Full Paper

Pharmacological Characterization of RS-1259, an Orally Active Dual Inhibitor of Acetylcholinesterase and Serotonin Transporter, in Rodents: Possible Treatment of Alzheimer's Disease

Yasuyuki Abe^{1,*}, Atsushi Aoyagi¹, Takao Hara¹, Kazumi Abe¹, Reina Yamazaki¹, Yoshihiro Kumagae¹, Shunji Naruto², Kazuo Koyama², Shinji Marumoto², Keiko Tago², Narihiro Toda², Kazuko Takami², Naho Yamada², Mayuko Ori², Hiroshi Kogen², and Tsugio Kaneko¹

¹Neuroscience and Immunology Research Laboratories and ²Exploratory Chemical Research Laboratories, Sankyo Co., Ltd., 2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo 140-8710, Japan

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Abstract. A dual inhibitor of acetylcholinesterase (AChE) and serotonin transporter (SERT), RS-1259 (4-[1*S*]-methylamino-3-(4-nitrophenoxy)]propylphenyl *N,N*-dimethylcarbamate (fumaric acid)_{1/2} salt), was newly synthesized. RS-1259 simultaneously inhibited AChE and SERT in the brain following an oral administration in mice and rats. Actual simultaneous elevation of extracellular levels of 5-HT and ACh in the rat hippocampus was confirmed by microdialysis. The compound was as effective as SERT inhibitors such as fluoxetine and fluvoxamine in a 5-hydroxytryptophan-enhancing test in mice. Spatial memory deficits in the two-platform task of a water maze in aged rats were ameliorated by RS-1259 as well as donepezil. Both RS-1259 and donepezil increased the awake episodes in the daytime electroencephalogram of rats. Although RS-1259 was weaker than donepezil in enhancing central cholinergic transmission, as observed by ACh elevation in the hippocampus and memory enhancement in aged rats, the efficacy of RS-1259 on the consciousness level, which reflects the whole activity in the brain, was almost the same as that of donepezil. These results suggest that both cholinergic and serotonergic systems are involved in maintaining brain arousal and that a dual inhibitor of AChE and SERT may be useful for the treatment of cognitive disorders associated with reduced brain activity such as in Alzheimer's disease.

Keywords: acetylcholinesterase, serotonin transporter, cerebral activation, Alzheimer's disease, rodent

Introduction

The central cholinergic system is known to play a fundamental role in cognitive function, and extensive evidence indicates that a disruption of the cholinergic function leads to characteristic signs of aging and Alzheimer's disease (AD) (1, 2). Today, acetylcholinesterase (AChE) inhibitors such as tacrine (3), donepezil (4), rivastigmine (5), and galanthamine (6) are the only approved medication for AD patients. However, their clinical usefulness is limited, largely because of adverse events arising from excessive activation of cholinergic

systems (7).

Recent research suggests that cholinergic dysfunction does not provide a complete account of age-related cognitive deficits (8, 9). Hypofunction of several other neuronal systems such as the glutamatergic, GABAergic, and monoaminergic systems in the brain of AD patients, which has also been reported (10), may be partly responsible for the cognitive decline. Accordingly, there are many experimental reports suggesting that several neurotransmitters, including serotonin, norepinephrine, dopamine, and GABA, appear to play a role in cognitive processes and interact with the cholinergic systems (11). Moreover, AD patients often exhibit several behavioral abnormalities such as irritability, anxiety, and depres-

*Corresponding author. FAX: +81-3-5436-8572
E-mail: yasuae@shina.sankyo.co.jp

sion, as well as cognitive impairment (12–14). It is reported that depression is present in about 40% of patients with dementia (15). This has led to several clinical trials being conducted for psychotropic drugs to treat the behavioral symptoms of AD patients (16–19). Co-administration of sertraline, a selective inhibitor of serotonin transporter (SERT), with donepezil has been proven to be favorable over placebo in treating AD patients with behavioral symptoms (20). However, a major disadvantage of the combination of varying drug classes is that compounds with different pharmacodynamic and pharmacokinetic properties may be difficult to manage clinically. Moreover, they may cause a detrimental drug-drug interaction as well (21, 22).

To circumvent these disadvantages, we attempted to develop a new agent that simultaneously inhibits AChE and SERT for the treatment of AD (23). So far, two potent cholinesterase inhibitors that show moderate activities on monoaminergic systems *in vitro* have been reported (24, 25), but no compound has been reported to show simultaneous activation of the cholinergic and monoaminergic transmissions in the brain *in vivo*, probably due to the toxic overstimulation of the cholinergic neurotransmission prior to the appearance of the monoaminergic effect. Here, we report the pharmacological properties of RS-1259, a newly synthesized dual inhibitor of AChE and SERT in rodents. Preliminary results have been presented in an abstract form (26).

Materials and Methods

Experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society and also in accordance with Sankyo's Code of Ethical Research.

Animals

Male ddY mice (4-week-old; SLC, Shizuoka) were used for the *ex vivo* determination of AChE and SERT inhibition in the brain and the 5-hydroxy-L-tryptophan (5-HTP)-enhancing test. Male Sprague-Dawley (8-week-old; Charles River Japan, Yokohama), male Wistar (10–13-week-old, SLC) and male F344 (24–25-month-old, Charles River Japan) rats were used for the microdialysis of the hippocampus, the daytime electroencephalogram (EEG), and the water maze, respectively. The animals were housed for at least one week before use in breeding rooms (lighted: 7:00–19:00) in which the room temperature and humidity were maintained at 22–24°C and 50–70%, respectively. Food and water were consumed *ad libitum*.

RS-1259:
4-[(1S)-methylamino-3-(4-nitrophenoxy)]propylphenyl
N,N-dimethylcarbamate (fumaric acid)_{1/2} salt

Fig. 1. Chemical structure of RS-1259.

Chemicals

RS-1259 (4-[(1S)-methylamino-3-(4-nitrophenoxy)]propylphenyl *N,N*-dimethylcarbamate (fumaric acid)_{1/2} salt; Fig. 1) was synthesized in the Exploratory Chemical Research Laboratories, Sankyo Co., Ltd. Donepezil HCl and rivastigmine tartrate were synthesized at Chemtech Labo., Inc. (Tokyo). Fluoxetine HCl and fluvoxamine maleate were purchased from Tocris Cookson, Ltd. (Avonmouth, UK). *S*-(-)-carbidopa and 5-HTP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The compounds were dissolved in distilled water or suspended in 0.5% tragacanth gum (Wako Pure Chemical Industries, Ltd., Osaka) solution for oral administration and were dissolved in physiological saline for injection. The systemic doses were expressed as the salt forms. Drug solutions were administered at a constant volume of 1 and 10 ml/kg of body weight to rats and mice, respectively. [³H]Serotonin, [³H]noradrenaline, and [³H]dopamine were purchased from New England Nuclear (Boston, MA, USA). [³H]Citalopram was purchased from NEN Life Science Products (Boston, MA, USA).

AChE inhibition in the brain homogenate of mice and rats

AChE activity was measured in duplicate by the spectrophotometric method of Ellman et al. (27) with some modifications. Brain homogenate was used as the enzyme source. The whole brain except for the cerebellum was homogenized in 9 volumes of 100 mM sodium phosphate buffer (pH 7.0). Homogenates were stored at –80°C until analysis. The test compounds were dissolved in dimethyl sulphoxide (DMSO) and 10 µl of the solution was added to 3 ml of the buffer containing 10 µl of the brain homogenate. The mixture was incubated for 10 and 20 min at room temperature before and after adding 50 µl of dithiobisnitrobenzoate (DTNB),

respectively. The absorbance at 412 nm was measured (U-1080; Hitachi Ltd., Tokyo) immediately and 8 min after adding 50 μ l of acetylthiocholine (ATC). The AChE activity was expressed as a change in OD at 412 nm.

Uptake inhibition of serotonin (5-HT), noradrenaline (NA), and dopamine (DA) in the rat synaptosome

The whole brain except for the cerebellum was homogenized in 100 mM sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 1,000 $\times g$ for 10 min and the supernatant was recentrifuged at 20,000 $\times g$ for 20 min at 4°C. The pellet was suspended in 100 mM ice-cold sodium phosphate buffer (pH 7.0). After pre-incubation of 1 ml of the synaptosome suspension with 10 μ l of the test compound (in DMSO) at 37°C for 5 min, 10 μ l of [3 H]5-HT (100 nM), [3 H]NA (100 nM), or [3 H]DA (100 nM) was added and the mixture was incubated at 37°C for another 5 min. Adding 4 ml of ice-cold saline solution terminated the uptake of transmitters into the synaptosome. The reaction mixture was filtrated on a Whatman GF/B filter that was then washed with 4 ml of ice-cold saline solution once. The radioactivity retained on the filter was measured by a liquid scintillation counter. The blank was determined by measuring the uptake of [3 H]5-HT, [3 H]NA, or [3 H]DA at 4°C for 5 min. All the determinations were performed in duplicate.

The in vitro profiles of RS-1259 with respect to receptor binding, transporter inhibition, and enzyme inhibition were evaluated in MDS Pharma Services as a contract study.

Inhibition of brain AChE and SERT following oral administration

Animals were instantly decapitated at 0.25, 0.5, 1, 2, 4, 8, and 24 h after oral administration of test compounds. Forebrains were removed and stored at -80°C until analysis. All determinations were performed in duplicate.

The brain was homogenized in 1.6 volumes of 100 mM sodium phosphate buffer (pH 7.0) for the determination of AChE activity by the spectrophotometric method. Of the homogenate, 100 μ l was incubated at room temperature for 60 s with 10 μ l of 60 mM ATC or 100 μ l of 1 N perchloric acid (PCA) (used as a blank). The reaction was terminated by adding 100 μ l of PCA or 10 μ l of ATC, respectively, and the homogenate was centrifuged at 10,000 $\times g$ for 10 min at 20°C. In a 96-well plate, 5- μ l samples of each supernatant were incubated with 150 μ l of DTNB for 20 min at room temperature and then the absorbance at 415 nm was measured.

The brain was homogenized in 3 volumes of 50 mM Tris-HCl buffer (pH 7.7) for the SERT inhibition measurement as determined by the displacement of [3 H]citalopram binding. Of the homogenate, 250 μ l was incubated with 10 μ l of [3 H]citalopram (0.77 nM) at 25°C for 60 min with or without 1 mM of fluvoxamine. The incubated mixture was centrifuged at 3,000 $\times g$ for 6 min at 4°C after adding 2.5 ml of Tris-HCl buffer (pH 7.7). The pellet was resuspended in 2.5 ml of the buffer and the suspension was centrifuged at 3,000 $\times g$ for 6 min at 4°C. Then, the pellet was suspended in 1 ml of the buffer and the radioactivity was measured by a liquid scintillation counter.

Microdialysis of the rat hippocampus

Rats were anesthetized with an intraperitoneal injection of 50 mg/kg of pentobarbital Na (Nembutal[®]; Abbott Laboratories, Abbott Park, IL, USA) and fixed to a stereotaxic apparatus. A probe (CMA/10; BAS, Inc., Tokyo) was implanted into the hippocampus according to the brain atlas of Paxinos and Watson (28) (AP -5.5, ML +5.0, depth -6.0 from the dura mater). One day after the probe-implantation, the rat was fixed to the microdialysis device under anesthesia with isoflurane (FORANE[®], Abbott). Modified Krebs-Ringer solution (145 mM NaCl, 1.2 mM CaCl₂, 2.7 mM KCl, 1.0 mM MgCl₂, 0.2 mM ascorbic acid, and 2 mM phosphate buffer (pH 7.4)) was perfused into the dialysis probe and the dialysate was collected every 60 min after the anesthesia had been ceased for more than 1 h, with a flow rate of 2 μ l/min. Then the test compounds were administered orally. ACh and 5-HT were assayed using an HPLC system (LC-10AD; Shimadzu Co., Kyoto) equipped with an electrochemical detector (LC-4C, BAS Inc.). For the determination of 5-HT, 20 μ l of the collected dialysate was injected into the HPLC system. An Eicompak CA-5ODS column with a pre-column (4.6 $\phi \times$ 150 mm; Eicom Co., Kyoto) was used and kept at 30°C. The mobile phase consisted of 0.1 M phosphate buffer (pH 6.0), 50 mg/l EDTA, 240 mg/l sodium 1-octanesulfonate, and 20% methanol; and the flow rate was 0.23 ml/min. The potential of the detector was set at 400 mV. For the determination of ACh, the internal standard, 30 μ l of 0.5 μ M ethyl homocholine, was added to 90 μ l of the dialysate and a 100- μ l aliquot of the mixture was injected into the HPLC system. A separation column with a pre-column (AC-Gel, Eicom Co.) and an enzyme column (AC-Enzympak, Eicom Co.) kept at 35°C were used. The mobile phase consisted of 0.1 M phosphate buffer (pH 8.5), 240 mg/l sodium 1-decanesulfonate, and 65 mg/l tetramethylammonium chloride; and the flow rate was 0.6 ml/min. The potential of the detector was

set at 450 mV.

5-HTP-enhancing test in mice

Mice were separately placed in transparent acrylic resin-cages (19-cm W, 19-cm L, and 20-cm H) that were set in a locomotion counting apparatus (Scanet MV-10TW; Toyosangyo Co., Toyama). Mice were allowed to explore their surroundings beforehand for more than 30 min. *S*-(–)-Carbidopa (20 mg/kg, i.p.) and 5-HTP (50 mg/kg, s.c.) were injected 15 min and 30 min after an oral administration of the test compound, respectively. The locomotion count, which was defined as passing a set of infrared beams, of each mouse was measured for 120 min after the 5-HTP injection. The mice were allocated to four groups of 8 mice to prepare the control and 3 dosage groups for each test compound. The locomotion count measured every 30 min in the 4 groups was statistically analyzed by the ANOVA of repeated measures followed by Fisher's PLSD method for a comparison between the control and each dose group. The StatView + Graphics4.1J (Abacus Concepts, Inc., Berkeley, CA, USA) was used for the statistical processing.

Water maze test in aged rats

Pretraining: Thirteen rats were used in the behavioral tests. Pretraining for the two-platform task (TPT) was conducted as previously described (29, 30), starting at 2 months of age. In brief, after 2 days of daily handling, rats were trained in the place navigation task (PNT); that is, they were trained to find a submerged platform (diameter: 12 cm) in a circular pool (diameter: 1.5 m) at a rate of 6 trials per day. The platform location was pseudorandomly changed each day, so that on the first trial of the day, the rats had no information about where the platform was located. This pretraining shortened the period necessary for the following TPT training and improved the selection accuracy. Within 12 days of the PNT training, all rats fulfilled the criterion of reaching the platform by swimming a distance less than 300 cm in Trials 2–6 for 3 consecutive days.

TPT: The TPT was conducted as previously described (29, 30) with some modifications. Briefly, two visible platforms of identical appearance (diameter: 12 cm), one fixed and the other floating, were simultaneously placed at the center of two non-neighboring quadrants (e.g., north and south) in the same pool as that in the pretraining. The surfaces of the platforms were set 0.5-cm above the water surface. A trial began by releasing a rat into the water facing the wall of the pool from one of the remaining two quadrants. The various patterns of platform locations and start positions are shown in Fig. 5A. One session consisting of 6 trials was given

each day. Rats were released alternately from the 2 start positions. The trial ended when the rat reached either platform. The selection accuracy was defined as the percentage of correct responses (i.e., choosing the fixed platform first) in Trials 2–6 for each session. The first trial served as an informational trial for the location of the fixed platform. More than 20 sessions of the TPT training were done for each rat, and the success rate of all 13 animals increased asymptotically with a selection accuracy of 75–80%. The selection accuracy established for each rat could be maintained by periodical trainings (approximately 3 sessions per month) for 16 months. At 18–20 months of age, the selection accuracy gradually declined with age as previously reported (31). At 24–25 months of age, 11 out of 13 rats showing a decline in the TPT performance were divided into one vehicle and three treatment groups of the two test compounds at different doses and used on a rotational basis for two experiments. There was at least a 2-day washout period between each drug test to ensure the absence of influence of the previous drug. The swimming distance, the swimming speed, the floating time, and the swimming time were measured simultaneously for each trial. The tests for the vehicle and three drug-treated groups were carried out over four days. The results of the four tests for each group (vehicle and dose groups) were pooled and analyzed by the one-tailed Wilcoxon's Ranked-Sums test.

Consciousness levels of freely-moving rats in daytime EEG

Under anesthesia with pentobarbital-Na (Nembutal®; 50 mg/kg, i.p.), rats were fixed to a stereotaxic apparatus. A pair of twisted wire electrodes insulated except at their tips (diameter: 0.17 mm, interpolar distance: 0.5 mm) was inserted into the dorsal hippocampus according to the brain atlas by Fikova and Marsala (32). Another pair of stainless wire electrodes (diameter: 0.15 mm) covered with silicone tubing except for 5–10 mm of length from the tip was inserted into the neck muscle to record the electromyogram (EMG). In addition, a pair of silver-ball electrodes (diameter: 0.5–1.0 mm) was implanted into the skull over the sensory-motor cortex. These electrodes and an anchor screw were fixed on the skull with dental cement and acrylic resin.

After a recovery period of at least one week, the EEG and EMG were recorded (EEG-3118, Nihon Kohden) from 9:00 to 16:00 continuously. The animals were placed in a cage, which was set in a sound-proof, shielded room with food and water, 16–17 h prior to the recording. The general behavior of each animal was recorded on a video tape recorder (AG220D; Matsushita

Electric, Osaka). After an hour of baseline recordings, the test compound or distilled water was orally administered. The consciousness level of the animals defined as an awake, slow wave sleep or paradoxical sleep episode was determined based on the EEG and EMG patterns.

Statistical analyses

Pharmacological results were represented as the mean and S.E.M. The difference between the means was determined by ANOVA followed by Dunnett's *t*-test unless otherwise stated. The statistical significance level was set at $P < 0.05$.

Results

In vitro profiles of RS-1259

AChE inhibition was measured in the homogenate of the mouse brain. The 50% inhibitory concentration (IC_{50}) of RS-1259 was 105 nM, which was 10–20 times weaker than that for donepezil. The IC_{50} of rivastigmine was extremely high in the assay system. For the uptake of 5-HT, NA, and DA measurement in the rat synaptosome, RS-1259 specifically inhibited 5-HT uptake with an IC_{50} of 90 nM, which was twice as potent as that of fluoxetine. The IC_{50} values of RS-1259 for the NA and DA uptake were more than 1 μ M, whereas those of imipramine and GBR12909 were 46 and 4 nM in the assay system (Table 1).

The binding affinity for receptors such as nicotinic and muscarinic ACh, 5-HT, NA, DA, histamine, glutamate, GABA, glycine, and opiate receptors and the IC_{50} values of RS-1259 against choline, adenosine, GABA transporters, and choline acetyltransferase (ChAT) were more than 10 μ M. RS-1259 was reported by MDS Pharma Services to show weak affinities to NA transporters ($IC_{50} = 7.7 \mu$ M), L-type Ca^{2+} channels (3.6 μ M), sigma receptors (2 μ M), and Na^+ channels

(5.1 μ M) (data not shown).

Ex vivo inhibition of AChE and SERT

RS-1259 inhibited AChE and SERT in the brain of mice (Fig. 2A and Table 2) and rats (Table 2) dose-dependently following oral administration. The doses necessary for 50% efficacy (ED_{50}) of RS-1259, donepezil, and rivastigmine were larger in rats than those in mice, although the potency order of compounds was the same in each species. RS-1259 was as potent as fluoxetine and more potent than fluvoxamine for SERT inhibition *ex vivo* in mice.

After oral administration, RS-1259 inhibited AChE and SERT in the mouse brain in a similar manner over time. The inhibition of AChE and SERT reached a maximum at 1–2 h, was still observed at 8 h and fully disappeared by 24 h after administration. The time to reach maximum AChE inhibition and the duration of the inhibitory effect by donepezil were much shorter than that of RS-1259 in mice (Fig. 2B).

Microdialysis in the rat hippocampus

RS-1259 simultaneously elevated extracellular levels of ACh and 5-HT in the hippocampus of freely moving rats. Donepezil and fluoxetine only increased ACh and 5-HT levels, respectively (Fig. 3: A and B). ACh was increased to a similar extent by RS-1259 and donepezil at doses of 64 and 16 mg/kg, respectively. At such high doses, all rats showed cholinergic symptoms such as muscle fasciculation, salivation, and lacrimation. The rate of 5-HT increase induced by fluoxetine at a dose of 32 mg/kg was slower and the overall 5-HT values were lower than those by RS-1259 at 64 mg/kg. The flat body posture elicited by fluoxetine could not be distinguished from the abnormal posture resulting from the cholinergic symptom that was observed when rats were treated with RS-1259.

Table 1. In vitro activities (IC_{50}) of RS-1259 in comparison with those of reference compounds

	IC_{50} values (nM)			
	AChE	5-HT uptake	NA uptake	DA uptake
	mouse brain	rat synaptosome		
RS-1259	105	90	>1000	>1000
Donepezil	5–10 [#]	n.d.	n.d.	n.d.
Rivastigmine	11,000	n.d.	n.d.	n.d.
Fluoxetine	n.d.	180	840	n.d.
Imipramine	n.d.	n.d.	46	n.d.
GBR12909	n.d.	n.d.	n.d.	4

n.d.: not determined. [#]Donepezil was always added as an internal standard of the AChE inhibition assay; the numbers represent a range of IC_{50} values.

Fig. 2. Dose-dependency (A) and time course (B) of inhibition of AChE and SERT in the mouse brain following oral administration of RS-1259, donepezil, and fluoxetine. Ex vivo inhibition was measured at 60 min (A) and at other time points (B) after administration. Symbols represent the mean of 5 animals, with the S.E.M.

5-HTP-enhancement in mice

RS-1259 enhanced 5-HTP-induced locomotor activity in a bell-shaped dose-response relationship with maximum enhancement at 8 mg/kg (Fig. 4A). The locomotor

Fig. 3. Increase in extracellular concentrations of ACh (A) and 5-HT (B) in the hippocampus induced by RS-1259, donepezil, and fluoxetine in rats. Compounds or the vehicle was orally administered just after the first sampling (–60–0 h). Each symbol represents the mean of 3–4 animals, with the S.E.M. indicated by a bar. Where S.E.M. bars are not shown, they lie within the dimensions of the symbols. The values indicated by #, * and ** are statistically different ($P<0.1$, 0.05, and 0.01, respectively) from those of the vehicle control at the corresponding time (ANOVA).

activity in mice treated with doses larger than 8 mg/kg was reduced as the cholinergic symptoms appeared. Fluoxetine (Fig. 4B) and fluvoxamine (Fig. 4C) were

Table 2. Inhibition of AChE and SERT in the brain 60 min after oral administration of RS-1259, AChE inhibitors, and SERT inhibitors in mice and rats

ED ₅₀ values (mg/kg, p.o.)				
AChE	mice		rats	
RS-1259	13	(11–15, n = 5)	43	(31–75, n = 5)
Donepezil	5.1	(4.6–5.6, n = 10)	16	(12–21, n = 10)
Rivastigmine	0.70	(0.64–0.77, n = 10)	4.1	(2.7–7.5, n = 10)
5-HT uptake	mice		rats	
RS-1259	2.4	(1.9–2.9, n = 5)	<8	(66% inhibition at 8 mg/kg, n = 5)
Fluoxetine	2.2	(2.0–2.5, n = 10)	n.d.	
Fluvoxamine	4.6	(4.2–4.9, n = 10)	n.d.	

n.d.: not determined. Number represents the ED₅₀ value with the 95% confidence interval in parentheses.

Fig. 4. 5-HTP-enhancing effect by RS-1259 (A), fluoxetine (B), and fluvoxamine (C) in mice. Each symbol represents the mean of 8 mice, with S.E.M. indicated by a bar. ANOVA of repeated measures and a post-hoc analysis, Fisher's PLSD, indicated that oral doses at 4, 8, and 16 mg/kg of RS-1259 and at 30 mg/kg of fluoxetine and fluvoxamine enhanced 5-HTP-induced locomotor activity ($P < 0.05$).

effective in the test with a longer duration of action for fluoxetine than for fluvoxamine.

Amelioration of memory deficits of aged rats

Short-term memory is severely affected early in the course of AD as well as in normal aging (33), and it is reported that short-term memory is preferentially impaired in aged animals (34). To examine whether RS-1259 could reverse age-related cognitive decline, we evaluated spatial short-term memory using the TPT of a water maze in aged rats. At 24–25 months of age, 11 rats showing a significant decline in the TPT performance were orally administered AChE inhibitors or the vehicle 60 min before each test. In the first test, both donepezil (0.5 mg/kg) and RS-1259 (1 mg/kg, but not 0.5 mg/kg) significantly ameliorated the aged-related short-term memory impairment (Fig. 5B). In the second test, at half the dose, donepezil (0.25 mg/kg) also improved the memory impairment, although at twice the dose (1 mg/kg) it did not (Fig. 5C). A higher dose of RS-1259 (2 mg/kg) was also effective in recovering the memory deficit (Fig. 5C). The parameters to evaluate

sensorimotor activities, that is, the swimming distance, the swimming speed, the floating time, and the swimming time of rats, were not affected by the drug treatments (data not shown).

Elevation of daytime consciousness level in rats

The daytime consciousness level of rats in a familiar cage was low. The total awake (10–20 min), slow wave sleep (35–45 min), and paradoxical sleep (about 5 min) episodes for every 60 min were stable during the recording period except for the initial 1 h when the awake episode increased and the other episodes decreased due to the handling necessary for oral administration. RS-1259 and donepezil, at all doses tested, increased the awake episode and decreased slow wave sleep as well as paradoxical sleep episodes in the initial 1 h following oral administration (Fig. 6). The consciousness level remained elevated up to 3 h after the highest dose of RS-1259 was administered, whereas it returned to control levels after 2–3 h in rats given the highest dose of donepezil. RS-1259 was also effective for the elevation of daytime consciousness level in rats where

Fig. 5. Amelioration of memory deficits in aged rats by RS-1259 and donepezil. A: Four representative patterns of the platform locations and start positions are shown. Locations of the fixed platform and floating platform were randomly changed at each session. Closed circles, fixed platform; open circles, floating platform; S₁ and S₂, start positions. Panels B and C represent the results of two separate drug tests. Animals were orally treated with AChE inhibitors or distilled water 60 min before a test session. Each column represents the mean accuracy of 11 rats, with the S.E.M. indicated by a bar. As determined by Wilcoxon's analysis, an amelioration of the reduced accuracy by RS-1259 at 1 and 2 mg/kg and donepezil at 0.25 and 0.5 mg/kg was detected ($P < 0.05$).

Fig. 6. Effects of RS-1259 and donepezil on the daytime consciousness level of rats. The total time of awake episodes was calculated every 60 min after a single oral administration of AChE inhibitors or the vehicle. Each symbol represents the mean awake episode of 5 rats during 60 min, with the S.E.M. indicated by a bar. ** $P < 0.01$ vs vehicle control (ANOVA).

the serotonergic neurons in the brain had been degenerated by an intracerebroventricular injection of 5,7-dihydroxytryptamine (35), except for the minimum effective dose of 4 mg/kg (Table 3). No abnormal behavior was observed throughout the recording period at any dose tested.

Discussion

RS-1259 is the first reported compound that inhibits both AChE and SERT in the brain following oral administration. The balanced inhibition of AChE and SERT by RS-1259 demonstrated the unique property of this compound and its potentially high efficacy in vivo. RS-1259 showed little affinity to various transporters including NA, DA, and choline transporters; to receptors including cholinergic and monoaminergic receptors; and to ChAT in vitro. A single oral administration of RS-1259 inhibited AChE and SERT in the central nervous system in a similar time frame, as expected.

Table 3. Effects of RS-1259 on the daytime consciousness level in the intact rats and serotonergic denervated rats with 5,7-DHT

	Dose (mg/kg)	Awake episode (min)	
		Intact rats (n = 5)	5,7-DHT-treated rats (n = 5)
Vehicle	—	51.0 ± 5.4	101.0 ± 7.4
RS-1259	1	76.2 ± 6.0	Not tested
	2	82.4 ± 9.3*	131.2 ± 13.0
	4	106.6 ± 9.8**	155.4 ± 7.3††

Numbers represent the mean of total time of awake episodes in the period of 180 min after a single oral administration of RS-1259, with the S.E.M. * $P < 0.05$, ** $P < 0.01$ vs vehicle control in the intact rats. †† $P < 0.01$ vs vehicle control in 5,7-DHT treated rats. The statistical differences were calculated in each group of intact and 5,7-DHT-treated rats independently with Dunnett's *t*-test.

Both the ex vivo measurements of AChE and SERT inhibition and the microdialysis experiment indicated that RS-1259 was 3–4 times weaker than donepezil and almost equipotent to fluoxetine in exerting central cholinergic and serotonergic effects, respectively.

RS-1259 was as effective as the serotonin-selective re-uptake inhibitors in the 5-HTP enhancement test in mice, suggesting an anti-depressant effect could be expected of the compound. It was somewhat surprising that RS-1259 was effective at doses lower than the effective dose of fluoxetine, since the potencies of both compounds were similar in the ex vivo measurement of SERT inhibition in the brain. Such low doses of RS-1259 alone did not increase the locomotor activity in mice and fluoxetine-induced 5-HTP enhancement was not affected by a co-administration of donepezil at doses that do not induce cholinergic symptoms. The above inconsistency may be due to the difference in the pharmacokinetic properties between RS-1259 and fluoxetine. The doses of RS-1259 and donepezil eliciting similar increases of ACh in the hippocampus correlated well with their ED₅₀ for the ex vivo inhibition of brain AChE. An oral dose four times higher than donepezil was necessary for RS-1259 to achieve a similar inhibition level of the brain AChE in mice and rats. Thus, it was concluded that the potency of RS-1259 following oral administration was approximately a quarter of that of donepezil with respect to AChE inhibition, whereas it was equipotent to fluoxetine with respect to SERT inhibition in the central nervous system in vivo.

It has been reported that not only cholinergic but also serotonergic impairments in the brain are involved in causing cognitive deficits in aged animals (36) and in humans (37). The effectiveness of RS-1259 in improving these deficits related to the central nervous system was compared to that of donepezil by evaluating two models, i.e., memory deficits in aged rats and the

daytime consciousness level of rats with nocturnal habits (38). In the memory test, both RS-1259 and donepezil ameliorated the low TPT performance of aged rats, but the effective dose of RS-1259 was approximately four times higher than that of donepezil, suggesting that no advantage was obtained by a dual activation of cholinergic and serotonergic transmissions over a solely cholinergic activation in the brain with respect to memory enhancement. TPT is reported to be highly sensitive to cholinergic deficits (30). We failed to observe any increase in extracellular levels of ACh and 5-HT in the rat hippocampus by fluoxetine and donepezil, respectively, even at high doses. A positive interaction between cholinergic and serotonergic transmissions in the central nervous system may not exist, at least not in the hippocampus. A higher TPT score by RS-1259 was probably obtained solely by improvements in the cholinergic transmission and not the serotonergic one. The simultaneous serotonergic activation by RS-1259 seemed not to affect the cholinergic transmission involved in the memory system.

The consciousness level is associated with several neurotransmitter systems in the brain including cholinergic and serotonergic systems. Dringenberg and Diavolitsis (39) reported that a co-activation of the cortical cholinergic and serotonergic transmissions restored the EEG activation abolished by a monoaminergic-cholinergic blockade. The authors used a complicated pharmacological procedure to induce the blockade and a co-activation of cholinergic and serotonergic transmissions in the cortex, without considering any possible drug-drug interaction between the compounds used. Using the dual inhibitor of AChE and SERT, we were able to evaluate the pharmacological effects of a simultaneous activation of both cholinergic and serotonergic transmissions in the brain without having to take into account any drug-drug interaction. We compared the effect of RS-1259 with that of don-

epezil on the daytime consciousness level of rats, which is low when they are in a familiar surrounding. Both compounds increased the consciousness level at similar doses. Considering the fact that RS-1259 inhibited brain AChE with a potency four times weaker than that of donepezil, this result clearly indicates that a simultaneous activation of the central serotonergic system could reduce the dose of AChE inhibitor needed to maintain an active consciousness level. This notion was confirmed by the observation that a larger dose of RS-1259 was required for the elevation of consciousness level in rats subjected to serotonergic denervation than that in normal rats.

In summary, an orally active dual inhibitor of AChE and SERT, RS-1259, was newly synthesized. Using this dual inhibitor, we confirmed that the degree of cholinergic activation required in maintaining the brain active could be reduced by a concurrent activation of the central serotonergic transmission. Thus, it is anticipated that cognitive disorders such as AD would be effectively and safely treated with dual inhibitors of AChE and SERT.

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