Pharmacologic Characterization of a Novel Non-Benzodiazepine Selective Anxiolytic, DN-2327

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Accepted November 19, 1988

Abstract—DN-2327, 2-(7-chloro-1,8-naphthyridin-2-yl)-3-[(1,4-dioxo-8-azaspiro[4.5]dec-8-yl)carbonylmethyl]isoindolin-1-one, produced anxiolytic, taming and anti-convulsive effects when administered orally to several species of animals. DN-2327 produced few of the sedative-hypnotic and muscle-relaxant effects observed with diazepam. The durations of the anxiolytic and anti-convulsive activities of DN-2327 were much longer than those of diazepam. Tolerance to DN-2327 did not develop when it was administered daily for 14 days in an anti-conflict test (Vogel conflict test). DN-2327 showed potent displacement activity against [^3H]diazepam binding. The binding affinity of DN-2327 for benzodiazepine receptors was about twenty times that of diazepam. Furthermore, the affinity of DN-2327 for benzodiazepine receptors was not enhanced by the presence of GABA. There is a wide margin between the doses of DN-2327 that cause the anxiolytic effects and its sedative-hypnotic/muscle-relaxant effects. These results suggest that DN-2327 has more marked anxiolytic properties compared with the benzodiazepines.

The benzodiazepines are the most widely prescribed of the anti-anxiety drugs for the treatment of neurotic patients. However, they have sedative-hypnotic and muscle-relaxant activities in addition to their anxiolytic action, and these side effects limit their utility, especially in geriatric patients. Furthermore, these drugs significantly potentiate the sedative properties of other CNS depressants (1, 2). In addition, problems involving a tendency for drug dependence (3) and amnesia (4) following their administration have been raised recently. For this reason, many neuro-psychopharmacologists have been searching for anxiolytics that are devoid of or exhibit markedly reduced side effects. Our pharmacologic screening of a non-benzodiazepine chemical series has revealed that 2-(7-chloro-1,8-naphthyridin-2-yl)-3-[(1,4-dioxo-8-azaspiro[4.5]dec-8-yl)carbonylmethyl]isoindolin-1-one (5), DN-2327, has anxiolytic properties. In the present study using several species of animals, DN-2327

was tested pharmacologically and neuro-chemically in comparison with diazepam for its anti-conflict, taming, anti-hyperemotionality, anti-aggressiveness, anti-convulsive, muscle-relaxant, and sedative actions and its affinity for benzodiazepine receptors.

Materials and Methods

I. Anxiolytic activity

(1) Vogel conflict test

1) **Potency:** Male rats (Jcl:Wistar, 140–160 g) were used. The experimental procedures used were similar to those described by Vogel et al. (6). Rats were deprived of water for 48 hr before the test. After the first 24 hr of water deprivation, the rat was placed in the test box and given water ad libitum without any electric shock for 30 sec. The test box (light compartment: 34×25×25 cm, dark compartment: 10×10×10 cm) was enclosed in a sound-proof box and equipped with a grid floor of stainless steel and a drinking bottle containing water. After another

24 hr of water deprivation, the rat was again placed in the test box. Test agents were administered orally 1 hr before the test. Upon placement into the test box, each animal was allowed to drink water and to complete 20 licks or 2 sec of licking before the application of an electric shock (1.5 mA, 2 sec). During the subsequent 3-min test period, shocks were delivered every 20th lick. The number of shocks tolerated during a 3-min period was recorded.

2) Duration of action: The duration of action was studied using the same procedure as described above except that the test agents were administered 3, 6, 12, 16 and 24 hr before the test.

3) Repeated administration: DN-2327 was given orally once a day for 14 days to investigate changes in its anxiolytic activity related to chronic administration. The test was conducted 1 hr after the drug had been administered on the 14th day.

(2) Geller conflict test

The procedure used was based on that described by Geller and Seifter (7). Male rats (Jcl:Wistar, 30–48 weeks old, 242–266 g) were used. They were partially deprived of food in order to maintain their body weight at 80% of the initial body weight. The rats were trained in an operant chamber to lever-press for a food reward (9% powdered milk solution with 1% sugar added) on a multiple variable interval of 30 sec/continuous reinforcement (VI30/CRF) schedule. The two schedule components were alternated during 60-min sessions. Each session began with a 12-min VI period followed by a 3-min CRF period signaled by a tone as a cue, in which every response was reinforced with food but accompanied by an electric shock (0.2–0.4 mA). This period is referred to as the “conflict period”. The rats were given a food reward on a VI30 schedule in the safety period without an electric shock. The control values for the conflict and safety periods were estimated after administration of saline before the drug-administration studies.

II. Anti-hyperemotationality

(1) Anti-fighting effect in mice

The procedure employed was essentially the same as that described by Tedeschi et al. (8). Pairs of male mice (Jcl:ICR, 5 weeks old)

were selected at random and placed under an inverted 2-liter glass beaker on a grid-floor composed of stainless steel rods. A foot-shock of 2 mA (AC) in intensity was delivered for 3 min from a power supply through a scrambler. The frequency of fighting episodes during the 3-min period was determined, and pairs exhibiting 5 or more fighting episodes were used for the drug tests. One or two days after the selection, 6 pairs for each dose level were given the test compound orally, and were then subjected to one 3-min fighting test 30 min later. Since all pairs showed 5 or more fighting episodes in the control test, occurrence of 4 or fewer fighting episodes after administration of the drug was regarded as a significant drug effect. The anti-fighting ED₅₀ value was calculated as the dose that significantly suppressed the fighting behavior in half of the pairs.

(2) Anti-hyperemotationality in rats with septal lesions

Rats with septal lesions (Jcl:Wistar, male, 9 weeks old, 250–300 g) were prepared using the procedure described elsewhere (9). The degree of hyperemotationality before and after intraperitoneal injection of the test agent was scored according to the method of King and Meyer (10). Reduction in the score after administration of a drug was expressed as the percentage decrease compared with the pre-drug score. ED₅₀ values were calculated as the dose that suppressed the score by 20% or more in half of the animals tested.

(3) Anti-muricidal effect in rats with olfactory bulbectomy

Male rats (Jcl:Wistar, 9 weeks old, 250–300 g) were anesthetized with pentobarbital. The top of the skull was removed, and the exposed bilateral olfactory bulbs were ablated by aspiration. The muricidal response of rats thus treated was evaluated by the procedure described by Nakajima et al. (9). The rats were assigned to groups of 6, and each animal was presented with a mouse for 10 min before and at 0.5, 1, 1.5 and 2 hr after drug administration. ED₅₀ values were calculated as the dose that suppressed the muricidal response in half of the rats tested. Each rat was used several times, but only tested once a week.

III. Taming effect in monkeys and beagle dogs

(1) Cynomolgus monkeys

Eight consistently hostile cynomolgus monkeys (male, 4.5–7 kg) were used. Three animals were used for each dose level. The same animals were used several times for drug testing, but at two-week intervals. The behavior of animals was rated according to a check-list devised by Norton (11) with some modifications, before and at 0.5, 1, 2, 4, 6 and 24 hr after oral administration of the test agent. In this study, the taming, sleep-inducing and muscle-relaxant effects of DN-2327 were compared with those of diazepam.

(2) Beagle dogs

Three male beagle dogs that showed anxious and timid behavior when confronted with the observer were used repeatedly for each dose level, but at 2-week intervals, for the drug test. The behavior of the animals was rated according to a scoring sheet devised by ourselves (Table 1), before and at 0.5, 1, 2, 3, 5, 7 and 24 hr after oral administration of the test drug. A control experiment in which glucose was administered was carried out on the same animals before administration of the test drug.

IV. Anti-convulsive effect

(1) Pentylenetetrazol-induced convulsion

Groups of 6 rats (Jcl:Wistar, male, 6 weeks old, 130–161 g) pretreated with the test agent were injected with 150 mg/kg, s.c. of pentylenetetrazol-HCl. The ED₅₀ values of the test agents were evaluated as the dose that prevented death in 50% of the rats during 60 min after the pentylenetetrazol challenge. The test agents were administered orally 1 hr before the pentylenetetrazol challenge. Additionally, in order to study the duration of action of the test agents, the pretreatment time was varied using periods of 0.5, 1, 2, 4, 6, 16 and 24 hr.

(2) Bicuculline-induced convulsion

Groups of 6 rats (Jcl:Wistar, male, 6 weeks old, 133–181 g) pretreated with the test agent were injected subcutaneously with 3 mg/kg of bicuculline. The ED₅₀ values of the test agents were evaluated as the dose that prevented death in 50% of the rats during 60 min after the bicuculline challenge. The test agents were given orally 1 hr before the bicuculline challenge.

(3) Maximum electroshock-induced convulsion

Maximum electroshock seizure (MES) was produced essentially as described by Swinyard et al. (12). Mice (Jcl:ICR, male, 4 weeks old) were challenged with a supramaximal electroshock (75 mA, 0.2 sec) delivered through bilateral corneal electrodes 30 min after administration of the test agent either orally or intraperitoneally. The ED₅₀ value was calculated as the dose that prevented death in half of the mice. Eight mice were used per dose.

V. Muscle relaxation

(1) Rota-rod test

Rats (Jcl:Wistar, male, 6–7 weeks old, 127–250 g) previously trained to remain on a rota-rod 3.5 cm in diameter (8 revolutions/min) for more than 1 min were used. The animals were subjected to this test at 15- or 30-min intervals after administration of the test agent either orally or intraperitoneally; the ED₅₀ value of the test agent was determined as the dose that caused 50% of the rats to fall off the rota-rod within 1 min. Six rats were used per dose level.

(2) Inclined screen test

Male mice (Jcl:ICR, 4 weeks old, 22–25 g) were placed on a 60° inclined screen (a tetron-gauze covered plate) before and at 0.25, 0.5, 1, 1.5 and 2 hr after oral administration of the test agent. The ED₅₀ value of each test agent was determined as the dose that caused 50% of the mice to slide off the screen within 1 min. Ten mice were used per dose.

VI. Effect on CNS depressants

(1) Pentobarbital potentiation

A non-hypnotic dose of pentobarbital Na (25 mg/kg) was given intraperitoneally to groups of 8 mice (Jcl:ICR, male, 4 weeks old, 21–24.5 g) 30 min after oral administration of the test agent at various doses. Thereafter, the number of mice that showed loss of the righting reflex for more than 3 min within 30 min of receiving the pentobarbital were counted, and the ED₅₀ value of the test agent was taken as the dose producing hypnosis in half of the mice.

(2) Ethanol anesthesia potentiation

Twenty-five percent ethanol was given intraperitoneally at 0.2 ml/10 g body weight 30 min after oral administration of the test agent at various doses to groups of 8 or 10 mice (Jcl:ICR, male, 4 weeks old, 23–26.5 g).

Table 1. Scoring sheet for dog behavior

Time Post-Drug (min)				
Observation Item (Score)				
FAMILIARITY (0–8)				
Can be touched (0, 1)				
Lick hand friendly (0–2)				
Come forward friendly (0–2)				
Eat food from hand (0, 1)				
Wagging tail (0–2)				
CONTENTMENT (0–4)				
Playing (0–2)				
Eating in front of observer (0, 1)				
Eating in absence of observer (0, 1)				
EXCITEMENT (0–6)				
Barking (0–2)				
Tail elevation (0–2)				
Moving around (0–2)				
DEFENSIVENESS (0–8)				
Withdrawing (0–2)				
Crouch in corner (0–2)				
Staring at observer (0–2)				
Tail between legs (0–2)				
Defecation				
DROWSY & ASLEEP (0–3)				
ATAXIA (0–3)				
BODY POSITION				
Standing				
Sitting				
Prostrate				
Recumbent				

Loss of the righting reflex for a period of more than twice as long as the mean anesthetic duration in a saline-treated group was considered to be significant potentiation of ethanol anesthesia by the test agent. The ED50 value was calculated as the dose

producing potentiation of ethanol anesthesia in 50% of the mice.

VII. Spontaneous motor activity

Each rat (Jcl:Wistar, male, 8 weeks old, 210–250 g) was placed in a Perspex cage (44×24×19 cm) mounted on top of an

Animex meter (Muromachi Kikai Co., Ltd.). Spontaneous motor activity, mainly large movements, was counted for 3 hr after drug administration. All experiments were performed between 13:00 hr and 17:00 hr at an ambient temperature of $24 \pm 1^\circ\text{C}$.

VIII. Effect on body temperature

Groups of 8 mice (Jcl:ICR, male, 4 weeks old) were given various doses of DN-2327 and diazepam orally or intraperitoneally. The rectal temperature was measured using an electronic thermistor (Takara Kogyo, D221) at 0.5, 1, 1.5, 2 and 4 hr after drug administration. The experiments were performed between 13:00 hr and 17:00 hr at an ambient temperature of $24.0\text{--}24.5^\circ\text{C}$.

IX. Neurochemical study

(1) Affinity for benzodiazepine receptors in rat brain

Experiments were performed according to the method of Braestrup and Squires (13). After decapitation of each animal, the cerebral cortex was immediately excised and homogenized in 20 vol. of ice-cold 0.32 M sucrose. The supernatant obtained by centrifugation at $2,000 \times g$ for 5 min was recentrifuged at $30,000 \times g$ for 10 min. The sediment was suspended in 50 mM Tris-HCl buffer (pH 7.4, 25°C) and used for the receptor-binding experiments. One-milliliter aliquots of the membrane fraction, 10 μl of a test drug and 20 μl [^3H]diazepam (final concentration: 2 nM) were incubated for 20 min at 0°C . Non-specific binding was estimated in the presence of 100 μM diazepam.

(2) GABA ratio

GABA ratio is the ratio of the affinity of benzodiazepine receptor ligands for the benzodiazepine receptor in the absence of GABA over that in the presence of GABA ($\text{IC}_{50}[(\text{without GABA})/(\text{with GABA})]$).

The effect of DN-2327 on the inhibition of [^3H]diazepam binding, as described above, was studied in the presence or absence of 100 μM GABA. In this study, non-specific binding was estimated in the presence of 10 μM diazepam.

X. Drugs

The following drugs were used: DN-2327, 2-(7-chloro-1,8-naphthyridin-2-yl)-3-[(1,4-dioxo-8-azaspiro[4.5]dec-8-yl)carbonylmethyl]isoindolin-1-one (Fig. 1), synthesized

Fig. 1. Chemical structure of DN-2327.

in our Chemistry Laboratories; diazepam (Cercine®, Takeda); pentylenetetrazol HCl (Sigma); bicuculline (Pierce Chem.); glucose (Wako Pure Chemical Ind., Ltd.); pentobarbital Na (Nakarai Chemicals); ethanol (Wako Pure Chemical Ind., Ltd.); gamma-aminobutyric acid (GABA, Sigma); [^3H]diazepam (specific activity, 76.8 Ci/mmol, New England Nuclear). DN-2327 and diazepam were suspended in 5% gum arabic saline. Bicuculline was dissolved in 5 mM HCl. Pentobarbital Na and ethanol were dissolved in and diluted with saline, respectively.

XI. Statistical analysis

Student's *t*-test was used to evaluate the results of the Vogel conflict test, spontaneous motor activity and body temperature. The paired *t*-test was used for evaluation of the Geller conflict test. Unless otherwise specified, ED₅₀ values were determined by Finney's probit analysis (14).

Results

I. Anxiolytic activity

(1) Vogel conflict test

1) Potency: Animals dosed with DN-2327 at 2.5, 5, 10 and 20 mg/kg, p.o., tolerated a greater number of shocks than did control saline-treated rats (Fig. 2). The anxiolytic activity of DN-2327 was equipotent to that of diazepam at the minimum effective dose (10 mg/kg, p.o.) of each drug.

2) Duration of action: As shown in Table 2, the duration of action of DN-2327 in this test was at least 12 hr (at 20 mg/kg, p.o.), whereas diazepam administered at 10 and 20 mg/kg, p.o., 3 and 6 hr before the test had no anti-conflict activity.

3) Repeated administration: Repeated administration of DN-2327 (2.5, 5 and 20 mg/kg/day, p.o.) for 14 days did not attenuate

Fig. 2. Anti-conflict action of DN-2327 and diazepam in the Vogel conflict test in rats. DN-2327 or diazepam was given 1 hr before the test. * $P<0.05$, ** $P<0.01$, compared with the saline control (Student's t -test). Each group was comprised of 8–12 rats.

Table 2. Duration of anti-conflict and anti-pentylenetetrazol convulsive actions of DN-2327 compared with those of diazepam in rats

A) Anti-conflict action

Drug	Dose (mg/kg, p.o.)	Punished response in Vogel conflict test (% control)					
		pretreatment time (hr)					
		1	3	6	12	16	24
Saline	—	100	100	100	100	100	100
DN-2327	2.5	206	209	150	105	—	—
	5	258	293*	238*	160*	134	83
	10	318*	289*	188	145	209*	92
	20	427**	402**	315**	220**	189	109
Diazepam	10	482**	93	—	—	—	—
	20	—	—	160	—	—	—

* $P<0.05$, ** $P<0.01$ vs. saline control (Student's t -test). —: not tested

B) Anti-convulsive action

Drug	ED50 Value for protection against death induced by pentylenetetrazol						
	pretreatment time (hr)						
	0.5	1	2	4	6	16	24
DN-2327	3.4 ^a	0.95	1.5	1.9	2.9	14.7	163
Diazepam	6.1	13.9	22.0	—	59.1	—	—

Pentylenetetrazol: 150 mg/kg, s.c. —: not tested. ^a mg/kg, p.o.

its effect on the number of shocks tolerated. Significant increases were observed at doses of 5 and 20 mg/kg, p.o., of DN-2327. Diazepam (10 mg/kg/day, p.o.) also pro-

duced a significant increase in the number of shocks tolerated (data not shown).

(2) Geller conflict test

As shown in Fig. 3, the anti-conflict effect

Fig. 3. Effect of DN-2327 and diazepam on punished bar-pressing behavior in rats (Geller conflict test). Open columns represent the number of responses in the conflict period. Hatched columns indicate the saline control. Lines with solid circles represent the response rate (% of control) in the safety period. The drugs were administered 30 min before the test. * $P < 0.05$, ** $P < 0.01$, compared with the saline control (paired t -test). Each group was comprised of 12–14 rats.

of DN-2327 was dose-dependent, without affecting significantly the lever-press responses in the safety period. The minimum effective dose of both DN-2327 and diazepam was 2.5 mg/kg, p.o.

II. Anti-hyperemotonicity

(1) Anti-fighting effect in mice

DN-2327 suppressed the fighting episodes in a dose-dependent manner. The ED₅₀ values for DN-2327 and diazepam were 18.1 mg/kg and 3.2 mg/kg, p.o., respectively (Table 3).

(2) Anti-hyperemotonicity in rats with septal lesions

As shown in Table 3, the ED₅₀ values of DN-2327 and diazepam for hyperemotonicity in rats with septal lesions were 7.6 mg/kg and 2.8 mg/kg, i.p., respectively.

(3) Anti-muricidal effect in rats with olfactory bulbectomy

The ED₅₀ values of DN-2327 and diazepam required to suppress muricidal behavior in rats with olfactory bulbectomy were 99.1 mg/kg and 16.6 mg/kg, i.p., respectively (Table 3).

III. Taming effect in monkeys and beagle dogs

(1) Cynomolgus monkeys

DN-2327 dose-dependently decreased the scores for excitement, defensive and aggressive behavior. These effects were observed 30 min to 1 hr after drug administration and continued for about 6 hr, although they were not observed 24 hr after administration. Unlike diazepam, DN-2327 (5 and 20 mg/kg, p.o.) did not cause drowsiness nor sleep-inducing and muscle-relaxant effects (Fig. 4).

(2) Beagle dogs

DN-2327, at 5 and 20 mg/kg, p.o., produced very friendly behavior from 1 hr after drug administration. The peak effect of DN-2327 occurred at 90 min to 2 hr; and thereafter, the effect declined gradually, but continued for at least 7 hr after drug administration. Twenty-four hours later, the animals had returned to their previous anxious and timid state. Neither sleep-inducing nor ataxic effects were observed during a 7-hr period after drug administration (Fig. 5).

IV. Anti-convulsive effect

Table 3. Comparative pharmacological profiles of DN-2327 and diazepam in various tests

Test	Animal	Adminis- tration Route	ED50 (95% confidential limits, mg/kg)	
			DN-2327	Diazepam
Anti-aggressive effect				
Foot shock fighting	mouse	p.o.	18.1 (9.7–30.9)	3.2 (1.6–5.9)
Hyperemotionality in rats with septal lesion	rat	i.p.	7.6 (4.7–10.1)	2.8 (1.1–5.1)
Anti-muricide in rats with olfactory bulbectomy	rat	i.p.	99.1 (57.9–216.9)	16.6 (8.4–23.5)
Anti-convulsive effect				
Pentylenetetrazol	rat	p.o.	0.95 (0.34–1.7)	6.1 (3.7–10.9)
Bicuculline	rat	p.o.	6.5 (1.8–23.4)	6.4 (2.5–17.4)
MES	mouse	p.o.	>160	14.1 (9.5–26.9)
Muscle relaxant effect				
Rota-rod test	rat	p.o.	>160	17.8 (9.6–42.2)
		i.p.	>160	NT
Inclined screen test	mouse	p.o.	>160	14.0 (9.2–33.6)
Inhibition of SMA	rat	p.o.	>40	10 (MED)
Effect on CNS depressants				
Pentobarbital potentiation	mouse	p.o.	>200	1.1 (0.6–1.7)
Ethanol anesthesia potentiation	mouse	p.o.	>200	0.11 (0.06–0.17)
Effect on body temperature	mouse	p.o.	>40	2.5 (MED)
		i.p.	>20	NT
Inhibition of [³ H]diazepam binding (IC50, nM)	rat brain	—	0.38	8.6
Effect on GABA ratio	rat brain	—	1.1	3.0

MES: maximum electroshock induced convulsion, MED: minimum effective dose, NT: not tested, SMA: spontaneous motor activity.

(1) Anti-pentylenetetrazol convulsion

1) Potency: As shown in Table 3, DN-2327 dose-dependently suppressed the number of animal deaths induced by pentylenetetrazol challenge. The ED₅₀ values for DN-2327 and diazepam were 0.95 and 6.1 mg/kg, p.o., respectively (pretreatment time: 1 hr and 0.5 hr, respectively).

2) Duration of action: DN-2327 showed a peak effect when given 1 hr before pentylenetetrazol administration. Its action lasted longer than that of diazepam (Table 2).

(2) Bicuculline-induced convulsion

DN-2327 and diazepam dose-dependently suppressed the number of rat deaths induced by bicuculline. The ED₅₀ values for DN-2327 and diazepam were 6.5 and 6.4 mg/kg, p.o., respectively (Table 3).

(3) Maximum electroshock-induced convulsion

DN-2327 (160 mg/kg, p.o. or i.p.) did not protect against maximum electroshock-induced convulsion, whereas the ED₅₀ value of diazepam was 14.1 mg/kg, p.o. (Table 3).

V. Muscle relaxation

(1) Rota-rod test

DN-2327 did not cause muscle relaxation at a dose of 160 mg/kg, p.o. or i.p. However, diazepam caused muscle relaxation with an ED₅₀ value of 17.8 mg/kg, p.o. (Table 3).

(2) Inclined screen test

As shown in Table 3, DN-2327 did not cause muscle relaxation even at 160 mg/kg, p.o. Diazepam, on the other hand, clearly induced muscle relaxation with an ED₅₀ value of 14.0 mg/kg, p.o.

Fig. 4. Taming, sleep-inducing and ataxic effects of DN-2327 and diazepam in cynomolgus monkeys. Each score after administration of the drug represents the total score for 3 monkeys. The precontrol scores were set at 0. Numbers in parentheses represent the allotted points in the respective behavior.

VI. Effect on CNS depressants

(1) Pentobarbital potentiation

In contrast to diazepam, DN-2327 did not cause pentobarbital potentiation, even at a high dose of 200 mg/kg, p.o. The ED₅₀ value of diazepam was 1.1 mg/kg, p.o. (Table 3).

(2) Ethanol anesthesia potentiation

As shown in Table 3, DN-2327 showed a tendency to potentiate ethanol anesthesia at a high dose of 200 mg/kg, p.o. Diazepam had

an ethanol-potentiating effect at low doses; the ED₅₀ value was 0.11 mg/kg, p.o.

VII. Spontaneous motor activity

DN-2327, 10 and 40 mg/kg, p.o., did not produce any significant decrease in spontaneous motor activity counts. On the other hand, diazepam at 10 mg/kg, p.o. or more caused a significant decrease in spontaneous motor activity counts during the first 30 min and the first 60 min following diazepam ad-

Fig. 5. Effect of DN-2327 (5 mg/kg, p.o.) on the behavior of beagle dogs that appeared anxious and timid. Each score, before or after drug administration, represents the mean score obtained from 3 dogs. Numbers in parentheses represent the allotted points in the respective behavior.

ministration, respectively (Table 3).

VIII. Effect on body temperature

DN-2327 (10, 20 and 40 mg/kg, p.o.) did not produce hypothermia during a 4-hr period after its administration. Also, intraperitoneal injection of DN-2327 at 10 and 20 mg/kg produced no hypothermia. Diazepam (1.25, 2.5 and 5 mg/kg, p.o.) caused dose-dependent hypothermia. A significant change was observed at 2.5 mg/kg or more (Table 3).

IX. Neurochemical study

(1) Affinity for benzodiazepine receptors in rat brain

DN-2327 showed a potent affinity for the benzodiazepine receptors in rat brain; its affinity (IC_{50} : 0.38 ± 0.08 nM, $n=5$) was approximately 20 times that of diazepam (IC_{50} : 8.6 ± 2.7 nM, $n=5$, Table 3).

(2) GABA ratio

The GABA ratio of DN-2327 was 1.1 ± 0.1 ($n=5$), whereas that of diazepam was 3.0 ± 0.7 ($n=5$, Table 3).

Discussion

In the rat conflict tests, DN-2327 produced an anticonflict activity, an effect similar to that produced by diazepam. DN-2327 administered repeatedly for 14 days produced the same anti-conflict activity as that observed after a single administration; the activity was similar to that of diazepam (15). This observation suggests that there might be no development of tolerance to the anxiolytic activity of DN-2327. Furthermore, in the Vogel conflict test, the anti-conflict activity of DN-2327 was longer lasting than that of diazepam. It was also demonstrated that DN-2327 exerted a potent and long-lasting anti-convulsive action against pentylenetetrazol-induced convulsion. Such properties as the ability to inhibit [^3H]diazepam binding, to increase the punishment response in a conflict situation, and to inhibit pentylenetetrazol-induced convulsions are highly characteristic of anxiolytic drugs, and have been used as preclinical screening parameters for predicting anxiolytic activity (16). The present findings on DN-2327 suggest the possibility that it could be of therapeutic value in the treatment of anxiety.

The benzodiazepines produce sedative-hypnotic and muscle-relaxant effects in addition to their anti-conflict effects. The purpose of this study was to compare the separation ratios between the anti-conflict activity and sedative or muscle-relaxant activity of DN-2327 and diazepam. As can be seen in Table 4, DN-2327 showed a better separation ratio between its anti-conflict effect and muscle-relaxant or sedative-hypnotic action than did diazepam.

The anti-convulsive potency of DN-2327 against bicuculline-induced convulsions was as much as that of diazepam. By contrast, DN-2327 was more potent and longer lasting than diazepam in its ability to protect against the effects of pentylenetetrazol. CGS9896 (17) and suriclone (18) have been reported to possess good separation ratios between their anxiolytic and muscle-relaxant actions, and it has also been shown that these two compounds have a more potent protective effect against pentylenetetrazol-induced convulsion than against bicuculline- or picrotoxin-induced convulsion. In the neurochemical study, DN-2327 showed a more potent affinity for benzodiazepine receptors than did diazepam. However, DN-2327, like CGS 9896 (19), did not alter the GABA ratio. Thus, the degree of interaction with the GABAergic system may be a critical factor in separating the anxiolytic action of a drug from its sedative or muscle-relaxant action. These results suggest that the pharmacologic activities of DN-2327 may be mediated via a subpopulation or a specific conformation of the benzodiazepine receptors which are not susceptible to modulation by GABA.

Wood et al. (19) reported that the non-sedating benzodiazepine agonist/antagonist CGS9896 had a GABA ratio of 1.1 and that its stimulation of [^3S]butyl bicyclopophosphorothionate (TBPS) and [^3H]muscimol binding was only about 20% and 10%, respectively, of the levels of classical benzodiazepine agonists. These authors suggested that CGS9896 possesses a partial agonistic property for benzodiazepine receptors. Since, as previously mentioned, DN-2327 did not alter the GABA ratio, this drug may possess a

Table 4. Comparison of the separation ratio of DN-2327 and diazepam (differentiation between anti-conflict effect and muscle-relaxant or sedative effect)

	Separation ratio	
	DN-2327	Diazepam
Muscle relaxation ¹⁾		
Anti-conflict ²⁾	>16	1.8
Sedative effect ³⁾		
Anti-conflict ²⁾	>20	0.1

Separation ratios were calculated from the respective minimum effective dose or ED₅₀ values. ¹⁾ Rota-rod test, ²⁾ Vogel conflict test, ³⁾ pentobarbital potentiation.

partial agonistic property for benzodiazepine receptors. However, the precise mechanisms of action of DN-2327 remain unknown. Pharmacologic data have also indicated that multiple benzodiazepine receptors may be involved in the anxiolytic, anti-convulsant, muscle-relaxant and sedative-hypnotic actions of the benzodiazepines (20). The mixed agonist/antagonist agents CL218872 (21), quazepam (22), premapepam (23) and CGS9896 (24) exert an agonistic action on one class of benzodiazepine receptors and an antagonistic action on another class. It has been suggested that these compounds have affinities for the benzodiazepine receptors of the cerebellum that are different from those for the cerebral cortex receptors and that they have good separation ratios between their anxiolytic action and side effects. However, it is difficult to explain the relation between the pharmacologic effects and the sites of binding of CL218872 (21), as this compound showed a higher affinity for the benzodiazepine receptors in the cerebellum than those in other regions of the brain, but had reduced muscle-relaxant action. Also, it is presently unclear whether the modes of CGS9896 binding differ between the cerebellum and forebrain. There is a possibility that DN-2327 may have different modes of binding for benzodiazepine receptors in various regions of the brain, since the agent has anxiolytic properties with less marked sedative and muscle-relaxant effects.

The anti-hyperemotionality, anti-aggressiveness, anti-fighting and anti-muricidal actions of DN-2327 were less potent than those of diazepam. However, the dose of diazepam required to produce these actions was higher than that for its anxiolytic effect. These effects of diazepam may be considered to depend partly on its muscle-relaxant or sedative effect. In cynomolgus monkeys, DN-2327 reduced aggressiveness, excitement and defensive behavior with no motor incoordination or drowsiness and sleep induction. Furthermore, DN-2327 produced very friendly behavior in beagle dogs that had previously shown anxious and timid behavior, without producing motor incoordination or drowsiness and sleep induction. With regard to spontaneous motor activity and body temperature,

DN-2327 did not produce the significant changes observed with diazepam. This lack of CNS-depressant effect of DN-2327 would appear to be beneficial for medication. The number of lever-pressings in the non-punishment period of the Geller conflict test was increased by smaller doses of diazepam, which may be related to the disinhibitory action of this drug (25).

In conclusion, DN-2327, a new non-benzodiazepine highly selective anxiolytic drug with a novel pharmacologic profile, holds promise for the treatment of anxiety without producing sedation and muscle-relaxation as side effects. Further investigations to elucidate the mechanism of action of DN-2327 are currently in progress.

Acknowledgments: The technical assistance of Ms. F. Wada and Messrs. K. Ikeda, H. Nishikawa and K. Gomaibashi is gratefully acknowledged. The authors wish to thank Dr. Y. Nagawa for valuable comments on the manuscript.

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