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Short communication

Synthesis of 9-substituted 2,3,4,9-tetrahydro-1*H*-carbazole derivatives and evaluation of their anti-prion activity in TSE-infected cells

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ABSTRACT

2,3,4,9-Tetrahydro-9-[2-hydroxy-3-(1-piperidinyl)propyl]-6-methyl-1*H*-carbazol-1-one (GJP14) is a novel anti-prion compound that we previously discovered by *in silico* screening and cellular assay. In this study, a variety of GJP14 derivatives were prepared using pyrrole derivatives, (haloalkyl)oxiranes, and amines, and their anti-prion activity was evaluated in TSE-infected cells. It was found that the tricyclic aromatic ring, a hydroxy group at the 2-position and an amino group at the 3-position of the *N*-propyl group were the basic requirements for anti-prion activity. The derivatives bearing an *N*-*ortho*-halobenzyl group exhibited an improved activity, and the most potent derivative was 8 times as effective as the original lead compound, GJP14.

1. Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a family of invariably fatal neurodegenerative disorders that affect humans and animals [1–3]. Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia, and kuru in humans, scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease in deer and elk are well-known representatives of prion diseases. These disorders are characterized by the spongiform degeneration of the central nervous system. The epidemic of BSE and the appearance of a new variant CJD provoked great public concern and necessitated the development of suitable treatments for prion diseases because no effective curative therapy for the diseases exists thus far [4,5].

The causative agent of prion diseases is thought to be a pathogenic isoform of cellular prion protein (PrP^C), designated PrP^{Sc}. PrP^{Sc} is formed via the conformational conversion of PrP^C and acts as a seed that provides a template for converting PrP^C to PrP^{Sc}. Therefore, PrP^{Sc} is one of the potential drug targets. However, PrP^{Sc} forms heterogeneous aggregates, and the diverse prion strains make rational drug design difficult [6]. The structurally well-defined PrP^C is an alternative target molecule that consists of a flexible N-terminal region containing an octapeptide repeat domain and a globular C-terminal region comprising three α-helices and one anti-parallel β-sheet. A chemical chaperone that binds to slowly fluctuating regions (hot spots) of PrP^C and stabilizes its conformation might be effective in inhibiting the accumulation of PrP^{Sc} [7–9]. A virtual screening that was focused on the hot spots of PrP^C was performed in the ZINC database containing approximately 600 000 commercially available compounds using AutoDock software, and we found a novel anti-prion compound, 2,3,4,9-tetrahydro-9-[2-hydroxy-3-(1-piperidinyl)propyl]-6-methyl-1*H*-carbazol-1-one (GJP14, Fig. 1) [10]. Surface plasmon resonance

Abbreviations: TSEs, transmissible spongiform encephalopathies; CJD, Creutzfeldt–Jakob disease; GSS, Gerstmann–Sträussler–Scheinker syndrome; PrP^C, cellular form of prion protein; PrP^{Sc}, infectious isoform of prion protein; PrP^{res}, proteinase K-resistant prion protein.

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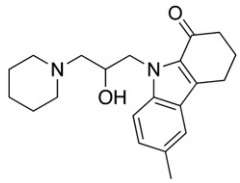


1

IC_{50}: 8.54 ± 0.28 μM

Fig. 1. Chemical structure of **1** (GJP14).

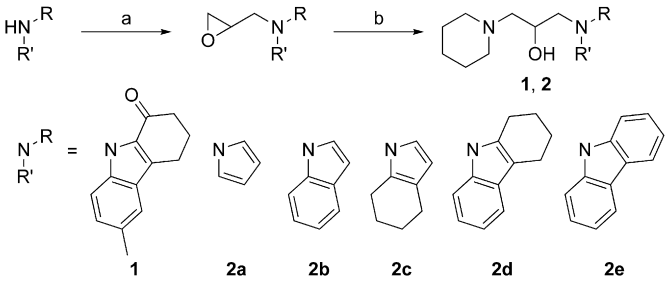
binding assay of GJP14 for PrP^C revealed that it had a capacity for binding to PrP^C. Herein, we report the synthesis of GJP14 derivatives and the evaluation of their anti-prion activity in TSE-infected cells.

2. Chemistry

Lead compound **1** (GJP14) is composed of a 6-methyl-2,3,4,9-tetrahydro-1*H*-carbazol-1-one scaffold, a piperidine unit, and a 2-hydroxypropylene tether (Fig. 1). To identify the structural features responsible for anti-prion activity and to improve the activity of lead compound **1**, a series of GJP14 derivatives **2**–**5** were prepared, and their anti-prion activity was tested in GSS-infected mouse neuronal cells. Our strategy for drug design mainly relies on trial-and-error testing of a series of derivatives on TSE-infected cells. Physicochemical properties of compounds such as molecular weight and ClogP were also taken into account.

GJP14 derivatives **2**, in which the 6-methyl-2,3,4,9-tetrahydro-1*H*-carbazol-1-one scaffold was replaced with other units, were prepared from a series of pyrrole derivatives, epichlorohydrin, and piperidine (Scheme 1) [11,12]. The pyrrole derivatives were treated with sodium hydride, and the resulting sodium amides were reacted with epichlorohydrin to produce *N*-(2,3-epoxypropyl)arenes. Subsequently, a ring-opening reaction of the *N*-(2,3-epoxypropyl)arenes with piperidine afforded the derivatives **2** in a total yield of 42–71% for the entire process.

GJP14 fragments **3b** and **3c** were prepared by alkylation of 2,3,4,9-tetrahydro-1*H*-carbazole **3a** with 1-bromopropane and 2-bromoethanol, respectively (Scheme 2). The alkyl chain-elongated derivative **3d** was synthesized from **3a**, 1-bromo-3,4-epoxybutane, and piperidine in a similar manner as employed for the synthesis of **2**. Alcohol **2d** was converted to the corresponding methyl ether **3e** by methylation of sodium alkoxide with methyl iodide. Finally, derivatives **4** and **5**, in which the terminal 1-piperidinyl group of **2d** was replaced with other amino groups, were prepared by reacting 1-(2,3-epoxypropyl)-2,3,4,9-tetrahydro-1*H*-carbazole **2d′** with a variety of primary and secondary amines in 27–87% yield (Scheme 3).



Scheme 1. Synthesis of **1** (GJP14) and its derivatives **2**. Reagents and conditions: (a) (i) NaH, DMF; (ii) epichlorohydrin, DMF; (b) piperidine, EtOH.

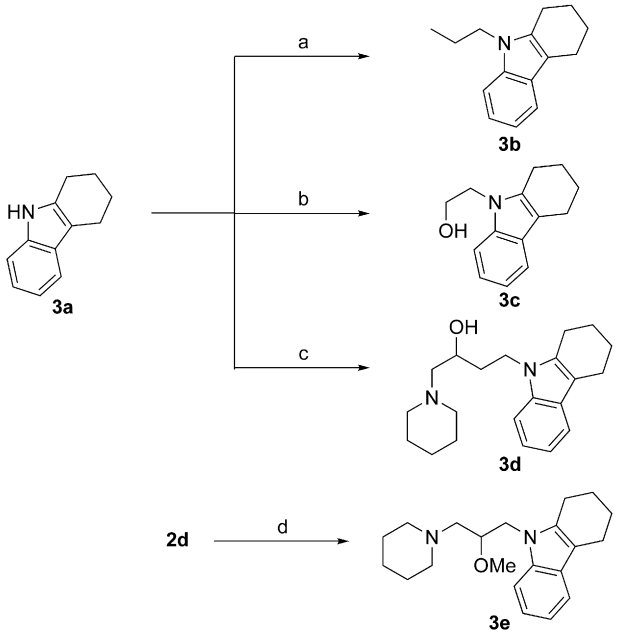
3. Anti-prion activity assay

The anti-prion activities of compound **1** and its derivatives **2**–**5** were assayed in GT + FK cells that are mouse neuronal cells (GT1-7) persistently infected with mouse-adapted GSS agent (Fukuoka-1 strain) [13,14]. The ability of the compounds to inhibit the accumulation of PrP^{Sc} was assessed by quantification of proteinase K-resistant prion protein (PrP^{res}) following treatment of the cells with the compounds. The percentage of PrP^{res} relative to the negative control at a concentration of 10 μM is presented in Fig. 2. The IC_{50} value is the concentration of the compound that caused 50% inhibition of PrP^{res} accumulation. The original lead compound **1** inhibited the accumulation of PrP^{res} with an IC_{50} value of 8.54 ± 0.28 μM.

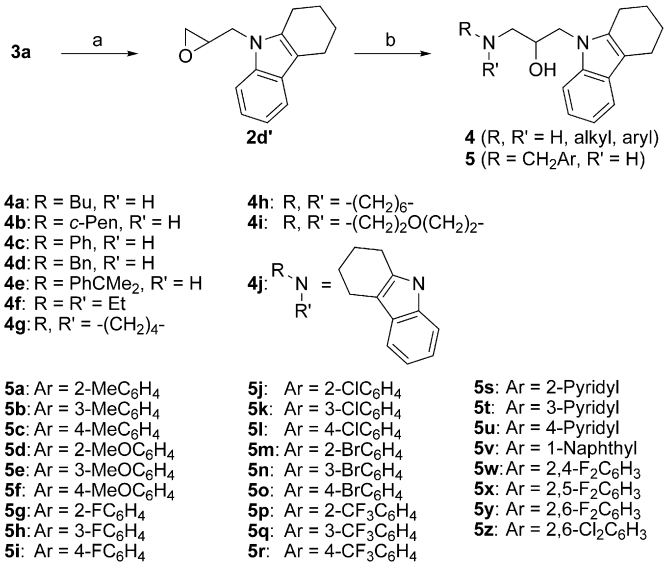
4. Results and discussion

Initially, the anti-prion activities of a series of derivatives **2** were tested in GT + FK cells (Scheme 1 and Fig. 2). When the 6-methyl-2,3,4,9-tetrahydro-1*H*-carbazol-1-one scaffold of compound **1** was replaced with pyrrole (**2a**), indole (**2b**), and 4,5,6,7-tetrahydro-1*H*-indole (**2c**), anti-prion activity was lost. However, PrP^{res} accumulation was inhibited by the use of compounds derived from 2,3,4,9-tetrahydro-1*H*-carbazole (**2d**) and carbazole (**2e**) to a similar extent as compound **1**. These results indicate that the presence of an aromatic tricyclic component is crucial for anti-prion activity, while a methyl group at the 6-position and carbonyl group at the 1-position in **1** are less important.

Second, the anti-prion activities of fragments **3a**–**c** and analogs **3d** and **3e** were examined (Scheme 2 and Fig. 2). 2,3,4,9-Tetrahydro-1*H*-carbazole (**3a**) had no anti-prion activity. The truncation of functional groups from the *N*-[2-hydroxy-3-(1-piperidinyl)propyl] group of **2d** led to a significant decrease in activity. The above results suggest that both the amino group and the hydroxy group are the essential structural features responsible for anti-prion activity. The hydroxy group appeared to act as a hydrogen bond donor because the replacement of the hydroxy group of **2d** with a methoxy group resulted in a loss of activity.



Scheme 2. Synthesis of GJP14 derivatives **3**. Reagents and conditions: (a) (i) NaH, DMF; (ii) PrBr, DMF; (b) $HOCH_2CH_2Br$, KOH, DMSO; (c) (i) NaH, DMF; (ii) 1-bromo-3,4-epoxybutane, DMF; (iii) piperidine, EtOH; (d) (i) NaH, THF; (ii) MeI, THF.



Scheme 3. Synthesis of GJP14 derivatives **4** and **5**. Reagents and conditions: (a) (i) NaH, DMF; (ii) epichlorohydrin, DMF; (b) RR'NH, MeOH or EtOH.

Elongation of the alkyl chain led to a slight decrease in activity, indicating that the propylene linker is adequate to maintain the proper distance between the tricyclic ring and the functional groups.

Third, the derivatives **4**, in which the terminal 1-piperidinyl group of **2d** was replaced with various *N*-substituted and *N*,*N*-di-substituted amino groups, were investigated (Scheme 3 and Fig. 2). Compounds derived from primary amines (**4a**, **4b**, and **4d**) with the exception of **4c** were found to be more potent than those derived from secondary amines (**2d** and **4f**–**j**). The morpholine derivative **4i** was less effective than the piperidine derivative **2d**. These results indicate that the hydrogen atom of the terminal amino group acts as a hydrogen bond donor in the case of compounds derived from primary amines and that the modest hydrophobic property of the substituent on the nitrogen atom is required for activity. Of the derivatives **4**, the *N*-benzyl derivative **4d** had the best terminal amino group.

Finally, the benzyl group of compound **4d** was refined comprehensively (Scheme 3 and Fig. 2). The anti-prion activities of derivatives bearing an electron-donating group such as a methyl group (**5a** and **5b**) or a methoxy group (**5d**) were comparable to that of **4d**. Intriguingly, derivatives bearing a fluoro, chloro, or bromo group at the *ortho*-position of the phenyl group (**5g**, **5j**, and **5m**) had improved activity, with relative PrP^{res} levels of 10–12%, while the introduction of such groups at the *meta*- or *para*-positions (**5h**, **5i**, **5k**, and **5n**) did not lead to an increase in activity. The derivatives bearing a (trifluoromethyl)phenyl group (**5p** and **5r**), a pyridyl group (**5s**–**u**), or a 1-naphthyl group (**5v**) were less active than **4d**. The introduction of a second halo group to the aromatic ring (**5w**–**z**) led to retained activity or a slight loss in activity. The derivatives **5e** and **5q**, and the *para*-substituted derivatives **5c**, **5f**, **5l**, and **5o** were cytotoxic at 10 μM.

The increase in activity observed in the case of *ortho*-halobenzyl derivatives such as **5g**, **5j**, and **5m** has been attributed to the additional halogen–oxygen interaction between the *ortho*-halo group and the biomolecule rather than the electronic effect of the *ortho*-halo group on the aromatic ring [15]. In fact, the introduction of a halo group at the *meta*- and *para*-positions did not necessarily improve the activity of the compounds tested here. This is additionally supported by our results showing that derivatives with a more electron-deficient aromatic ring (**5w**–**z**) did not show a further increase in activity.

The GJP14 derivatives bearing a hydroxy group possess a chiral center that gives rise to two enantiomers. To compare the anti-prion activity of the *R*- and *S*-enantiomers, optically active *R*-**5g** and *S*-**5g** were prepared from *R*- and *S*-epichlorohydrins, and their

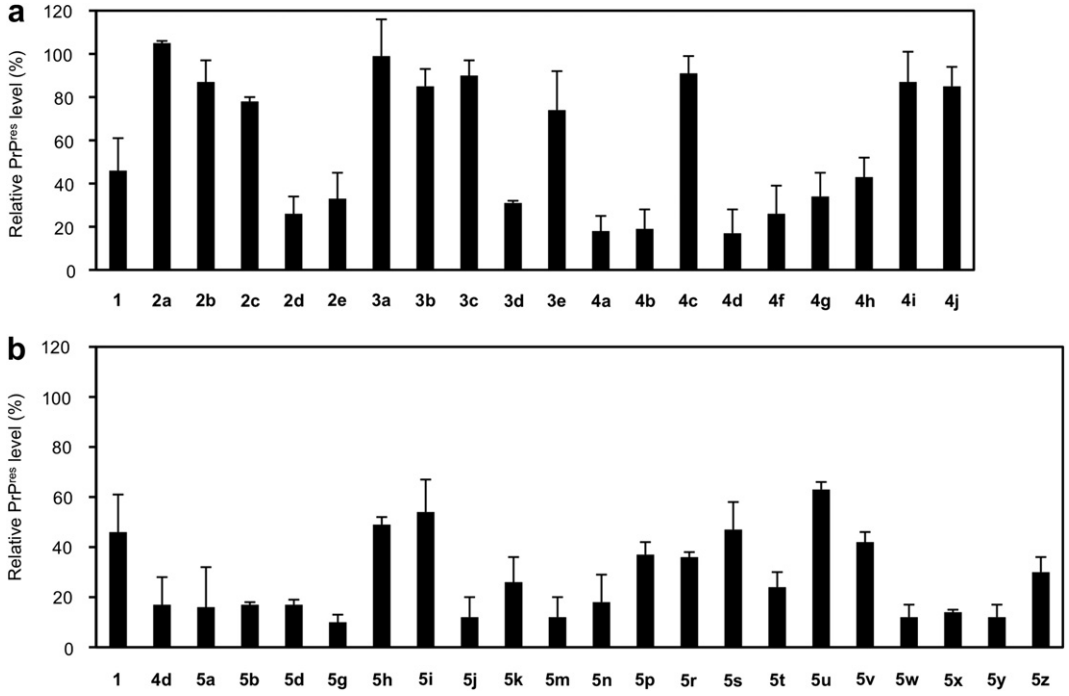
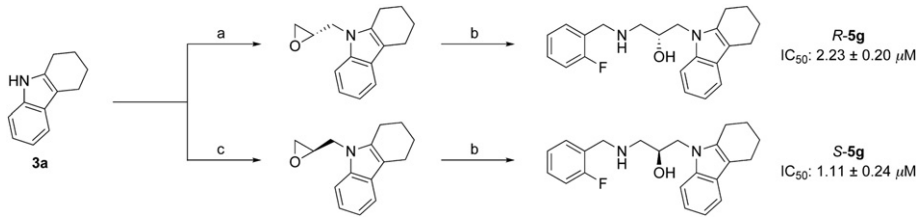


Fig. 2. (a) Anti-prion activity of **1**–**4** in GT + FK cells at 10 μM. (b) Anti-prion activity of **1**, **4d** and **5** in GT + FK cells at 10 μM. Bars represent means ± SD. The derivatives **4e**, **5c**, **5e**, **5f**, **5l**, **5o**, and **5q** were cytotoxic at 10 μM. Relative PrP^{res} levels for these compounds at 5 μM were as follows: **4e**, 34 ± 1%; **5c**, 32 ± 3%; **5e**, 21 ± 2%; **5f**, 23 ± 7%; **5l**, 18 ± 5%; **5o**, 25 ± 5%; **5q**, 40 ± 5%. Relative PrP^{res} levels for **4d** and GN8 at 5 μM were as follows: **4d**, 28 ± 3%; GN8, 34 ± 3%.



Scheme 4. Synthesis of optically active GJP14 derivatives *R*-**5g** and *S*-**5g**. Reagents and conditions: (a) (i) NaH, DMF; (ii) (*R*)-epichlorohydrin, DMF; (b) 2-fluorobenzylamine, MeOH. (c) (i) NaH, DMF; (ii) (*S*)-epichlorohydrin, DMF.

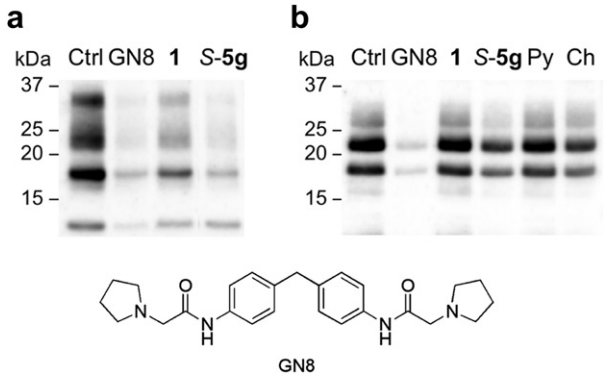


Fig. 3. (a) Western blotting of PrP^{res} in GT + FK cells after treatment with GN8, **1**, and *S*-**5g** at 10 μM. (b) Western blotting of PrP^{res} in ScN2a-3-Ch cells after treatment with GN8, **1**, *S*-**5g**, 3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazol-5(4*H*)-one (Py), and chrysoidine (Ch) at 5 μM. Relative PrP^{res} levels for these compounds were as follows: GN8, 6%; **1**, 82%; *S*-**5g**, 39%; Py, 74%; Ch, 48%.

anti-prion activity was evaluated in GT + FK cells (Scheme 4). Both *R*-**5g** and *S*-**5g** have anti-prion activity, and *S*-**5g** (IC_{50}: 1.11 ± 0.24 μM) was more potent than *R*-**5g** (IC_{50}: 2.23 ± 0.20 μM), with a eudysmic ratio of 2 ($P < 0.01$, Student's *t*-test). Derivative *S*-**5g** was 8 times more effective than the original lead compound **1**, and its anti-prion activity was comparable to that of GN8 (1.4 μM) [16]. The expression level of PrP^{C} remained intact in GT + FK cells treated with *S*-**5g**, as evidenced by a cellular assay without proteinase K digestion.

In this study, we used GT + FK cells as a cell culture model of human prion disease. Unlike ScN2a cells, GT + FK cells produce significant amounts of PrP^{res} as well as PrP^{C} [14]. Hence, the assay system using GT + FK cells is reliable, reproducible and less sensitive to minor perturbations such as those due to culture conditions.

To date, a variety of compounds have been identified that inhibit the accumulation of PrP^{res} in TSE-infected cells [17,18]. However, the strain-dependent activity of such compounds is a troublesome matter in drug development for prion diseases. Indeed, anti-prion compounds that were effective in scrapie-infected cells were not necessarily effective in our assay system using GSS-infected cells [19]. In contrast, the derivative *S*-**5g** exhibited anti-prion activity in both GSS-infected cells and scrapie-infected cells (ScN2a-3-Ch cells) [20], as shown in Fig. 3. It is noteworthy that the anti-prion activity of *S*-**5g** in ScN2a-3-Ch cells was higher than those of an edaravone derivative [21] and chrysoidine [22] that were reported to be effective at low nanomolar concentrations in ScNB cells and ScN2a cells.

5. Conclusion

A total of 47 derivatives were prepared by the epoxyalkylation of pyrrole derivatives and subsequent ring-opening reaction with amines. The (*S*)-*N*-*ortho*-fluorobenzyl derivative *S*-**5g** was found to be the most active compound among these derivatives, with an IC_{50} value of 1.11 μM. The derivative *S*-**5g** was 8-fold more effective than the original lead compound **1**. A tricyclic aromatic scaffold, a hydroxy group, and a terminal amino group were determined to be the basic requirements for anti-prion activity. Further investigations *in vitro* and *in vivo*, as well as molecular modeling studies, are currently ongoing and will be reported in due course.

6. Experimental protocols

6.1. Chemistry

6.1.1. General methods

All manipulations were performed under an argon atmosphere. Argon gas was dried by passage through P_2O_5. 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. The reactions were carried out in a temperature-controlled personal organic synthesizer (PPS-1510, Eyela). The progress of the reactions was monitored using thin-layer chromatography with a silica gel $60F_{254}$ TLC glass plate (Merck), with UV light as a visualizing agent. Chromatographic purification of the compounds was performed by medium-pressure liquid chromatography (MPLC) using a YFLC AI-580 system (Yamazen) equipped with a silica gel high-flash column or by gel permeation chromatography (GPC) using an LC-9201 system (Japan Analytical Industry).

6.1.2. Spectroscopic methods

NMR spectra were recorded on an Inova 500 spectrometer (Varian) and an Avance 500 (Bruker) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C acquisitions. The chemical shifts of protons were reported in δ values referred to Me_4Si as an internal standard. The chemical shifts of ^{13}C were reported in δ values referred to $CDCl_3$ (77.05 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.

6.1.3. Analytical methods

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 were left uncorrected.

6.1.4. General procedure for the synthesis of GJP14 and its derivatives **1**–**5**. A representative procedure for the synthesis of 1-(1-piperidinyl)-3-(1,2,3,4-tetrahydro-9H-carbazol-9-yl)-2-propanol (**2d**)

A solution of 2,3,4,9-tetrahydro-1*H*-carbazole (**3a**, 171 mg, 1.00 mmol) in DMF (1 mL) was added to a suspension of sodium hydride (26 mg, 1.1 mmol) in DMF (4 mL) at 0 °C, and the mixture

was stirred at 25 °C for 1 h. Epichlorohydrin (102 mg, 1.10 mmol) was added to the resulting solution at 0 °C, and the mixture was stirred at 25 °C for 12 h. The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by MPLC on a silica gel using hexane/$CHCl_3$ as the eluent and GPC using $CHCl_3$ as the eluent to produce 1-(2,3-epoxypropyl)-2,3,4,9-tetrahydro-1*H*-carbazole (**2d′**, 185 mg, 0.814 mmol, 81% yield) as a pale yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.84–1.96 (m, 4H), 2.46 (dd, J = 2.6, 4.9 Hz, 1H), 2.71–2.76 (m, 5H), 3.19–3.22 (m, 1H), 4.14 (dd, J = 4.8, 15.7 Hz, 1H), 4.30 (dd, J = 3.6, 15.7 Hz, 1H), 7.08 (dt, J = 0.9, 7.6 Hz, 1H), 7.08 (dt, J = 1.1, 7.6 Hz, 1H), 7.29 (d, J = 7.6 Hz, 1H), 7.47 (d, J = 7.7 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 21.0, 22.2, 23.1, 23.3, 44.2, 45.5, 51.0, 108.6, 110.0, 117.9, 119.0, 120.9, 127.5, 135.6, 136.6. IR (ATR): 1256 cm^{-1}. MS (EI): m/z 227 (M^+). HRMS calcd for $C_{15}H_{17}NO$: 227.1310. Found: 227.1323. Piperidine (104 mg, 1.22 mmol) was added to a solution of **2d′** (185 mg, 0.814 mmol) in EtOH (5 mL) at 25 °C, and the mixture was stirred under reflux for 12 h. After the solvent was evaporated, the residue was purified by MPLC on a silica gel using $CHCl_3$/MeOH as the eluent to give **2d** (221 mg, 0.707 mmol, 87% yield) as a colorless solid. Mp: 65–67 °C (dec). ^{1}H NMR ($CDCl_3$): δ 1.41–1.59 (m, 6H), 1.83–1.96 (m, 4H), 2.29–2.36 (m, 4H), 2.53 (br, 2H), 2.68–2.84 (m, 4H), 4.00–4.10 (m, 3H), 7.05 (dt, J = 1.0, 7.7 Hz, 1H), 7.12 (dt, J = 1.1, 7.7 Hz, 1H), 7.28 (d, J = 7.7 Hz, 1H), 7.45 (d, J = 7.7 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 21.1, 22.6, 23.2, 23.4, 24.2, 26.1, 47.1, 54.6, 62.5, 66.4, 109.0, 109.6, 117.7, 118.7, 120.6, 127.5, 135.9, 136.7. IR (ATR): 3260 cm^{-1}. MS (EI): m/z 312 (M^+). HRMS calcd for $C_{20}H_{28}N_2O$: 312.2202. Found: 312.2175.

6.2. Anti-prion activity assay

The anti-prion activity assay was performed as described in our preceding paper [10]. We used an immortalized neuronal mouse cell line that was persistently infected with the human TSE agent (Fukuoka-1 strain) [13]. This cell line was grown and maintained at 37 °C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Equitech-bio, Kerrville, TX), 50 U/mL penicillin G sodium, and 50 μg/mL streptomycin sulfate (Invitrogen, Carlsbad, CA). Confluent cells were passaged every 7 days using 0.25% trypsin and 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrasodium salt (Invitrogen, Carlsbad, CA), and the cell concentration was adjusted to 0.5 × 10^5 cells/mL with medium. Compounds were dissolved in dimethyl sulfoxide at a stock concentration of 10 mM. Approximately 3 × 10^5 cells were plated in each well of a six-well plate, and 15 h later, the media was replaced with fresh media containing the appropriate dilution of the 10 mM compound stock. Control cells were treated with medium containing solvent alone (0.1% dimethyl sulfoxide). Three days after the addition of the compound, cells were lysed in 150 μL of 1 × Triton-DOC lysis buffer (150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 50 mM Tris–HCl [pH 7.5]) [14]. After 1 min of centrifugation at 11200×*g*, the supernatant was collected, and its total protein concentration was measured by the BCA protein assay (Thermo Fisher Scientific, Rockford, IL) and adjusted with lysis buffer to 1 mg/mL. The samples were digested with 20 μg of proteinase K per mL for 30 min at 37 °C, and the digestion was stopped with 3 mM Pefabloc SC (Roche Applied Science, Indianapolis, IN). The samples were centrifuged at 21952×*g* for 45 min at 4 °C, and the pellets were resuspended in sample buffer and then loaded onto a 15% polyacrylamide gel just after boiling. Western blotting for PrP^{res} was performed as described previously [10]. As a primary antibody, the PrP M-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect PrP^{res}. The signals were visualized using a SuperSignal reagent (Thermo Fisher Scientific, Rockford, IL) and scanned using a LAS-1000 UV mini analyzer (Fujifilm, Tokyo, Japan). The total density of the PrP^{res} bands in each sample was measured and compared to that of the control treated with medium containing solvent alone using Multi Gauge software (Fujifilm, Tokyo, Japan). IC_{50} values were determined by densitometry of four or five concentration points measured from the immunoreactive bands observed by Western blot. Two or three independent experiments were performed to determine the IC_{50}.

The anti-prion activity assay in ScN2a-3-Ch cells was performed in a manner similar to that in GT + FK cells. Approximately 1 × 10^5 cells were plated in each well of a six-well plate in the assay using ScN2a-3-Ch cells.

Acknowledgments

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.08.039.

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