The Cyanine Dye NK-4 Improves Scopolamine-Induced Memory Impairments in Mice

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Received April 24, 2012; accepted July 9, 2012

The aim of this study is to evaluate the effects of NK-4, a kind of cyanine dye, on cholinergic memory deficits in mice. We examined whether NK-4 could reverse scopolamine-induced amnesia in mice since NK-4 displays a potent and selective inhibitory effect on acetylcholinesterase (AChE) in vitro. Intraperitoneal administration of NK-4 significantly reversed scopolamine-induced cognitive impairments in mice in the Y maze and the passive avoidance tests, and NK-4 also improved spatial learning ability in the Morris water maze test. Despite NK-4 displaying remarkable AChE inhibitory activity in vitro, we could not detect a significant reduction of AChE activity in brain homogenates of NK-4-treated mice. Although the mechanism through which NK-4 reverses cognitive impairments in scopolamine-treated mice remains unclear, these data suggest that NK-4 may have potential as a therapeutic agent for the treatment of dementia.

Key words cyanine dye; scopolamine; acetylcholinesterase inhibitor; behavioral test

NK-4, a kind of cyanine dye, exhibits a variety of biological activities, including antiviral,1) macrophage-activating,2) and anticancer properties.3) Recently, we found that NK-4 is a potent scavenger of free radicals and displays remarkable neurotrophic and neuroprotective activities in vitro.4–6) Furthermore, NK-4 effectively prevented ischemia-induced brain injury in rats7) and improved motor coordination in genetically ataxic hamsters.5,6) In our most recent study, long-term administration of NK-4 for 9 months significantly attenuated the impaired cognitive function observed in a transgenic mouse model of Alzheimer’s disease (AD).5,7)

Impairments of learning and memory, being the most characteristic manifestation of dementia, can be induced chemically in experimental animals via administration of scopolamine. Scopolamine is a tropane alkaloid drug that exhibits competitive antagonism at muscarinic acetylcholine receptors by interfering with cholinergic transmission in the central nervous system.8) Since the cholinergic system plays an important role in learning and memory,9) this animal model has been frequently used in research to screen for drugs with potential therapeutic value in dementia.10–12) Furthermore, the cognitive deterioration observed in scopolamine-treated animals resembles the memory disturbances seen in patients with AD. Consequently, the scopolamine-treated amnesic mouse has also been used as an experimental model for AD-type dementia.13,14) Based on the cholinergic hypothesis for the etiology of AD, it has been suggested that elevations in acetylcholine levels might help to improve cognitive deficits observed in AD.15) In fact, the most common therapy for AD is administration of acetylcholinesterase (AChE) inhibitors, such as donepezil, galantamine, and rivastigmine, which temporarily increase the availability of acetylcholine at cholinergic synapses.16)

In the present study, we evaluated the inhibitory effect of NK-4 on the activity of cholinesterases (ChEs) in vitro and showed that NK-4 is a potent and selective inhibitor of AChE. Subsequently, we examined the effect of NK-4 on scopolamine-induced cognitive impairments in mice using a set of behavioral tests. We also measured brain AChE activity in NK-4-treated mice to address the underlying mechanism of action.

MATERIALS AND METHODS

Chemicals and Reagents NK-4 {4,4′-[3-[2-(1-ethyl-4(1H)-quinolylidene)ethylidene]propenylene]bis(1-ethylquinolinium iodide)} (Fig. 1, purity >99%) was synthesized by Hayashibara Co., Ltd. (Okayama, Japan). NK-4 was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, U.S.A.) (5.0 mg/mL) and stored in the dark at room temperature. Scopolamine hydrobromide (scopolamine) was purchased from Tocris Bioscience (Ellisville, MO, U.S.A.). Acetyltiocholine iodide and butyryltiocholine iodide were purchased from Tokyo Kasei (Tokyo, Japan). All other reagents were from Wako Pure Chemicals (Osaka, Japan) unless otherwise indicated.

Animals This study was approved by the Laboratory Animal Care Committee of Hayashibara Co., Ltd. (permission number LN. 0171), and all animal experiments were conducted in accordance with the Guidelines of Care and Use of Laboratory Animals at Hayashibara Co., Ltd. Male ICR mice, weighing 28–34 g, and male F344 rats were purchased from Charles River Japan (Atsugi, Japan). Animals were individually caged and were allowed ad libitum access to water and standard diet.

Measurement of Cholinesterase Activity Enzymatic activities of AChE and butyrylcholinesterase (BChE) were determined at 37°C based on the Ellman method17) with modifications for microplate assay as described by Groner et al.18) Crude rat AChE and BChE were prepared from erythrocytes and plasma of male F344 rats, respectively, according to the description of Greig et al.19) Purified human AChE
(Sigma-Aldrich) or BChE (Sigma-Aldrich) were used at a final concentration of 0.02 units/mL.

Each well (total 250 µL) contained 50 µL of sample dye, 50 µL of AChE or BChE, 0.25 mM 5,5′-dithiobis 2-nitrobenzoic acid (DTNB), and 1 mM acetylthiocholine iodide or butryrylthiocholine iodide in 100 mM phosphate buffer (pH 8.0) containing bovine serum albumin (0.05%), Na3N (0.01%), and 1 mM ethylenediaminetetraacetic acid (EDTA)-2Na. For measuring brain AChE activity in mice, the enzyme solution and dye sample were replaced by the brain homogenate and 100 mM phosphate buffer (pH 8.0), respectively. Absorbance was measured spectrophotometrically at 405 nm.

**Drug Administration** NK-4 and scopolamine were diluted with saline before use and administered intraperitoneally to mice according to the schedule shown in Fig. 2. The control group received saline instead of NK-4 solution. NK-4 (200 or 500 µg/kg) was administered to mice 60 min before behavioral tests. In subsequent experiments, amnesia was induced by scopolamine (1.0 mg/kg), which was administered 30 min after NK-4 injection.

**Y Maze Test** Mice were initially placed within one arm of the Y-maze field (opaque plastic, arm length; 40 cm, path width; 3 cm, wall height; 12 cm), and the sequence (e.g., ABC, CAB, etc.) and number of arm entries were recorded for each mouse over an 8-min period. Alternation behavior was determined from successive entries into three different arms (e.g., ABC, CAB, or BCA). An arm entry by the mice was defined as placing all four paws within a boundary of the arm. The percentage of successive alternations was defined by the following equation: Spontaneous alternation (%)=([number of alternations]/(total arm entries−2))×100. The number of total arm entries also served as an indicator of locomotor activity.

**Step-through Passive Avoidance Test** A white compartment (14×25×20 cm) was illuminated by a 40 W bulb. The floor of another black, non-illuminated compartment (30×30×20 cm) was covered with copper strips spaced 1 cm apart. These two compartments were separated by a guillotine-type door (5×3.5 cm). In the acquisition trial, a mouse was placed in the illuminated compartment for habituation and the door was opened 20 s later. When the mouse entered the non-illuminated compartment, the door was immediately closed and an electrical foot shock (AC, 25 V) of 3 s duration was delivered. Twenty-four hours later, the mouse was placed again in the illuminated compartment for the retention trial. The latency to enter the dark compartment was measured in both trials with a maximum cutoff time of 180 s. An entry was defined as entering of the abdomen of the mouse into the non-illuminated compartment.

**Morris Water Maze Test** A circular pool (ϕ130 cm, height; 30 cm) was filled to a depth of 10 cm with water (20±2 °C) opacified with milk and was placed in a room with visual cues. The day before the first training trial was dedicated to swimming training for 120 s in the absence of the platform. Over the following four consecutive days (Day 1–Day 4), the mice were given two training trials each day with an intertrial interval of 30 min. A clear platform (ϕ9 cm) was placed at the midpoint of one quadrant, submerged 1 cm below the water surface and fixed in the same place throughout the training trials. The point of entry of the mouse into the pool was changed on each trial and day. When a mouse located the platform, it was permitted to remain on it for 10 s. If the mouse could not locate the platform within 120 s, it was gently navigated to the platform and remained on the platform for 30 s. A probe trial was carried out on the next day after the last training trial. The platform was removed and the mouse was allowed to swim freely for 120 s. The time spent in target and opposite quadrants was measured separately for each mouse.

**Measurement of Brain AChE Activity** Male ICR mice were intraperitoneally administered NK-4 (500 µg/kg/d) for four consecutive days. One hour after the final injection, mice were sacrificed and the brains were removed. The hippocampus and cerebral cortex were dissected and separately homogenized by a motor-driven Potter-type teflon homogenizer for 30 strokes with 4 volumes of ice-cold sodium phosphate buffer (100 mM, pH 7.4). The homogenate was then centrifuged at 7940×g for 10 min at 4°C, and the resulting supernatant was used as a source of enzyme for the assay. Protein concentration of the supernatant was determined by the Bradford method.

**Statistical Analysis** One-way analysis of variance (ANOVA) with a subsequent Tukey–Kramer test was used to determine the significance of differences across multiple comparisons (Y maze test). A t-test was used for comparisons between two groups (passive avoidance test and AChE inhibitory activity of brain region homogenates). Morris water maze test data were analyzed by repeated measures two-way ANOVA followed by Tukey test. Statistical significance was set at p<0.05.

**RESULTS AND DISCUSSION**

**ChE Inhibitory Activity of NK-4 in Vitro** First, we examined whether NK-4 displayed inhibitory effects on AChE and BChE activity. As shown in Fig. 3, NK-4 was found to be a potent inhibitor of AChE (IC50=22 nM, B; IC50=88 nM) but not BChE, suggesting that NK-4 is a selective inhibitor of
AChE. At the present time, enhancement of cholinergic neurotransmission via AChE inhibition is the most effective therapeutic approach for dementia. A comparison with other known AChE inhibitors showed that the AChE inhibitory activity of NK-4 was weaker than that of donepezil (IC50 = 6.7 nM), but comparable or superior to tacrine (IC50 = 77 nM), and much more potent than galantamine (IC50 = 1200 nM).21 As this suggested that NK-4 may ameliorate conditions of dementia, we next examined the effectiveness of NK-4 on amnesia in mice induced by scopolamine treatment.

**Effect of NK-4 on the Y Maze Test** The Y maze test is used for evaluating non-spatial working memory, a form of short-term memory.22 Reportedly, scopolamine treatment significantly reduces spontaneous alteration in the Y maze test.22 Initially, we examined the efficacy of a single injection of NK-4 (200 µg/kg, intraperitoneally (i.p.)) in scopolamine-treated mice in the Y maze test. However, a single administration of NK-4 did not significantly improve spontaneous alternation behavior in scopolamine-treated amnestic mice (data not shown), suggesting that a single treatment is insufficient for NK-4 to improve cognitive impairments. Daily, intraperitoneal administration in mice (500 µg/kg, 1 week) gradually increases NK-4 concentrations to nM levels in the brain (our unpublished data). Thus, we next tested a pretreatment protocol of NK-4 starting from 2 d before the behavioral evaluation. In this protocol, NK-4 (200 µg/kg/d) significantly reversed the disturbed spontaneous alternation induced by scopolamine (Fig. 4A left, p<0.05). Administration of NK-4 alone (500 µg/kg) to normal mice did not produce a significant change in spontaneous alternation. These results indicated that NK-4 ameliorated the scopolamine-induced memory deficits, but did not alter short-term memory function in non-impaired mice. This effect of NK-4 was not attributable to a change in locomotor activity since NK-4 administration did not affect the number of total arm entries in the Y maze test (Fig. 4A right).

Administration of NK-4 alone did not affect non-impaired normal mice, thus we did not set such a group in the following behavioral tests.

**Effect of NK-4 on the Passive Avoidance Test** Retention latency in the passive avoidance test is known to reflect long-term memory functions in rodents.23 Therefore, we tested the effect of NK-4 on scopolamine-induced memory deficits using the step-through passive avoidance test. During the acquisition trial, no significant differences in step-through latency were observed among all three groups (Fig. 4B black bars). During the retention trial, the latency time for all the saline-treated mice reached 300 s, the maximum cut off time, whereas scopolamine treatment remarkably reduced the latency (p<0.01, vs. control group). In comparison, NK-4 treatment significantly ameliorated the scopolamine-induced memory deficits (p<0.01, vs. scopolamine group). We also examined the effects of NK-4 by using the step-down-type passive avoidance apparatus and obtained similar results (data not shown). These data suggested that NK-4 ameliorated impairments of long-term memory in addition to working memory.

**Effect of NK-4 on the Morris Water Maze Test** The effect of NK-4 on hippocampus-dependent spatial learning ability and long-term spatial memory was evaluated using the Morris water maze test.24 The saline-treated mice showed a general decrease in overall escape latency throughout the training trials, while scopolamine-treated mice exhibited a significantly longer latency compared to the scopolamine group. We also examined the effect of NK-4 on the Morris water maze test (Fig. 4C) (left). In scopolamine-treated mice, NK-4 treatment shortened escape latencies throughout the training trials. A high dose of NK-4 (500 µg/kg) significantly reduced the escape latency compared to the scopolamine-treated group (Group×time F[2,99]=3.350, p<0.05). These data suggest that NK-4 augmented spatial learning ability in scopolamine-treated mice. Figure 4C (right) shows the results of the probe trial. Scopolamine-treated mice tended to decrease their time spent in the target quadrant and increase their time spent in the opposite quadrant compared to the control mice. A higher dose of NK-4 (500 µg/kg), but not a lower dose (200 µg/kg), produced a mild improvement; however, there was no significant difference in time distribution among all four groups. One possibility for this is that the
acquisition training, which was composed of two trials each day for four consecutive days, was not sufficient to establish complete spatial memories. Although the effects of NK-4 on long-term spatial memory appeared modest, NK-4 improved the spatial learning ability of scopolamine-treated amnestic mice in the Morris water maze test.

**AChE Inhibitory Activity of NK-4 in Brain** Finally, we compared the AChE activity within the brain of NK-4-treated (500 μg/kg, i.p.) versus untreated mice to examine the AChE inhibitory effect of NK-4 *in vivo*. AChE activity in both the hippocampus and cerebral cortex were measured. AChE activity was not significantly inhibited by NK-4 in either region, although NK-4 tended to decrease AChE activity in the hippocampus (Table 1).

In the brain, we could not detect a significant reduction of AChE activity by NK-4 administration. However, our unpublished research has demonstrated that the peripheral administration of NK-4 penetrates the brain–blood barrier and is detectable at nM levels in the brain. Therefore, there is still a possibility that NK-4 might function locally on cholinergic synapses. Other approaches including hippocampal microdialysis may reveal differences in the local availability of acetylcholine following NK-4 administration.

Recently, we found that NK-4 induces phosphorylation of Akt via activation of phosphatidylinositol 3-kinase (PI3K) in PC12 cells. It has been reported that activation of Akt induces phosphorylation of cAMP response element binding protein (CREB) which plays an important role in learning and long-term memory formation. Since scopolamine reduces phosphorylation of CREB in the hippocampus of mice, it is possible that NK-4 reverses the scopolamine-induced amnesia in mice through the PI3K-Akt-CREB signaling cascade, independent of cholinergic enhancement. In this

### Table 1. Effect of NK-4 on AChE Activity in the Hippocampus and Cerebral Cortex

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<thead>
<tr>
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<th>AChE activity (mU/mg protein)</th>
<th>*-Test</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NK-4</td>
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<tr>
<td>Hippocampus</td>
<td>6.57±0.91</td>
<td>5.79±0.59</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>7.81±0.96</td>
<td>7.23±1.47</td>
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Each value represents the mean±S.E.M. (n=8/group).
regard, we do not have decisive data yet. It is an important problem to be solved.

In this study, we could not elucidate the precise molecular mechanisms of NK-4 action, although we believe that NK-4 can be considered as a potential therapeutic agent for cognitive impairment.

REFERENCES