



Letter

Oral toxicity study of an antiprion compound *N,N'*-[(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] in rats and cynomolgus monkeys

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ABSTRACT — *N,N'*-[(Cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] (**1**) is a novel antiprion compound, termed a designer molecular chaperone, that we recently developed. The administration of compound **1** prolonged the survival time of prion-infected mice and slowed the development of neurological and psychological symptoms in prion-infected macaques. The aim of this study was to investigate the oral toxicity of compound **1** to rats and cynomolgus monkeys. Compound **1** was administered orally to rats at doses of 31.3, 125, and 500 mg/kg. Although two of ten rats died at a dose of 500 mg/kg, no serious safety problems were identified at doses of 31.3 and 125 mg/kg. Repeated oral administration of compound **1** to rats at a dosage of 31.3 mg/kg/day for a week led to no significant toxic effects in the rats. An acute toxicity test in cynomolgus monkeys revealed that the administration of compound **1** at a dose of 60 mg/kg induced vomiting and fecal abnormalities. The monkeys did not die even at a dose of 250 mg/kg. A dose-dependent increase in the plasma concentrations of compound **1** in the cynomolgus monkeys as measured by LC/MS/MS analysis indicated that compound **1** migrated into the bloodstream. These results suggest that compound **1** might have potential as a therapeutic agent for prion diseases.

Key words: Prion diseases, Acute toxicity, Subacute toxicity, Oral administration, Lowest observed adverse effect level, Plasma concentration

INTRODUCTION

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are invariably fatal neurodegenerative disorders that affect humans and animals (Prusiner, 1998; Prusiner *et al.*, 1998; Vana *et al.*, 2007). They include Creutzfeldt-Jakob disease, Gerstmann-Sträussler-

Scheinker syndrome (GSS), fatal familial insomnia in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease in deer and elk. These diseases are characterized by the accumulation of the infectious isoform of prion protein (PrP^{Sc}) in the central nervous system. Although prion diseases are rare disorders, research on therapeutics

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for prion diseases is necessary since no suitable therapies exist thus far. To date, numerous compounds that inhibit the accumulation of PrP^{Sc} in TSE-infected cells have been identified (Cashman and Caughey, 2004; Sim and Caughey, 2009). However, only a limited number of compounds, including amphotericin B and its derivative (Demaimay *et al.*, 1997), pentosan polysulfate (Doh-ura *et al.*, 2004), and porphyrin derivatives (Kocisko *et al.*, 2006), have been reported to be effective in TSE-infected animals. The inadequacy of most antiprion compounds as therapeutic agents can be attributed to their strain-dependent activity, low blood–brain barrier permeability, and toxicity (Zerr, 2009).

Previously, we discovered an antiprion compound, *N,N'*-(methylenedi-4,1-phenylene)bis[2-(1-pyrrolidinyl)acetamide] (GN8), through a virtual high-throughput screening and cellular assay (Fig. 1) (Kuwata *et al.*, 2007). GN8 exhibited antiprion activity in GSS-, scrapie-, and BSE-infected cells. The administration of GN8 improved the survival of TSE-infected mice. A nonclinical safety assessment of GN8 in rats and dogs revealed that it had no severe adverse effects at the concentration necessary to exert its antiprion activity (Hosokawa-Muto *et al.*, 2012). Furthermore, a positron emission tomography study using a ¹¹C-labeled GN8 derivative in rats unambiguously demonstrated that it has the ability to cross the blood–brain barrier (Kimura *et al.*, 2013). During the course of our recent lead optimization study, we developed a more potent antiprion compound, GN8 derivative *N,N'*-(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] (**1**) (Fig. 1) (Yamaguchi *et al.*, 2019). Although the *in vivo* absorption, distribution, and excretion profiles of this compound upon intravenous administration were investigated, the safety and toxicological features of a novel compound **1** via oral

administration remain to be clarified. Herein, we report a nonclinical toxicity study of antiprion compound **1** in rats and cynomolgus monkeys via oral administration.

MATERIALS AND METHODS

Preparation of the test substance

All manipulations were performed under an argon atmosphere. Argon gas was dried by passage through P₂O₅. Commercially available reagents and solvents were purchased from Aldrich, Tokyo Chemical Industry, Wako Pure Chemical Industries, and Nacalai Tesque and were used without further purification. Chromatographic purifications of the compounds were performed by medium-pressure liquid chromatography using a YFLC AI-580 system (Yamazen) equipped with a silica gel high-flash column.

NMR spectra were recorded on an Inova 500 spectrometer (Varian) and an Avance 500 (Bruker) operating at 500 MHz for ¹H and 125 MHz for ¹³C spectra. Chemical shifts of protons were reported in δ values referenced to Me₄Si as an internal standard. Chemical shifts of ¹³C were reported in δ values referenced to CDCl₃ (77.05 ppm) or DMSO-*d*₆ (39.70 ppm) as an internal standard. IR spectra were obtained on a Nicolet iS10 FT-IR (Thermo Scientific) in ATR mode. Mass spectra (EI) and high-resolution mass spectra (EI) were obtained on a JEOL JMS-700/GI spectrometer. Combustion analysis was performed at the Laboratory for Organic Elemental Microanalysis of Kyoto University. Melting points were measured on a Yanaco micro melting point apparatus (MP-J3) and are uncorrected.

Large-scale synthesis of *N,N'*-(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] dihydrochloride (**1**·2HCl)

Cyclohexanecarboxaldehyde (11.2 g, 100 mmol) was added to a solution of aniline hydrochloride (13.0 g, 100 mmol) in aniline (27.9 g, 300 mmol) at 25°C, and the mixture was stirred at 120°C for 48 hr. The reaction mixture was poured into an aqueous solution of NaOH (approximately 1 mol/L, 500 mL) and extracted with CH₂Cl₂ (500 mL × 2). The organic layer was washed with water (500 mL × 2), dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The residue was purified by medium-pressure liquid chromatography on silica gel using hexane/AcOEt as the eluent to yield 4,4'-(cyclohexylmethylene)dianiline (14.1 g, 50.4 mmol, 50%) as a pale-yellow solid. Mp: 103–105°C (dec). ¹H NMR (CDCl₃): δ 0.77–0.85 (m, 2H), 1.07–1.23 (m, 3H), 1.60–

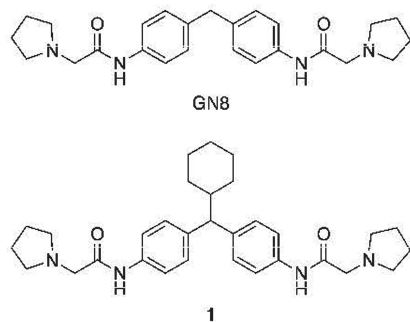


Fig. 1. Chemical structures of *N,N'*-(methylenedi-4,1-phenylene)bis[2-(1-pyrrolidinyl)acetamide] (GN8) and *N,N'*-(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] (**1**).

1.67 (m, 5H), 1.94 (tq, $J = 2.9, 10.8$ Hz, 1H), 3.25 (d, $J = 10.8$ Hz, 1H), 3.42 (br, 4H), 6.56 (d, $J = 8.4$ Hz, 4H), 7.00 (d, $J = 8.4$ Hz, 4H). ^{13}C NMR (CDCl_3): δ 26.5, 26.7, 32.2, 41.5, 57.7, 115.3, 128.8, 135.5, 144.1. IR (ATR): 3339, 3421 cm^{-1} . MS (EI): m/z 280 (M^+). HRMS (EI) calculated for $\text{C}_{19}\text{H}_{24}\text{N}_2$: 280.1939. Found: 280.1935.

A solution of bromoacetyl bromide (22.2 g, 110 mmol) in CH_2Cl_2 (500 mL) was added to a solution of 4,4'-(cyclohexylmethylene)dianiline (14.0 g, 50.0 mmol), pyridine (9.49 g, 120 mmol), and 4-(dimethylamino)pyridine (147 mg, 1.2 mmol) in CH_2Cl_2 (2.0 L) dropwise over a period of 1 hr at 0°C , and the mixture was stirred at that temperature for 1 hr. The resulting precipitates were collected by filtration and washed with CH_2Cl_2 (100 mL \times 3) to give crude N,N' -[(cyclohexylmethylene)di-4,1-phenylene]bis(2-bromoacetamide) (25.6 g) as a colorless solid. Pyrrolidine (10.7 g, 150 mmol) was added to a suspension of N,N' -[(cyclohexylmethylene)di-4,1-phenylene]bis(2-bromoacetamide) (25.6 g) and K_2CO_3 (27.6 g, 200 mmol) in THF (1.0 L) at 25°C , and the mixture was stirred at 60°C for 12 hr. After the solvent was evaporated, the residue was poured into water (500 mL) and extracted with CHCl_3 (500 mL \times 2). The organic layer was dried over Na_2SO_4 , filtered, and concentrated under reduced pressure. The residue was purified by medium-pressure liquid chromatography on silica gel using $\text{CHCl}_3/\text{MeOH}$ as the eluent to give N,N' -[(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] (21.6 g, 43.0 mmol, 86% yield over 2 steps) as a colorless solid. Mp: $76\text{--}78^\circ\text{C}$ (dec). ^1H NMR (CDCl_3): δ 0.80–0.88 (m, 2H), 1.09–1.24 (m, 3H), 1.59–1.67 (m, 5H), 1.83 (br, 8H), 2.04 (tq, $J = 2.9, 11.0$ Hz, 1H), 2.66 (br, 8H), 3.25 (s, 4H), 3.43 (d, $J = 11.0$ Hz, 1H), 7.21 (d, $J = 8.5$ Hz, 4H), 7.45 (d, $J = 8.5$ Hz, 4H), 9.00 (s, 2H). ^{13}C NMR (CDCl_3): δ 24.0, 26.3, 26.5, 32.0, 41.2, 54.5, 58.3, 59.7, 119.7, 128.5, 135.5, 140.3, 169.0. IR (ATR): 1682 cm^{-1} . MS (EI): m/z 502 (M^+). HRMS (EI) calculated for $\text{C}_{31}\text{H}_{42}\text{N}_4\text{O}_2$: 502.3308. Found: 502.3309. Analysis calculated for $\text{C}_{31}\text{H}_{42}\text{N}_4\text{O}_2$: C, 74.07; H, 8.42; N, 11.15. Found: C, 73.83; H, 8.41; N, 11.12.

Hydrogen chloride (approximately 1.0 mol/L in Et_2O , 120 mL, 120 mmol) was added to a suspension of N,N' -[(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] (20.1 g, 40.0 mmol) in MeOH (400 mL) dropwise at 0°C , and the mixture was stirred at that temperature for 1 hr. Removal of the solvent under reduced pressure gave N,N' -[(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] dihydrochloride ($\mathbf{1}\cdot 2\text{HCl}$, 22.1 g, 38.4 mmol, 96%) as a colorless solid. Mp: $165\text{--}167^\circ\text{C}$ (dec). ^1H NMR ($\text{DMSO}-d_6$): δ 0.80 (q, $J = 10.8$ Hz, 2H), 1.07–1.21 (m, 3H), 1.46

(d, $J = 11.0$ Hz, 2H), 1.59 (br, 3H), 1.88 (br, 4H), 1.98 (br, 4H), 2.12 (q, $J = 10.7$ Hz, 1H), 3.11 (br, 4H), 3.46 (d, $J = 10.7$ Hz, 1H), 3.59 (br, 4H), 4.27 (d, $J = 4.0$ Hz, 4H), 7.29 (d, $J = 8.5$ Hz, 4H), 7.55 (d, $J = 8.5$ Hz, 4H), 10.63 (br, 2H), 11.08 (s, 2H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 23.0, 25.8, 26.2, 31.7, 40.5, 54.0, 55.7, 57.5, 119.7, 128.3, 136.2, 140.4, 163.4. IR (ATR): 1686 cm^{-1} . Analysis calculated for $\text{C}_{31}\text{H}_{44}\text{Cl}_2\text{N}_4\text{O}_2\cdot\text{H}_2\text{O}$: C, 62.72; H, 7.81; N, 9.44. Found: C, 62.46; H, 7.94; N, 9.34.

Three solutions of **1** (concentrations of 1.57, 6.25, and 25 mg/mL) were prepared for the toxicity test in rats. For the toxicity test in cynomolgus monkeys, five solutions of **1** were prepared (concentrations of 3, 6, 12, 25, and 50 mg/mL). The test substance, $\mathbf{1}\cdot 2\text{HCl}$, was dissolved in distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), and the pH of each solution was adjusted to 5.0 with 0.1 M aqueous NaOH.

Animals and housing conditions

All treatments and procedures using rats were performed at Nihon Bioresearch Inc. (Hashima, Japan), and those using cynomolgus monkeys were performed at Bozo Research Center Inc. (Tokyo, Japan), and all procedures were in compliance with the Act on Welfare and Management of Animals and the Basic Guidelines for the Use of Experimental Animals in Institutions under the Jurisdiction of the Japanese Ministry of Health, Labour and Welfare. Crl:CD (Sprague-Dawley) rats and cynomolgus monkeys were purchased from Charles River Laboratories Japan Inc. (Hino, Japan) and Nafovanny (Vietnam), respectively.

The rats were reared in the cages at $24 \pm 1^\circ\text{C}$ and $54 \pm 10\%$ relative humidity with a 12-hr light/dark cycle with filtered fresh air changed 12 times per hour. In the single administration study, the rats were allowed free access to solid feed (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water except in the 18 hr before the administration of **1** and the 6 hr after the administration of **1**. In the repeated administration study, the rats were allowed free access to the pellets and tap water throughout the day.

A pair of male and female cynomolgus monkeys were kept independently in cages at $23 \pm 5^\circ\text{C}$ and $55 \pm 25\%$ relative humidity with a 12-hr light/dark cycle with filtered fresh air changed 9–15 times per hour. The cynomolgus monkeys were fed 150 g of solid feed daily (PS; Oriental Yeast Co., Ltd., Tokyo, Japan) and were allowed free access to tap water. On administration days, the monkeys were fed 2 hr after the administration of **1**.

Experimental design

Acute toxicity test in rats via single oral administration

Forty rats were divided into four groups based on the average weight of each group. Each group comprised five male and five female rats. A solution of test substance **1** was administered orally to the 5-week-old rats at doses of 0 (vehicle-only control), 31.3, 125, and 500 mg/kg. The administration volumes were derived from the body weights of the rats (male rats: 134–145 g; female rats: 107–114 g; 20 mL/kg). A stainless steel feeding needle (Fuchigami Kikai Ltd., Kyoto, Japan) connected to a disposable polypropylene syringe (Terumo, Tokyo, Japan) was used for the oral administration of **1**. The general conditions of the rats were monitored daily for 14 days, and the body weights of the rats were measured using an electronic balance PG2002-S (Mettler Toledo International Inc., Greifensee, Switzerland) at days 0, 1, 3, 7, 10, and 14 after administration. When the rats died, a necropsy was carried out as soon as possible. Upon termination of the observation period, surviving rats were anesthetized by the intraperitoneal injection of 4% pentobarbital sodium solution (40 mg/kg), exsanguinated via the abdominal aorta, and then subjected to necropsy.

Subacute toxicity test in rats via repeated oral administration

Twenty rats were divided into two groups based on the average weight of each group. A solution of test substance **1** was administered orally to five male and five female 6-week-old rats at doses of 0 (vehicle-only control) and 31.3 mg/kg once a day for 7 days. The administration volumes were derived from the body weights of the rats (male rats: 214–234 g; female rats: 153–166 g; 20 mL/kg). The general conditions of rats were checked twice daily, and the body weights of the rats were measured at days 1, 2, 4, 7, and 8 after administration. One day after the final administration, the rats were sacrificed under anesthesia and subjected to necropsy. The organ weights of the brain, pituitary, thyroid, thymus, lungs, heart, liver, spleen, kidneys, adrenal glands, testes/ovaries, and prostate/uterus were measured using an electronic balance AB204 (Mettler Toledo International Inc., Greifensee, Switzerland).

Acute toxicity test in cynomolgus monkeys via single oral administration

A pair of male and female cynomolgus monkeys were employed for all tests. A solution of test substance **1** was administered orally to the monkeys at doses of 15, 30, 60,

and 125 in this order at one-week intervals. Twenty-two days after the last administration, substance **1** was administered to the monkeys at a dose of 250 mg/kg as an additional dose. The administration volumes were derived from the body weights of the monkeys (5 mL/kg). A soft catheter (Terumo, Tokyo, Japan) connected to a polypropylene syringe (Terumo, Tokyo, Japan) was used for the oral administration of **1**.

The various measurements were retaken for 7 days after administration. The body weights of the monkeys were measured weekly before administration. Body temperature, electrocardiogram, and blood pressure of the monkeys were recorded before administration and 0.5, 1, 3, and 4 hr after administration. The rectal temperature of the monkeys was taken as the body temperature using a digital electronic thermometer (Terumo-Finer; Terumo, Tokyo, Japan). Electrocardiographic measurements were conducted without anesthesia under monkey-chair restraint using an electrocardiograph (LABO-SYSTEM ZM-5012; Fukuda M•E Kogyo Co., Ltd., Tokyo, Japan). Heart rate, P-R interval, Q-T interval, QRS interval, QTC value, and QRS axis were recorded. The diastolic and systolic blood pressures of the chair-restrained and unanesthetized monkeys were measured noninvasively with a sphygmomanometer (BP-8800; Omron Colin, Tokyo, Japan).

Hematology tests and blood chemical analyses were carried out weekly starting 4 days after administration. One milliliter of blood was collected from the femoral vein of the monkeys into a blood collection tube containing dipotassium ethylenediaminetetraacetate (SB-41; Sysmex Corporation, Hyogo, Japan). Red blood cell (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte, platelet, and white blood cell (WBC) were analyzed using an ADVIA 120 hematology system (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). To a blood collection tube containing sodium citrate (Venoject II VP-C052K; Terumo, Tokyo, Japan), 1.8 mL of collected blood sample was added, and the sample was centrifuged at $1600 \times g$ for 10 min using a centrifuge separator (LC-122; Tomy Seiko Co., Ltd., Tokyo, Japan). Prothrombin time, activated partial thromboplastin time, and the amount of fibrinogen in the resulting blood plasma were measured using a hemostasis system (ACL Elite Pro; Instrumentation Laboratory, Bedford, MA). A blood sample (3 mL) from the monkeys was added to a blood collection tube containing heparin sodium (Venoject II VP-H050K; Terumo, Tokyo, Japan), and the tube was centrifuged at $1600 \times g$ for 10 min. The blood

plasma thus obtained was analyzed with respect to alkaline phosphatase (ALP), total cholesterol, triglyceride, phospholipid, total bilirubin, glucose, urea nitrogen, creatinine, sodium, potassium, chloride, calcium, phosphorus, total protein, albumin, A/G ratio, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) using a TBA-120FR (Toshiba Medical Systems Corporation, Tochigi, Japan).

Seven days after the final administration, the monkeys were anesthetized by an intramuscular injection of ketamine hydrochloride solution (10 mg/kg, Daiichi Sankyo, Tokyo, Japan), exsanguinated from the axillary artery and vein, and then subjected to necropsy. The organ weights of the heart, lungs, liver, kidneys, spleen, and brain were measured.

Toxicokinetic study in cynomolgus monkeys

Blood samples (0.8 mL) from the administered monkeys were collected from the saphenous vein before each administration and 0.5, 1, 2, 4, 8, and 24 hr after each administration. The collected blood samples were added to blood collection tubes containing heparin sodium (Venoject II VP-H052K; Terumo, Tokyo, Japan), and the tubes were centrifuged at $1600 \times g$ for 10 min to afford the blood plasma (approximately 300 μ L). Quantitative analysis of **1** in the blood plasma samples was carried out using an LC/MS/MS system [HPLC: 2690 separation module (Waters Corporation, Milford, MA), MS/MS: API4000 (AB SCIEX, Tokyo, Japan), control software: Analyst 1.5 (AB SCIEX)] equipped with a reversed-phase column CAPCELL PAK C18 AQ (2.0 mm I. D. \times 150 mm, 5 μ m, Shiseido Co., Ltd., Tokyo, Japan). A mixture of H₂O and MeOH (3:2 v/v) containing 0.1% HCO₂H was used as the eluent at a flow rate of 0.2 mL/min.

A standard curve and an internal standard were used for the quantification of compound **1**. *p*-Acetamidophenol was adopted as an internal standard, and solutions of *p*-acetamidophenol in methanol were prepared at concentrations of 100 and 101 ng/mL. Ten standard solutions of **1**·2HCl in methanol were prepared at concentrations of 5, 10, 20, 50, 100, 200, 500, 800, 1000, and 10000 ng/mL.

A solution of internal standard (20 μ L) and methanol (400 μ L) were added to the sample prior to treatment, and the mixture was stirred vigorously. After the mixture was centrifuged at $15000 \times g$ for 5 min at 4°C, the supernatant was collected. The supernatant was concentrated by drying under N₂, and the residue was redissolved in a mixture of H₂O/MeOH (100 μ L, 3:2 v/v) containing 0.1% HCO₂H. After the insoluble parts were filtered off, 10 μ L of the solution was subjected to LC/MS/MS analysis. The analysis was performed in ESI positive ion mode

under multiple reaction monitoring conditions. C₅H₁₀N (m/z 84.15) was selected as the target ion.

The time of maximum concentration (T_{\max}), maximum drug concentration (C_{\max}), and area under the plasma concentration-time curve ($AUC_{0-24\text{hr}}$) were derived from the plasma concentrations of compound **1**. If the concentrations were under 5 ng/mL, they were treated as 0 ng/mL. $AUC_{0-24\text{hr}}$ was measured by the trapezoidal method.

RESULTS

Preparation of compound **1**

Antiprion compound **1** was synthesized on a large scale from commercially available materials for 4 steps (Fig. 2). Initially, 4,4'-(cyclohexylmethylene)dianiline was prepared by reacting cyclohexanecarboxaldehyde with aniline in the presence of aniline hydrochloride. The diamine was then treated with bromoacetyl bromide under basic conditions to give bis(2-bromoacetamide). The substitution of bis(2-bromoacetamide) with pyrrolidine in the presence of K₂CO₃ afforded compound **1**. Finally, compound **1** was converted to the dihydrochloric acid salt (**1**·2HCl) by treatment with hydrogen chloride. The dihydrochloric acid salt (**1**·2HCl) was soluble in water.

Acute toxicity test in rats via single oral administration

To estimate the lowest lethal dose and the lowest

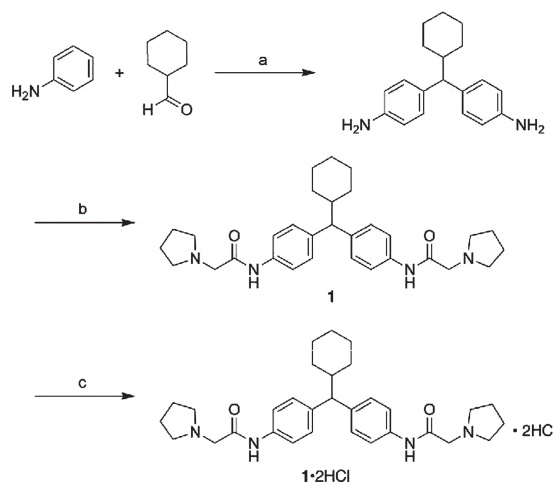


Fig. 2. Synthesis of *N,N'*-[(cyclohexylmethylene)di-4,1-phenylene]bis[2-(1-pyrrolidinyl)acetamide] dihydrochloride (**1**·2HCl). Reagents and conditions: (a) PhNH₂Cl, aniline, 120°C, 48 hr; (b) (i) BrCH₂COBr, pyridine, DMAP, CH₂Cl₂, 0°C, 1 hr; (ii) pyrrolidine, K₂CO₃, THF, 60°C, 12 hr; (c) HCl, MeOH/Et₂O, 0°C, 1 hr.

observed adverse effect level (LOAEL) of **1**, solutions compound **1** were administered orally to rats at doses of 0 (vehicle-only control), 31.3, 125, and 500 mg/kg, and the rats were observed for two weeks. The observations included general status, clinical signs, mortality, body weights, and necropsy findings. There were no abnormalities in clinical indicators or necropsy in the group treated with compound **1** at a dose of 31.3 mg/kg (Table 1). When compound **1** was administered to rats at a dose of 125 mg/kg, salivation (3 males and 2 females of the 10 rats), diarrhea (3 males of the 10), and loose stool (1 female of the 10) appeared within 1 hr. These symptoms disappeared within one day, and none of the treated rats died during the observation period. All organs and tissues remained intact in the necropsy. In the highest dose group (500 mg/kg), salivation (3 males and 5 females of the 10), a decrease in locomotor activity (3 males and 3 females of the 10), and diarrhea were observed just after administration, and dirt around the nose (2 males and 3 females of the 10) appeared within 1 day. Although 8 rats recovered from the abnormal status by 5 days after administration, the one male rat that exhibited hypothermia and bradypnea and the one female rat that exhibited nasal noise and abdominal distention died 4 and 11 days after administration, respectively. The reduction of the thymus and thinning of the forestomach mucous membrane were observed in the necropsy of the dead male rat. In the case of the dead female rat, distention of the digestive tract and a reduction of the thymus and spleen

were identified. A thickened forestomach mucous membrane was observed in a surviving female rat. There was no difference in body weight changes between the control group and the treated group at a dose of 31.3 mg/kg. Mild weight loss (8.2%) was observed 1 day after administration in male rats treated with **1** at a dose of 125 mg/kg, and a significant weight loss (male: 30.8%; female: 21.7%) was observed 3 days after administration in the highest dose group (Fig. 3).

Subacute toxicity test in rats via repeated oral administration

As a daily dose for the subacute toxicity test in rats, we chose a dose of 31.3 mg/kg/day based on the results of the single oral toxicity test in which the treated rats exhibited no abnormal status. Compound **1** was administered repeatedly to rats once a day for 7 days. One male and one female rat of the ten rats in the treatment group showed salivation at days 4–7 after administration. No significant decrease in weight gain was observed in the treated group compared to that of the vehicle-only control group. Abnormal necropsy findings were not identified. Although the organ weight of the spleen was slightly higher (21%) in the treated group, other organ weights were within the normal limit.

Acute toxicity test in cynomolgus monkeys via single oral administration

To estimate the LOAEL of compound **1** in cynomol-

Table 1. General symptoms observed in the acute toxicity test in rats via single oral administration of **1**.

Dose(mg/kg)	Status	Hours after administration				Days after administration											
		0–1	1–2	2–4	4–6	1	2	3	4	5–7	8	9	10	11	12–14		
0	Normal	10*	10	10	10	10	10	10	10	10	10	10	10	10	10		
31.3	Normal	10	10	10	10	10	10	10	10	10	10	10	10	10	10		
125	Normal	3	5	10	10	9	10	10	10	10	10	10	10	10	10		
	Salivation	5	1	0	0	0	0	0	0	0	0	0	0	0	0		
	Diarrhea	3	3	0	0	1	0	0	0	0	0	0	0	0	0		
	Loose stool	1	1	0	0	0	0	0	0	0	0	0	0	0	0		
500	Normal	0	0	3	2	0	4	5	7	8	8	8	8	8	8		
	Salivation	8	0	0	0	0	0	0	0	0	0	0	0	0	0		
	Decrease in locomotor activity	6	6	0	0	2	1	0	0	0	0	0	0	0	0		
	Diarrhea	10	10	5	8	9	5	1	0	0	0	0	0	0	0		
	Dirt around the nose	0	0	0	0	5	3	5	1	0	0	0	0	0	0		
	Hypothermia	0	0	0	0	0	1	1	0	0	0	0	0	0	0		
	Bradypnea	0	0	0	0	0	1	1	0	0	0	0	0	0	0		
	Nasal noise	0	0	0	0	0	1	1	1	1	1	0	0	0	0		
	Abdominal distention	0	0	0	0	0	0	0	0	0	1	1	1	1	0		
	Death	0	0	0	0	0	0	0	1	0	0	0	0	1	0		

* The number of rats that revealed each status.

Oral toxicity of an antiprion compound in rats and cynomolgus monkeys

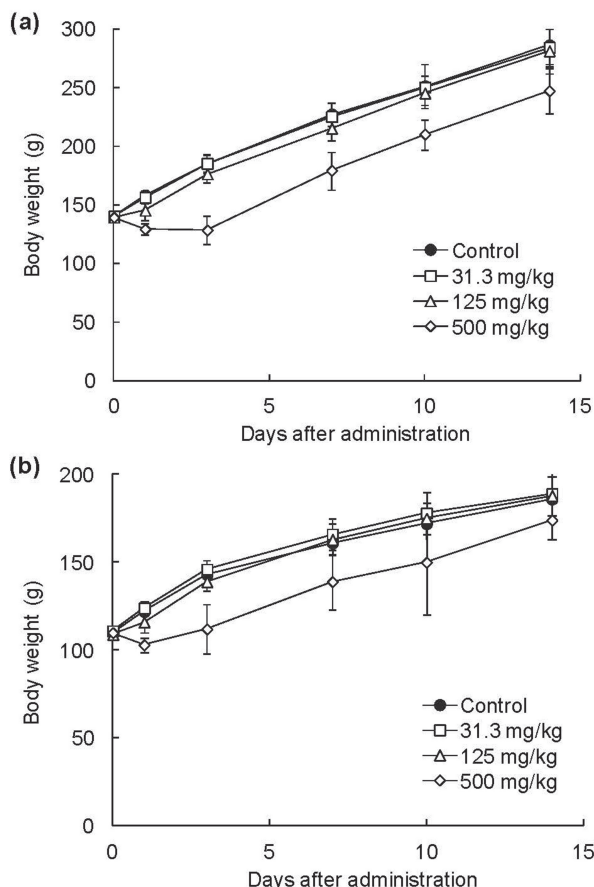


Fig. 3. Body weight changes in male (a) and female (b) rats treated compound **1** at doses of 31.3 mg/kg (white squares), 125 mg/kg (white triangles), and 500 mg/kg (white diamonds) for 14 days. Distilled water was administered orally to the control rats (black circles). Each value represents the mean and standard deviation of 5 rats.

gus monkeys, compound **1** was administered orally to a pair of male and female monkeys at doses of 15, 30, 60, 125, and 250 mg/kg at predetermined intervals. There were no abnormal findings in the monkeys treated with compound **1** at doses of 15 and 30 mg/kg. A male monkey showed vomiting and soft stool 2–4 hr after administration at doses of 60 mg/kg and higher. When compound **1** was administered to a female monkey at a dose of 60 mg/kg, vomiting appeared 6 hr after administration. At a dose of 125 mg/kg, a female monkey showed salivation and vomiting 5–90 min after administration. At a dose of 250 mg/kg, a female monkey showed vomiting, soft stool, and watery stool 1.5, 4–48, and 2 hr after administration, respectively. The male and

female monkeys did not die even at a dose of 250 mg/kg. No remarkable abnormal findings were observed in the body weight, body temperature, electrocardiogram, blood pressure, blood chemical analysis, necropsy, and organ weight of the treated monkeys. In the hematological tests, the values of RBC, Hb, and Ht of the male and female monkeys decreased with increasing dose up to 125 mg/kg because of frequent blood sampling. When compound **1** was administered to two monkeys at a dose of 250 mg/kg 22 days after the last administration, these values returned to the level observed following the first treatment.

Toxicokinetic study in cynomolgus monkeys

To elucidate the toxicokinetic profile of compound **1**, the quantitative analysis of compound **1** in the blood plasma samples of the treated monkeys was carried out using LC/MS/MS. The plasma concentrations of compound **1** in male and female cynomolgus monkeys increased with increasing dosage of compound **1** (Fig. 4). There was little decline in plasma concentration of this compound 8 hr after administration. In terms of the toxicokinetic parameters, the values of AUC_{0-24hr} tended to increase with increasing dosage of compound **1** (Table 2). The half-life could not be determined because of the small number of blood samples collected within 8 hr after administration.

DISCUSSION

Prion diseases are progressive neurodegenerative disorders that are incurable and invariably fatal. Since the epidemic of BSE and the appearance of a new variant Creutzfeldt-Jakob disease, much effort has been devoted to developing a therapeutic treatment for prion diseases. Unfortunately, there is still no established treatment for the diseases thus far. For example, an oral administration of quinacrine to patients with prion diseases transiently improved their manifestation (Collinge *et al.*, 2009). However, the continuous administration of quinacrine led to liver dysfunction, and the treatment had to be discontinued (Nakajima *et al.*, 2004). As a continuation of our rational antiprion drug discovery and development studies (Hosokawa-Muto *et al.*, 2009; Ishikawa *et al.*, 2009; Kimura *et al.*, 2011a; Kimura *et al.*, 2011b; Kuwata *et al.*, 2007; Yamamoto and Kuwata, 2009), we found that the administration of GN8 derivative **1** prolonged the survival time of TSE-infected mice and slowed the development of neurological and psychological symptoms in TSE-infected macaques (Yamaguchi *et al.*, 2019). Since **1** is a promising candidate for antiprion agent development, it is of great importance to identify its toxicological features from the standpoint of the practical use of compound **1**.

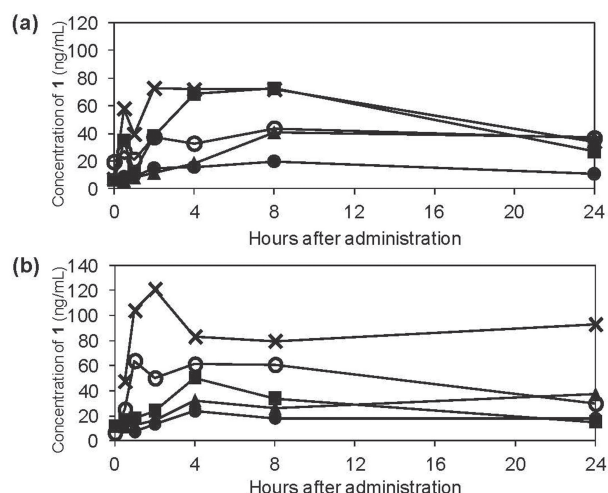


Fig. 4. The time course of plasma concentrations of compound **1** in male (a) and female (b) cynomolgus monkeys treated at doses of 15 mg/kg (black circles), 30 mg/kg (black triangles), 60 mg/kg (black squares), 125 mg/kg (white circles), and 250 mg/kg (crosses) over 24 hr following oral administration.

Table 2. Toxicokinetic parameters in male and female cynomolgus monkeys treated compound **1**.

	Dose (mg/kg)	T _{max} (hr)	C _{max} (ng/mL)	AUC _{0-24hr} (ng·hr/mL)
Male	15	8	19.8	364
	30	8	40.5	786
	60	8	72.5	1230
	125	8	43.6	918
	250	2	72.6	1380
Female	15	4	23.7	418
	30	24	37.0	692
	60	4	50.2	670
	125	1	63.8	1160
	250	2	121	2070

In the acute toxicity test in rats via single oral administration, compound **1** was found to have no adverse effects at a dose of 31.3 mg/kg, whereas several symptoms, such as salivation, diarrhea, and loose stool, were observed in the rats administered doses of 125 and 500 mg/kg. From these results, the LOAEL was presumed to be between 31.3 and 125 mg/kg. After the compound was administered intraperitoneally to mice at a dose of 30 mg/kg, systemic adverse effects were not observed (Yamaguchi *et al.*, 2019). When the rats were treated at a dose of 125 mg/kg, adverse effects were not observed for all rats and faded over time, indicating that they were not serious.

The mortality rate of rats treated at 500 mg/kg was 20%. Therefore, the lowest lethal dose was estimated to be less than 500 mg/kg and would be close to 500 mg/kg. When compound **1** was administered repeatedly to rats at a dose of 31.3 mg/kg, there were no abnormal findings, suggesting that compound **1** can be safely used repeatedly at that dose.

The acute toxicity test in cynomolgus monkeys revealed that the administration of compound **1** at doses of 60 mg/kg or higher led to vomiting and fecal abnormalities. The LOAEL of compound **1** for cynomolgus monkeys was estimated to be 30–60 mg/kg. The monkeys did not die at a dose of 250 mg/kg, suggesting that the lowest lethal dose of **1** for the monkeys was higher than 250 mg/kg.

After oral administration to cynomolgus monkeys, we observed an increase in the plasma concentrations of compound **1**. Additionally, an increase in plasma concentrations of ¹⁴C-labeled **1** was found after intraperitoneal administration into mice (Yamaguchi *et al.*, 2019). Migration of **1** from the periphery to the bloodstream was observed. A toxicokinetic study revealed that the plasma concentration of **1** was maintained after T_{max}, although the migration efficiency of **1** into the blood was low, indicating that **1** would be a long-acting compound.

In conclusion, we conducted single and repeated oral toxicity tests of antiprion compound **1** in rats and a single oral toxicity test in cynomolgus monkeys. The LOAELs of compound **1** were estimated to be 31.3–125 mg/kg and 30–60 mg/kg in rats and cynomolgus monkeys, respectively. The lowest lethal doses of compound **1** in rats and cynomolgus monkeys were determined to be approximately 500 mg/kg and higher than 250 mg/kg, respectively. Although the administration of **1** at the highest dose led to fecal abnormalities in both rats and cynomolgus monkeys, these effects were not serious. Daily repeated oral administration of compound **1** to rats at a dose of 31.3 mg/kg for one week resulted in no abnormal findings. GN8 derivative **1** (a designer molecular chaperone) has potential as a therapeutic agent but requires further research in clinical trials using oral administration.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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