Activation of Cerebral Function by CS-932, a Functionally Selective M_1 Partial Agonist: Neurochemical Characterization and Pharmacological Studies

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ABSTRACT—A newly synthesized agonist for muscarinic acetylcholine (ACh) receptors CS-932, (*R*)-3-(3-iso-xazoloxy)-1-azabicyclo-[2.2.2]octane hydrochloride, showed a relatively higher affinity for M_1 than M_2 receptors expressed in Chinese hamster ovary (CHO)-cells in comparison with ACh. CS-932 elevated the intracellular Ca^{2+} level only in M_1 -CHO cells, although ACh increased the level in both M_1 - and M_3 -CHO cells. CS-932 and ACh reduced forskolin-stimulated accumulation of cAMP in M_2 -CHO cells by 20% and 80%, respectively. This neurochemical profile of CS-932 indicates that the compound can activate M_1 -receptor-mediated functions selectively. CS-932 increased firing of cholinceptive neurons in rat hippocampal slices, and this excitation was antagonized by pirenzepine, but not by AF-DX 116. CS-932 increased awake and decreased slow wave sleep episodes of daytime EEG in free-moving rats. It counteracted scopolamine-induced slow waves in rat cortical EEG. CS-932 also increased the power of α - and β -waves, but decreased δ -wave of the cortical EEG in anesthetized monkeys. It ameliorated scopolamine-induced impairment of working memory in rats. Orally administered CS-932 had the best penetration into the brain among the muscarinic agonists tested and caused the least salivary secretion among the cholinomimetics examined. These results indicate that CS-932 has potential as a cognitive enhancer with fewer side effects in therapy for Alzheimer disease.

Keywords: M_1 agonist, Acetylcholine, Cerebral activation, Alzheimer disease

Davis and Maloney first reported that central cholinergic neurons are selectively lost in Alzheimer's disease patients (1). White et al. showed that a presynaptic marker in patients with non-vascular senile dementia of the Alzheimer's type is selectively depleted (2). Since then, a large amount of work has been carried out and now it has been well established that cholinergic cell bodies in the nucleus basalis of Meynert degenerate in this disease.

In 1986, Summers et al. demonstrated that oral treatment with tetrahydroaminoacridine (THA) significantly improved the cognitive function of Alzheimer's patients in a double-blind, placebo-controlled crossover study (3), although the number of patients was limited. Now cholinesterase inhibitors (ChE-Is), THA (4, 5) and donepezil

(6–8), have been clinically proven to be effective for patients with Alzheimer's disease, particularly in the early to moderate stages.

Among the five acetylcholine (ACh) muscarinic receptor subtypes M_1 – M_5 , the functions and distributions of the M_1 – M_3 receptor subtypes are clearly elucidated. M_1 receptors are abundant in postsynaptic membranes in the cerebral cortex and the hippocampus and are involved in cognitive function. M_2 receptors are located at presynaptic terminals and inhibit ACh release in the brain. They are also distributed in the heart, where they cause bradycardia. M_3 receptors facilitate secretion from glands. According to Mash et al., the number of M_1 receptors is unaltered in Alzheimer's patients even though cholinergic innervation of the cerebral cortex is reduced (9). Furthermore, M_1 agonists (10, 11), but not ChE-Is (12), increase release of soluble amyloid precursor proteins and decrease β -amyloid

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Fig. 1. Chemical structure of CS-932.

proteins. Thus, selective M₁-receptor agonists are expected to be potential cognitive enhancers with much lower incidence of adverse events than ChE-Is.

In agreement with this expectation, a muscarinic M₁ agonist, xanomeline (3-(4-hexyloxy 1,2,5-thiadiazol-3-yl)1,2,5,6-tetrahydro 1-methylpyridine) (13, 14), has been clinically shown to be effective in Alzheimer's patients (15), although its clinical use is still limited by its side effects such as nausea, vomiting, anorexia and hypersalivation seen in the therapeutic doses (15). As a result of these studies, more selective M₁ agonists have been expected as the next generation of agents for Alzheimer's disease.

CS-932 ((*R*)-3-(3-isoxazoloxy)-1-azabicyclo[2.2.2]octane hydrochloride) (Fig. 1) is a newly synthesized compound that was aimed to be a functionally selective M₁ partial agonist. In this report, we describe the neurochemical and cerebroactivating properties of this compound as a cognitive enhancer in comparison with other M₁ partial agonists and ChE-Is.

MATERIALS AND METHODS

Animals

Male adult Sprague-Dawley or Wistar rats (250–300 g) were used to assay the receptor bindings. Male ddY mice (31–38 g) were used for determination of the ex vivo receptor bindings, the penetration rates of compounds into

the brain, and salivary secretion. Wistar rats (325–600 g) purchased from Charles River Japan were used for recording neuronal firing in the hippocampal slices. Wistar rats (250–300 g) were also used to record cortical EEG under freely-moving conditions as well as to record scopolamine-induced slow waves in the cortical EEG. In addition, this strain of rats (120–200 g) was used for memory and learning studies. Male cynomolgus monkeys (4.2–5.8 kg) were used for recording cortical EEG. All these animals were purchased from SLC Japan (Hamamatsu) unless otherwise mentioned. The animals were housed for at least one week before use in breeding rooms (lighted: 7:00–19:00) in which room temperature and humidity were maintained at $23 \pm 1^\circ\text{C}$ and $55 \pm 5\%$. Food and water were consumed ad libitum.

Compounds

[³H]*N*-Methyl-scopolamine (NMS; NET-636, 2.96 TBq/mmol), [³H]oxotremorine-M (oxo-M; NET-671, 3.22 TBq/mmol) and all the other tritiated ligands (Table 1) were purchased from New England Nuclear (Boston, MA, USA). ACh, carbachol, oxotremorine sesquifumarate, pilocarpine HCl, arecoline HBr, pirenzepine 2HCl, scopolamine HCl, NMS, atropine sulfate and its methyl bromide salt and physostigmine salicylate were from Sigma (St. Louis, MO, USA). (±)-Quinuclidinyl benzilate (QNB) was from RBI (Natick, MA, USA). M₁ agonists (CS-932, WAL-2014 ((*R*)-3-(propynyloxy) 1-aza-bicyclo-(2.2.2)octane(*E*)-butenedioate), xanomeline, sabcomeline ([*R*-(*Z*)]-(+)-α-(methoxyimino)-1-azabicyclo[2.2.2]octane-3-acetonitrile) (16) and AF-102B ((+)-*cis*-methyl-spiro(1,3-oxathiolane-5,3')quinuclidine), ChE-Is (THA, donepezil, rivastigmine ((*S*)-*N*-ethyl-3-[(1-dimethyl-amino)ethyl]-*N*-methyl-phenyl-carbamate) and amiridin (9-amino 2,3,5,6,7,8-hexahydro 1*H*-cyclopenta-(*b*)-quinoline)) and AF-DX 116 (11-[2-[(diethylamino)methyl]-1-piperidinyl]acetyl-5,11-dihydro-6-*H*-pyrido-[2,3-*b*]-[1,4]-benzodiazepine-6-one) were synthesized at the Neuroscience and Immunology Research Labora-

Table 1. Summary of experimental procedures for receptor binding assay

Receptors	Ligands	Conditions of incubation			Nonspecific bindings	
		Final concentration (nM)	Temperature (°C)	Time (min)	Cold ligands	Final concentration (μM)
α ₁ -Adrenaline	[³ H]WB4101	0.4	25	40	prazosine	100
α ₂ -Adrenaline	[³ H]clonidine	1.0	25	40	clonidine	10
β-Adrenaline	[³ H]DHA	1.0	23	40	1- <i>α</i> -prenolol	10
5-HT ₁	[³ H]5-HT	2.0	37	10	5-HT	10
5-HT ₂	[³ H]ketanserin	0.5	25	40	ketanserin	1
D ₁	[³ H]SCH 23390	0.2	25	45	(+)-butaclamol	1
D ₂	[³ H]domperidone	0.5	25	45	(+)-butaclamol	1
GABA _A	[³ H]muscimol	1.0	0	10	muscimol	0.1
M ₁ -ACh	[³ H]pirenzepine	1.0	25	60	atropine sulfate	1

tories, Sankyo. Pancuronium bromide (Mioblock®) was purchased from Organon Teknika (Boxtel, Netherlands) and lidocaine HCl (Xylocaine®) was from Fujisawa (Osaka). Pentobarbital-Na (Nembutal®) was purchased from Abbott (Abbott Park, IL, USA); and α -chloralose, urethane and ketamine (Ketalar®) were from Wako (Osaka), Tokyo Kasei (Tokyo) and Warner-Lambert (Morris Plains, NJ, USA), respectively.

For the neurochemical studies, the compounds were dissolved in DMSO (10% or 100%) unless otherwise stated. For the pharmacological studies, the compounds were dissolved in physiological saline (for injection or bath application for the hippocampal slices) or distilled water (for oral administration). When the compounds were systemically administered, the concentrations were adjusted so as to be administered at 10 or 1 ml/kg of the body weight of the mice or rats, respectively. The systemic doses were expressed as salts. The compounds were orally administered unless otherwise described.

Binding and functional assays with M_1 -, M_2 - and M_3 -CHO cells

Cell culture: Infected Chinese hamster ovary (CHO) cells were cultured in α -minimum essential medium (α -MEM; Gibco, Grand Island, NY, USA) containing glutamine (4 mM) and antibiotics (penicillin and streptomycin) in 5% CO₂ / 95% air at 37°C.

Preparation of membranes: The cells were rinsed and harvested in *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) solution (20 mM, pH 7.4) containing EDTA (10 mM), and homogenized with a Polytron in the same buffer. The homogenate was centrifuged (14,000 $\times$ g, 4°C) for 10 min, and then the pellet was suspended in HEPES solution containing EDTA (0.1 mM) and washed twice by centrifugation. The resulting membrane preparation (1 mg protein/ml) was kept at -80°C until use.

Receptor binding assays: Membrane preparations obtained from M_1 - (15 μ g protein/ml), M_2 - (60 μ g protein/ml) or M_3 - (10 μ g protein/ml) CHO cells were diluted with HEPES solution containing NaCl (100 mM) and MgCl₂ (10 mM). The diluted membrane preparation (1 ml) was incubated with [³H]NMS (10 μ l) and a muscarinic agonist (10 μ l) at 30°C for 2 h. For Scatchard analysis, 0.01–1 nM of [³H]NMS was used for the M_1 and M_3 receptors and 0.03–3 nM of [³H]NMS, for the M_2 receptors. For the displacement experiments, final concentrations of 0.15 (M_1 -CHO), 0.4 (M_2 -CHO) or 0.2 nM (M_3 -CHO) of [³H]NMS were used for the binding assays. After incubation for 2 h, the reaction mixture was quickly filtered through a Whatman GF/B filter (Whatman, Maidstone, UK) that was presoaked with 0.1% polyethylenimine with a cell harvester. The filter was washed twice with 2 ml of distilled water, and the radioactivity remaining on the filter paper

was counted with a liquid scintillation counter. Non-specific binding was determined in the presence of QNB (1 μ M).

The IC₅₀ values of the M_1 agonists for inhibiting [³H]NMS binding to the M_1 – M_3 receptors were calculated with SigmaPlot (SPSS Inc., Chicago, IL, USA), and K_i values were derived using the Cheng-Prusoff equation (17).

Functional assays

Measurement of free [Ca^{2+}]_{in} in M_1 - and M_3 -CHO cells: M_1 - or M_3 -CHO cells were detached from the flask with PBS containing 0.5% trypsin and 0.2% EDTA 4Na and then collected by centrifugation (150 $\times$ g) for 5 min. The cells were suspended in α -MEM (10 ml) containing newborn calf serum (10%) and incubated with acetoxymethyl form of fura 2 (fura 2 AM, 5 μ M) for 30 min at 37°C in the dark. The resulting fura 2-loaded cells were collected by centrifugation, washed twice with cold Locke's solution and resuspended in 1 ml of Locke's solution (3–5 $\times$ 10⁸ cells/ml).

Free [Ca^{2+}]_{in} was measured with a fluorometer (F-2000; Hitachi, Tokyo). Twenty microliters of the fura 2-loaded CHO cell solution was incubated with 1.96 ml of Locke's solution at 37°C for 3 min. After adding 20 μ l of CS-932 solution at various concentrations, the CHO cells were incubated for another 2 min. Fluorescence at 510 nm derived from 340 or 380 nm excitation waves was recorded. Free [Ca^{2+}]_{in} was estimated according to the method of Grynkiewicz et al. (18), in which all data was fitted to the following equation: Effect = E_{max} / {1 + ([Ago.] / EC₅₀)^b}, where [Ago.] and b represent the concentration of the agonist and an independent variable, respectively.

Measurement of cAMP levels in M_2 -CHO cells: M_2 -CHO cells were detached from the flask with PBS containing 0.5% trypsin and 0.2% EDTA 4Na and then collected by centrifugation. The cells were suspended in Locke's solution (0.5 $\times$ 10⁶ cells/ml). After incubation for 10 min at 37°C, forskolin (10 μ M), isobutylmethyl-xanthine (IBMX, 200 μ M) and a muscarinic agonist were added to the cell-suspended solution (1 ml) and incubated for another 10 min. The reaction mixture was centrifuged (1,000 $\times$ g) for 10 min, and the supernatant was mixed with a half volume of 2 M KHCO₃ and kept at -80°C until the cAMP assay, which was carried out by means of a cAMP kit (Yamasa Shoyu, Choshi).

Binding assays of rat M_1 (brain) and M_2 (heart) receptors in vitro

After decapitation, the cerebral cortices or the hearts of rats were homogenized in ice-cold sodium potassium phosphate buffer (10 mM, pH 7.4). The homogenate was centrifuged (1,000 $\times$ g) for 10 min. The supernatant was further centrifuged (49,000 $\times$ g for the cortices or 17,500 $\times$ g

for the hearts) for 10 min. The pellet was suspended in the buffer and stored at -80°C until use.

Aliquots of the membrane preparation, the compound and [^3H]-ligand solutions were incubated for 1 h (cerebral cortex) or 2 h (heart) at 30°C . The assay mixture was quenched with ice-cold buffer, filtered through a Whatman GF/B filter and washed twice. Radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was determined with atropine ($20\text{ }\mu\text{M}$).

M-agonist index and M₁ index: The M-agonist index (an index of muscarinic agonist efficacy) and the M₁ index (an index of M₁ selectivity) were determined according to Sauerberg et al. (19) with some modifications;

$$\text{M-agonist index} = \text{IC}_{50}[\text{QNB, Cx}] / \text{IC}_{50}[\text{oxo-M, Cx}]$$

$$\text{M}_1 \text{ index} = \text{IC}_{50}[\text{QNB, heart}] / \text{IC}_{50}[\text{pirenzepine, Cx}],$$

in which Cx indicates the cerebral cortex (Table 4).

Bindings with other receptors

After decapitation, the cerebral cortices were dissected and weighed. The tissue was homogenized in 7–8 vol of ice-cold TBS (50 mM, pH 7.7). The homogenate was diluted to 17–20 vol with the buffer and was centrifuged ($1,000 \times g$, 4°C) for 10 min. The supernatant was centrifuged ($49,000 \times g$, 4°C) for 10 min, frozen in the buffer and stored at -80°C until assayed.

A $5\text{-}\mu\text{l}$ aliquot of the test compound solution was added to $485\text{ }\mu\text{l}$ of the crude membrane preparation (0.412 mg protein/ml) and $10\text{ }\mu\text{l}$ of [^3H]-ligand solution. After the mixed solution was incubated (Table 1), 4 ml of ice-cold Tris buffer saline (TBS) was added to the reaction mixture and rapidly filtered (Whatman GF/B). The radioactivity remaining on the filter paper was measured by a scintillation counter. Non-specific radioactivity was obtained by adding an excessive amount (10^{-5} or 10^{-4} M) of a cold ligand to the reaction mixture (Table 1). Specific binding was obtained by subtracting the non-specific binding from the total binding.

Ex vivo muscarinic receptor binding assays: The assays were performed according to Freedman et al. (20) with slight modifications. Twenty minutes prior to the oral administration of the agonists, the mice were treated with NMS bromide (1 mg/kg, i.p.). One hour after the administration, the forebrain was removed and stored at -80°C .

The tissue was homogenized in ice-cold HEPES solution (20 mM, pH 7.4) containing MgCl_2 (1 mM). Aliquots of the homogenate were incubated with [^3H]oxo-M solution (final concentration: 2.4 nM) for 40 min at 30°C . The assay mixture was quenched with ice-cold buffer, filtered through a Whatman GF/C filter and washed twice. The level of radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was determined with atropine (15 μM).

Rate of penetration into the mouse brain: The penetra-

tion rate (%) of the muscarinic agonist into the brain was calculated based on the ex vivo receptor bindings. The amount of the test compound present in the mouse forebrain was calculated as a percentage of the administered amount.

Recordings of neuronal firing in hippocampal slices

The hippocampus of the rat was isolated under anesthesia with pentobarbital-Na (50 mg/kg, i.p.) or a combination of urethane (1 g/kg, i.p.) and α -chloralose (25 mg/kg, i.p.). Hippocampal slices, 400 μm in thickness, were prepared using a vibrating slicer. Slices were incubated in artificial cerebrospinal fluid (CSF), bubbled with 95% O_2 and 5% CO_2 , for more than an hour. The artificial CSF consisted of NaCl (124 mM), KCl (5 mM), CaCl_2 (2.4 mM), MgSO_4 (1.3 mM), NaHCO_3 (26 mM), KH_2PO_4 (1.24 mM) and glucose (10 mM).

A hippocampal slice was mounted on a recording chamber and perfused with bubbling CSF ($35\text{--}37^{\circ}\text{C}$) with a flow rate of 2 ml/min. The activity of neurons in the CA1 region was recorded extracellularly with a glass micropipette filled with 1 M NaCl (approximately 1 M Ω). A unit discharge was amplified (AVB-11; Nihon Kohden, Tokyo), displayed on an oscilloscope (VC-11, Nihon Kohden) and isolated with a window discriminator (MET-1100, Nihon Kohden). Output from the discriminator was recorded and analyzed with MacLab Chart (AD Instruments, Castle Hill, Australia).

Antagonism of scopolamine-induced slow waves in rat cortical EEG

Rats with chronic electrodes implanted on the skull were fixed to a wooden board. Under anesthesia with ether, the trachea was cannulated and a polyethylene catheter was inserted into the femoral artery for monitoring the blood pressure. After the surgical procedure was finished, ether anesthesia was discontinued. The animal was paralyzed with pancuronium bromide (0.4 mg/body, s.c.) and was maintained under artificial respiration (90/min). The wounded portion was locally anesthetized with lidocaine solution (1%), which was applied every 0.5 h. Body temperature was kept at 37°C with a heating pad. After at least an hour after discontinuation of the anesthesia, the EEG recording (EEG-4314, Nihon Kohden) was started. After stable EEG was recorded, scopolamine HCl (1 mg/kg, i.p.) was injected. A test compound was given (i.p.) about 30 min later and changes in the cortical EEG were determined by visual inspection.

Conscious levels of freely-moving rats

Under anesthesia with pentobarbital-Na (50 mg/kg, i.p.), rats were fixed onto a stereotaxic apparatus. A pair of twisted wire electrodes that were insulated except for their

tips (diameter: 0.17 mm, interpolar distance: 0.5 mm) was inserted into the dorsal hippocampus according to the brain atlas by Fifkova and Marsala (21). Another pair of stainless wire electrodes (diameter: 0.15 mm) covered with a silicone tube except for 5–10 mm of length from their tips 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.

At least one week later, the animals were placed in a cage with food and water, which was set in a shielded room with sound proofing and constant temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$). EEG and EMG were recorded (EEG-4314) from 9:00 to 16:00 continuously. All this data was fed into a data recorder (NFR 4,000; Sony, Tokyo) for further analysis. Gross behavior of each animal was recorded on a video tape recorder (AG220D; Matsushita, Osaka). After 1 h of baseline recordings, vehicle (for control) or compound solution was orally administered.

The conscious level of the animals was determined as awake (AW), slow wave sleep (SWS) or paradoxical sleep (PS) episode based on the EEG and EMG patterns. The total time of each episode was calculated during the first hour after administration of the test compound or vehicle.

EEG recordings from the cerebral cortex of lightly anesthetized cynomolgus monkeys

Three monkeys were used repeatedly with an interval of more than 2 weeks. Monkeys were fasted from the previous evening throughout the experiment. Animals were anesthetized initially with ketamine (5 mg/kg, i.p.) and a single dose of pentobarbital-Na (15 mg/kg, i.v.). Atropine methyl bromide (0.5 mg/kg, s.c.) was injected to prevent respiratory accidents. Each monkey was mounted on a monkey chair and a pair of needle electrodes was bilaterally inserted into the head skin over the frontal bone. Cortical EEG was monitored (EEG-4314) and stored on magnetic tapes by a data recorder (RD-180T; TEAC, Tokyo) for analysis later. The EEG for 10 s, sampled every 2 min, was

transformed to a frequency spectrum and divided into 4 frequency bands (δ : 1.0–3.5 Hz, θ : 3.5–7.5 Hz, α : 7.5–13.0 Hz and β : 13.0–30.0 Hz) using a minicomputer (ATAC-450, Nihon Kohden). The relative power of each band was calculated against the total power. After recording the baseline EEG for 10 min, CS-932 (5 mg/kg) or saline (0.33 ml/kg) was subcutaneously injected.

Scopolamine-induced working memory deficit model in the rat

The three-panel runway performance (22–24) was used. Briefly, the apparatus was composed of a start box, a goal box and four consecutive choice points consisting of a gate with three panels. Rats could pass through only one (the correct panel) of the three panels in each gate and could not return to the start box or a previous choice point. When the rats reached the goal box, they received two food pellets (about 50 mg each) as positive reinforcement. Six consecutive trials per day (one session) were conducted. The correct panels were changed in each session. Trials were repeated at 2-min intervals and water was freely available in the home cage between trials. Rats attaining the criteria (errors less than 8 from trial 2 through trial 6 for three consecutive sessions) were used for drug evaluation. CS-932 (0.032, 0.1 or 0.32 mg/kg, p.o.) was administered 5 min before scopolamine (0.32 mg/kg, i.p.). The test was started 30 min after the administration of scopolamine.

Determination of salivary secretion

The amount of saliva secreted was determined according to the method of Freedman et al. (20), which was slightly modified. At 10–20 min after oral administration of the

Table 2. Binding parameters of [^3H]NMS in membranes from CHO cells transfected with human muscarinic receptor subtype genes

Cell type	B_{max} (fmol/mg protein)	K_d (nM)
M_1	9310 ± 380	0.14 ± 0.01
M_2	1760 ± 20	0.47 ± 0.05
M_3	4880 ± 200	0.16 ± 0.00

The values indicate the mean $\pm$ S.E.M. of 3–4 determinations.

Table 3. K_i values (μM) of muscarinic agonists in membranes from CHO cells transfected with human muscarinic receptor subtype genes, derived from [^3H]NMS binding experiments

Compounds	M_1		M_2		M_3	
ACh	20.4 ± 0.8	(1.0)	0.0447 ± 0.0049	(456.38)	7.70 ± 0.45	(2.65)
CS-932	5.55 ± 0.12	(1.0)	0.762 ± 0.034	(7.28)	6.14 ± 0.31	(0.90)
AF-102B	3.78 ± 0.30	(1.0)	1.23 ± 0.07	(3.07)	5.57 ± 0.31	(0.68)
WAL-2014	27.9 ± 2.6	(1.0)	3.20 ± 0.14	(8.72)	44.2 ± 2.8	(0.63)
Xanomeline	0.0299 ± 0.0026	(1.0)	0.00832 ± 0.00088	(3.59)	0.0250 ± 0.0009	(1.20)
Sabcomeline	0.115 ± 0.030	(1.0)	0.0231 ± 0.0024	(4.98)	0.0637 ± 0.0183	(1.81)

The values indicate the mean $\pm$ S.E.M. of 5 determinations. Relative affinities of compounds against that of the M_1 receptor are given in parentheses.

test compound to the mice, a pair of pre-weighed cotton buds were placed into both sides of the buccal cavity every 2 min under anesthesia with pentobarbital-Na (50 mg/kg, i.p.). The amount of saliva secreted for 10 min was determined by subtracting total dry weights of the cotton buds from their total wet weights.

Statistical analyses

Significant difference was determined by ANOVA followed by Dunnett's *t*-test unless otherwise stated. In unit firing recordings, firing rates with or without agonists were compared by means of a one-tailed paired *t*-test. In all cases, a significant difference was noted when the *P* value was less than 0.05.

RESULTS

Binding affinities of M₁ agonists to human M₁-, M₂- and M₃-CHO cells

The B_{max} of [³H]NMS binding to membranes with M₁–M₃ receptors ranged from 1760 to 9310 fmol/mg protein, while K_d values were from 0.14 to 0.47 nM in the present study. The B_{max} of the M₂ receptors was the smallest, while the K_d value at the M₂ receptors was the largest. On the other hand, the B_{max} of the M₁ receptors was the largest and the K_d values at the M₁ and M₃ receptors were almost the same (Table 2).

As summarized in Table 3, the bindings of CS-932 and AF-102B to M₁ receptors were 3–5 times more potent than that of ACh, while that of WAL-2014 was almost equal to that of ACh. The bindings of xanomeline and sabcomeline were 680 and 180 times more potent than that of ACh, respectively. As to the binding activity to M₃ receptors, CS-932 and AF-102B were roughly equal to ACh, while WAL-2014 was approximately 6 times weaker than ACh. The bindings of xanomeline and sabcomeline to the M₃ receptors were 310 and 120 times more potent than that of ACh, respectively.

ACh had highest affinity for M₂ receptors, which was approximately 450 times that for M₁ receptors. Although CS-932, AF-102B, WAL-2014, xanomeline and sabcomeline also had higher affinities to M₂ receptors than to M₁ or M₃ receptors in the NMS binding studies, their affinity ratios for M₂ receptors over M₁ receptors were less than 10.

Functional assays of M₁- and M₃-CHO cells

In M₁-CHO cells, basal [Ca²⁺]_{in} was 119 ± 14 and maximal [Ca²⁺]_{in}, which was stimulated by a maximum concentration of ACh (10^{−5} M), was 1063 ± 113 nM (mean ± S.E.M., *n* = 18). The basal and maximal concentrations of [Ca²⁺]_{in} in M₃-CHO cells were 104 ± 7 and 908 ± 106 nM (*n* = 19), respectively. Net elevation of [Ca²⁺]_{in} was obtained by subtracting the basal from the total [Ca²⁺]_{in}

stimulated by ACh or CS-932. Since the basal and stimulated [Ca²⁺]_{in} varied among the experiments, the effect of CS-932 was represented as a relative potency against the maximal concentration (10^{−5} M) of ACh. CS-932 increased [Ca²⁺]_{in} in M₁-CHO cells concentration-dependently starting from 10^{−6} M and attained maximal effect (43 ± 5%, *n* = 7) at 10^{−4} M (EC₅₀: 3.7 μM) (Fig. 2, top panel). ACh increased [Ca²⁺]_{in} with increasing concentrations up to

Fig. 2. Effects of CS-932 (●) and ACh (○) on free [Ca²⁺]_{in} in M₁- or M₃-CHO cells and cAMP levels stimulated by forskolin (10^{−5} M) in M₂-CHO cells. Abscissae: log concentration of CS-932 (M). Ordinate: relative net increases of free [Ca²⁺]_{in} in M₁- (top panel) and M₃-CHO cells (middle panel), and relative net decrease in cAMP levels in M₂-CHO cells (bottom panel). Each symbol represents the mean of 3–4 determinations, with the S.E.M. indicated. Where S.E.M. bars are not shown, they lie within the dimensions of the symbols.

Table 4. IC₅₀ values, M-agonist- and M₁-indices of muscarinic agonists and antagonists

Compounds	IC ₅₀ (μM)				M-agonist index	M ₁ index
	[³ H]Oxo-M(Cx) ¹⁾	[³ H]QNB(Cx) ²⁾	[³ H]Pz(Cx) ³⁾	[³ H]QNB(H) ⁴⁾		
Agonists						
Carbachol	0.084	>1,000	83	12	>12,000	0.14
Arecoline	0.10	180	7.2	12	1,800	1.7
Oxotremorine	0.014	18	1.3	1.2	1,300	0.92
Pilocarpine	0.17	64	3.6	11	380	3.1
WAL-2014	1.0	140	7.1	61	140	8.6
Sabcomeline	0.0052	0.57	0.039	0.36	110	9.2
AF-102B	0.42	45	1.2	11	100	9.2
CS-932	0.52	52	1.2	11	100	9.2
Xanomeline	0.0069	0.67	0.037	0.48	97	13
Antagonists						
Pirenzepine	0.40	1.3	0.19	2.1	3.3	110
QNB	0.00061	0.0028	0.00032	0.00022	4.6	0.69
Atropine	0.0015	0.0079	0.00050	0.0039	5.3	7.8

¹⁾⁻⁴⁾: Final concentrations of [³H]Oxo-M, [³H]QNB and [³H]Pz were 3, 0.12 and 1 nM, respectively. M-agonist index = [³H]QNB(Cx) / [³H]Oxo-M(Cx), M₁ index = [³H]QNB(H) / [³H]Pz(Cx).

10⁻⁵ M without saturation (Fig. 2, top panel). On the other hand, CS-932 barely influenced [Ca²⁺]_{in} in M₃-CHO cells up to 10⁻⁴ M, while ACh increased the level with increasing concentrations up to 10⁻⁵ M without saturation (Fig. 2, middle panel).

CS-932 slightly decreased the cAMP level in M₂-CHO cells, which were stimulated by forskolin (10 μM); only a 20% decrease was observed evenly from 10⁻⁵ M to 10⁻³ M (Fig. 2, bottom panel). ACh increased the cAMP level concentration-dependently starting from 10⁻⁹ M and attained its plateau effect (about 20% of the control) at 10⁻⁶ M (Fig. 2, bottom panel).

M-agonist index and M₁-index

IC₅₀ values of muscarinic agonists for inhibition of [³H]oxo-M (agonist) binding were lower than their IC₅₀ values for inhibition of [³H]-labeled antagonists binding ([³H]QNB or [³H]pirenzepine). However, the IC₅₀ values of the antagonists (pirenzepine, QNB and atropine) for inhibition of binding of the tritiated agonists or antagonists did not show any consistent tendency (Table 4).

Among the muscarinic agonists examined, sabcomeline and xanomeline had the lowest IC₅₀ values for inhibition of [³H]oxo-M binding to muscarinic receptors prepared from the cerebral cortex. Oxotremorine, carbachol, pilocarpine

Fig. 3. Excitatory effect of CS-932 (▲, in left panel) upon cholinergic neurons in the rat hippocampal slices and antagonism by pirenzepine (●, in right panel), but not by AF-DX 116 (○, in right panel). Cholinergic neurons were identified by excitation following application of carbachol (3×10^{-7} M). Abscissae: log concentration of CS-932 (left panel) and that of the antagonists (right panel). Ordinates: firing rate by CS-932 relative to that by carbachol (3×10^{-7} M, left panel) and relative firing rate elicited by CS-932 (10^{-5} M) with co-administration of an antagonist (right panel). Each symbol represents the mean of 3–7 neurons, with the S.E.M. indicated. The relative firing rate by CS-932 alone in the right panel is shown by a horizontal line (the mean of 7 neurons) with S.E.M. (dotted zone). * in the left panel: $P < 0.01$, for an increasing effect of CS-932 (one-tailed t -test). # in the right panel: $P < 0.01$ for antagonistic effect of pirenzepine.

and arecoline had 20–30 times higher IC₅₀ values than those of sabcomeline and xanomeline, while CS-932 and AF-102B had IC₅₀ values about 100 times that of sabcomeline. The IC₅₀ value of WAL-2014 was the highest, which was about twice that of CS-932. These results indicate that the affinity order of these agonists for muscarinic receptors prepared from the rat cerebral cortex was: sabcomeline = xanomeline > carbachol > arecoline = pilocarpine > AF-102B = CS-932 > WAL-2014 *in vitro*.

The M-agonist index of carbachol was extremely high. The M-agonist indices of arecoline (1,800) and oxotremorine (1,300) were much higher than those of other agonists (97–380). On the other hand, the M-agonist indices of 3 muscarinic antagonists (pirenzepine, QNB and atropine) were less than 10.

The M₁ index of pirenzepine was as high as 110. The M₁ indices of CS-932, xanomeline, AF-102B and WAL-2014 were between 8.6 and 13. Compounds with the higher M-agonist indices had the lower M₁ indices.

Bindings to other receptors

CS-932 did not inhibit ligand bindings to the various kinds of receptors listed in Table 1 up to 10 μ M, although it displaced ligand bindings to 5-HT₃ (45%) and nicotinic receptors (56%) at maximal concentration of 10 μ M.

M₁-receptor-mediated firing of the rat hippocampal neurons *in vitro*

Most of the neurons in the CA1 region of the rat hippocampal slices increased the firing rate following application of carbachol (3×10^{-7} M) by $5.0 \pm 0.9/s$ ($n=38$) and returned to the baseline firing rate after washing with normal CSF.

CS-932 increased the firing rate of these cholinceptive neurons in a concentration-dependent manner (Fig. 3, left panel). No cells showed a decrease in their firing rates following application of CS-932 in 38 neurons examined. The increase in firing rate by 10^{-5} M CS-932 was antagonized by pirenzepine concentration-dependently, but not by AF-DX116 even at concentration as high as 10 μ M (Fig. 3, right panel).

Ex vivo muscarinic receptor binding

Among the muscarinic agonists administered orally, oxotremorine inhibited [³H]oxo-M binding at the smallest dose, and sabcomeline was the second most potent (Fig. 4). Other muscarinic agonists, except for WAL-2014, had a potency roughly in the same range. More specifically, in order to give more than 40% displacement, CS-932, xanomeline, AF-102B and WAL-2014 required doses of 30, 100, 100 and 300 mg/kg, respectively (Fig. 4). Thus CS-932 was slightly more potent than AF-102B, xanomeline and WAL-2014 in ex vivo binding studies when orally administered.

Fig. 4. Inhibition of ex vivo binding of [³H]-oxo-M in mouse forebrain by muscarinic agonists. Abscissa: % inhibition of [³H]-oxo-M bindings. Ordinate: dose (mg/kg, p.o.). Each symbol represents the mean of 4–6 animals, with the S.E.M. indicated. ○, CS-932; ●, Xanomeline; ■, WAL-2014; □, Sabcomeline; △, AF-102B; ▲, Pilocarpine; ×, Oxotremorine.

Penetration rates of the M₁ partial agonists into the brain

Approximately 0.2% of orally administered CS-932 was identified in the brain, which was the highest rate among the muscarinic agonists examined (Table 5). The penetration rate of AF-102B, WAL-2014 and xanomeline was about 55%, 40% and 4% of that of CS-932, respectively. The penetration rate of pilocarpine and oxotremorine was 30–50% of that of CS-932 (Table 5). Thus orally administered CS-932 and sabcomeline penetrated into the brain better than xanomeline, AF-102B, WAL-2014, pilocarpine and oxotremorine.

Antagonism by cholinomimetics against scopolamine-induced slow waves in the rat cortical EEG

As shown in Fig. 5, scopolamine (1 mg/kg, i.p.) stably induced slow waves in the rat cortical EEG. Physostigmine (1 mg/kg, i.p.) antagonized the scopolamine effect immedi-

Table 5. Penetration of muscarinic agents into mouse forebrain

Compounds	% Penetration
CS-932	0.18 ± 0.03
Sabcomeline	0.12 ± 0.01
AF-102B	0.10 ± 0.01*
WAL-2014	0.078 ± 0.017*
Xanomeline	0.0065 ± 0.0014*
Pilocarpine	0.051 ± 0.012*
Oxotremorine	0.083 ± 0.007*

The penetration of each compound was estimated by comparing the inhibition of ex vivo [³H]oxotremorine-M binding with the calibration curve. The values are the mean ± S.E.M. ($n=4-5$). * $P<0.05$ vs CS-932 (ANOVA and Dunnett's *t*-test).

Before administration

Fig. 5. Antagonism by physostigmine on scopolamine-induced slow waves in the rat cortical EEG. Upper and lower traces: cortical EEG and heart rates, respectively. Physostigmine (1 mg/kg, i.p.) was injected immediately after recording at 90 min. Calibrations: as indicated.

Before administration

Fig. 6. Effect of CS-932 on scopolamine-induced slow waves in the cortical EEG of the rat. CS-932 was administered approximately 30 min after scopolamine. Calibrations: as indicated.

Table 6. Effects of M_1 partial agonists and ChE-Is on scopolamine-induced slow waves in rat cortical EEG

	Dose (mg/kg, i.p.)						
	0.3	1	3	10	30	50	100
M_1 Agonists							
CS-932	nt	nt	nt	0/4	4/4	4/4	nt
Xanomeline	nt	nt	nt	0/4	0/4	nt	1/5
WAL-2014	nt	nt	nt	0/4	1/4	nt	2/4
AF-102B	nt	nt	nt	0/4	2/4	2/4	4/4
Sabcomeline	0/4	4/4	4/4	nt	nt	nt	nt
Cholinesterase inhibitors							
THA	nt	0/4	1/4	4/4	nt	nt	nt
Donepezil	nt	0/4	4/5	3/4	nt	nt	nt
Amiridin	nt	nt	0/5	2/5	4/4	nt	nt

number of animals antagonized / number of animals tested. nt: not tested.

ately after administration, although this antagonistic effect lasted only for about 30 min. This antagonism was confirmed in all 5 rats examined. The effect of physostigmine was not always observed at doses less than 0.3 mg/kg (i.p.).

As demonstrated by the example shown in Fig. 6, CS-932 antagonized the scopolamine-induced slow waves, as was observed with physostigmine, at a dose of 30 mg/kg (i.p.) and higher (Table 6). The effect of CS-932 lasted throughout the observation period of 1 h. Xanomeline and WAL-2014 antagonized the scopolamine effect in 1/5 and 2/4 of the rats examined, respectively, at a large dose of 100 mg/kg (i.p.). Sabcomeline antagonized the scopolamine effect most potently (Table 6).

ChE-Is (THA, donepezil and amiridin) antagonized the scopolamine-induced slow waves at smaller doses than the M_1 agonists (CS-932, xanomeline, WAL-2014 and AF-102B) except for sabcomeline (Table 6).

Fig. 7. Effects of M₁ agonists on AW (upper panel) and SWS (lower panel) during the first hour of oral administration in freely moving rats. Ordinates: total duration (min) of AW or SWS episodes during the first hour of administration of vehicle (indicated as dose 0) or a compound. Abscissae: dose (mg/kg, p.o.). Each column represents the mean of 5 animals with S.E.M., indicated. Each upper column (AW) and its corresponding lower column (SWS) were obtained from the same group of animals. * $P < 0.05$, ** $P < 0.01$, vs vehicle for each experiment.

Fig. 8. Effects of ChE-Is upon AW and SWS during the first hour of oral administration in freely-moving rats. All other details are the same as those in Fig. 7.

Table 7. Minimal effective doses of M₁ agonists and ChE-Is to activate rat cortical EEG

	Minimal effective dose (mg/kg)	
	Scopolamine-induced slow waves (i.p.)	Daytime consciousness (p.o.)
M₁ agonists		
CS-932	10–30	0.3
Sabcomeline	1	0.03
AF-102B	30	30
WAL-2014	100	10
Xanomeline	>100	3
Cholinesterase Inhibitors		
THA	3–10	10
Donepezil	1–3	10
Amiridin	10	3
Rivastigmine	nt	3

nt: not tested.

Activation of daytime consciousness level by cholinomimetics in the freely-moving rat

In all rats treated with the M₁ partial agonist or ChE-Is, the total AW increased significantly, while the total SWS episodes decreased during the first hour of oral administration (Figs. 7 and 8). These effects were observed at 0.03 (sabcomeline), 0.3 (CS-932), 3 (xanomeline, rivastigmine and amiridin), 10 (WAL-2014, THA and donepezil) or 30 mg/kg (AF-102B) (Figs. 7, 8 and Table 7). The PS episodes differed from experiment to experiment, and the total PS episodes during the first hour ranged between 0.6 ± 0.4 (mean $\pm$ S.E.M., $n=5$) and 2.8 ± 0.9 min ($n=5$) after oral administration of vehicle. All the cholinomimetics examined in the present study reduced the PS episodes dose-dependently during the first hour of oral administration. The significant effects on the PS episodes were obtained at the same doses that caused significant effects on the AW and/or SWS episodes by most cholinomimetics (data not shown).

Activation of spontaneous cortical EEG by CS-932 in the anesthetized cynomolgus monkey

As demonstrated in Fig. 9, spontaneous cortical EEG of the anesthetized monkey mainly (approximately 80%) consisted of δ -waves. This EEG pattern was unaltered by vehicle treatment during the observation period of about 1 h (open circles). CS-932, at 3 mg/kg only, decreased the δ -waves and increased the α - and β -waves (triangles). There was no effect at 1 mg/kg (squares). The significant effect of CS-932 (filled triangles) appeared 20–30 min after the subcutaneous injection and lasted throughout the observation period of 1 h. θ -Waves, however, changed little following injection of CS-932 (1 or 3 mg/kg).

Fig. 9. Effects of CS-932 upon spontaneous cortical EEG in cynomolgus monkeys anesthetized lightly with ketamine and pentobarbital-Na. Abscissa: time (min) after an injection (s.c.) of vehicle (○) or CS-932 (1 mg/kg, □; 3 mg/kg, △). Filled marks indicate significant changes (vs saline, ANOVA). Ordinates: relative amounts (%) of power in δ -, θ -, α - and β -wave components of the cortical EEG to the total power. Each symbol represents the averaged power with the S.E.M. of each component over a 10-min measurement period. Where S.E.M. bars are not shown, they lie within the dimensions of the symbols.

Effects of CS-932 on scopolamine-induced impairment of working memory in the rat

Scopolamine (0.32 mg/kg, i.p.) markedly prolonged the goal time and increased the number of errors passing through the correct panels. CS-932 significantly reduced the scopolamine-induced errors (Fig. 10, left panel) and shortened the goal time that was prolonged by scopolamine (Fig. 10, right panel) at doses of 0.1 and 0.32 mg/kg (p.o.).

Salivary secretion

Mice treated with distilled water hardly secreted any saliva during the observation period of 10 min. As demonstrated in Fig. 11A, sabcomeline secreted saliva as severely as oxotremorine and pilocarpine. Out of the six animals treated with 15 or 30 mg/kg (p.o.) of donepezil, three or six mice died, respectively, within an hour of administration. The other muscarinic agonists (Fig. 11A) and ChE-Is (Fig. 11B), including CS-932, induced saliva secretion to almost the same degree. CS-932 increased salivary secretion in a dose-dependent manner between 3 and 60 mg/kg (p.o.) with a lower slope than the others examined.

Fig. 10. Ameliorating effect of CS-932 on impairment of working memory produced by scopolamine in a three-panel runway task in rats. The deficits were measured as the increase in the number of errors and the elongation of goal time in trials 2 to 6. Each symbol represents the mean with S.E.M. ○, control; ●, scopolamine alone (0.32 mg/kg, i.p., $n=9$); △, scopolamine with CS-932 (0.032 mg/kg, p.o., $n=5$); ▲, scopolamine with CS-932 (0.1 mg/kg, p.o., $n=8$); ■, scopolamine with CS-932 (0.32 mg/kg, p.o., $n=8$). * $P < 0.05$ vs control; ** $P < 0.05$, *** $P < 0.01$ vs scopolamine alone (ANOVA followed by Fisher's PLSD method).

Fig. 11. Salivary secretion by muscarinic agonists (A) and ChE-Is (B) in mice. The amount of saliva (ordinate, mg) secreted during 10 min was determined at 10–20 min after administration (p.o.) of the compound in mice under anesthesia with pentobarbital. Abscissa: dose (mg/kg). Each symbol represents the mean of 3–6 animals, with the S.E.M. indicated. In panel A: ●, CS-932; ○, Xanomeline; ▲, WAL-2014; ■, Sabcomeline; △, AF-102B; □, Pilocarpine; ×, Oxotremorine. In panel B: ○, THA; □, Donepezil; △, Rivastigmine; ▲, Amiridin.

DISCUSSION

Since ACh neurons in the nucleus basalis Meynert degenerate in patients with Alzheimer's disease, particularly in the early stages (9), several strategies have been attempted to increase ACh content or stimulate M₁ receptors in the brain in order to treat this disease. The present study demonstrates that ACh has an affinity for M₂ receptors

more than 450 times greater than that for M₁ receptors and that it also activates M₃ receptors as potently as M₁ receptors. Thus agents increasing ACh content can not avoid the side effects elicited by stimulation of the heart (M₂ receptors) or the glands (M₃ receptors) at doses close to those enhancing cognitive functions (M₁ receptors). Therefore selective M₁ agonists are expected to lack adverse events and are thought to be preferable as agents for Alzheimer's

disease.

CS-932 was clearly demonstrated to activate M_1 receptors in a dose-dependent manner, but not M_2 and M_3 receptors. The effect of CS-932 to increase $[Ca^{2+}]_{in}$ in M_1 -CHO cells, however, reached a plateau (approximately 40% of the maximum response caused by ACh) at a concentration of 10^{-5} M, indicating that CS-932 is an M_1 partial agonist. On the other hand, CS-932 decreased the cAMP level by only about 20% even at the maximal concentration (10^{-3} M), unlike ACh, which decreased cAMP levels substantially. The selectivity of CS-932 to M_1 receptors relative to M_2 receptors was also confirmed in the rat cerebral cortex and heart by calculating the M_1 index. Both the M -agonist index and the M_1 index of CS-932 were roughly the same as those of WAL-2014, xanomeline, sabcomeline and AF-102B *in vitro*. These results indicate that CS-932 is as potent as these M_1 partial agonists and that the selectivity of CS-932 to M_1 receptors relative to M_2 receptors is roughly the same as those of these M_1 partial agonists *in vitro*.

According to Schwarz (25), M_1 partial agonists such as AF-102B, xanomeline, WAL-2014 and sabcomeline are well tolerated and do not elicit any changes in blood pressure, heart rate, electrocardiogram (ECG) and respiration at therapeutic doses. Thus CS-932 is expected not to cause any adverse events in the cardiovascular system like other M_1 partial agonists at therapeutic doses. In fact, CS-932 did not cause any changes in blood pressure, heart rate or ECG up to 30 mg/kg in rats (p.o.) or dogs (i.d.). This dose is 100–300 times larger than doses for improving the scopolamine-induced amnesia (0.1 mg/kg, p.o.) or for activating the daytime consciousness level (0.3 mg/kg, p.o.) in the rat.

Free $[Ca^{2+}]_{in}$ levels in M_3 -CHO cells were almost unaltered even at a large concentration (10^{-3} M) of CS-932, suggesting that CS-932 hardly activates M_3 receptors. This could explain why the salivary secretion following CS-932 was the least even at a high dose of 30 mg/kg (p.o.) in the mouse among the cholinomimetics examined.

Regarding the cerebroactivating effect, CS-932 excited cholinceptive neurons in the hippocampal slices, and this excitatory effect was well antagonized by pirenzepine, but not by AF-DX116. This indicates that CS-932 excited hippocampal cholinceptive neurons by activation of M_1 receptors.

Considering all these results together, including the low affinity of CS-932 for the other 27 receptors examined, it was concluded that CS-932 is a functionally selective M_1 partial agonist.

It has been known for a long time that muscarinic antagonists elicit sleep-like EEG patterns both in animals (26) and humans (27), although they do not actually fall asleep. Since muscarinic antagonists cause amnesia (28), the sleep-

pattern-like cortical EEG elicited by scopolamine has been suggested to be related to cognitive impairment (26). CS-932 activated the rat daytime consciousness level, which is low in nature as they are nocturnal animals, and antagonized the slow waves in the cortical EEG induced by scopolamine (rats) or pentobarbital-Na (monkeys). ChE-Is antagonized the scopolamine-induced slow-waves in the rat cortical EEG at smaller doses than the M_1 partial agonists, except for sabcomeline. It generally seems that ACh replaces scopolamine already bound to M_1 receptors more easily than the M_1 partial agonists. However, this potency order was not observed consistently with the study on activation of the daytime consciousness level. It can be stated, from the EEG studies, that systemically administered CS-932 exerted cerebroactivating effects in rats and monkeys presumably due to the activation of M_1 receptors.

The cerebroactivating effect of CS-932 was also confirmed by studying the ameliorating effect of this compound on the scopolamine-induced impairment of working memory in the rat. Numerous papers report that working memory is impaired in Alzheimer's disease (29–35). Cholinomimetics have also been reported to improve impairment of working memory in animal models (36–40) and in patients with Alzheimer's disease (41, 42). Although the radial maze (43, 44) has been often used as a model of working memory, the three-panel runway task was used in the present study in order to discriminate working memory more clearly from reference memory. The three-panel runway task is considered to depend more on working memory because the combination of correct panels in the four gates to pass through was changed every day in this task (22–24). The present study demonstrates that scopolamine impairs working memory as has been observed in previous works (22–24) and that a small dose of CS-932 ameliorates this impaired working memory.

As summarized in Table 8, a safety margin was calculated using the minimum effective dose to activate the daytime consciousness level and the dose to secrete 100 mg of saliva for each compound. The safety margin of CS-932 was the largest among the cholinomimetics examined, although the neurochemical properties of CS-932 were quite similar to those of WAL-2014 and AF-102B, and the affinity and efficacy of CS-932 to M_1 receptors were much less than those of xanomeline and sabcomeline in the *in vitro* studies. The better penetration into the brain of CS-932 than xanomeline, WAL-2014 and AF-102B could explain the discrepancy, at least partly. Furthermore, the high functional selectivity of CS-932 to M_1 receptors could be the major reason for its largest safety margin, although the detailed mechanism remains to be elucidated.

From these results, it is concluded that CS-932 is expected to be used as a novel M_1 partial agonist for improving cognitive function of patients with Alzheimer's disease

Table 8. Safety margins of cholinomimetics

Compounds	MED to activate daytime consciousness (mg/kg, p.o.) ¹⁾	Dose to secrete 100 mg of saliva (mg/kg, p.o.) ²⁾	Safety margin 2)/1)
CS-932	0.3	13	43
Sabcomeline	0.03	0.006	0.2
Xanomeline	3	9	3
WAL-2014	10	13	1
AF-102B	30	8	0.3
THA	10	16	2
Donepezil	10	15	2
Rivastigmine	3	10	3
Amiridin	3	15	5

MED: minimum effective dose. ²⁾: The values are determined from the dose-response curve for each drug in Fig. 11.

with less adverse events than ChE-Is and with a larger safety margin than other M₁ agonists.

REFERENCES

- Davis P and Maloney AJ: Selective loss of central cholinergic neurons in Alzheimer disease [letter]. *Lancet* **2**, 1403 (1976)
- White P, Hiley CR, Goodhardt MJ, Carrasco LH, Keet JP, Williams IE and Bowen DM: Neocortical cholinergic neurons in elderly people. *Lancet* **1**, 668–671 (1977)
- Summers WK, Majovski LV, Marsh GM, Tachiki K and Kling A: Oral tetrahydroaminoacridine in long-term treatment of senile dementia, Alzheimer's type. *N Eng J Med* **315**, 1241–1245 (1986)
- Sahakian BJ, Owen AM, Morant NJ, Eagger SA, Boddington S, Crayton L and Crockford HA: Further analysis of the cognitive effects of tetrahydroaminoacridine (THA) in Alzheimer's disease: assessment of attentional and mnemonic function using CAN-TAB. *Psychopharmacology* **110**, 395–401 (1993)
- Soares JC and Gershon S: THA – historical aspects, review of pharmacological properties and therapeutic effects. *Dementia* **6**, 225–234 (1995)
- Kawashima K, Sato A, Yoshizawa M, Fujii T, Fujimoto K and Suzuki T: Effects of the centrally acting cholinesterase inhibitors tetrahydroaminoacridine and E2020 on the basal concentration of extracellular acetylcholine in the hippocampus of freely moving rats. *Naunyn Schmiedberg Arch Pharmacol* **350**, 523–528 (1994)
- Giacobini E, Zhu XD, Williams F and Sherman KA: The effect of the selective reversible acetylcholinesterase inhibitor E2020 on extracellular acetylcholine and biogenic amine levels in rat cortex. *Neuropharmacology* **35**, 205–211 (1996)
- Bryson HM and Benfield P: Donepezil. *Drugs Aging* **10**, 234–239 (1997)
- Mash DC, Flynn DD and Potter LT: Loss of M2 muscarinic receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. *Science* **228**, 1115–1117 (1985)
- Eckols K, Bymaster FP, Mitch CH, Shannon HE, Ward JS and DeLapp NW: The muscarinic M1 agonist xanomeline increases soluble amyloid precursor protein release from Chinese hamster ovary-M1 cells. *Life Sci* **57**, 1183–1190 (1995)
- Muller DM, Mendla K, Farber SA and Nitsch RM: Muscarinic M1 receptor agonists increase the secretion of the amyloid precursor protein ectodomain. *Life Sci* **60**, 985–991 (1997)
- Lahiri DK, Farlow MR, Sambamurti K and Nall C: The effect of tacrine and leupeptin on the secretion of the beta-amyloid precursor protein in HeLa cells. *Life Sci* **61**, 1985–1992 (1997)
- Bymaster FP, Wong DT, Mitch CH, Ward JS, Calligaro DO, Schoepp DD, Shannon HE, Sheardown MJ, Olesen PH, Suzdak PD, Swedberg MDB and Sauerberg P: Neurochemical effects of the M1 muscarinic agonist xanomeline (LY246708/NNC11-0232). *J Pharmacol Exp Ther* **269**, 282–289 (1994)
- Shannon HE, Bymaster FP, Calligaro DO, Greenwood B, Mitch CH, Sawyer BD, Ward JS, Wong DT, Olesen PH and Sheardown MJ: Xanomeline: a novel muscarinic receptor agonist with functional selectivity for M1 receptors. *J Pharmacol Exp Ther* **269**, 271–281 (1994)
- Bodick NC, Offen WW, Levey AI, Cutler NR, Gauthier SG, Satlin A, Shannon HE, Tollefson GD, Rasmussen K, Bymaster FP, Hurley NR, Potter WZ and Paul SM: Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. *Arch Neurol* **54**, 465–473 (1997)
- Loudon JM, Bromidge SM, Brown F, Clark MS, Hatcher JP, Hawkins J, Riley GJ, Noy G and Orlek BS: SB 202026: a novel muscarinic partial agonist with functional selectivity for M1 receptors. *J Pharmacol Exp Ther* **283**, 1059–1068 (1997)
- Cheng Y and Prusoff WH: Relationship between the inhibition constant (K_i) and the concentration of an inhibitor which cause 50 percent inhibition (IC₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**, 3099–3108 (1973)
- Gryniewicz G, Poenie M and Tsien RY: A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* **260**, 3440–3450 (1985)
- Sauerburg P, Falch E, Meier E, Lembo HL and Krogsgaard-Larsen P: Heterocyclic muscarinic agonists. Synthesis and biological activity of some bicyclic sulfonium arecoline bioisosters. *J Med Chem* **31**, 1312–1316 (1988)
- Freedman SB, Harley EA and Patel S: Direct measurement of muscarinic agents in the central nervous system of mice using ex vivo binding. *Eur J Pharmacol* **174**, 253–260 (1989)
- Fifkova E and Marsala J: Stereotaxic atlas for the cat, rabbit and rat. In *Electrophysiological Methods in Biological Research*, Edited by Petran BJ and Zachar J, pp 653–731, Academic Press, New York and London (1967)
- Ohno M, Yamamoto T and Watanabe S: Effects of intrahippocampal injections of *N*-methyl-D-aspartate receptor antagonists and scopolamine on working and reference memory assessed in rats by a three-panel runway task. *J Pharmacol Exp Ther* **263**, 943–950 (1992)
- Yamamoto T, Ohno M, Kitajima I, Yatsugi S and Ueki S: Ameliorative effects of the centrally active cholinesterase inhibitor, NIK-247, on impairment of working memory in rats. *Physiol Behav* **53**, 5–10 (1993)
- Yamamoto T and Ohno M: Pharmacological application of a three-panel runway task to elucidate learning and memory in rats. *Methods Find Exp Clin Pharmacol* **18**, Suppl A, 5–15 (1996)
- Schwarz RD: Muscarinic receptor agonists under development for the treatment of Alzheimer's disease. *Alzheimer Dis ID Res Alert* **2**, 459–465 (1997)
- Votava Z, Benesova O, Bohdanecky Z and Grofova O: Influence of atropine, scopolamine and benactyzine on the physostigmine arousal reaction in rabbits. *Prog Brain Res* **28**, 40–47 (1968)
- Sagales T, Erill S and Domino EF: Differential effects of scopol-

- amine and chlorpromazine on REM and NREM sleep in normal male subjects. *Clin Pharmacol Ther* **10**, 522–529 (1969)
- 28 Pazzagli A and Pepeu G: Amnesic properties of scopolamine and brain acetylcholine in the rat. *Int J Neuropharmacol* **4**, 291–299 (1964)
 - 29 Kopelman MD: Rates of forgetting in Alzheimer's type dementia and Korsakoff's syndrome. *Neuropsychologia* **23**, 623–638 (1985)
 - 30 Morris RG and Baddeley AD: Primary and working memory of functioning in Alzheimer's-type dementia. *J Clin Exp Neuropsychol* **10**, 279–296 (1988)
 - 31 Baddeley AD, Bressi S, Della Sala S, Logie R and Spinnler H: The decline of working memory in Alzheimer's disease. A longitudinal study. *Brain* **114**, 2521–2542 (1991)
 - 32 Greene JD, Hodges JR and Baddeley AD: Autoradiographical memory and executive function in early dementia of Alzheimer's type. *Neuropsychologia* **33**, 1647–1670 (1995)
 - 33 Belleville S, Peretz I and Malenfant D: Examination of working memory components in normal aging and in dementia of the Alzheimer's type. *Neuropsychologia* **34**, 195–207 (1996)
 - 34 Kemper S: Metalinguistic judgements in normal aging and Alzheimer's disease. *J Gerontol B Psychol Sci Soc Sci* **52**, 147–155 (1997)
 - 35 Waters GS and Caplan D: Working memory and on-line sentence comprehension in patients with Alzheimer's disease. *J Psycholinguist Res* **26**, 377–400 (1997)
 - 36 Mastropaolo J, Nadi NS, Ostrowski NL and Crawley JN: Galanin antagonizes acetylcholine on a memory task in basal forebrain-lesioned rats. *Proc Natl Acad Sci USA* **85**, 9841–9845 (1988)
 - 37 Fisher A, Brandeis R, Karton I, Pittel Z, Gurwitz D, Haring R, Sapir M, Levy A and Heidman E: ($\pm$)-*cis*-2-Methyl-spiro(1,3-oxathiolane-5,3')quinuclidine, an M1 selective cholinergic agonist, attenuates cognitive dysfunctions in an animal model of Alzheimer's disease. *J Pharmacol Exp Ther* **257**, 392–403 (1991)
 - 38 Fisher A, Brandeis R, Chapman S, Pittel Z and Michaelson DM: M1 muscarinic agonist treatment reverse cognitive and cholinergic impairments of apolipoprotein-E deficient mice. *J Neurochem* **70**, 1991–1997 (1998)
 - 39 Weinstock M: Possible role of the cholinergic system and disease models. *J Neural Transm Suppl* **49**, 93–102 (1997)
 - 40 Iversen SD: Behavioural evaluation of cholinergic drugs. *Life Sci* **60**, 1145–1152 (1997)
 - 41 Alhainen K and Riekkinen PJ Sr: Discrimination of Alzheimer's patients responding to cholinesterase inhibitor therapy. *Acta Neurol Scand Suppl* **149**, 16–21 (1993)
 - 42 Robbins TW, McAlonan G, Muir JL and Ekeritt BJ: Cognitive enhancers in theory and practice: studies of the cholinergic hypothesis of cognitive deficits in Alzheimer's disease. *Behav Brain Res* **83**, 15–23 (1997)
 - 43 Magni S, Krekule I and Bures J: Radial maze type as a determinant of the choice behavior of rats. *J Neurosci Methods* **1**, 343–352 (1979)
 - 44 Watts J, Stevens R and Robinson C: Effects of scopolamine on radial maze performance in rats. *Physiol Behav* **26**, 845–851 (1981)