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Effect of 2-hydroxypropyl-β-cyclodextrin on the solubility, stability, and pharmacological activity of the chemical delivery system of TRH analogs

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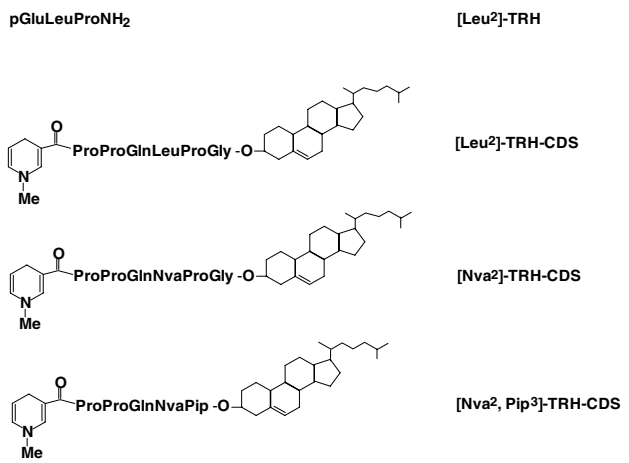
To improve the aqueous solubility and stability of the chemical delivery system (CDS) of the thyrotropin-releasing hormone (TRH) analogs, 2-hydroxypropyl-β-cyclodextrin (HPBCD) has been attempted. TRH analogs were [Leu^2]-TRH, [Nva^2]-TRH and [Nva^2, Pip^3]-TRH. Excess amount of CDS was added in various HPBCD in water solutions (0% ~ 50%, pH 6.5). The mixture was saturated by ultra-sonication for 1 h at 15 °C and filtered. The concentration of CDS in the filtrate (solubility) was determined with UV detector, and subsequently the stability was investigated. By HPBCD complexation, the aqueous solubility and stability (half-life) of CDS were significantly improved from undetectable levels to about 15 mg/ml and 30 h, respectively. In pH 6.5 and 7.4 HPBCD solution, the degradation of CDS was mainly via acid catalyzed water addition reaction, thus, e.g. [Leu^2]-TRH-CDS was more stable in pH 7.4 than in pH 6.5 aqueous solutions. After lyophilizing the saturated CDSs in 50% HPBCD complex solutions, the amount of CDS in the complex was determined as 26.22, 26.79, and 30.34 mg/g for [Leu^2]-TRH, [Nva^2]-TRH and [Nva^2, Pip^3]-TRH, respectively. The half-life of [Leu^2]-TRH-CDS/HPBCD solid complex at 25 °C, 4 °C and −15 °C was about 100 days, 440 days and no detectable change in three months, respectively. Argon protected condition did not improve the stability of lyophilized [Leu^2]-TRH-CDS/HPBCD complex. Dimethyl sulfoxide although increased the solubility of [Leu^2]-TRH-CDS in the 50% HPBCD solution by 1.3 times, significantly decreased its stability by 6.6 times. After intravenous administration of CDS (in 30% HPBCD) at a dose of 10 μmole/kg in mice, compared to the vehicle control or the same dose of [Leu^2]-TRH (in 30% HPBCD), a significant increase in pharmacological effect (decrease in barbiturate-induced sleeping time) was observed. These results demonstrate the usefulness of cyclodextrin in the formulation of the CDSs of TRH analogs.

1. Introduction

Chemical delivery systems (CDSs) have been found to improve the CNS delivery of various peptides, such as enkephalin, kyotorphin, and TRH analogs including [Leu^2]-TRH, [Nva^2]-TRH and [Leu^2, Pip^3]-TRH and [Nva^2, Pip^3]-TRH [1–6]. However, the aqueous solubility and stability of these CDSs remain problems for parenteral pharmaceutical formulations. In this study, the "molecular encapsulation" method using 2-hydroxypropyl-β-cyclodextrin (HPBCD) was attempted to solve these problems. Cyclodextrin can form inclusion complexes with drugs which provide their structure, or part of it, to fit in the cavity of cyclodextrin. The formation of inclusion complex generally involves a simple, spatial entrapment of a single guest molecule without formation of any covalent bonds. However, it can affect the physical and chemical properties of the guest molecules [7–10]. For the preliminary pre-formulation of the CDSs of TRH analogs, in this study HPBCD was chosen as dissolution enhancing and stabilizing agent. The solubility and stability of the CDSs in various concentrations of HPBCD aqueous solutions and the effect of pH and DMSO were investigated. To improve the shelf life and the convenience of the use of CDS, lyophilization of CDS/HPBCD complex was attempted, and the effect of temperature and air on the stability of the CDS in this complex was also evaluated. To assure the pharmacological activity of CDS after HPBCD complexation, the CDS/HPBCD complex solution was intravenously administered to mice to evaluate the antagonism of barbiturate-induced sleeping time, which is an index of the cholinergic neurons activation effect of the CNS delivered TRH analogs [11, 12].



2. Investigations, results and discussion

2.1. Solubility of CDS in aqueous HPBCD solutions

In order to determine the concentration of CDS in the samples, the standards were developed for each CDS. As an example, the standard for [Leu^2]-TRH-CDS is displayed in Fig. 1. At UV 355 nm, a linear relationship with a correlation coefficient of 0.997 was observed between the concentration of CDS (0 ~ 1.8 mg/ml, in 30% HPBCD) and the UV absorbency. Since the HPBCD concentration (10% ~ 50%), pH (6.5 or 7.4) and DMSO (4%) did not affect the absorbency of CDSs at UV 355 nm, these standards were used for all of the quantitative analysis of the compounds in this report.

In Table 1, after ultra-sonication, the solubility of CDS alone in water was not detectable (detection limit 0.05 mg/ml). By forming a CDS/HPBCD complex, a remarkable increase in the aqueous solubility was observed. In 50% HPBCD water solution, the solubility of [Leu^2]-TRH-CDS, [Nva^2]-TRH-CDS and [Nva^2, Pip^3]-TRH-CDS in 50% HPBCD/water solutions were 13.80, 14.10 and 15.97 mg/ml, respectively. The effect of HPBCD displayed a concentration dependency. Thus, a larger effect was obtained when a higher concentration of HPBCD was

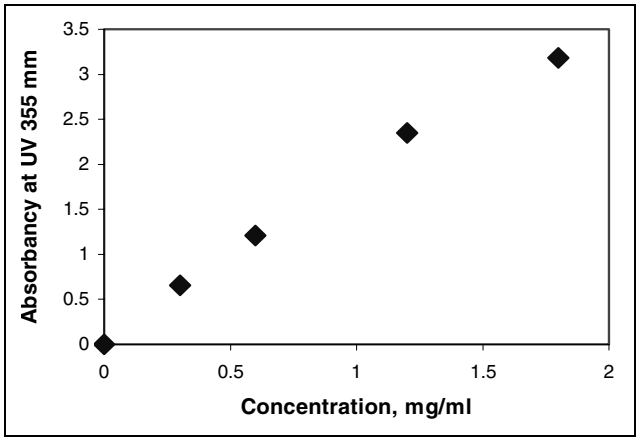


Fig. 1: Correlation between [Leu^2]-TRH-CDS concentration and absorbency at UV 355 nm

Table 1: Solubility of CDS in aqueous HPBCD solution at pH 6.5

Compd.	HPBCD concentration (%)	Solubility (mg/ml)
[Leu^2]-TRH-CDS	0	< Detection limit*
	10	2.56
	20	4.66
	30	6.38
	40	9.70
	50	13.80
[Nva^2]-TRH-CDS	50	14.10
[Nva^2, Pip^3]-TRH-CDS	50	15.97

* Detection limit was 0.05 mg/ml; values are mean of two experiments

used, as shown in Fig. 2. In these solubility experiments, the de-ionized water, instead of buffer solution, was used in order to avoid the effect of ion strength on the solubility of CDS in HPBCD solution therefore the pH of the HPBCD solution was 6.4.

2.2. Stability of [Leu^2]-TRH-CDS in aqueous HPBCD solution

Stability studies were performed with [Leu^2]-TRH-CDS only, due to the limited amounts of the other CDSs. In Table 2, the stability was evaluated by comparing the pseudo-first order rate constant of the disappearance of the

Table 2: Stability of [Leu^2]-TRH-CDS in pH 6.5 and 7.4 aqueous HPBCD solution at 25 °C

HPBCD % (w/w)		k (hr^{-1})	$t_{1/2}$ (h)	t_{90} (h)	r^2
pH 6.5	0	Undetectable	–	–	–
	30	0.0764	9.07	1.37	0.999
	40	0.0646	10.73	1.63	0.998
	50	0.0334	20.72	3.14	0.994
pH 7.4	0	Undetectable	–	–	–
	10	0.0374	18.51	2.81	0.998
	20	0.0283	24.46	3.71	0.998
	30	0.0258	26.82	4.06	0.998
	40	0.0237	29.30	4.44	0.997
	50	0.0231	30.06	4.55	0.999

Detection limit was 0.05 mg/ml; Values are mean of two experiments

compound in the solution, k (h^{-1}), and half-life ($t_{1/2}$, h). The results indicate a significant increase in half-life of [Leu^2]-TRH-CDS by HPBCD ($10 \sim 50\%$) from undetectable levels to 21 h at pH 6.5 and 30 h at pH 7.4.

Although there are various possible pathways related to the degradation of CDS in the body, in the aqueous HPBCD solution, without enzymatic hydrolysis, mainly two pathways, the oxidation reaction and the water addition reaction as shown in the Scheme, are expected [1–6]. In the present study, the degradation of the CDS mainly resulted in the product of water addition reaction. Since water addition reaction is acid-catalyzed, the CDS (in the HPBCD complex) in pH 7.4 solution was more stable than in pH 6.5 solution. As displayed in Fig. 3, by adjusting the pH from 6.5 to 7.4, the water addition reaction was significantly reduced, and a decreased pseudo-first order rate constant, k (h^{-1}) was observed.

2.3. Lyophilization of CDS/HPBCD complex

To improve the stability and the convenience of the use of CDS/HPBCD complex, lyophilization was performed. In Table 3, CDSs in 50% HPBCD solution were freeze-dried, and the amount of [Leu^2]-TRH-CDS, [Nva^2]-TRH-CDS and [Leu^2, Pip^3]-TRH-CDS, in the lyophilized complex was determined as 26.22, 26.79 and 30.34 mg/g, respectively. Subsequently, the stability of the [Leu^2]-TRH-CDS/HPBCD complex was determined and displayed in Table 4. The complex was kept at −15 °C, 4 °C or 25 °C, either exposed to air, or protected by argon to prevent oxidation reaction. The results indicate that stability of [Leu^2]-TRH-CDS/HPBCD complex was not af-

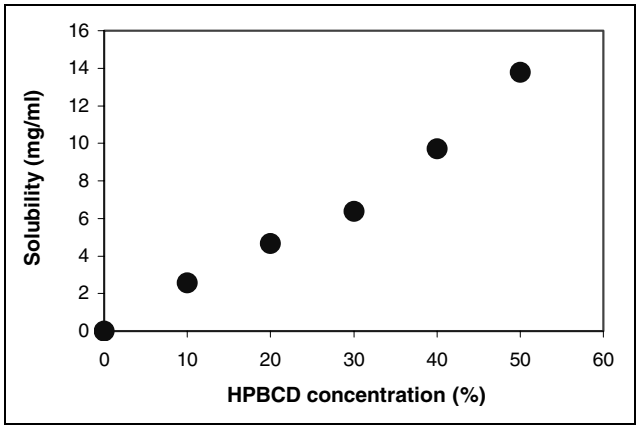


Fig. 2: Solubility of [Leu^2]-TRH-CDS in aqueous HPBCD solution at pH 6.5, 20 °C

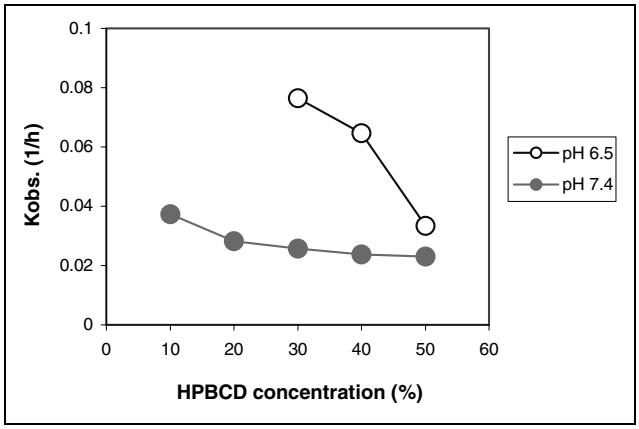
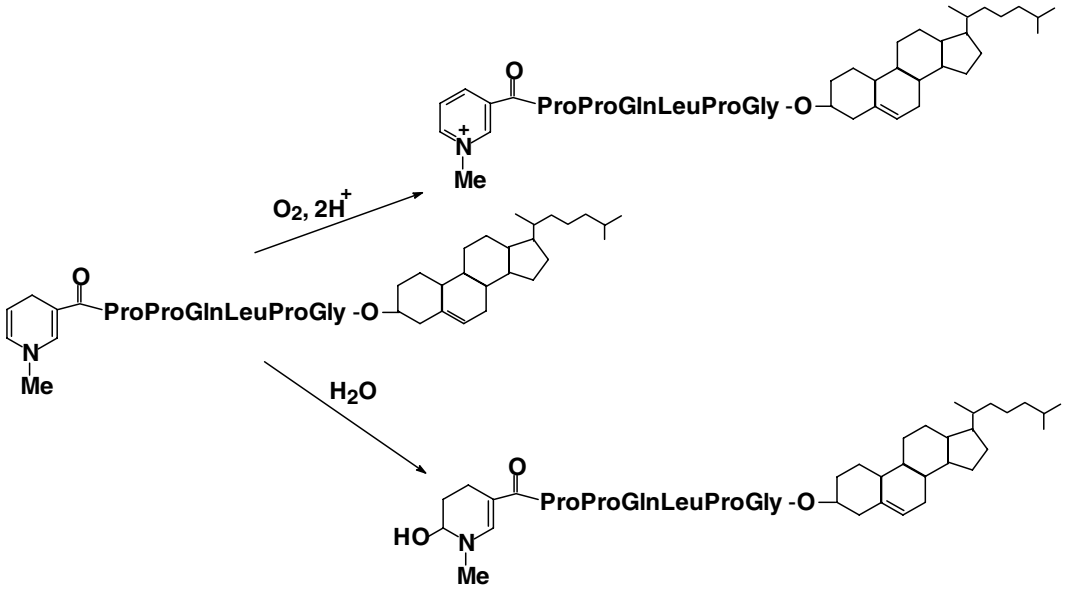


Fig. 3: Stability of [Leu^2]-TRH-CDS in pH 6.5 and 7.4 aqueous HPBCD solution at 25 °C

Scheme



fected by air exposure, but significantly affected by changing temperature, and it was quite stable (no detectable change found in three months) when stored in the freezer (−15 °C).

Table 3: Amount of CDS in lyophilized CDS/HPBCD complex

Compd.	Content (mg/g)
[Leu^2]-TRH-CDS	26.22
[Nva^2]-TRH-CDS	26.79
[Nva^2, Pip^3]-TRH-CDS	30.34

Values are mean of two experiments

Table 4: Stability of lyophilized [Leu^2]-TRH-CDS/HPBCD complex

HPBCD % (w/w)	k (h^{-1})	$t_{1/2}$ (h)	t_{90} (h)	r
Argon protected				
−15 °C	No detectable change in 3 month			
4 °C	0.00166	417	63	0.998
25 °C	0.00666	104	16	0.917
Air exposed				
−15 °C	No detectable change in 3 month			
4 °C	0.00152	457	69	0.962
25 °C	0.00759	91	14	0.930

Detection limit was 0.05 mg/ml; values are mean of two experiments

Table 5: Effect of dimethyl sulfoxide on the solubility and stability of [Leu^2]-TRH-CDS in aqueous 50% HPBCD solution

	Solubility (mg/ml)	Stability	
		k (h^{-1})	$t_{1/2}$ (h)
Without DMSO	5.89	0.0334	20.72
With DMSO	7.74	0.207	3.34

Values are mean of two experiments

2.4. Effect of dimethyl sulfoxide on the solubility and stability of CDS in HPBCD solution

DMSO is frequently used for assisting the dissolution of the compounds in the laboratory. In this investigation, by adding 4% of DMSO in [Leu^2]-TRH-CDS/HPBCD (50%) solution, the solubility of [Leu^2]-TRH-CDS was increased by ∼1.3 times, from 5.89 mg/ml to 7.74 mg/ml. However, the stability ($t_{1/2}$) was largely reduced (by 6.2 times), from 20.72 h to 3.34 h, as shown in Table 5. The degradation of [Leu^2]-TRH-CDS was the same as in other aqueous solutions described before, mainly via water addition reaction.

2.5. Pharmacological studies

To confirm the pharmacological activities of CDSs in the body after cyclodextrin complexation, the antagonism of barbiturate-induced sleeping time in mice was investigated to assess the activation effect of CNS delivered TRH analogs on mice cholinergic neurons. Firstly, [Nva^2]-TRH-CDS was used to evaluate the dose-effect relationship. After intravenous administration of various doses, the CNS activity of [Nva^2]-TRH was evaluated. As shown in Fig. 4 and 5, the sleeping time and the percentage increase in the sleeping time were plotted against the concentration. Compared to the vehicle, [Nva^2]-TRH-CDS significantly reduced the pentobarbital-induced sleeping time in mice. The maximum sleeping time reducing activity (47%) was achieved at a dose of 2 μmol/kg. The doses higher than 2 μmol/kg did not further improve the CNS activity.

Subsequently, the pharmacological activity of other CDSs was compared with vehicle and [Leu^2]-TRH. In Table 6, after intravenous administration of 10 μmol/kg, all CDSs showed a significantly higher pharmacological activity. Compared to the vehicle (94.19 min) and [Leu^2]-TRH (79.24 min), the sleeping times after [Leu^2]-TRH-CDS, [Nva^2]-TRH-CDS and [Nva^2, Pip^3]-TRH-CDS were 50.85 min, 49.84 min and 41.39 min, respectively. This represents a 46 ∼ 56% decrease in the barbiturate-induced sleeping time, and demonstrates a successful delivery and effective release of the active TRH analogs in the brain by means of CDSs/HPBCD complex.

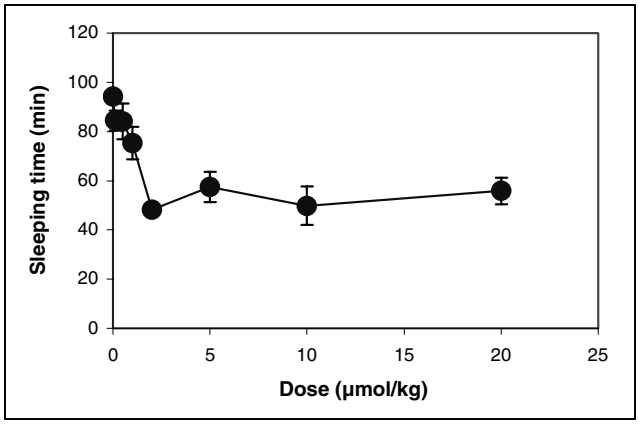


Fig. 4: Dose response after intravenous injection of [Nva^2]-TRH-CDS in mice

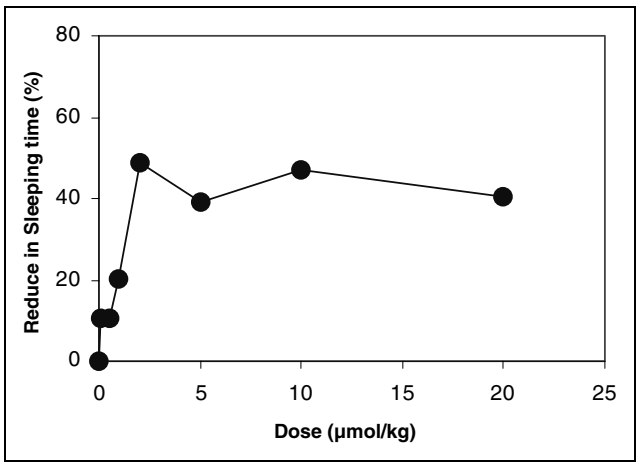


Fig. 5: Percent reduced in sleeping time after intravenous injection of [Nva^2]-TRH-CDS in mice

Table 6: A comparison of the pentobarbital induced sleeping time in mice after the administration of vehicle, [Leu^2]-TRH and CDSs

Compd.	Sleeping time (min)	Reduced in sleeping time (%)
Vehicle	94.19 ± 2.48	–
[Leu^2]-TRH	79.24 ± 4.88	15.85
[Leu^2]-TRH-CDS	50.85 ± 2.52*	46.00
[Nva^2]-TRH-CDS	49.84 ± 7.77*	47.07
[Nva^2, Pip^3]-TRH-CDS	41.39 ± 2.63*	56.05

Ten min after intravenous injection of the compound (10 μmole/kg), pentobarbital, 60 mg/kg, was i.p. injected in the animal. Sleeping time was recorded as the time elapsed from the onset of loss of the righting reflex until the reflex was regained. Reduced in sleeping time indicates the per cent reduced sleeping time compared to the control. A 30% hydroxypropyl-β-cyclodextrin solution was used as vehicle. Eight to nine Swiss Webster mice (30 ± 3 g) were used for the testing of each compound, and table entries are mean ±SE. * By student t-test, $p < 0.005$ when compared to vehicle control or [Leu^2]-TRH.

In conclusion, by forming a cyclodextrin inclusion complex, the aqueous solubility and stability of the CDSs of TRH analogs were significantly improved. The stability can be further improved by changing temperature or pH. Lyophilization of the CDS/HPBCD complex markedly increased the shelf life of the CDS and the convenience of the use of CDS. In aqueous solution, at neutral pH, CDS degraded mainly via water addition reaction, thus, more stable at pH 7.4 than at pH 6.5. Significant pharmacological activity was achieved by intravenous administration of CDS/HPBCD complex solution, demonstrating the usefulness of cyclodextrin in the pharmaceutical formulation of the CDSs of TRH analogs.

3. Experimental

3.1. Materials and animals

[Leu^2]-TRH-CDS, [Nva^2]-TRH-CDS and [Leu^2, Pip^3]-TRH-CDS were freshly prepared in our laboratory. All of the compounds were characterized by NMR and elemental analysis. 2-Hydroxypropyl-β-cyclodextrin (HPBCD) was purchased from CERESTAR USA Inc. Swiss Webster mice (body weight, 30 ± 3 g) were obtained from HARLAN (Indiana, USA).

3.2. Solubility and stability

Saturated CDS in HPBCD solutions were made by adding excess amounts of CDS in 10 ~ 50% (w/w) cyclodextrin in water solution. The pH of solutions were either 6.5 (not adjusted) or 7.4 (adjusted by NaOH). The mixtures were placed in an ultra-sonicator for 20 min at 10 °C. De-aerated water was used and the operation was under air protection (with argon) at all time to minimize the side-reactions, such as oxidation, of the compound during the operation process. After equilibration at room temperature for 10 min, the complex solutions were filtered through Millipore HV membrane filters (pore size 0.45 μm). Aliquots of the filtrates were diluted 10 times with HPBCD/water solution and analyzed by UV spectrophotometer, immediately or after appropriate time intervals, to determine the solubility and stability of the compounds in the solutions. The stability of CDS in HPBCD solution was evaluated by half-life ($t_{1/2}$, h or day) and pseudo-first order rate constant (k, h^{-1}) of the disappearance of the compound in the aqueous solution.

3.3. Lyophilization

Previously mentioned CDS in 50% HPBCD solutions were freeze-dried at −45 °C, 100 millitorr. The resulting solid mixture was reconstituted with water and analyzed, to determine the CDS content in the solid CDS/HPBCD complex. The solid complex was then kept at −15 °C, 4 °C, and 25 °C, under either argon protected or air exposed condition. At appropriate time intervals, samples were taken, reconstituted with water and analyzed.

3.4. Effect of dimethyl sulfoxide

A 4% of DMSO was included in 50% HPBCD. To this solution, excess amount of [Leu^2]-TRH-CDS was added and the mixture was sonicated, then the solubility and stability of the compound was determined as described in section 3.2.

3.5. UV spectrophotometry

PEKIN ELMER, UVNIS spectrometer lambda II was used for the quantitative determination of CDS in HPBCD complex. The UV absorbency peak height at 355 nm was used to compare the concentrations. A standard curve was developed for quantifying each CDS.

3.6. Pharmacological studies

Swiss Webster mice (body weight, 30 ± 3 g) were used. Freeze dried CDS/HPBCD complex was reconstituted by adding an appropriate amount of water to obtain the desired concentration of CDS in 30% HPBCD aqueous solution. Vehicle only (30% HPBCD, 3.0 ml/kg) or compounds at a dose of 10 μmol/kg (or 0.1 ~ 20 μmol/kg for dose response investigations) were injected in the animals through tail vein to compare the CNS activities. Ten minutes after intravenous administration, each animal received an intraperitoneal injection of sodium pentobarbital solution (30 mg/ml) at a dose of 60 mg/kg. The sleeping time was recorded as the time elapsed from the onset of loss of the righting reflex until the reflex was regained. A group of 8 ~ 9 animals were used for each test. Student's t-test was used for statistical evaluation.

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References

1 Bodor, N.; Prokai, L.; Wu, W. M.; Farag, H.; Jonnalagada, G.; Kawamura, M.; Simpkins, J.: Science **257**, 1698 (1994)
2 Prokai, L.; Ouyang, X.; Wu, W. M.; Bodor, N.: J. Am. Chem. Soc. **116**, 2643, (1994)

3 Simpkins, J.; Ouyang, X.; Prokai, L.; Bodor, N.: Neuroprotocols: A comparison to methods in neurosciences, **4**, 225 (1994)
4 Chen, P.; Bodor, N.; Wu, W. M.; Prokai, L.: J. Med. Chem. **41**, 3773 (1998)
5 Prokai, L.; Prokai, K.; Ouyang X.; Kim, H.; Wu, W. M.; Zharikova, A.; Bodor, N.: J. Med. Chem. **42**, 4563 (1999)
6 Yoon, S.; Wu, J.; Wu, W. M.; Prokai, L.; Bor, N.: Bioorg. Med. Chem. **8**, 1059 (2000)
7 Loftsson, T.; Brewster, M.: J. Pharm. Sci. **85**, 1017 (1996)
8 Stella, V. J.; Rajewski, R. A.: Pharm. Res. **14**, 556 (1997)
9 Thompson, D. O.: Crit Rev Ther Drug Carrier Syst. **14**, 1–104 (1997)
10 Brewster, M. E.; Hora, M. S.; Simpkins, J. W.; Bodor, N.: Pharm. Res. **8**, 792 (1991)
11 Horita, A.; Corona, M.; Lai, H.: Fed. Proc. **45**, 795 (1986)
12 Santori, E.; Schmidt, D.: Regul. Pept. **1**, 69 (1980)

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