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# Synthesis of GN8 derivatives and evaluation of their antiprion activity in TSE-infected cells

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ABSTRACT

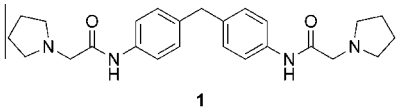
A series of GN8 derivatives were synthesized from various diamines, carboxylic acid derivatives, and nitrogen nucleophiles, and their antiprion activity was tested in TSE-infected mouse neuronal cells. We found that two ethylenediamine units, hydrophobic substituents on the nitrogen atoms, and the diphenylmethane scaffold were essential structural features responsible for the activity. Seven derivatives bearing substituents at the benzylic position exhibited an improved antiprion activity with the $IC_{50}$ values of 0.51–0.83 μM. Conformational analysis of model compounds suggested that the introduction of the substituent at the benzylic position restricted the conformational variability of the diphenylmethane unit.



Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are invariably fatal neurodegenerative disorders of mammals characterized by the accumulation of a pathogenic isoform ($PrP^{Sc}$) of cellular prion protein ($PrP^{C}$) in the central nervous system.[1,2] These disorders include Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia, and kuru in humans, scrapie in sheep and goat, bovine spongiform encephalopathy in cattle, and chronic wasting disease in deer and elk. There is no cure for prion diseases to date.

Since the appearance of a new variant Creutzfeldt–Jakob disease, considerable effort has been devoted to developing a therapeutic treatment for the disease.[3,4] Consequently, a number of compounds have been identified that inhibit $PrP^{Sc}$ accumulation in prion-infected cells.[5] However, most of these compounds are inadequate for use as therapeutic agents for the following reasons: (i) their antiprion activity is not sufficient and the chemical structure is unsuitable for the appropriate derivatization necessary for lead compound optimization; (ii) low blood–brain barrier permeability results in a limited antiprion effect and serious adverse effects in vivo; and (iii) strain-dependent activity restricts the drug's use to a small range of prion diseases.[6] Recently, we discovered a novel antiprion compound, *N*,*N*′-(methylenedi-4,1-phenylene)bis[2-(1-pyrrolidinyl)acetamide] (GN8),[7–10] through a virtual screening based on the structure of $PrP^{C}$ (Fig. 1).[11,12] GN8 is a promising antiprion lead compound because it is strain-independent, and when administered subcutaneously it prolonged the survival time of TSE-infected mice. Herein, we report the synthesis of GN8 derivatives and evaluation of their antiprion activity in TSE-infected cells.

GN8 (**1**) is composed of a diphenylmethane scaffold and two 2-(1-pyrrolidinyl)acetylamino groups at the 4,4′-positions of diphenylmethane (Fig. 1). To identify the chemical structure responsible for the antiprion activity and to optimize lead compound **1**, a series of GN8 derivatives **2–7** were prepared. GN8 fragments **2b–f** and derivatives **2g–i** were synthesized from 4,4′-diaminodiphenylmethane **2a** as a starting material (Scheme 1). Acetylation of **2a** with acetic anhydride gave bis(acetamide) **2b**. Dehydrative condensation of **2a** with *N*-Boc-glycine followed by deprotection with HCl provided bis(2-aminoacetamide) **2c**. As a key precursor for the synthesis of unsymmetrical derivatives, compound **2d** was prepared via mono-N-protection of diamine **2a**. Acetamide **2e** and 2-aminoacetamide **2f** were prepared from **2d** in a similar manner as that for the syntheses of **2b** and **2c**, respectively. *N*,*N*′-Dimethyl-4,4′-diaminodiphenylmethane was prepared by formylation of **2a** with ethyl formate and a subsequent reduction of the resulting bisformamide with $LiAlH_4$. The reaction of the diamine with bromoacetyl bromide and pyrrolidine gave



**1**

**Figure 1.** Chemical structure of **1** (GN8).

*Abbreviations:* $PrP^{C}$, cellular form of prion protein; $PrP^{Sc}$, infectious isoform of prion protein; $PrP^{res}$, proteinase K-resistant prion protein.

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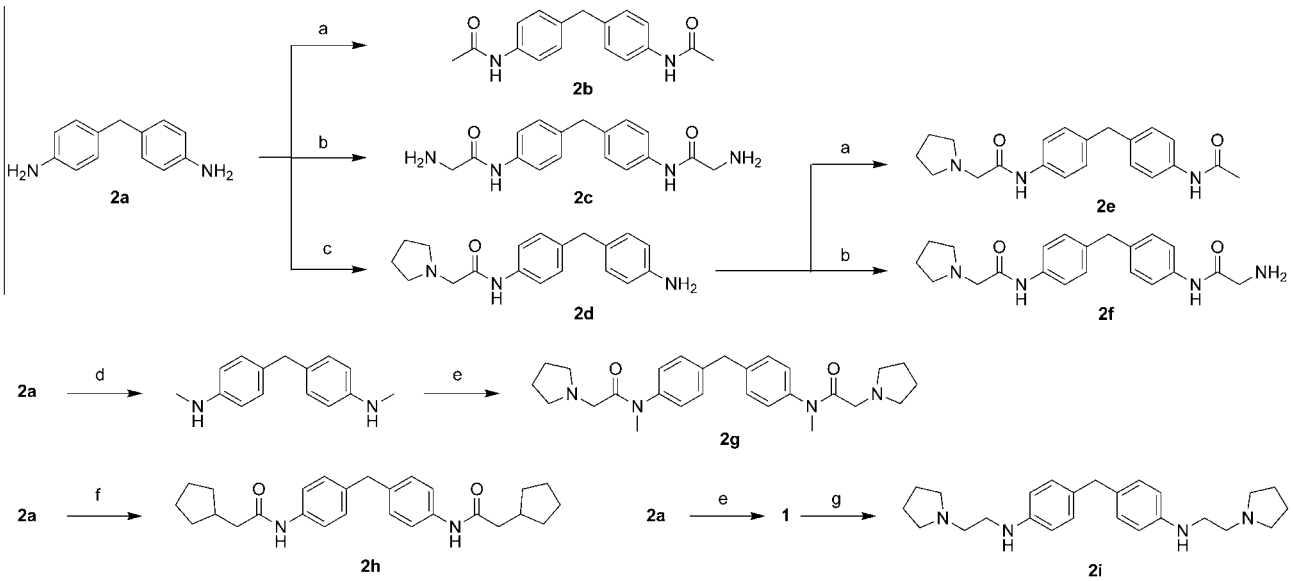
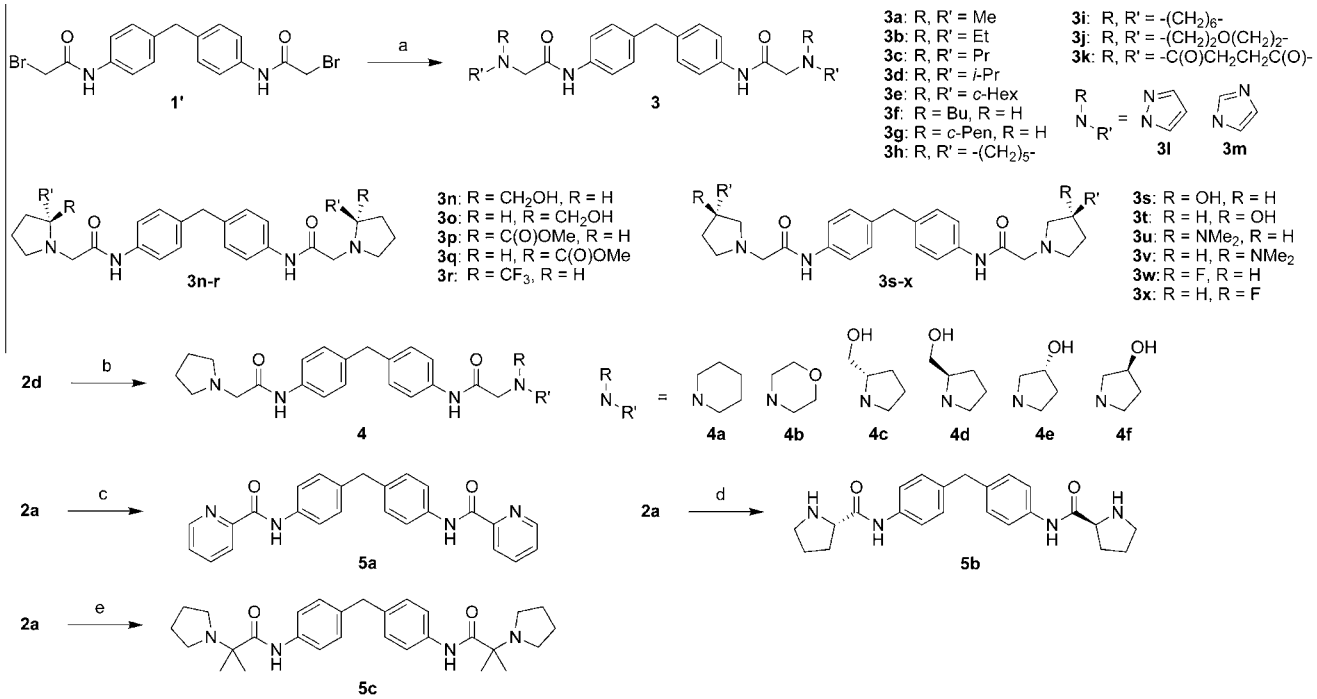



**Scheme 1.** Synthesis of GN8 derivatives **2b**–**i**. Reagents and conditions: (a) $Ac_2O$, pyridine, DMAP, $CH_2Cl_2$; (b) (i) *N*-Boc-glycine, EDCI, $CH_2Cl_2$; (ii) HCl, MeOH/AcOEt; (c) (i) $(Boc)_2O$, $Et_3N$, THF; (ii) $BrCH_2C(O)Br$, pyridine, DMAP, $CH_2Cl_2$; (iii) pyrrolidine, $K_2CO_3$, THF; (iv) HCl, MeOH/AcOEt; (d) (i) $HCO_2Et$, THF; (ii) $LiAlH_4$, THF; (e) (i) $BrCH_2C(O)Br$, pyridine, DMAP, $CH_2Cl_2$; (ii) pyrrolidine, $K_2CO_3$, THF; (f) *c*-$PenCO_2H$, EDCI, $CH_2Cl_2$; (g) $LiAlH_4$, THF.

*N*,*N*′-dimethyl derivative **2g**. Condensation of **2a** with cyclopentanecarboxylic acid gave bis(cyclopentanecarboxamide) **2h**. Reduction of **1** with $LiAlH_4$ afforded bis(ethylenediamine) **2i**.

The synthesis of derivatives **3** and **4**, in which one or two pyrrolidinyl groups in **1** were replaced with other N-substituted amino groups, is illustrated in Scheme 2. Symmetrical derivatives **3** were prepared by the substitution reaction of bis(2-bromoacetamide) **1**′ with primary and secondary amines in 39–97% yield. For the synthesis of unsymmetrical derivatives **4**, a key precursor **2d** was used as the starting material. The reaction of **2d** with bromoacetyl bromide and secondary amines provided unsymmetrical derivatives **4** in 40–87% yield. The reaction of **2a** with 2-pyridinecarboxylic acid in the presence of EDCI gave bis(2-pyridinecarboxamide) **5a**, and condensation of **2a** with *N*-Boc-L-proline followed by deprotection with HCl provided bis(2-pyrrolidinecarboxamide) **5b**. Bis[2-(1-pyrrolidinyl)-2-methylpropanamide] **5c** was prepared by the reaction of bis(2-bromo-2-methylpropanamide) with pyrrolidine in the presence of $Ag_2O$.[13]



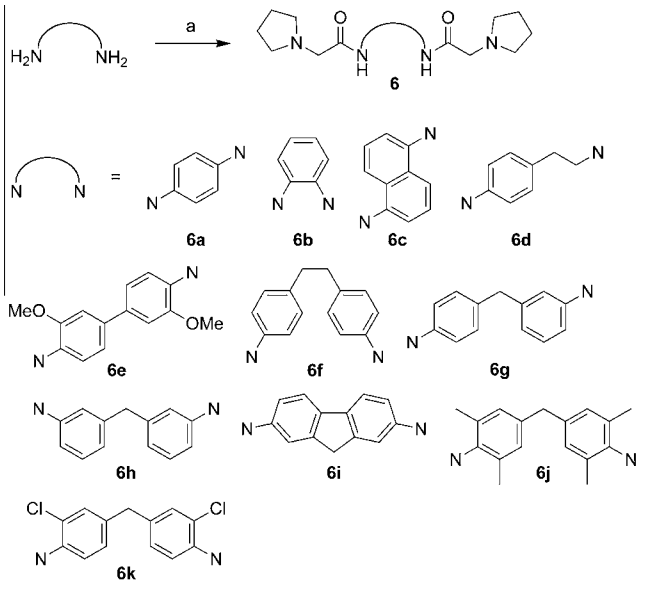

**Scheme 2.** Synthesis of GN8 derivatives **3**–**5**. Reagents and conditions: (a) HNRR′, $K_2CO_3$, THF or HNRR′ NaH, DMF; (b) (i) $BrCH_2C(O)Br$, pyridine, DMAP, $CH_2Cl_2$; (ii) HNRR′, $K_2CO_3$, THF; (c) 2-$PyCO_2H$, EDCI, $CH_2Cl_2$; (d) (i) *N*-Boc-L-proline, EDCI, $CH_2Cl_2$; (ii) HCl, MeOH/AcOEt; (e) (i) $BrCMe_2C(O)Br$, pyridine, DMAP, $CH_2Cl_2$; (ii) pyrrolidine, $Ag_2O$, $MeCN/H_2O$.

The derivatives **6** and **7**, in which the diphenylmethane unit of **1** was replaced with other scaffolds, were synthesized by the reaction of the appropriate diamines with bromoacetyl bromide and subsequent substitution of the bromo groups with pyrrolidine (Schemes 3–5). Monosubstituted and disubstituted 4,4′-diaminodiphenylmethanes for the synthesis of **7h–l** were prepared by the reaction of carbonyl compounds with aniline in the presence of aniline hydrochloride.[14] Reduction of ketone **7c** with $NaBH_4$ gave benzhydryl alcohol **7d**.

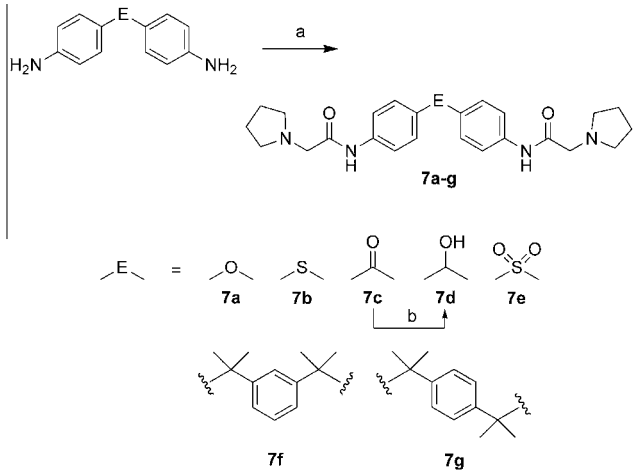
To evaluate the antiprion activity of GN8 derivatives **2**–**7**, a mouse neuronal cell culture (GT1-7) persistently infected with mouse-adapted human GSS agent (Fukuoka-1 strain), designated GT + FK, was used as a cell culture model of prion disease.[15] $PrP^C$ is overexpressed in the GT1-7 cells, and the $PrP^C$ level in the GT1-7 cells is eight times that in N2a cells.[16] The amount of proteinase K-resistant prion protein ($PrP^{res}$) was quantified and compared to that in the negative control. Percentages of $PrP^{res}$ level relative to the negative control at a concentration of 10 μM and effective concentrations for 50% inhibition of $PrP^{res}$ ($IC_{50}$) are shown in Figures 2–5, respectively. The $IC_{50}$ value of **1** was 1.4 μM, and the percentage of $PrP^{res}$ at 10 μM was 25%.

Initially, antiprion activity of a series of GN8 derivatives **2** were tested in GT + FK cells (Scheme 1, Fig. 2). Truncation of one or two tetramethylene units, pyrrolidine units, or 2-(1-pyrrolidinyl)acetyl units resulted in a significant decrease in activity compared to that of **1**. Introduction of methyl groups to the amide nitrogen atoms and replacement of the nitrogen atoms of two pyrrolidine rings with methine units led to a decrease in activity, whereas use of bis(ethylenediamine) **2i** led to retained activity. These results suggest that the overall structure of **1** contributes to the activity and that two 2-(1-pyrrolidinyl)ethylamino groups are essential along with the amide N–H and nitrogen atoms of the pyrrolidine rings that act as H-bond donors and acceptors, whereas the carbonyl groups are less important.

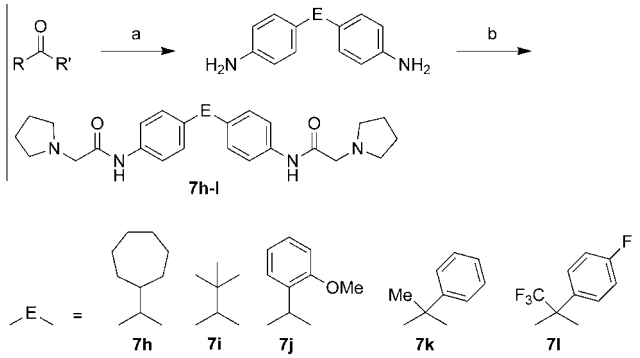
Second, the GN8 derivatives bearing a variety of monosubstituted and disubstituted amino groups at the α-position of carbonyl groups **3**–**5** were prepared to investigate the effects of the substituents on the nitrogen atoms of the amino groups on antiprion activity (Scheme 2 and Figs. 3 and 4). Most of the compounds derived from acyclic and cyclic alkyl amines (**3a–d**, **3f–i**, **4a**, and **5b**)




**Scheme 3.** Synthesis of GN8 derivatives **6**. Reagents and conditions: (a) (i) $BrCH_2C(O)Br$, pyridine, DMAP, $CH_2Cl_2$; (ii) pyrrolidine, $K_2CO_3$, THF.




**Scheme 4.** Synthesis of GN8 derivatives **7a**–**g**. Reagents and conditions: (a) (i) $BrCH_2C(O)Br$, pyridine, DMAP, $CH_2Cl_2$; (ii) pyrrolidine, $K_2CO_3$, THF; (b) $NaBH_4$, MeOH.




**Scheme 5.** Synthesis of GN8 derivatives **7h**–**l**. Reagents and conditions: (a) $PhNH_2$, $PhNH_3Cl$; (b) (i) $BrCH_2C(O)Br$, pyridine, DMAP, $CH_2Cl_2$; (ii) pyrrolidine, $K_2CO_3$, THF.

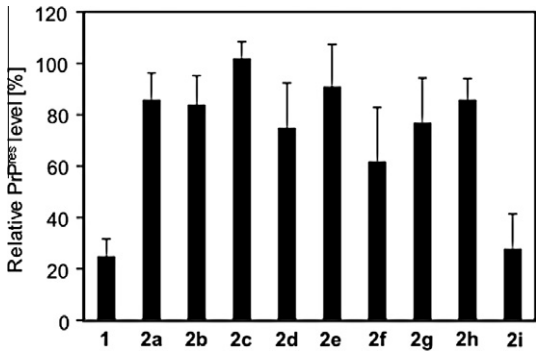



**Figure 2.** Antiprion activities of **1** and **2** at 10 μM. Bars represent means ± SD.

had comparable antiprion activity to **1**. In contrast, the derivatives having hydrophilic substituents on the pyrrolidine rings showed weak or no antiprion activity at 10 μM. For example, the derivatives bearing a hydroxymethyl group (**3n**, **3o**, **4c**, and **4d**) or a methoxycarbonyl group (**3p** and **3q**) at the 2-position of the pyrrolidine ring and those bearing a hydroxy group at the 3-position of the pyrrolidine ring (**3s**, **3t**, **4e**, and **4f**) were less active than **1**. When one or two pyrrolidinyl groups in **1** was replaced with hydrophilic morpholinyl groups, the gradual loss of activity was observed in the order **1** (relative $PrP^{res}$ level = 25%) to the monomorpholinyl derivative **4b** (relative $PrP^{res}$ level = 77%) and the

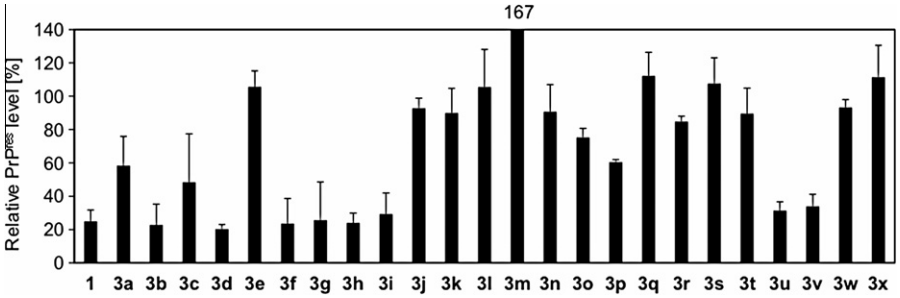



**Figure 3.** Antiprion activities of **1** and **3** at 10 μM. Bars represent means ± SD.

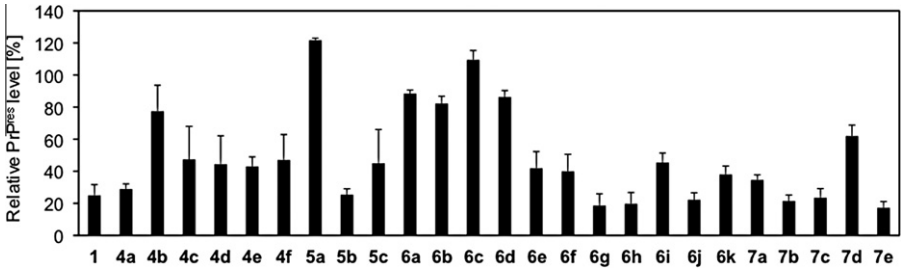



**Figure 4.** Antiprion activities of **1** and **4**–**7** at 10 μM. Bars represent means ± SD.

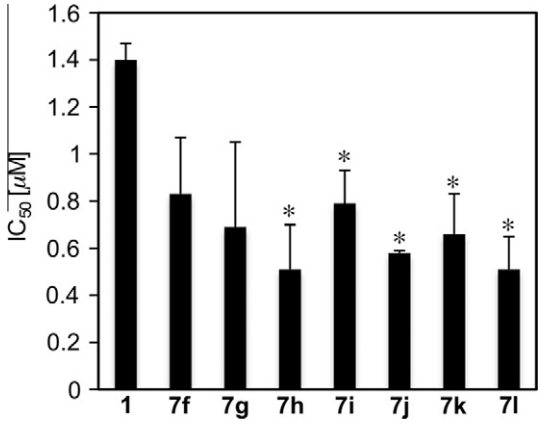



**Figure 5.** $IC_{50}$ values of **1** and **7f**–**l** in GT + FK cells. Bars represent means ± SD. *$IC_{50}$ values of **7h**–**l** were significantly different from that of **1** ($P$ <0.01, Student's $t$-test).

bis-morpholinyl derivative **3j** (relative $PrP^{res}$ level = 93%). The above results indicate that the hydrophobic property of the substituents on the nitrogen atoms of both terminal amino groups plays a crucial role in the ligand–protein interaction. $PrP^{res}$ accumulation was facilitated by the addition of imidazolyl derivative **3m**. The introduction of methyl groups to the α-position of carbonyl groups did not improve antiprion activity.

Next, we turned our attention to the synthesis of derivatives **6** from various diamines rather than from **2a** (Scheme 3 and Fig. 4). The derivatives containing phenylene or naphthylene units (**6a–d**) were inactive at 10 μM. Deletion or elongation of the central methylene unit led to a slight decrease in activity. On the other hand, the derivatives containing a diphenylmethane unit such as the regioisomers of **1** (**6g** and **6h**) and *ortho*-substituted diphenylmethane derivatives (**6j** and **6k**) showed a similar order of activity to that of **1**. These results suggest that the diphenylmethane scaffold is an essential component that acts as a core structure to interact with $PrP^{C}$ and to maintain proper distance between two acylamino groups.

Finally, the GN8 derivatives **7**, in which the methylene tether of two benzene rings was replaced with other linkers, were investigated (Schemes 4 and 5 and Figs. 4 and 5). Replacement of the central methylene tether with ether, sulfide, carbonyl, or sulfone linkers did not change the level of activity, whereas the introduction of a hydroxy group led to a slight decrease in activity. Intriguingly, derivatives **7f–l** bearing one or two alkyl or aryl substituents on the methylene tether exhibited an improved antiprion activity with $IC_{50}$ values of 0.51–0.83 μM (Fig. 5, see Table S1 in Supplementary data). Among them, the most active derivatives, **7h** and **7l**, were approximately three times more potent than **1**. No cytotoxicity was observed in the cellular assay with **7h** and **7l** at 2 μM. The $PrP^{C}$ expression level was not affected by the addition of antiprion active derivatives, as evidenced by the bioassay without proteinase K digestion (see Supplementary data).

As mentioned above, various compounds have been reported to be active at low nanomolar concentrations in scrapie-infected cells. However, these compounds showed weak or no antiprion activity in our GT + FK cells (Fig. 6). For example, Cp-60,[17] edaravone derivative,[18] chrysoidine,[19] D-penicillamine,[20] and indole-3-glyoxylamides[21] were inactive even at 10 μM. The $IC_{50}$ value of Congo red[22] was 5.5 μM and that of the most effective compound, quinacrine,[23] was 1.1 μM. Therefore, the derivatives **7f–l** constitute a class of the most potent antiprion compounds in GT + FK cells reported so far.

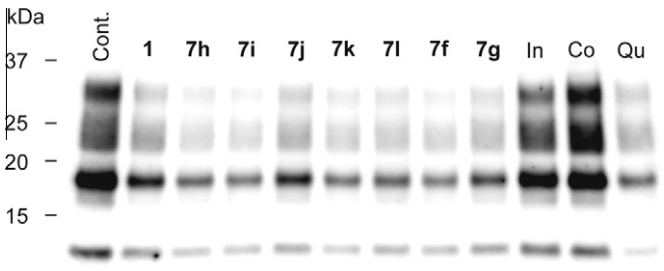



**Figure 6.** Western blotting of $PrP^{res}$ in GT + FK cells after treatment with **1**, **7f**–**l**, *N*-(4-chlorophenyl)-2-(1*H*-indol-3-yl)-2-oxoacetamide (In), Congo red (Co), and quinacrine (Qu) at 2 μM.

Thermal denaturation study using the circular dichroism indicated that the cellular form of the prion protein is significantly stabilized upon binding with **7l** (Fig. 7). The degree of stabilization by **7l**, $\Delta\Delta H$ = 14.2 kcal/mol, was approximately twice compared with that by GN8, $\Delta\Delta H$ = 6.7 kcal/mol,[7] essentially consistent with their $IC_{50}$ values, 0.51 and 1.40 μM, respectively. The increase in $\Delta\Delta H$ may be due to the additional interaction between $PrP^C$ and substituent at the benzylic position. Binding affinity of **7l** for $PrP^C$ was also elucidated by a surface plasmon resonance (SPR) binding assay.[7] The derivative **7l** had the ability to bind to $PrP^C$ with the RU value of 32, which was larger than that of **1** (RU = 15). These results indicate that the antiprion effect of these derivatives is attributed to the binding with the prion protein and stabilization of its conformation.

The conformational flexibility of the compound is one of the most important factors in the process of lead compound optimization. The improved activity of **7f–l** would be attributable to the conformational rigidity provided by the substituents at the benzylic position rather than additional interactions between $PrP^C$ and the substituents, based upon the fact that all of these derivatives showed increased activity regardless of the properties of their substituents. To elucidate the effects of the substituents at the benzylic position on the conformation of the diphenylmethane unit, conformational analysis[24,25] of model compounds diphenylmethane (**1′**) and 2,2-dimethyl-1,1-diphenylpropane (**7i′**) was performed using the 6-31G(d) basis set at the B3LYP level with the GAUSSIAN 03 program (Fig. 8).[26] The energy surface of **1′** had a large flat-bottomed area. In contrast, the conformational space of **7i′** was confined to a narrow valley due to the steric repulsion between the ortho hydrogen atoms of the phenyl rings and the *tert*-butyl group. Therefore, the substituents at the benzylic position restricted the dihedral angles of the phenyl groups to a relevant orientation accessible to the binding site of $PrP^C$. Reduction of the degree of freedom of the compound in the unbound state would be advantageous in terms of the binding affinity because of the resulting smaller entropic loss upon binding to the biomolecule.

In summary, a total of 64 derivatives were systematically synthesized by the reaction of diamines with carboxylic acid derivatives and nitrogen nucleophiles. It was found that two ethylenediamine units, hydrophobic substituents on the nitrogen atoms, and the diphenylmethane scaffold are the basic requirements for antiprion activity. Seven derivatives bearing one or two substituents at the benzylic position were found to be more potent than the original lead compound **1**, and the most effective derivatives (**7h** and **7l**) in the series exhibited antiprion activity with the $IC_{50}$ value of 0.51 μM. Introduction of the substituents at the benzylic position restricted the conformational variability of the diphenylmethane unit, increased the stability of the prion protein upon binding, and led to an improvement of antiprion activity. Further investigations focused on the synthesis and evaluation of a series of GN8 derivatives with substituents at the benzylic position as well as exhaustive conformational analyses of the derivatives are currently ongoing and will be reported in due course.

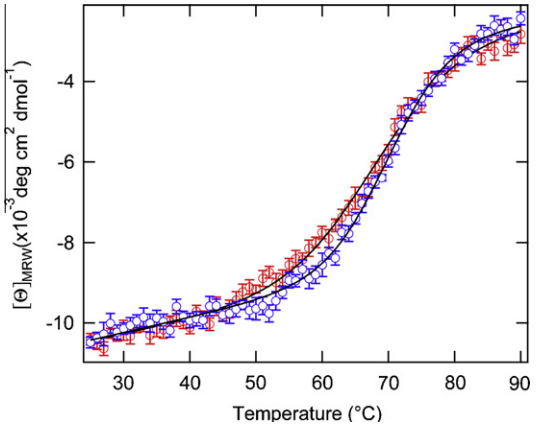



**Figure 7.** Thermal unfolding profiles of recombinant mouse PrP (amino acids 121–231, 10.7 μM) without (red) or with (blue) **7l** (100 μM) in acetate buffer pH 4.8 using CD spectroscopy. Parameter sets obtained by a nonlinear fit, that is, melting temperature ($T_m$) and enthalpy change ($\Delta H$), without **7l** were 68.8 ± 0.4 °C and 34.7 ± 1.7 kcal/mol, respectively, whereas those with **7l** were 70.3 ± 0.2 °C and 48.9 ± 2.1 kcal/mol, respectively.

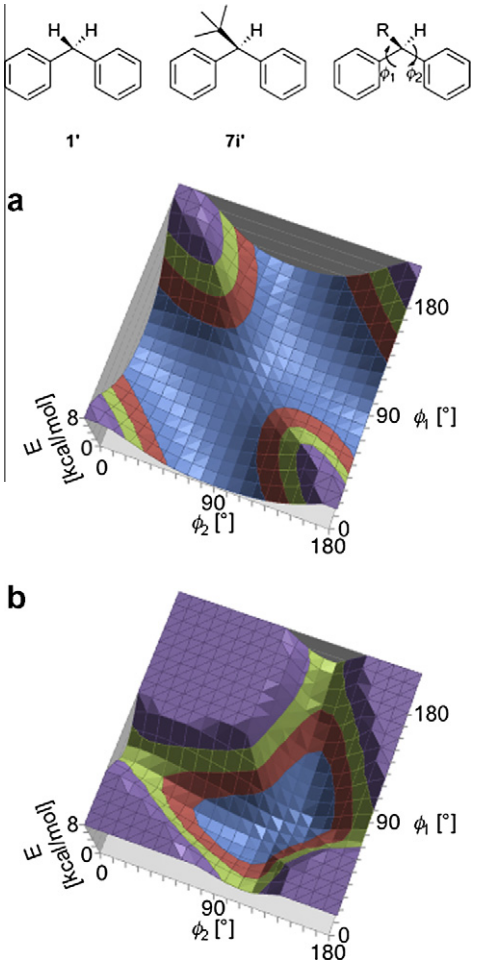



**Figure 8.** Three-dimensional conformational energy surfaces of model compounds diphenylmethane [**1′**, (a)] and 2,2-dimethyl-1,1-diphenylpropane [**7i′**, (b)] generated by the rotation of two phenyl rings (blue: 0–2 kcal/mol; orange: 2–4 kcal/mol; green: 4–6 kcal/mol; purple 6–8 kcal/mol). Dihedral angles $\varphi_1$ and $\varphi_2$ were defined as $C_{ortho1}$–$C_{ipso1}$–CHR–$C_{ipso2}$ and $C_{ortho2}$–$C_{ipso2}$–CHR–$C_{ipso1}$, respectively.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.132.

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