JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1275–1282

Received February 19, 2004
Accepted July 9, 2004
DOI 10.1211/0022357044391
ISSN 0022-3573

Asiatic acid derivatives enhance cognitive performance partly by improving acetylcholine synthesis

So Ra Kim, Kyung Ah Koo, Mi Kyeong Lee, Hyeung-geun Park, Sang-sup Jew, Kyung-hoe Cha and Young Choong Kim

Abstract

Thirty-six semi-synthesized derivatives of asiatic acid were examined to determine if they had cognitive-enhancing activity in a passive avoidance test. Among the compounds tested, AS-2, AS-2-9-006 and AS-9-006 significantly alleviated scopolamine-induced memory impairment at doses of 1 and 10 mg kg^{-1}. Furthermore, AS-2 and AS-2-9-006 (1 mg kg^{-1} administered four times daily) enhanced cognitive performance as determined in a water maze test. These three asiatic acid derivatives did not show any significant effect on the learning process in active avoidance tests. AS-2, AS-2-9-006 and AS-9-006 enhanced cholineacetyltransferase activity in a cholinergic neuroblastoma cell line, S-20Y, in-vitro. Therefore, AS-2, AS-2-9-006 and AS-9-006 may have therapeutic value in alleviating certain memory impairment observed in dementia.

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder and is the most common cause of senile dementia in people aged 65 years or older. The characteristics of Alzheimer's disease include a global impairment of higher mental function and loss of memory (Palmer 2002). The most consistent features of Alzheimer's disease are cholinergic deficits (Francis et al 1999; Frolich 2002) including: (i) decreased numbers of cholinergic neurons in the basal forebrain nuclei; (ii) decreased acetylcholine (ACh) production; (iii) enhanced levels of acetylcholinesterase (AChE); and (iv) reduced levels of cholineacetyltransferase (ChAT) in the frontal and temporal cortices. As such, treatment strategies have focused on replacement therapy for deficits in central cholinergic neurotransmission (Davidson et al 1991; Palmer 2002).

Centella asiatica (L.) Urban (Umbelliferae) has been used for centuries as a wound-healing agent and a brain tonic for the mentally challenged (Appa Rao et al 1977). Asiaticoside, a triterpene glycoside of *C. asiatica*, has been patented by Hoechst Aktiengesellschaft as a cognitive enhancer useful in treating dementia (De Souza et al 1992). However, asiaticoside is required at high doses and has to be given long-term to achieve its cognitive-enhancing activity as a putative anti-amnesic drug. We modified the chemical structure of asiatic acid, an aglycone of asiaticoside, in an attempt to prepare cognitive-enhancing compounds that are more effective than asiaticoside itself. We obtained 36 asiatic acid derivatives (AADs). In preliminary studies of the 36 AADs, only AS-2, AS-2-9-006 and AS-9-006 showed significant cognitive-enhancing activity in a scopolamine-induced memory impairment model.

In the present study, we attempted to verify the cognitive-enhancing activity of AS-2, AS-2-9-006 and AS-9-006 using three different tests in-vivo: the passive avoidance test, the water maze, and active avoidance test. We also tried to elucidate the underlying mechanisms for their anti-amnesic activities by determining their effects on cholinergic systems in-vitro. We examined cholinergic receptors, AChE activity, ChAT activity and choline uptake.

College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, San 56-1, Shillim-Dong, Kwanak-Gu, Seoul 151-742, Korea

So Ra Kim, Kyung Ah Koo, Mi Kyeong Lee, Hyeung-geun Park, Sang-sup Jew, Young Choong Kim

Dong Kook Pharmaceutical Co. Ltd, Central Research Institute, Jukhyun-Li 488-5, Kwanghyewon-Myeon, Jincheon-Gun, Chungcheongbuk-Doo 365-830 Korea

Kyung-hoe Cha

Correspondence: Young Choong Kim, College of Pharmacy, Seoul National University, San 56-1, Shillim-Dong, Kwanak-Gu, Seoul 151-742, Korea. E-mail: youngkim@plaza.snu.ac.kr

Funding: This research was supported by Dong Kook Pharmaceutical Co. Ltd via the Research Center of New Drug Development at Seoul National University.

Material and Methods

Animals

Male ICR mice (Experimental Animal Breeding Center of Seoul National University, Seoul, Korea), 25–30 g, were used after a 1-week adaptation period ($22 \pm 2°C$, $55 \pm 5\%$ humidity, 12-h light cycle from 0700 to 1900 hours, food and water freely available). The present experimental procedures were approved and conducted in accordance with the standards established by the Guide for the Care and Use of Animal Center for Pharmaceutical Research in the College of Pharmacy, Seoul National University and the US National Institutes of Health (NIH publication 85-23, 1985).

Materials

AADs were synthesized by chemically modifying asiatic acid, the major component of *C. asiatica*. Details of the chemical process are reported elsewhere (Jew et al 1998, 2000). The S-20Y neuroblastoma cell line was kindly donated by Dr Tae H. Oh (University of Maryland, MD, USA). Transfected Chinese hamster ovary cells (CHO-K1) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). [^{3}H]-Acetyl-CoA (specific activity 200 mCi mmol^{-1}), [^{3}H]*N*-methyl scopolamine (specific activity 75 Ci mmol^{-1}) and [methyl-^{3}H] choline chloride (specific activity 73 Ci mmol^{-1}) were purchased from Du Pont-New England Nuclear (Boston, MA, USA). All other compounds and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Passive avoidance test

Training and testing of passive avoidance behaviour were carried out in two identical light and dark square boxes (Gemini San Francisco Inc., CA, USA) as described previously (Kim et al 1999). In brief, mice received either one of the 36 AADs or velnacrine by intraperitoneal injection before trial training. At 60 min after AADs or velnacrine were administered, memory deficit was induced in mice with scopolamine (1 mg kg^{-1}, s.c.). After a further 30 min, mice were placed initially in the light chamber and the guillotine door between the compartments was opened 10 s later. When mice entered the dark compartment, the door automatically closed and an electrical foot shock (0.1 mA/10 g body weight) of 2 s duration was delivered. At 24 h after the training trial, the mice were again placed in the light compartment. The retention time of staying in the light compartment was checked as a parameter of memory.

Water maze

This spatial memory test was performed according to the method of Morris (1984). The water maze used was a white circular pool (90 cm in diameter and 45 cm in height) with a featureless inner surface. The pool was placed in a room with fixed furniture and posters capable of exerting extra-maze cues. The circular pool was filled to a height of 30 cm with water ($22 \pm 1°C$), in which 500 mL of milk was mixed. The pool was divided into four quadrants of equal area. A glass platform (6 cm in diameter) was placed 1 cm below the water surface, midway between the centre and rim of the pool in the target quadrant so that it was invisible at the water surface. On the first day of an experimental set, the mice were introduced for 60 s without the submerged platform. In the following days, the mice were given two trials daily (the inter-trial interval was 5 min) for 4 consecutive days without an inter-trial interval. The entry point of the mice into the pool and the location of the escape platform remained unchanged between trials 1 and 2, but the entry point was changed each day. In all procedures, swimming paths were recorded on a map of the pool by an experimenter who was unaware of the treatments. The escape latencies of mice were recorded with a stopwatch by the same observer. Once the mice found the platform and placed themselves on it, they were permitted to remain for 10 s. If a mouse did not find the platform within 120 s, the mouse was placed on the platform for 10 s and then removed from the pool. This parameter was averaged for each session of trials and for each mouse. The decrease in escape from day to day in the mean of trials 1 and 2 represents long-term memory.

At 90 min before entry into the pool, the mice received 5% Tween 80 (as vehicle, w/v), AS-2, AS-2-9-006 or AS-9-006 (1 mg kg^{-1} in 5% Tween 80) by intraperitoneal injection each day. After 60 min, memory impairment was induced in mice with scopolamine (1 mg kg^{-1}, s.c.). All mice were tested for spatial memory 30 min after the injection of scopolamine or 5% Tween 80.

Active avoidance test

Training for and testing of active avoidance behaviour were carried out in two boxes identical to those used in the passive avoidance tests. The mice were initially placed in one chamber with the overhead room lights off. After 10 s, the combined stimuli of small light and sound were simultaneously added for 5 s as a conditioned shock. After 2 s, an electrical foot shock (0.1 mA/10 g bodyweight) of 2 s duration was delivered through the stainless steel rods as an unconditioned shock. If the mice entered the other compartment during unconditioned shock, the response was considered as an escape response. If the mice entered the other compartment during conditioned shock, the response was defined as an avoidance response. If the mice did not enter the other compartment until the end of conditioned or unconditioned shocks, the response was regarded as no response.

Mice were given 30 training sessions daily, 5 days a week. At the start of daily training, AADs and scopolamine were injected into mice as described above. The increment in avoidance response of a mouse for 5 days was monitored as a parameter of learning by training repetition (Molchan et al 1992).

Cell culture

Cell monolayers of mouse neuroblastoma S-20Y cells (for choline uptake and ChAT activity assay) were plated on 75-cm^2 tissue culture flasks (Falcon, Heidelberg, Germany) and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air/5% CO_2. CHO-K1 cells transfected with human m1-m3 receptors (for muscarinic ACh receptor binding assay) were maintained in a Ham's F12 medium with 10% fetal bovine serum and 0.1 mg mL^{-1} G418 at 37°C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every 48 h. For the experiments, cells were released by exposure to 0.25% trypsin and plated on 60-mm tissue culture dishes at a density of 1×10^5 cells/dish.

Muscarinic ACh receptor binding assay

The transfected CHO-K1 cells were plated on 12-well plates at a density of 1×10^4 cells/well and maintained in serum-free media. After 24 h, AADs or 1 mM atropine was added to the culture media for 30 min at 37°C. After the incubation, [^{3}H]*N*-methyl scopolamine was added to each well at a final concentration of 1 nM and then further incubated for 30 min at 37°C. The cells were washed three times with ice-cold phosphate-buffered saline, and dissolved in 0.3 mL of 1% Triton X-100 (w/v). The radioactive pellets were then counted in 3 mL of HydroSol scintillation cocktail in a Wallac system 1400 counter (EG&G Co., Finland). Muscarinic ACh receptor binding affinity was calculated from the specific activity of the [^{3}H]*N*-methyl scopolamine (Shannon et al 1997; Tsuga et al 1998).

ChAT activity assay

ChAT activity was determined by the method of Fonnum (1975). At 40 h after plating S-20Y cells on 60-mm culture dishes, cells were washed with Hank's Balanced Salt Solution and 10 or 100 nM AADs was added to serum-free media. After incubation for 24 h, cells were washed twice with phosphate-buffered saline and collected. After centrifugation at 100 *g* for 5 min, the pellets were suspended in 0.3 mL of 50 mM sodium phosphate (pH 7.4) and then sonicated at an output of 7 in a Branson 5510 ultrasonicator (Branson Power Company, Danbury, CT, USA). For measurement of ChAT activity, 0.2 mL of the homogenate was incubated with 0.2 mL of reaction mixture containing: 150 mM NaCl, 5 mM EDTA, 5 mM choline, 0.1 mM neostigmine, 0.2 mM acetyl-CoA, and 0.25 μCi of [^{3}H]acetyl-CoA at 37°C for 30 min. The reaction was terminated by the addition of 0.2 mL of 1.5% tetraphenylboron in 3-heptanone. After vortexing the mixture, 50 μL of the upper phase, which contained the [^{3}H]ACh, was removed, dissolved and counted in 3 mL of HydroSol scintillation cocktail in the Wallac system 1400 counter. ChAT activity was calculated from the specific activity of the [^{3}H]ACh. The protein content was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Choline uptake

Cell monolayers of mouse neuroblastoma S-20Y were prepared as described above at a density of approximately 1×10^5 cells/dish. The cells collected in Tris-HEPES buffer (pH 7.4) were pre-incubated in the presence of AAD or 0.3 mM HC-3 as an inhibitor of choline uptake for 15 min at 37°C. Choline uptake was initiated by transferring 0.3 mL of the cell suspension to microcentrifuge tubes containing [methyl-^{3}H]choline chloride (final concentration 0.1 mM). After incubation for 30 min at 37°C, cells were immediately harvested and centrifuged. The radioactive pellets were washed twice with ice-cold Tris-HEPES buffer (pH 7.4), and then dissolved and counted in 3 mL of HydroSol scintillation cocktail in the Wallac system 1400 counter. Net uptake of choline was determined from the specific activity of the choline content of the homogenate (Sawada et al 1999).

AChE activity assay

AChE activity was determined by the modified method of Ellman et al (1961). AChE (from electronic eel, Type V-S; Sigma Chemical Co.) was diluted to 4.3 units mL^{-1} in phosphate buffer (pH 8.0, 0.1 M). The colour reagent, 5, 5′-dithiobisnitrobenzoic acid, AADs and authentic AChE were added to a spectrophotometric cuvette and pre-incubated at 25°C. Acetylcholine iodide was added as a substrate and incubation continued for 3 min. AChE activity was terminated by the addition of neostigmine. The absorbance at 412 nm was determined.

Statistical analysis

All data are expressed as mean ± s.e.m. Passive avoidance latencies were analysed by one-way analysis of variance and, if significant, group means were compared by post-hoc analysis using Tukey's multiple comparison of means. Morris water maze latencies were analysed by two-way analysis of variance with the day as one variable and the treatment as the other. The data were considered to be statistically significant at a value of $P < 0.05$.

Results

We examined the effects of 36 AADs on scopolamine-induced memory impairment using step-through passive avoidance tests. Among the 36 AADs, AS-2, AS-2-006, AS-5, AS-6-005, AS-18, AS-27, AS-lac-7 and SM-21 showed significant cognitive-enhancing activity at a dose of 1 mg kg^{-1} in preliminary tests (see Table 1). However, only AS-2, AS-2-006 and AS-9-006 (Figure 1) showed significant and effective cognitive-enhancing activity at doses ranging from 0.1 mg kg^{-1} to 10.0 mg kg^{-1} (Table 2). Saline-treated control mice stayed in the light compartment for 180.0 ± 1.2 s after passive avoidance training, whereas for the mice receiving 1 mg kg^{-1} scopolamine, the retention time in the light compartment was reduced to 31.4 ± 2.9 s. However, when the mice received AS-2, AS-2-9-006 or

Table 1 Effects of asiatic acid derivatives on scopolamine-induced dementia in passive avoidance tests

Compound	Retention time (s)	Relative effect (%)
Control	180.0 ± 3.9	100.0
Scopolamine	34.5 ± 1.8	0.0
Asiatic acid	55.5 ± 5.6	14.4
Madecassic acid methyl ester	32.5 ± 2.2	−1.4
AS-1	51.1 ± 3.4	11.4
AS-2	145.5 ± 6.4***	75.4
AS-2-9-006	153.6 ± 3.4***	81.9
AS-3	43.2 ± 2.6	6.0
AS-3-a	49.2 ± 4.1	10.1
AS-5	66.2 ± 3.5***	21.8
AS-5-alc	44.0 ± 2.5	3.2
AS-5-ald	41.5 ± 1.4	4.8
AS-6	33.5 ± 5.4	−0.7
AS-6-005A	30.2 ± 2.9	−3.0
AS-6-005	63.9 ± 6.0***	20.2
AS-6-007	44.2 ± 3.0	6.7
AS-8	32.3 ± 4.1	−1.5
AS-9	49.4 ± 1.3	10.2
AS-9-006	95.5 ± 4.9***	42.0
AS-13	33.9 ± 2.3	−0.4
AS-15	35.8 ± 3.4	0.9
AS-16	22.5 ± 1.8	−8.2
AS-17	35.3 ± 4.7	0.7
AS-18	66.7 ± 9.2***	22.1
AS-19	22.3 ± 2.6	−8.4
AS-19-005	21.6 ± 1.3	−8.9
AS-19-006	15.7 ± 2.5	−12.9
AS-27	60.5 ± 4.1***	17.9
AS-33-005	35.8 ± 2.1	0.9
AS-58	31.3 ± 6.2	−2.2
AS-59	22.9 ± 2.8	−8.0
AS-lac-7	70.9 ± 4.0***	25.0
Ester 4	50.9 ± 3.2	11.3
SM-08	57.1 ± 2.4	15.5
SM-18	35.8 ± 3.2	0.9
SM-21	73.5 ± 3.9***	26.8
SM-22	28.2 ± 3.1	−4.3
SM-25	34.4 ± 2.9	−0.1
Velnacrine	92.7 ± 5.0***	40.0

At 90 min before the training trial, mice received one of 36 asiatic acid derivatives or velnacrine at a dose of 1.0 mg kg^{-1}. After 60 min, amnesia was induced with scopolamine (1 mg kg^{-1}, s.c.). At 24 h after the training trial, the mice were again placed in the light compartment. The time they stayed in the light compartment was measured. Values are the mean retention times ± s.e.m. of three experiments (five animals per experiment) except for AA, AS-2, AS-2-9-006 and AS-9-006 (six experiments for these groups). The scopolamine-treated group was significantly different compared with the saline control, $P < 0.001$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ significantly different compared with the scopolamine-treated group (analysis of variance and Tukey's test).

AS-9-006 at a dose of 10.0 mg kg^{-1} 1 h before scopolamine administration, their memory standards were increased to 154.4 ± 8.6 to 165.6 ± 5.3 s. These three AADs were therefore chosen for further studies.

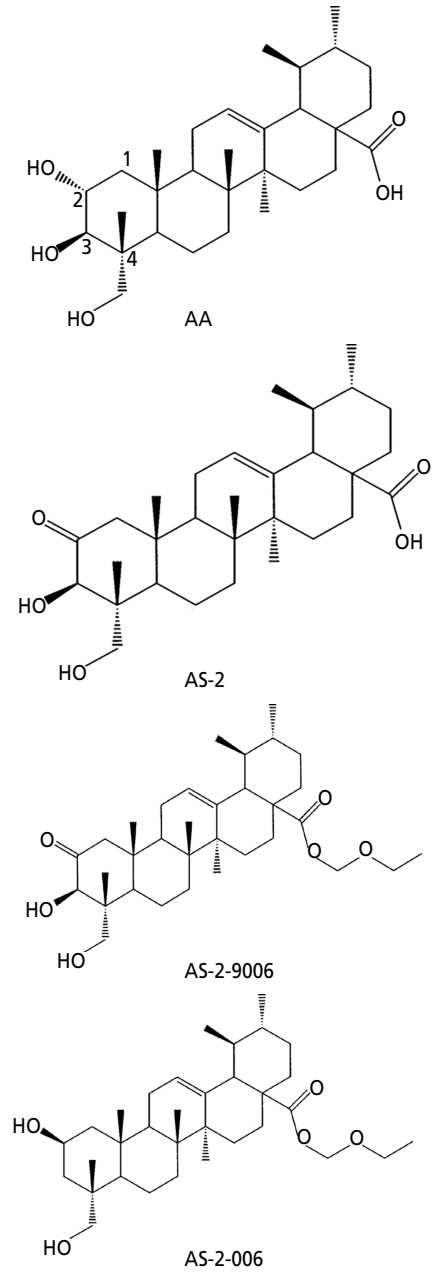

Figure 1 Structure of asiatic acid (AA) and its derivatives, AS-2, AS-2-9-006 and AS-9-006.

To test the cognitive-enhancing activity of the these three AADs, we determined if AS-2, AS-2-9-006 and AS-9-006 affected spatial memory using the Morris water maze, a test that can evaluate long-term memory. Saline-treated mice rapidly learned the location of the platform. This was demonstrated by a marked reduction in escape latency to platform in parallel with the repeated trials for 5 days in the Morris water maze (Figure 2). Furthermore, the swimming pathway required to find the submerged platform was simplified in groups given saline over days (data not shown). The shortened

Table 2 Effects of asiatic acid derivatives on scopolamine-induced memory impairment in a passive avoidance test

Compound	Dose ($mg\,kg^{-1}$)	Retention time (s)	Relative effect (%)
Control		180.0 ± 1.2	100.0
Scopolamine		31.4 ± 2.9	0.0
AA	0.1	20.0 ± 2.7	−7.7
	1.0	52.8 ± 5.2	14.4
	10.0	66.3 ± 2.2**	23.5
AS-2	0.1	39.8 ± 4.9	5.5
	1.0	143.4 ± 6.4***†	75.4
	10.0	163.7 ± 5.0***†	89.0
AS-2-9-006	0.1	75.9 ± 3.8*	30.0
	1.0	153.1 ± 3.4**†	81.9
	10.0	165.6 ± 5.3***†	90.3
AS-9-006	0.1	40.3 ± 3.3	6.0
	1.0	93.8 ± 4.9***	42.0
	10.0	154.4 ± 8.6***†	82.8
Velnacrine	0.1	61.6 ± 3.6**	20.3
	1.0	90.8 ± 5.0***	40.0
	10.0	79.4 ± 3.4***	32.3

At 90 min before the training trial, mice received appropriate asiatic acid derivatives or velnacrine at doses ranging from 0.1 to $10.0\,mg\,kg^{-1}$. Values are the mean units ± s.e.m. of three experiments (five animals per experiment) except for the $1.0\,mg\,kg^{-1}$ treated group. The retention time of the $1\,mg\,kg^{-1}$ treated group was taken from Table 1. The scopolamine-treated group was significantly different compared with the saline control, $P < 0.001$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ significantly different compared with the scopolamine-treated group; $^{\dagger}P < 0.001$ significantly different compared with the effect of velnacrine at the same concentration.

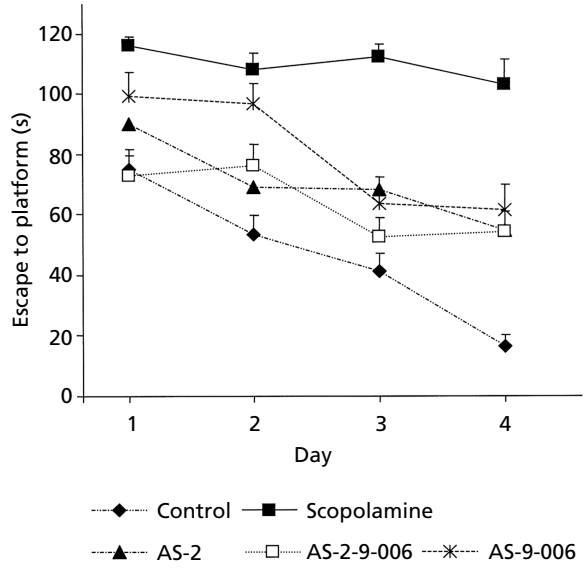

Figure 2 Effects of AS-2, AS-2-9-006 and AS-9-006 in the Morris water maze test. Mice were given two trials each day for 5 consecutive days. The swimming time required for the mouse to escape was recorded in each trial. Each day, the mice were injected with one of the three asiatic acid derivatives ($1\,mg\,kg^{-1}$, i.p.). After 60 min, memory impairment was induced by scopolamine ($1\,mg\,kg^{-1}$, s.c.). All mice were tested for spatial memory 30 min after the injection of scopolamine. The values shown are the mean latency for escape to platform ± s.e.m. of three experiments (five animals per experiment). The scopolamine-treated group was significantly different compared with the saline control for the entire experimental period, $P < 0.001$. The groups treated with asiatic acid derivatives were significantly different compared with the scopolamine-treated group from Day 3, $P < 0.05$.

swimming distance correlated well with the decrease in escape latency. In contrast, in the scopolamine-treated ($1.0\,mg\,kg^{-1}$) group, a characteristic swimming behaviour (circling around the pool) was observed; the escape latencies to platform remained essentially unchanged throughout all 4 days of the testing period. AS-2, AS-2-9-006 or AS-9-006 treatment ($1\,mg\,kg^{-1}$) significantly antagonized the effect of scopolamine on escape latency during the testing period (Figure 2). Furthermore, treatment with AADs also simplified the swimming pathway, which correlated well with the degree of shortened escape latency. The escape time to platform in groups given saline or scopolamine with or without AS-2, AS-2-9-006 or AS-9-006, showed highly significant effects with respect to day ($F[3, 140] = 28.8$, $P < 0.0001$), with respect to AAD treatment ($F[4, 140] = 61.1$, $P < 0.0001$), and with respect to day and AAD treatment interaction ($F[12, 140] = 2.7$, $P < 0.01$).

We also determined if AS-2, AS-2-9-006 and AS-9-006 affected the learning process using the active avoidance test. When saline-treated mice were trained using 30 daily trials for 5 consecutive days, the number of avoidance responses, a parameter for the improvement of learning, was significantly increased from 1.4 ± 0.4 on Day 1 to 16.3 ± 2.4 on Day 5. The number of 'no responses' was dramatically reduced from Day 1 (21.6 ± 4.1) to Day 2 (5.4 ± 2.1), and reached 0.3 ± 0.4 on Day 5. However, when the mice received scopolamine ($1\,mg\,kg^{-1}$), the number of avoidance responses was not significantly changed (1.8 ± 1.0 on Day 1 vs 6.7 ± 3.3 on Day 5). Furthermore, the number of escape responses was not significantly increased (9.4 ± 1.4 on Day 1 vs 10.4 ± 0.7 on Day 5). AAD administration did not significantly affect the change of avoidance responses (Figure 3). However, the number of escape responses was significantly increased on days when the mice received AS-2, AS-2-9-006 or AS-9-006 at $1.0\,mg\,kg^{-1}$ (Figure 3).

Since the scopolamine-induced memory impairment model is based on the 'cholinergic hypothesis' of Alzheimer's disease (Francis et al 1999; Frolich 2002), to elucidate the mechanism of action on cognitive-enhancing activity for AS-2, AS-2-9-006 and AS-9-006, we examined their effect on cholinergic systems using four different cholinergic parameters in-vitro: cholinergic receptors, AChE activity, ChAT activity and choline uptake. Among the four cholinergic parameters, AADs showed a significant effect only on ChAT activity in-vitro. In S-20Y neuroblastoma cells, at a concentration of 100 nM, AS-2, AS-2-9-006 and AS-9-006 significantly increased ChAT activity up to 1.25, 1.28 and 1.37-fold, respectively,

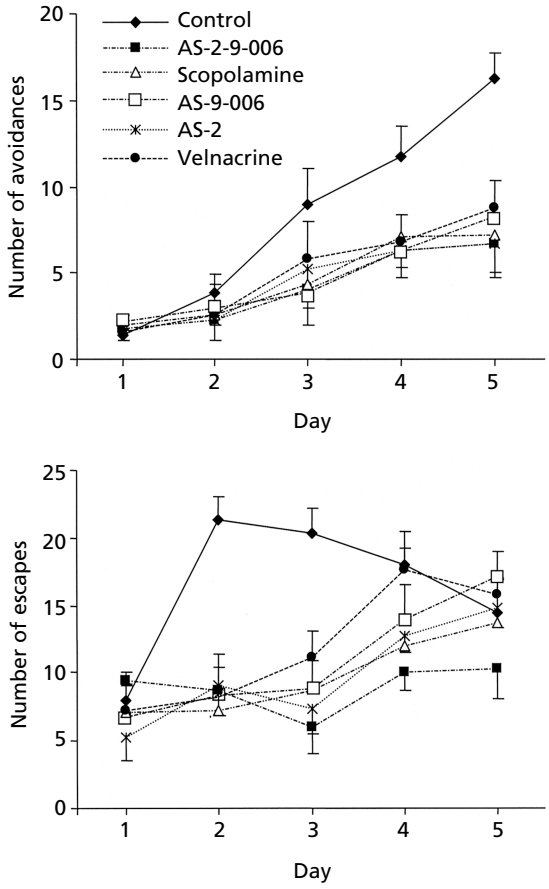

Figure 3 Effects of AS-2, AS-2-9-006 and AS-9-006 in the active avoidance test. Mice were given 30 trials daily for 5 consecutive days. The number of avoidances was recorded each day. Each day, the mice were injected with one of the asiatic acid derivatives (1 mg kg^{-1}, i.p.). After 60 min, memory impairment was induced by scopolamine (1 mg kg^{-1}, s.c.). All mice were tested for learning improvement 30 min after the injection of scopolamine. The values shown are the mean latency ± s.e.m. of three experiments (five animals per experiment). The number of escapes of the scopolamine-treated group was significantly different compared with the saline control for the entire experimental period, $P < 0.001$. The number of escapes of the groups treated with asiatic acid derivatives was significantly different compared with the scopolamine-treated group on Day 5, $P < 0.05$.

compared with controls (Table 3). However, these three AADs did not significantly affect [^{3}H]*N*-methyl scopolamine binding to its cholinergic receptors, choline uptake or AChE activity at concentrations ranging from 0.01 to 10.0 mM (data not shown).

Discussion

Natural products are used to treat neurodegenerative disorders such as senile dementia in nations such as China, Japan and Korea (Shanghai Academy of Science 1985). However, there is insufficient scientific evidence for their effectiveness and a systematic pharmaceutical screen of the components of these products has not been undertaken. The present study was performed in an attempt to evaluate the cognitive-enhancing activity of semi-synthetic derivatives of asiaticoside, a natural product of *C. asiatica*, for the possible prevention and/or treatment of memory deficits such as those seen in Alzheimer's disease. The cholinergic-neural system plays an important role in learning and memory in humans and animals (Lahiri et al 2003). Scopolamine, a muscarinic antagonist, has been known to disrupt hippocampal electrical activity by suppression of cholinergic function in this region of the brain (Stumpf 1965). When administered to animals, scopolamine is capable of transiently producing some of the deficits in the processes of learning acquisition and short-term memory considered as characteristic of Alzheimer's disease (Kopelman & Corn 1988). To examine the cognitive-enhancing activity of AADs, we used a step-through passive avoidance test paradigm in which scopolamine was used to induce memory impairment. Passive avoidance tests are a useful tool for estimating standard memory (retention) without the learning (acquisition) step.

Pre-treatment with AS-2, AS-2-9-006 or AS-9-006 dramatically increased standard memory compared with scopolamine-treated controls at doses ranging from 0.1 mg kg^{-1} to 10.0 mg kg^{-1}. The cognition-enhancing effects of these three AADs were superior to velnacrine, a tacrine derivative developed by a major drug manufacturer to treat Alzheimer's disease, at the same therapeutic concentration.

The Morris water maze has been used as a test of cholinergic disruption since 'cue' and 'place' learning are differentially sensitive to decreases in cholinergic function (Day & Schallert 1996; Kang et al 2003). We chose the hidden platform instead of the visible platform in the Morris water maze because cue learning is not disrupted by muscarinic receptor antagonists (Whishaw 1985; Whishaw et al 1985). In this study, the groups treated with AS-2, AS-2-9-006 or AS-9-006 at a dose of 1 mg kg^{-1} had significantly improved deficits in spatial learning and memory induced

Table 3 Effects of AS-2, AS-2-9-006 and AS-9-006 on cholineacetyltransferase (ChAT) activity in the S-20Y neuroblastoma cell line

Compound	Concentration (nM)	ChAT activity (pmole (mg protein)$^{-1}$)
Control		26.13 ± 0.64
AS-2	10	27.33 ± 0.48
	100	32.64 ± 0.61*
AS-2–9-006	10	29.81 ± 0.99
	100	33.56 ± 2.26*
AS-9-006	10	29.03 ± 1.49
	100	35.88 ± 1.40**

Values are the mean ± s.e.m., n = 3. *$P < 0.05$; **$P < 0.01$, significantly different compared with the control (analysis of variance and Tukey's test).

by scopolamine (Figure 3). The deficits in the swimming maze were antagonized by such centrally acting cholinergic drugs as physostigmine, oxotremorine and tacrine (Jackson & Soliman 1996). In contrast, peripherally acting cholinergic drugs such as neostigmine or nicotine are known to be inactive against scopolamine-induced spatial memory impairment (Riekkinen et al 1998). Therefore, the improved spatial learning and memory observed in the AAD-treated mice suggested that these AADs might act on the central cholinergic system, but not on the peripheral cholinergic system. Indeed, AS-2, AS-2-9-006 and AS-9-006 enhanced ChAT activity in S-20Y cells in-vitro (Table 3). ChAT, the key enzyme in ACh synthesis, is the most reliable marker of cholinergic neurons, which are thought to play a role in learning and memory (Dutar et al 1995). On the other hand, AS-2, AS-2-9-006 and AS-9-006 did not improve the learning process (Figure 3), which is generated by repeated experiences in an active avoidance test. Therefore, the increase in ChAT activity produced by AS-2, AS-2-9-006 and AS-9-006 may explain their ability to reverse the scopolamine-induced memory impairment in passive avoidance and water maze tests.

Since we previously reported that these AADs showed neuroprotective activity against glutamate-induced neurotoxicity in primary cultured rat cortical cells (Lee et al 2000), we cannot fully exclude the possibility that the cognitive-enhancing activity of AS-2, AS-2-9-006 and AS-9-006 might arise through a modification of cholinergic transmission by their action on the glutamatergic system. Recently, a number of studies on the interaction between cholinergic and glutamatergic systems has been reported (Dringenberg & Vanderwolf 1996; Iwase et al 2001). Therefore, the neuroprotective activity of AS-2, AS-2-9-006 and AS-9-006 could partly contribute to their cognitive-enhancing activity. However, the actual mechanisms of cognitive-enhancing activity of these AADs are still unclear.

Four AChE inhibitors have been approved by the Food and Drug Administration for the treatment of Alzheimer's disease: tacrine (Cognex), available by prescription since 1993; donepezil (Aricept), available since 1996; rivastigmine (Exelon), available since 2000; and galantamine (Reminyl), approved in 2001 (Bonner & Peskind 2002). They do not claim to cure Alzheimer's disease but rather they treat the symptoms. Prescriptions for tacrine have been restricted owing to its side-effects. AADs had no obvious signs of such cholinergic toxicity as tremor, convulsions, salivation, fasciculation, or lacrimation as observed after AAD administration in all our experiments in-vivo (data not shown). ADDs were not lethal at any of the doses tested in our experiments. These results suggest that the AADs, AS-2, AS-2-9-006 and AS-9-006, may be alternative candidates to compounds such as tacrine.

Conclusions

Using mice with amnesia induced by scopolamine and based on the results of passive avoidance, active avoidance and the Morris water maze tests, we found that AS-2, AS-2-9-006 and AS-9-006 had cognitive-enhancing activity. Recently, natural products and/or their synthetically developed active components, such as galanthamine and huperzine A, have received approval for the treatment of Alzheimer's disease or are under clinical study (Mantle et al 2000). The AADs, AS-2, AS-2-9-006 and AS-9-006, which exerted anti-amnesic activity in-vivo, may offer a useful therapeutic choice in the prevention and/or treatment for Alzheimer's disease. Based on our findings, we propose that the cognitive-enhancing activity of these three AADs may be attributed to the reinforcement of cholinergic systems via improvement in ACh synthesis.

References

Appa Rao, M. V. R., Srinivasan, K., Koteswara Rao, T. (1977) The effect of *Centella asiatica* on the general mental ability of mentally retarded children. *Indian J. Psychiatry* **19**: 54–59

Bonner, L. T., Peskind, E. R. (2002) Pharmacologic treatments of dementia. *Med. Clin. North Am.* **86**: 657–674

Davidson, M., Stern, R. G., Bierer, L. M., Horvath, T., Zemishlani, Z., Markofsky, R. C. (1991) Cholinergic strategies in the treatment of Alzheimer's disease. *Acta Psychiatr. Scand.* **366**: S47–S51

Day, L. B., Schallert, T. (1996) Anticholinergic effects on acquisition of place learning in the Morris water task: spatial mapping deficit or inability to inhibit nonplace strategies? *Behav. Neurosci.* **110**: 998–1005

De Souza, N. D., Shah, V., Desai, P. D., Inamdar, P. K., D'Sa, A., Ammonaman-chi, R., Dohadwalla, A. N., Lakdawala, A. D., Mandrekar, S. S., Blum-bach, J. (1992) 2,3,23-trihydroxy-urs-12-ene and its derivatives, processes for their preparation and their use. European Patent 0 383 171 A2

Dringenberg, H. C., Vanderwolf, C. H. (1996) Cholinergic activation of the electrocorticogram: an amygdaloid activating system. *Exp. Brain Res.* **108**: 285–296

Dutar, P., Bassant, M. H., Senut, M. C., Lamour, Y. (1995) The septohippocampal pathway: Structure and function of a central cholinergic system. *Physiol. Rev.* **75**: 393–427

Ellman, G. L., Courtney, K. D., Andre, V. Jr, Featherstone, R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**: 88–95

Fonnum, F. (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J. Neurochem.* **24**: 407–409

Francis, P. T., Palmer, A. M., Snape, M., Wilcock, G. K. (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J. Neurol. Neurosurg. Psychiatry* **66**: 137–147

Frolich, L. (2002) The cholinergic pathology in Alzheimer's disease – discrepancies between clinical experience and pathophysiological findings. *J. Neural Transm.* **109**: 1003–1013

Iwase, T., Ojika, K., Matsukawa, N., Nishino, H., Yamamoto, T., Okada, H., Fujimori, O., Ueda, R. (2001) Muscarinic cholinergic and glutamatergic reciprocal regulation of expression of hippocampal cholinergic neurostimulating peptide precursor protein gene in rat hippocampus. *Neuroscience* **102**: 341–352

Jackson, J. J., Soliman, M. R. (1996) Effects of tacrine (THA) on spatial reference memory and cholinergic enzymes in specific rat brain regions. *Life Sci.* **58**: 47–54

Jew, S. S, Lim, D. Y., Seo, S. K., Nam, T. G., Park, H. G., Kim, H. D., Kim, C. M., Lee, M. H., Paik, H. G., Lee, M. J., Jung, Y. H. (1998) Regioselective ring opening of steroidal epoxide with hydrides: formation of C(2)- and C(3)-deoxyasiatic acid derivatives. *Arch. Pharm. Res.* **21**: 793–795

Jew, S. S., Yoo, C. H., Lim, D. Y., Kim, H., Mook-Jung, I., Jung, M. W., Choi, H., Jung, Y. H., Kim, H., Park, H. G. (2000) Structure-activity relationship study of asiatic acid derivatives against beta amyloid (A beta)-induced neurotoxicity. *Bioorg. Med. Chem. Lett.* **10**: 119–121

Kang, S. Y., Lee, K. Y., Park, M. J., Kim, Y. C., Markelonis, G. J., Oh, T. H., Kim, Y. C. (2003) Decursin from *Angelica gigas* mitigates amnesia induced by scopolamine in mice. *Neurobiol. Learn. Mem.* **79**: 11–18

Kim, S. R., Hwang, S. Y., Jang, Y. P., Park, M. J., Markelonis, G. J., Oh, T. H., Kim, Y. C. (1999) Protopine has anticholinesterse and antiamnesic activities. *Planta Med.* **61**: 218–221

Kopelman, M. D., Corn, T. H. (1988) Cholinergic 'blockade' as a model of cholinergic depletion. A comparison of the memory deficits with those of Alzheimer-type dementia and the alcoholic Korsakoff syndrome. *Brain* **111**: 1079–1110

Lahiri, D. K., Farlow, M. R., Sambamurti, K., Greig, N. H., Giacobini, E., Schneider, L. S. (2003) A critical analysis of new molecular targets and strategies for drug developments in Alzheimer's disease. *Curr. Drug Targets* **4**: 97–112

Lee, M. K., Kim, S. R., Sung, S. H., Lim, D., Kim, H., Choi, H., Park, H. K., Jew, S. S., Kim, Y. C. (2000) Asiatic acid derivatives protect cultured cortical neurons from glutamate-induced excitotoxicity. *Res. Commun. Mol. Pathol. Pharmacol.* **108**: 75–86

Lowry, O. H., Robsebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275

Mantle, D., Pickering, A. T., Perry, E. K. (2000) Medicinal plant extracts for the treatment of dementia: a review of their pharmacology, efficacy and tolerability. *CNS Drugs* **13**: 201–213

Molchan, S. E., Martinez, R. A., Hill, J. L., Weingartner, H. J., Thompson, K., Vitiello, B., Sunderland, T. (1992) Increased cognitive sensitivity to scopolamine with age and a perspective on the scopolamine model. *Brain Res. Rev.* **17**: 215–226

Morris, R. G. (1984) Development of a water maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* **11**: 47–60

Palmer, A. M. (2002) Pharmacotherapy for Alzheimer's disease: progress and prospects. *Trends Pharmacol. Sci.* **23**: 426–433

Riekkinen, P. Jr, Ikonen, S., Riekkinen, M. (1998) D-cycloserine, a partial NMDA receptor-associated glycine-B site agonist, enhances reversal learning, but a cholinesterase inhibitor and nicotine has no effect. *Neuroreport* **9**: 3647–3651

Sawada, N., Takanaga, H., Matsuo, H., Naito, M., Tsuruo, T., Sawada, Y. (1999) Choline uptake by mouse brain capillary endothelial cells in culture. *J. Pharm. Pharmacol.* **51**: 847–852

Shanghai Academy of Science (1985) *Dictionary of Chinese Herbal Medicine*, Vol. 1–5. Shogukan Kan, Tokyo, Japan

Shannon, H. E., Sheardown, M. J., Bymaster, F. P., Calligaro, D. O., Delapp, N. W., Gidda, J., Mitch, C. H., Sawyer, B. D., Stengel, P. W., Ward, J. S., Wong, D. T., Olesen, P. H., Suzdak, P. D., Sauerberg, P., Swedberg, M. D. (1997) Pharmacology of butythio[2.2.2] (LY297802/NMC11-1053): a novel analgesic with mixed muscarinic receptor agonist and antagonist activity. *J. Pharmcol. Exp. Ther.* **281**: 884–894

Stumpf, C. (1965) Drug action on the electrical activity of the hippocampus. *Int. Rev. Neurobiol.* **8**: 77–138

Tsuga, H., Kameyama, K., Haga, T., Honma, T., Lameh, J., Sadee, W. (1998) Internalization and down-regulation of human muscarinic acetylcholine receptor m2 subtype. *J. Biol. Chem.* **273**: 5323–5330

Whishaw, I. Q. (1985) Cholinergic receptor blockade in the rat impairs locale but not taxon strategies for place navigation in a swimming pool. *Behav. Neurosci.* **99**: 979–1005

Whishaw, I. Q., O'Connor, W. T., Dunnett, S. B. (1985) Disruption of central cholinergic systems in the rat by basal forebrain lesions or atropine: effects on feeding, sensorimotor behavior, locomotor activity and spatial navigation. *Behav. Brain Res.* **17**: 103–115