Effects of N-[2-(1-Azabicyclo[3,3,0]octan-5-yl)ethyl]2-nitroaniline Fumarate (SK-946), a Novel Cognition Activator, on Learning and Memory in Rodent Models

Tsunemasa SUZUKI,* Kiyotaka HIROOKA, Kazumi KANDA, Hiroko HIROOKA, and Kenichi FURUSAWA

Pharmaceutical Laboratory, Sanwa Kagaku Kenkyusho Co., Ltd., 363 Shiosaki, Hokusei-cho, Inabe-gun, Mie 511–0406, Japan. Received December 1, 1997; accepted March 27, 1998

We examined the effects of N-[2-(1-azabicyclo[3,3,0]octan-5-yl)ethyl]2-nitroaniline fumarate (SK-946) on cognition in various rodent models. SK-946 slightly suppressed spontaneous motor activity, but had no effect on scopolamine-induced motor facilitation. SK-946 ameliorated scopolamine-, pirenzepine-, cycloheximide- and electric shock-induced passive avoidance deficits in rodents when administered before acquiring the training. In an active avoidance test, SK-946 accelerated avoidance acquisition in the later half of training without a marked increase in lever-pressing. In more reliable models of cognitive disorders, i.e., an AF64A intracerebroventricular infusion model using the step-through passive avoidance test, an aged rat model using the step-down passive avoidance test and methylazoxymethanol (MAM)-induced microencephalic rat model using the Morris water maze test, SK-946 ameliorated impaired learning and memory. These results suggest an ability of SK-946 to enhance cognitive functions.

Key words SK-946, cognition activator; passive avoidance; active avoidance; Morris water maze; animal model

The search for new drugs that improve cognitive dysfunction in Alzheimer’s disease (AD) and senile dementia of Alzheimer’s type (SDAT) has been accelerated by worldwide requirements. Although neurochemical deficits in AD and SDAT are varied and complex,1,2 the earliest, most marked and consistent neurochemical changes associated with these diseases result from the degeneration of the basal forebrain cholinergic neurons which project to the hippocampus and cortex.3-5 Given the central role played by the cholinergic system in AD and SDAT neuropathology, it may be possible to alleviate the cognitive and behavioral deficits of AD and SDAT by enhancing or supplementing cholinergic neurotransmission. In some clinical trials, cholinomimetic therapy has been shown to be of some benefit in AD and SDAT patients.6 However, it is not clear whether the cognition-enhancing effects of cholinomimetics such as acetylcholinesterase inhibitors and muscarinic agonists in humans are due to selective, potent, and safe muscarinic compounds or to the heterogeneity of the AD and SDAT population.

For the purpose of developing a novel compound with potential therapeutic activity against AD and SDAT, we have been screening a series of pyrrolizidine derivatives. SK-946 (N-[2-(1-azabicyclo[3,3,0]octan-5-yl)ethyl]2-nitroaniline fumarate, Fig. 1) is a new pyrrolizidine derivative which interacts with muscarinic receptors.7 In the present study, we examined the cognitive enhancing effects of SK-946 using various behavioral models in rodents.

MATERIALS AND METHODS

These studies were performed following the guiding principles for the care and use of laboratory animals approved by the Japanese Pharmacological Society.

Animals For the spontaneous motor activity measurement, a passive avoidance deficit model (except for the cycloheximide model), and active avoidance test, male ddY mice (Japan SLC) weighing 22—32 g were used. For the passive avoidance cycloheximide model, male ICR mice (Japan SLC) weighing 22—32 g were used. For the passive avoidance scopolamine model, male Wistar rats (Charles River Japan) weighing 160—200 g were used. F344 rats (Japan SLC) weighing 200—300 g were used for the AF64A model and as controls in the aged rat model. Aged F344 rats (Japan SLC) were 27—28 months. Microencephalic rats were born from pregnant Wistar rats (Japan SLC) treated with methylazoxymethanol (MAM, 20 mg/kg i.p.) at day 13 of pregnancy. All animals were housed with a 12 h light-dark cycle at a temperature of 23±2°C and a relative humidity of 50±20% and were allowed free access to food and water.

Chemicals and Drugs SK-946 and pirenzepine hydrochloride were synthesized at our laboratory (Mie, Japan). Scopolamine hydrobromide was purchased from Tokyo Kasei (Tokyo, Japan). Cycloheximide and methylazoxymethanol acetate were purchased from Sigma (St. Louis, U.S.A.). AF64A was prepared from acetylcholine mustard hydrochloride (Research Biochemicals International, MA, U.S.A.) according to Fisher et al.8

Spontaneous Motor Activity Spontaneous motor activity was measured in groups of three mice using Automex II (Columbus, U.S.A.). In the first experiment, SK-946 was administered orally after habituation for 50 min, then the activity was counted for 90 min. In another experiment, SK-946 was administered orally after habituation for 20 min, then 30 min later scopolamine (1 mg/kg) was given intraperitoneally, and motor facilitation was observed for 60 min.

Fig. 1. Chemical Structure of SK-946

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* To whom correspondence should be addressed.
Step-Through Passive Avoidance Tests in Mice and Rats

All tests were conducted using two-compartment (light/dark) boxes equipped with an electrified grid floor. Animals were placed in the light compartment and were allowed to move into the dark compartment. On the acquisition training day, scopolamine 0.25 mg/kg for mice and 2 mg/kg for rats, pirenzepine 50 mg/kg for mice, and cycloheximide 120 mg/kg for mice, were administered intraperitoneally 20, 60 and 15 min before the shock-exposure acquisition procedure, respectively. Electric shock via corneal electrodes was delivered immediately after acquisition. To test a possible amnesic effect of SK-946, this drug was administered p.o. (100 mg/kg) to mice instead of scopolamine.

Two weeks before the acquisition training, AF64A was infused into both sides of the lateral ventricle of the rat brain (AP: −0.8 mm, ML: ±1.4 mm, DV: −4.0 mm from bregma) under anesthesia.

SK-946 or purified water was administered orally 30 min before the training.

On the following day, for the retention test, retention was assessed by measuring latency (cut-off at 300 or 600 s) until animals stepped into the dark compartment.

Furthermore, in the AF64A model, rats were divided into two groups and given SK-946 or purified water after finishing the retention test. Thirty minutes after administration, rats were decapitated for hippocampal cholinesterase (CAT) and acetylcholine determinations. CAT activity was measured according to Fonnun et al. Acetylcholine content was measured using the HPLC-electron capture detector (ECD) acetylcholine analysis system (Waters, U.S.A. and Eicom, Japan).

Step-Down Passive Avoidance Test in Aged Rats

A transparent acrylic box equipped with an electrifiable grid floor was used. Aged or young control rats were placed on the escape platform set in a corner of the box and were allowed to step down onto the electrifiable floor. Acquisition training was continued until rats stayed on the platform for 300 s after experiencing several electric shocks. SK-946 or purified water was administered orally 30 min before the training. On the following day, for the retention test, retention was assessed by measuring latency (cut-off at 600 s) until animals stepped down to the floor.

Lever-Press Active Avoidance Test in Mice

A small operant box equipped with an electrifiable grid floor and a lever set vertically was used. Each animal was subjected to a session consisting of 120 trials over 2 weeks (total 10 sessions). The conditioning stimuli used was a buzzer for 5 s, and this was followed by a foot shock as unconditioned stimuli. SK-946 or purified water was administered orally immediately before the start of daily training.

Morris Water Maze Task in MAM-Induced Microencephalic Rats

Pups born from MAM-treated or purified water-treated rats were weaned 3 weeks after birth. Then, SK-946 or purified water was administered orally once a day, 5 d/week for 7 weeks.

The Morris water maze task was begun 6 weeks after the start of drug administration. Three days before the start of training, the rats were given a pretrained session in which they were allowed to swim freely in the pool (170 cm in diameter and 45 cm in height) for 60 s without the escape platform. In the training session, the transparent platform (11.5 cm in diameter) was submerged 2 cm below the surface of the water, which was maintained at 23 ± 1 °C. Daily training consisted of 3 trials in which rats started for the fixed goal from 3 different start points; this was conducted for 5 consecutive days. If the rat failed to find the platform within 120 s, the animal was placed on it for 30 s. Goal time was measured for each subject (latency before finding the platform). Daily administrations of SK-946 or purified water were done after the training. Three days after the Morris water maze task, rats were weighed and decapitated for the measurement of brain weight.

Statistics

In the passive avoidance test, animals showing a latency of over 300 or 600 s were regarded as good performers. The results were expressed as the percent of good performers per group (% retention), and the mean value of test latency (cut-off 300 or 600 s) combined with the individual value of test latency of poor performers with a latency of less than 300 or 600 s. The significance of difference was assessed using the Mann-Whitney U-test for cut-off data, Fisher's exact probability test for % retention of passive avoidance tests, and one-factor ANOVA followed by Fisher's protected least significant difference test for the others.

RESULTS

Spontaneous Motor Activity

Although no effect was produced by an oral high dose administration of SK-946 (1 and 10 mg/kg), 10 and 100 μg/kg of SK-946 tended to suppress spontaneous motor activity during the 90 min observation period (Fig. 2A). On the other hand, SK-946 did not affect scopolamine-induced motor facilitation at doses of 10—10000 μg/kg p.o. (Fig. 2B).

Passive Avoidance Deficit Models Involving Systemic Treatments of Drugs and an Electric Shock

The effects of SK-946 on various step-through passive avoidance models are summarized in Table 1.

Muscarnic antagonists such as scopolamine and pirenzepine produced passive avoidance deficits in mice and rats when administered before the training. SK-946 given 30 min before the acquisition training ameliorated muscarinic antagonist-induced deficits at doses of 0.1—1000 μg/kg p.o. Maximum effects of SK-946 were observed at doses of 10, 100 and 1000 μg/kg p.o. in the scopolamine mouse model, scopolamine rat model, and pirenzepine mouse model, respectively. SK-946 ameliorated cycloheximide-induced deficits at doses of 0.1—100 μg/kg p.o. with a maximum effect observed at 1 μg/kg p.o. An electric shock-induced deficit was recovered by SK-946 100 μg/kg p.o. treatment. Except in the pirenzepine model, no more than 100 μg/kg p.o. of SK-946 was tested (based on the effectiveness in preliminary experiments). In addition, SK-946 (100 mg/kg p.o.) did not induce any passive avoidance deficit in mice (% retention was 97%, n=15, data not shown) per se.

Lever-Press Active Avoidance Acquisition in Mice

Normal control mice gradually acquired lever-press behavior and the active avoidance response, but only about a 50% avoidance rate, at the highest, was achieved in the last session. SK-946 (1—100 μg/kg p.o.) dose-dependently accelerated improvement in avoidance rates in the 7th to 10th session, without a marked increase in lever-pressing (Fig. 3).

AF64A Intracerebroventricular Infusion Model in Rats
Penetration of AF64A into the perihippocampal area in rat brain was histochemically confirmed. AF64A-treated rats showed poor retention of passive avoidance behavior and low hippocampal CAT activity with decreasing acetylcholine content. SK-946 ameliorated the AF64A-induced deficit in passive avoidance at doses of 10—1000 μg/kg p.o. and, with a maximum effect observed at a dose of 100 μg/kg p.o. without any effect on hippocampal CAT activity or acetylcholine content (Table 2).

**Aged Rat Memory Deficit Model** Aged rats showed a
severe step-down passive avoidance deficit without any difference in first down latency. However, the number of step-downs was fewer in the aged than in young control rats until the animals had acquired an avoidance response, suggesting senile ataxia or insensitivity to electric shock (Table 3). The mice administered SK-946 (1—100 μg/kg p.o.) showed significant elongation in latency, with the most effective dose being 10 μg/kg p.o. which resulted in a significant increase in % retention.

**Learning Deficit Model in MAM-Induced Microencephalic Rats** MAM-induced microencephalic rats had small brains (about 30% loss) as compared with the controls, but there was little difference in body weight. The control rats reached the goal platform within 30 s after only 3 training sessions. Microencephalic rats, however, did not learn the fixed place of the goal as readily as the control rats did. SK-946 (100 μg/kg p.o.) significantly shortened the goal time by only the 5th session without affecting brain weight in microencephalic rats (Fig. 4).

**DISCUSSION**

Although *in vitro* binding studies indicate that SK-946 has a submicromolar affinity for muscarinic receptors (a moderate selectivity for M1 (m1) sites), a high dose (10 mg/kg p.o.) of SK-946 had no effect on spontaneous motor activity or passive avoidance acquisition, and did not produce any of the syndromes associated with muscarinic stimulation or blocking. SK-946 had little effect on rat cortical acetylcholinesterase activity (data not shown). However, 10 or 100 μg/kg p.o. of SK-946 tended to suppress the motor activity without any sedation. These behaviors resembled alert-

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### Table 2. Effect of SK-946 on the Amnesia Model Induced by AF64A Intracerebroventricular Infusion in Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (μg/kg p.o.)</th>
<th>n</th>
<th>Latency in retention test (s)</th>
<th>% retention</th>
<th>Hippocampal CAT activity (nmol Ach/min/mg protein)</th>
<th>Hippocampal acetylcholine content (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>—</td>
<td>10</td>
<td>531.8±43.2***</td>
<td>70***</td>
<td>1.73±0.07***</td>
<td>22.6±1.47 (5)</td>
</tr>
<tr>
<td>AF64A control</td>
<td>—</td>
<td>16</td>
<td>164.5±37.9</td>
<td>0</td>
<td>0.99±0.06</td>
<td>15.4±0.94 (8)</td>
</tr>
<tr>
<td>SK-946</td>
<td>1</td>
<td>15</td>
<td>294.4±53.8</td>
<td>13</td>
<td>0.94±0.12</td>
<td>13.1±0.85 (7)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>370.0±55.5**</td>
<td>27</td>
<td>1.01±0.12</td>
<td>14.5±0.81 (7)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15</td>
<td>388.0±51.5***</td>
<td>40**</td>
<td>1.05±0.10</td>
<td>15.2±0.80 (8)</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>13</td>
<td>363.5±54.1**</td>
<td>23</td>
<td>0.91±0.05</td>
<td>14.1±1.03 (7)</td>
</tr>
</tbody>
</table>

AF64A was infused into both sides of the lateral ventricle of rat brain at a dose of 4 nmol (2’2 nmol). Two weeks after infusion, a one-trial-step-through passive avoidance test was done. Cut-off latency was 600 s in this test. After finishing the retention test, rats were divided into two groups and decapitated for CAT and acetylcholine determinations (number of measurable samples is shown in parentheses). SK-946 was administered both 30 min before the acquisition trial and 30 min before the decapitation. Each value except for % retention represents the mean±S.E. *p<0.01, **p<0.001: significant difference from AF64A control (Mann-Whitney U-test). +p<0.05, ++p<0.01: significant difference from AF64A control (Fisher’s exact probability test). ***p<0.001: significant difference from AF-64A control (Fisher’s protected least significant difference test).

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### Table 3. Effect of SK-946 on Multi-Trial Step-Down Passive Avoidance Deficit in Aged Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (μg/kg p.o.)</th>
<th>Weight (g)</th>
<th>First down latency (s)</th>
<th>Number of step-downs</th>
<th>Latency in retention test (s)</th>
<th>% retention in acquisition test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young control</td>
<td>—</td>
<td>250.9±1.8***</td>
<td>12.2±3.4</td>
<td>3.5±0.4***</td>
<td>600.0</td>
<td>100.0***</td>
</tr>
<tr>
<td>Old control</td>
<td>—</td>
<td>401.9±10.0</td>
<td>16.4±2.9</td>
<td>1.9±0.2</td>
<td>118.1±43.3</td>
<td>7.7</td>
</tr>
<tr>
<td>SK-946</td>
<td>1</td>
<td>401.9±9.3</td>
<td>14.0±2.9</td>
<td>1.9±0.3</td>
<td>293.2±71.8*</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>400.7±13.4</td>
<td>13.5±3.1</td>
<td>2.4±0.2</td>
<td>380.6±69.4**</td>
<td>53.8*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>403.2±8.9</td>
<td>9.2±1.4</td>
<td>2.7±0.2</td>
<td>321.4±68.7*</td>
<td>38.5</td>
</tr>
</tbody>
</table>

Each value except for % retention represents the mean±S.E. of 13 animals. SK-946 was administered 30 min before training. Training was continued until rats stayed on the platform for 300 s. Cut-off latency was 600 s in this test, but all aged rats stepped down within 600 s in the retention test. +p<0.05, ++p<0.01, +++p<0.001: significant difference from old control (Fisher’s protected least significant difference test). *p<0.05, **p<0.01, ***p<0.001: significant difference from old control (Fisher’s exact probability test).
Fig. 4. Effects of SK-946 on the Learning Deficit of Morris Water Maze in MAM-Induced Microencephalic Rats

MAM (20 mg/kg i.p.) was injected to pregnant rats at day 13. Pups were weaned 3 weeks after birth, then SK-946 was given orally once a day, 5 d/week for 7 weeks. The training (3 trails/session) was done at the last week of drug administration; after that, the rats were decapitated. Each value represents the mean±S.E. (n=12). *p<0.05, **p<0.01, ***p<0.001; significant difference from MAM control (Fisher's protection: least significant difference test).

Non-mobile-behavior (ANMB) induced by cholinomimetics such as acetylcholinestrase inhibitors and muscarinic agonists. SK-946 did not affect scopolamine-induced motor facilitation at any dose tested, although it ameliorated scopolamine-induced deficits in mice and rats at a low dose range. These results indicate that SK-946 may be a cholinomimetic at a dose range as low as 10 or 100 μg/kg.

Pirenzepine, a selective antagonist for M1 receptor sites, produced a passive avoidance deficit in mice when very high doses were administered, indicating that pirenzepine poorly penetrates the blood brain barrier. Accordingly, there is no denying the possibility that the pirenzepine-induced deficit might be due to both a central and peripheral muscarinic blockade. This may be why higher doses of SK-946 were needed to obtain the maximum effect. In addition, SK-946 ameliorated other models induced by more general malfunctions in the brain, i.e., cycloheximide- and electric shock-induced deficit models in mice. Although cycloheximide is a protein synthesis inhibitor, its amnesic effect might be related to the marked inhibition of central catecholamine synthetic mechanisms rather than to the failure to synthesize the protein required for memory formation. In our preliminary studies, SK-946 partly decreased the brain monoamine contents of normal rats. Therefore, it seems that the antiamnesic effect of SK-946 in the cycloheximide-induced deficit model is not due to an improvement of brain monoaminergic systems. Since the latency in the retention test was shortened by electric shock applied immediately after training, the amnesia appears to be caused by interference with memory consolidation. SK-946 extended the latency in the retention test in the electric shock-induced deficit model. Therefore, SK-946 is considered to exert its antiamnesic effect by affecting this memory process.

Lever-press active avoidance response, an operant conditioning behavior, includes learning lever-pressing, a new behavioral repertoire, and negative reinforcement. Since SK-946 accelerated lever-press active avoidance acquisition in mice without a marked increase in lever-pressing, non-specific behavioral excitation may not be related to the avoidance accelerating effect of SK-946. This result indicates that SK-946 may improve learning ability under physiological conditions.

The results described above prompted us to examine whether or not SK-946 has an ameliorating effect in more reliable animal models of AD and SDAT. First we attempted to explore its effect on an AF64A intracerebroventricular infusion model in rats. AF64A has been claimed to selectively injure cholinergic nerve terminals owing to its ability to utilize the choline transport system. In the present experiment, hippocampal CAT activity was significantly reduced with a slight reduction of acetylcholine content. This condition resembles the pathological features in AD and SDAT. SK-946 dose-dependently ameliorated AF64A-induced passive avoidance deficit at doses of 1—100 μg/kg p.o. AF64A is a presynaptic chemical neurotoxin, capable of inducing a persistent deficiency in central cholinergic transmission. Since SK-946 did not affect lowered hippocampal CAT activity or acetylcholine content, its ameliorating effect may be due to stimulation at the remaining postsynaptic sites in the central cholinergic neuron. Although cognitive disturbance in aged animals is not always the same as that in aged humans, much less that in AD and SDAT patients, aged animals are used extensively as models of age-related cognitive disorders. In this experiment, aged rats displayed an apparent passive avoidance deficit with emotional or motor disturbance which were detected by the reduction in the number of step-downs in the training. Since SK-946 did not affect emotional and motor disturbance, this compound is considered to exhibit its effect by improving impaired cognitive function. This data supports the assertion of the cholinergic hypothesis, namely that the specific enhancement of cholinergic function may reverse geriatric cognitive deficits.

Although marked brain atrophy or ventricular dilation are among the most apparent features in AD and SDAT patients, few animal models display such features. From this point of view, we attempted to estimate the effects of SK-946 on memory impairment in MAM-induced microencephalic rats. The administration of MAM to pregnant rats induced a marked reduction in the weight of the offspring's brain. This aplasia could be ascribed to the antimitotic effect of MAM. MAM-induced microencephalic rats showed impaired acquisition in the Morris water maze task, indicating that this aplasia had disrupted the cognitive process. In contrast, these animals showed normal growth, and in general their behavior appeared normal. Although long-term treatment using SK-946 could not restore aplasia, rats treated with SK-946 found the hidden goal in a shorter time at the last session of train-
ing than did the MAM-control rats. However, the efficacy of SK-946 in this model was less than in any other model. MAM treatment produces an atrophic cortex relatively hyperinnervated by noradrenergic and cholinergic neurons. 21 Although SK-946 has a cholinomimetic effect, this defined efficacy of SK-946 in MAM-induced microencephalic rats may be related to adaptive cholinergic hyperinnervation in the atrophic cortex.

In some tests, the dose-effect relationships for SK-946 were bell-shaped. It is unclear whether this particular dose-effect function is due to specific mechanisms of the drug or is a common phenomena of cognition activators, as previously reported by many investigators. 22,23 In conclusion, SK-946 not only has an improving effect on impaired memory but also a facilitatory effect on the acquisition of learning without any adverse effect. These results suggest that SK-946 might be of therapeutic value for improving the cognitive function impaired by cerebral dysfunction in AD and SDAT patients. The mechanism underlying its ameliorating effects has been clarified by various neurochemical techniques. The results of our preliminary neurochemical investigation suggest that SK-946 may interact with the central cholinergic system. 24

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