsible for development of any anxiolytic-like effects. On the other hand, the present study revealed that 7-day administration of GBE (0.063−0.125 g/kg) resulted in an anxiolytic-like effect without causing any marked change in motor activity. At 0.125 g/kg, the GBE used in these experiments is equivalent to administration of 2.25 mg/kg ginkgolide-A, 1.06 mg/kg ginkgolide-B, 1.36 mg/kg ginkgolide-C, and 2.9 mg/kg bilobalide (Tokiwa Plant Chemical Institute, personal communication). Nerdner and Cathadi[22] reported anxiolytic-like activity for 5−20 mg/kg bilobalide following acute administration in rats. However, our experiments using mice did not confirm their findings. Ginkgolide-A (1 or 2 mg/kg) produced almost the same anxiolytic-like effect as that of 0.125 g/kg GBE. On the other hand, neither ginkgolide-B (1 mg/kg), ginkgolide-C (1 mg/kg), nor bilobalide (1 or 2 mg/kg) exhibited anxiolytic-like activity. When ginkgolide-A was administered daily, a significant anxiolytic-like effect was seen by the third administration. These results clearly indicate that ginkgolide-A is responsible for the anxiolytic-like effect of GBE. However, accumulation of ginkgolide-A during repeated administration is unlikely based on a pharmacokinetic study in rats where it was found that the half-life of ginkgolide-A was approximately 1.5 h.[23] The results presented here are analogous to the anxiolytic-like effects we previously reported for Saiboku-to, a Kampo medicine,[24,25] and its active constituent, honokiol.[26]

Table 6. Combined Effects of *Ginkgo biloba* Extract (GBE) and Ginkgolide-A with Caffeine and Diazepam on the Discrete Shuttle Avoidance Test in Mice

treatment[a]	shuttles/h[b]	shocks/h[b]
vehicle	199.8 ± 6.2	2.40 ± 0.29
GBE (1 g/kg)	130.8 ± 4.2*	11.30 ± 4.75*
caffeine (10 mg/kg)	428.9 ± 26.6*	3.40 ± 1.29
diazepam (1 mg/kg)	210.8 ± 17.1	3.20 ± 0.81
GBE + caffeine	178.0 ± 8.7[#]	6.10 ± 3.46
GBE + diazepam	146.4 ± 6.9*,[$]	9.80 ± 4.11*
vehicle	191.8 ± 8.0	1.32 ± 0.22
ginkgolide-A (1 mg/kg)	182.8 ± 14.8	1.60 ± 0.52
caffeine (10 mg/kg)	378.6 ± 42.4*	0.40 ± 0.31
diazepam (1 mg/kg)	181.4 ± 11.0	4.60 ± 2.23
ginkgolide-A + caffeine	348.0 ± 34.9*	0.40 ± 0.40
ginkgolide-A + diazepam	207.8 ± 13.7	2.00 ± 0.56

[a] GBE and ginkgolide-A were administered orally 30 min before the avoidance test, and caffeine and diazepam were given immediately before the test. [b] Values represent the mean ± standard error of the mean for 10 mice per treatment group; statistical significance compared to the corresponding vehicle-treated group was assessed by a one- or two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test: *, $P < 0.01$ vs the vehicle-treated group; [#], $P < 0.01$ vs the caffeine-treated group; [$], $P < 0.01$ vs the diazepam-treated group.

Administration of 0.25−1 g/kg GBE for 7 days produced less anxiolytic-like effect than 0.125 g/kg, while at the same time, no alterations in motor activity were observed for any of the doses. It is, therefore, doubtful that the observed decrease in anxiolytic-like activity with increasing dose is caused by nonspecific influence on motor activity. It is conceivable that unknown chemical(s) that have anxiogenic-like and/or antagonistic effects are present in the GBE preparation. Further study is required to examine this possibility.

At doses lower than 1 mg/kg, flumazenil, a benzodiazepine receptor antagonist, generally produces no marked effects, but it is able to reverse almost all of the pharmacological consequences of benzodiazepine anxiolytics.[27] The antagonistic relationship between diazepam and flumazenil was confirmed in our studies. There are two benzodiazepine receptor subtypes—central and peripheral—which are coupled and uncoupled, respectively, with the $GABA_A$-chloride ionophore complex.[28] It has been reported that GBE and ginkgolide-B interact with peripheral-type benzodiazepine receptors.[29,30] However, our experiments showed that flumazenil did not inhibit the anxiolytic-like effect of GBE. It is therefore highly probable that central-type benzodiazepine receptors are not involved in the anxiolytic-like effect of GBE even though a significant enhancement

of the anxiolytic-like effect was produced by the combined treatment of GBE and diazepam.

The known central depressive effect of diazepam can be demonstrated by the significant enhancement of pentobarbital-induced sleep at doses that are effective for anxiolytic-like effects. Consistent with our previous report,[31] in our current studies, diazepam did not suppress either motor activity or avoidance behavior and, furthermore, did not inhibit caffeine-induced increases in avoidance. A sedative effect produced by acute administration of relatively high doses of GBE was indicated by the suppression of motor activity and shuttle avoidance, potentiation of pentobarbital-induced sleep, and inhibition of caffeine-induced avoidance stimulation. However, it should be noted that the minimum dose for the sedative effect of GBE was 0.5 g/kg; that is, it was 4 times higher than the dose required to produce an anxiolytic-like effect following repeated administration. It is, therefore, possible that GBE might have a slight tendency to produce benzodiazepine-like side effects when used for treatment of anxiety-related symptoms. In contrast, ginkgolide-A did not cause sedation at doses up to 20 mg/kg, 20 times higher than the dose needed for anxiolytic-like efficacy. Observation of the mice indicated no abnormal signs following administration of ginkgolide-A. Thus, it can be expected that ginkgolide-A might exert a selective anxiolytic effect without producing benzodiazepine-like side effects such as sedation, muscle relaxation, ataxia, and/or enhancement of central-depressive drugs[32] if it is used for the treatment of anxiety-related symptoms.

Experimental Section

Animals. Male ddY mice (7 weeks old, 33−36 g; Japan SLC, Hamamatsu, Japan) were used for all experiments. The mice were randomly chosen and housed in groups of 10 in polycarbonate cages (20 cm × 30 cm × 15 cm with woodchip bedding). A standard solid diet (MF; Oriental Yeast Co., Tokyo) and tap water were freely given to the mice. The breeding room was carefully controlled for environmental conditions as follows: temperature, 23 ± 1 °C; relative humidity, 55 ± 3%; and a 12-h light-dark cycle (lights on at 7:00 a.m. and off at 7:00 p.m.). All 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". In each experiment described below, 5−30 drug-naive mice were used; all experimental treatments were carried out between 9:00 a.m. and 3:00 p.m. Animals used to assess anxiolytic-like activity on the elevated plus-maze were also subjected to activity testing on the tilting ambulometer, as described below. Separate groups of animals were employed for the pentobarbital-induced sleep tests. For both of these studies, each animal was used only once. For the discrete shuttle avoidance tests, two groups of well-trained mice were used repeatedly for the evaluation of drug effects. The first group was utilized for testing the single-administration regimens of GBE and ginkgolide-A (Table 5), and the second group for combined administration of GBE or ginkgolide-A with caffeine or diazepam (Table 6). Shuttle avoidance testing was conducted on Tuesdays and Fridays, in the order shown in Tables 5 and 6, respectively, with 10 mice tested each day.

Drugs. The crude extract of *G. biloba* L. used in our experiments (abbreviated as GBE) was prepared by hot water extraction of *G. biloba* leaves (4.5 mL water/1 g leaves) and contained 24% flavonoid glycosides, 6% terpene lactones (bilobalide, 2.32%; ginkgolide A, 1.80%; ginkgolide B, 0.85%; and ginkgolide C, 1.09%), and less than 1 ppm ginkgolic acids. This extract was purchased from Tokiwa Plant Chemical Institute (Sakura, Japan) as Ginkolon-24. We have found that GBE yields reproducible anxiolytic effects when stored for more than 2 months in aqueous solution at 4 °C. Ginkgolide-A (98+ %), ginkgolide-B (98+ %), ginkgolide-C (98+ %), and bilobalide (98+ %) were also obtained from Tokiwa. These GBE components were isolated by carbon chromatography of the hot water leaf extract, with elution by hot acetone solution, and purified by extraction and crystallization, as described.[33,34] Diazepam (Cercine Inj.; Takeda Chem. Ind., Osaka, Japan) and pentobarbital-Na (Nembutal Inj.; Abbott Laboratories, North Chicago, IL) were commercial preparations. Flumazenil was a generous gift from Hoffman-La Roche (Nutley, NJ).

GBE, ginkgolide-A, ginkgolide-B, ginkgolide-C, and bilobalide were suspended in distilled water with Arabic gum (0.1%). The injectable preparations of diazepam and pentobarbital-Na were diluted with physiological saline. Flumazenil was suspended in Arabic gum (0.1%)/physiological saline. The concentration of each drug solution or suspension was adjusted so that the volume administered was 0.1 mL/10 g mouse body weight. The animals in control groups were given the same volume of the corresponding vehicles.

Elevated Plus-Maze Test. The elevated plus-maze used here was the same as that used in our previous studies[24,25] and is a slightly modified version of the original device used for rats[35] and mice.[36] In our apparatus, two of the opposing arms have side walls (6 cm W × 30 cm L × 10 cm H) and like the central platform (8 × 8 cm) are painted a nontransparent gray color; the other opposing arms (6 cm W × 30 cm L) have transparent floors and no side walls. For testing, a mouse was placed on the central platform facing one of the closed-sided arms, and the cumulative time spent in the open-sided arms during the ensuing 5-min period was recorded by a trained observer. A mouse was defined as having entered an open-sided arm if all four paws crossed the border between the central platform and the open-sided arm.

Drug Administration. For experiments testing the effects of a single administration of GBE, the following doses were used: GBE, 0.25−1 g/kg, po; diazepam, 1 mg/kg, po. In accordance with the previous reports on the pharmacokinetic behavior of ginkgolides and bilobalide,[23] GBE was administered 1 h and diazepam 10 min before the plus-maze test. To evaluate the effects of a 7-day course of oral administration of test substance, the doses employed were as follows: GBE, 0.063−1 g/kg/day; ginkgolide-A, 1 or 2 mg/kg; ginkgolide-B, 1 mg/kg; ginkgolide-C, 1 mg/kg; and bilobalide, 1 or 2 mg/kg. In addition, the administration of ginkgolide-A (1 mg/kg) for 1, 2, 3, 5, and 7 days was also evaluated. The plus-maze test was carried out 24 h after the final drug administration.

Experiments were also conducted in which mice were treated for 7 days with GBE (0.125 g/kg, po) followed by a single dose of either diazepam (1 mg/kg, po; 10 min before testing) or flumazenil (0.3 mg/kg, ip; 10 min before testing). Single treatments with either diazepam or flumazenil and a combination of diazepam and flumazenil were also used.

Activity Test. The apparatus utilized was a tilting-type ambulometer with a bucket-style activity cage of 25 cm in diameter (SMA-1: O'Hara & Co., Tokyo, Japan) which selectively detects horizontal movement of the mouse. The ambulatory activity of each mouse was measured for 5 min immediately after the end of the plus-maze test.

Pentobarbital-Induced-Sleep Test. Mice were first orally treated with either GBE (0.063−1 g/kg, 30 min before), ginkgolide-A (0.5−20 mg/kg, 30 min), or diazepam (1 mg/kg, 10 min) followed by administration of pentobarbital-Na (50 mg/kg, ip). Subsequently, the time between loss and recovery of the righting reflex was recorded, and the difference was defined as the sleep time.

Discrete Shuttle Avoidance Test. Five separate units, each consisting of an experimental chamber (30 cm L × 9 cm W × 15 cm H; GT-8450; O'Hara & Co., Tokyo), a control module (De Cares GT-M5; O'Hara & Co.), and a data recording system (TIDP-10; O'Hara & Co.), were used for these experiments. In this way, five mice could be subjected to the avoidance test at the same time. The shuttle avoidance test[37] is a measure of learned behavior in which the test animal is trained to move from one side of the test chamber to the other in order to avoid a foot shock which is delivered at a specific

interval after an audible tone. For our studies, each avoidance trial consisted of the following: (1) a warning period of 5 s during which an 800 Hz tone signal was sounded; (2) a shock period of 1 s (100 V, 0.3 mA, 50 Hz AC); and (3) an inter-trial period of 24 s. In each session of 60 min, 120 avoidance trials were conducted, and the indices of avoidance behavior determined based on the number of shuttles (movement of the mouse completely across the chamber) and the number of shocks. Assessment of drug effects was carried out at intervals of 3−4 days using well-trained mice which consistently exhibited a stable number of shuttles (150−250/h) and a low level of shocks (less than 10) in each session of 120 trials. If the avoidance behavior was not deemed to be stable on the day before drug administration, the experiment was postponed for at least 3 days. Two different treatment regimens were evaluated. (1) Single administration was investigated in which GBE (0.125−2 g/kg, po) or ginkgolide-A (1 or 5 mg/kg, po) was administered 30 min before the avoidance test. When caffeine (10 mg/kg, po) or diazepam (1 mg/kg, po) was used, they were given immediately before testing. Control mice received the vehicle alone. (2) Combined administration was evaluated in which mice were treated with an additional drug after oral administration, as follows: GBE (1 g/kg) + caffeine (10 mg/kg); GBE + diazepam (1 mg/kg); ginkgolide-A (1 mg/kg) + caffeine (10 mg/kg); and ginkgolide-A + diazepam (1 mg/kg).

Statistical Analysis. Statistical significance was assessed through use of one- or two-way analysis of variance (ANOVA), followed by a Student-Neuman-Keuls test. Values of P less than 0.05 were considered significant.

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

References and Notes

(1) Corrigan, D. *Eur. J. Herb. Med.* **1995**, *1*, 16−18.
(2) McKenna, D. J.; Jones, K.; Hughes, K. *Altern. Ther.* **2001**, *7*, 70−90.
(3) White, H. L.; Scates, P. W.; Cooper, B. R. *Life Sci.* **1996**, *58*, 1315−1321.
(4) Beaubrun, G.; Gray, G. E. *Psychiatr. Serv.* **2000**, *51*, 1130−1134.
(5) Kleijnen, J.; Knipschild, P. *Lancet* **1992**, *340*, 1136−1139.
(6) Wong, A. H.; Smith, M.; Boon, H. S. *Arch. Gen. Psychiatry* **1998**, *55*, 1033−1044.
(7) Bauer, U. *Arzneim. Forsch.* **1984**, *34*, 716−721.
(8) Rudofsky, V. G. *Fortschr. Med.* **1987**, *105*, 397−400.
(9) Schneider, B. *Arzneim. Forsch.* **1992**, *42*, 428−436.
(10) AJNM Editorial Office. *Am. J. Nat. Med.* **1995**, *2*, 10−13.
(11) Topic, B.; Hasenohrl, R. U.; Hacker, R.; Huston, J. P. *Phytother. Res.* **2002**, *16*, 312−315.
(12) Topic, B.; Tani, E.; Tsiakitzis, K.; Kourounakis, P. N.; Dere, E.; Hasenohrl, R. U.; Hacker, R.; Mattern, C. M.; Huston, J. P. *Neurobiol. Aging* **2002**, *23*, 135−143.
(13) Chermat, R.; Brochet, D.; De Feudis, F. V.; Drieu, K. *Pharmacol. Biochem. Behav.* **1997**, *56*, 333−339.
(14) Hasenohrl, R. U.; Nichau, C. H.; Frisch, C. H.; De Souza Silva, M. A.; Huston, J. P.; Mattern, C. M.; Hacker, R. *Pharmacol. Biochem. Behav.* **1996**, *53*, 271−275.
(15) Hasenohrl, R. U.; Topic, B.; Frisch, C.; Hacker, R.; Mattern, C. M.; Huston, J. P. *Pharmacol. Biochem. Behav.* **1998**, *59*, 527−535.
(16) Satyan, K. S.; Jaiswal, A. K.; Ghosal, S.; Bhattacharya, S. K. *Psychopharmacology* **1998**, *136*, 148−152.
(17) Porsalt, R. D.; Martin, P.; Lenegre, A.; Fromage, S.; Drieu, K. *Pharmacol. Biochem. Behav.* **1990**, *36*, 963−971.
(18) Brochet, D.; Chermat, R.; De Feudis, F. V.; Drieu, K. *Gen. Pharmacol.* **1999**, *33*, 249−256.
(19) Dawson, G. R.; Tricklebank, M. D. *Trends Pharmacol. Sci.* **1995**, *16*, 33−36.
(20) Kulkarni, S. K.; Reddy, D. S. *Methods Find Exp. Clin. Pharmacol.* **1996**, *18*, 219−230.
(21) Kulkarni, S. K.; Shaema, A. C. *Methods Find Exp. Clin. Pharmacol.* **1991**, *13*, 573−577.
(22) Nerdner, M.; Cathadi, S. S. Japanese Patent No. 3188461, 2001.
(23) Biber, A.; Koch, E. *Planta Med.* **1999**, *65*, 192−193.
(24) Kuribara, H.; Maruyama, Y. *Jpn. J. Neuropsychopharmacol.* (Abstract in English) **1996**, *18*, 179−190.
(25) Kuribara, H.; Morita, M.; Ishige, A.; Hayashi, K.; Maruyama, Y. *Jpn. J. Neuropsychopharmacol.* (Abstract in English) **1996**, *18*, 179−190.
(26) Kuribara, H.; Stavinoha, W. B.; Maruyama, Y. *J. Pharm. Pharmacol.* **1998**, *50*, 819−826.
(27) Lee, C.; Rodgers, R. L. *Neuropharmacology* **1991**, *30*, 1263−1267.
(28) Gee, K. W.; Ehlert, F. J.; Yamamura, H. I. *J. Pharmacol. Exp. Ther.* **1983**, *225*, 132−137.
(29) Amri, H.; Drieu, K.; Papadopoulos, V. *Endocrinology* **1997**, *138*, 5415−5726.
(30) Amri, H.; Ogwuegbu, S. O.; Boujrad, N.; Drieu, K.; Papadopoulos, V. *Endocrinology* **1996**, *137*, 5708−5718.
(31) Kuribara, H.; Stavinoha, W. B.; Maruyama, Y. *J. Pharm. Pharmacol.* **1999**, *51*, 97−103.
(32) Woods, J. H.; Winger, G. *Psychopharmacology* **1995**, *118*, 107−115.
(33) Japanese Provisional Patent Publication No. 2002-519383, 2002.
(34) Okabe, K.; Yamada, S.; Yamamura, S.; Takada, S. *J. Chem. Soc. (C)* **1967**, *1967*, 2201−2206.
(35) Pellow, S.; Chopin, P.; File, S. E.; Briley, M. *J. Neurosci. Methods* **1985**, *14*, 149−167.
(36) Lister, R. G. *Psychopharmacology* **1987**, *92*, 180−185.
(37) Kuribara, H.; Tadokoro, S. *Jpn. J. Pharmacol.* **1986**, *40*, 303−310.

NP030122F