Cognitive Enhancing Properties and Tolerability of Cholinergic Agents in Mice: A Comparative Study of Nicotine, Donepezil, and SIB-1553A, a Subtype-Selective Ligand for Nicotinic Acetylcholine Receptors

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Several studies have demonstrated the importance of nicotinic mechanisms in the pathophysiology of neurodegenerative and cognitive disorders, warranting the search and development of novel nicotinic ligands as potential therapeutic agents. The present study was designed to assess whether the subtype-selective nicotinic acetylcholine receptor (nAChR) ligand SIB-1553A [(±)-4-[[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol hydrochloride], with predominant agonist activity at $\beta 4$ subunit-containing human nAChRs, and no activity at muscle nAChR subtypes, could enhance cognitive performance in rodents with a more desirable safety/tolerability profile as compared to the nonselective prototypic nAChR ligand nicotine. SIB-1553A was equi-efficacious to nicotine in improving working memory performance in scopolamine-treated mice as measured by increased alternation in a T-maze, and was more efficacious than nicotine in improving the baseline cognitive performance of aged mice. This effect on working memory was confirmed in a delayed nonmatching to place task using the eight-arm radial maze. SIB-1553A produced dose-dependent side effects (ie motor deficits and seizures), although these effects were observed at doses 12 to 640-fold above those required to increase cognitive performance. Overall, SIB-1553A was significantly less potent than nicotine in eliciting these undesirable effects. Thus, the subtype-selective profile of SIB-1553A appears to translate into a more efficacious and better tolerated nAChR ligand as compared to nicotine. In the present studies, cognitive enhancement induced by SIB-1553A was similar in magnitude to that produced by the clinically efficacious acetylcholinesterase inhibitor donepezil. Taken together, the present data confirm the importance of nAChR subtypes in modulating cognitive processes, and suggest that activation of nAChR subtypes by selective nAChR ligands may be a viable approach to enhance cognitive performance.

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INTRODUCTION

It has long been suggested that the cognitive deficits observed in normal aging and neurodegenerative disorders such as Alzheimer's disease (AD) are, at least in part, due to a decrease in cholinergic transmission (Bartus *et al*, 1982; Perry *et al*, 1987; Gallagher and Colombo, 1995; Lawrence and Sahakian, 1998; Sirviö, 1999).

Acetylcholinesterase (AChE) inhibitors such as donepezil (Aricept[®]) are currently the first line palliative treatment for AD (Rogers *et al*, 1998; Grutzendler and Morris, 2001).

At the cholinergic synapse, donepezil prevents the breakdown of acetylcholine (ACh) thus elevating and further maintaining its level to stimulate postsynaptic muscarinic and nicotinic receptors. However, AChE inhibitors have been less promising therapeutically as they produce only modest improvements in cognitive function (Bryson and Benfield, 1997; Greenberg *et al*, 2000; Mohs *et al*, 2001; Winblad *et al*, 2001) along with undesirable side effects (Wilkinson, 1999; Dunn *et al*, 2000; Imbimbo, 2001).

An alternative approach by which cholinergic neurotransmission could be augmented is to increase the release of ACh by direct activation of presynaptic nicotinic acetylcholine receptors (nAChRs). In addition to their cholinergic effects, nAChRs mediate and modulate a wide variety of neurochemical and behavioral functions, which have direct relevance to the symptomatology, progression, and treatment of AD (Whitehouse *et al*, 1986; Perry *et al*, 1998; Mihailescu and Drucker-Colin, 2000; Newhouse *et al*, 2001). Interestingly, some AChE inhibitors have also been

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demonstrated to be allosteric modulators of nAChRs (Barnes *et al*, 2000; Hellstrom-Lindhall *et al* 2000; Samochocki *et al*, 2000). Although substantial loss of cholinergic neurons has been demonstrated in AD (Francis *et al*, 1999), post-mortem studies have shown that some functional nAChRs remain, even at a late stage of the disease (Shimohama *et al*, 1986; Whitehouse *et al*, 1986; Schröder *et al*, 1991; Court *et al*, 2001; Perry *et al*, 2001). In agreement with this observation, nicotine, the nonselective prototypical ligand for nAChRs, improves certain cognitive functions in AD patients (Jones *et al*, 1992; Wilson *et al*, 1995; White and Levin, 1999; Newhouse *et al*, 2001; Rezvani and Levin, 2001). Unfortunately, nicotine also exhibits a broad spectrum of undesirable side effects (Benowitz, 1986; Watkins *et al*, 2000), which limits its use as a therapy.

The nAChRs belong to the family of ligand-gated ion channels. They are pentameric in structure and exist in a variety of forms composed of α ($\alpha 2$ – $\alpha 9$) and β ($\beta 2$ – $\beta 4$) subunits (Lindstrom, 1997), which serve different physiological functions, and are differentially expressed within the central and peripheral nervous systems. Thus, the molecular, anatomical, and functional diversity of nAChRs offer a potential for the development of subtype-selective ligands with a more desirable safety profile and greater efficacy as compared to nicotine.

SIB-1553A, ($\pm$)-4-[[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol hydrochloride, has been identified as a novel nAChR ligand with a unique pharmacological profile (Menzaghi *et al*, 1998; Vernier *et al*, 1999). Unlike nicotine, SIB-1553A exhibits predominant agonist activity at $\beta 4$ subunit-containing human nAChRs, with no activity at muscle nAChR subtypes (Menzaghi *et al*, 1998). *In vivo*, SIB-1553A induces a dose-dependent increase in ACh release in the frontal cortex and hippocampus in rats, with greater efficacy than nicotine (Menzaghi *et al*, 1998). To date, no other nAChR ligand has been reported that approaches the efficacy of SIB-1553A in releasing ACh *in vivo*, or that matches its selectivity profile for human nAChRs (Menzaghi *et al*, 1998). In addition, studies have shown that SIB-1553A induces the release of other neurotransmitters beside ACh, including dopamine and norepinephrine, from rat brain regions known to play an important role in learning and memory functions (Menzaghi *et al*, 1998). SIB-1553A has also been reported to improve the cognitive performance of aged nonhuman primates (Bontempi *et al*, 2001) and rodents with immunolesions of the cholinergic system (Menzaghi *et al*, 1998). This broad spectrum of activity suggests that SIB-1553A may be beneficial for the symptomatic treatment of impaired cognitive processes.

The purpose of the present study was to extend the characterization of SIB-1553A by comparing its relative efficacy to that of nicotine and donepezil in mice models of cognitive dysfunction. The effects of these three cholinergic agents on spatial working memory were characterized both in aged mice, and in young mice whose cholinergic system was compromised by scopolamine, a nonselective muscarinic antagonist. The acute tolerability profile of SIB-1553A was also characterized and compared to that of nicotine as a way to determine if the receptor subtype selectivity of SIB-1553A translated into a better tolerated compound as compared to nicotine.

MATERIALS AND METHODS

Animals

Young (23–30 g, 8–12 weeks) and aged (28–35 g, 24–26 months) male C57BL/6 mice (Charles River, Hollister, CA) were used in the present studies. All animals were maintained in a humidity- (50–55%) and temperature-controlled (22–24°C) facility on a 12:12 h light/dark cycle (lights on at 6:30 am) with free access to food (Harlan-Teklad 4% diet 7001) and water, except during behavioral testing. Animals were group-housed (one to five per cage) and were allowed a 1-week period of habituation to the animal room before testing. They were handled at least once during this period.

Compounds

Mecamylamine hydrochloride, (–)-nicotine hydrogen tartrate and (–)-scopolamine hydrobromide were obtained from Sigma Chemical Co. (St Louis, MO). Diazepam (5 mg/ml injectable solution) was obtained from Steris Laboratories Inc. (Phoenix, AZ). SIB-1553A and donepezil were synthesized at SIBIA Neurosciences, Inc. (now Merck Research Laboratories) (Vernier *et al*, 1999). Nicotine (1 μ mol/kg equivalent to 0.16 mg/kg base) was dissolved in saline and the pH adjusted to 7.0 by addition of 10 N NaOH. Mecamylamine (1 μ mol/kg equivalent to 2.04 mg/kg salt), scopolamine (1 μ mol/kg equivalent to 0.38 mg/kg salt), donepezil (1 μ mol/kg equivalent to 0.41 mg/kg salt), and SIB-1553A (1 μ mol/kg equivalent to 0.27 mg/kg salt) were dissolved in saline. Injectable diazepam solution (1 μ mol/kg equivalent to 0.28 mg/kg base) was diluted with distilled water and 1% Tween-80. Doses were expressed in micromoles per kilogram. Compounds were administered subcutaneously (s.c.) into the dorsal neck region in a volume of 10 ml/kg.

Behavioral and Physiological Procedures

Testing was carried out between 8:00 am and 5:30 pm each day (light cycle), according to protocols approved by the Institutional Animal Care and Use Committee at SIBIA Neurosciences, Inc.

Cognitive Studies

Spontaneous alternation. Spontaneous alternation is the innate tendency of rodents to alternate free choices in a T-maze over a series of successive runs (Dember and Fowler, 1958). This sequential procedure relies on working memory since the ability to alternate requires that the subject retain specific information, which varies from trial to trial. It is also sensitive to various manipulations (ie delay intervals, increased number of trials) and pharmacological treatments affecting memory processes (Tako *et al*, 1988; Ragozzino *et al*, 1994; Krazem *et al*, 2001; Stefani and Gold, 2001).

The T-maze was made of gray Plexiglas with a main stem (70 cm long $\times$ 10 cm wide $\times$ 20 cm high) and two arms (30 cm long $\times$ 10 cm wide $\times$ 20 cm high) positioned at 90° angle relative to the main stem. A start box (15 cm long $\times$ 10 cm wide) was separated from the main stem by a horizontal sliding door. Horizontal sliding doors were also

placed at the entrance of each arm. A halogen lamp positioned above the apparatus provided dimmed illumination (about 25 Lux). Cues (eg posters) were placed above the arms to serve as spatial reference points.

Mice were given a free exploration session consisting of 10 min in the apparatus 24 h prior to testing to allow familiarization to the testing apparatus. On the following day (test day), each subject was tested in a session of eight free trials. To begin a trial, the subject was placed in the start box for 30 s followed by the opening of the door to the stem. When the mouse entered one of the arms (left or right), the door to that arm was closed. The chosen arm and the elapsed time between the opening of the start box door and the choice of an arm (choice latency) were noted. After a 30 s confinement in the chosen arm, the mouse was removed and returned to the start box for a second free trial, identical to the first, and so on, until the completion of the testing session. Animals were allowed a maximum choice latency of 300 s to complete a trial. Urine and feces were removed from the maze between trials.

In the study investigating the effect of the compounds in young mice treated with scopolamine, scopolamine (3.9 $\mu\text{mol/kg}$) was injected 20 min prior to testing and was followed 1 min later by an injection of SIB-1553A, donepezil, or nicotine. In the study investigating the effect of the compounds in young mice treated with both scopolamine and mecamylamine (1.47 $\mu\text{mol/kg}$), both antagonists were concurrently injected 25 min prior to testing, followed by injection of SIB-1553A 5 min later. Aged mice were injected with SIB-1553A or saline 20 min prior to the alternation session.

The percentage of alternation over the eight trials was determined for each mouse and was used as an index of working memory performance. This percentage was defined as entry in a different arm of the T-maze over successive trials (ie left-right-left-right..., etc). In the present procedure, the maximum number of alternation sequences possible was 7, and the percentage of spontaneous alternation was equal to the ratio of actual alternations divided by 7×100 .

Delayed nonmatching to place (DNMTP) procedure. The DNMTP procedure has been developed by Jaffard and co-workers (Cho *et al*, 1992) and is similar to the delayed matching to sample (DMTS) procedure frequently used to evaluate working memory in monkeys. This procedure assesses the animals' ability to distinguish a novel stimulus from a familiar stimulus on the basis of a single presentation. Aged mice were trained and tested in a semi-automated elevated (88 cm above the floor) eight-arm radial maze based on that described by Olton *et al* (1979). The maze was made of black metal and consisted of an octagonal-shaped central platform (36 cm in diameter) from which eight arms (70 cm long $\times$ 10 cm wide) radiated in a symmetrical fashion (Lafayette Instrument, Indiana, IN). A circular food pellet cup was located at the end of each arm. Clear Plexiglas vertical doors were at the entrance of each arm and were individually controlled (WCB-ARAM 1000, West Coast Biotech, La Mesa, CA). A remote control box and a closed-circuit video system allowed the experimenter to activate the doors of the maze and to observe the behavior of each animal from an adjacent room. The maze

was located in the center of a dimmed room (about 100 Lux) with various pictures and objects placed around the room to serve as spatial cues.

Food-deprived animals were first allowed free exploration sessions on 2 successive days. During these sessions, the eight arms of the maze were baited (Noyes Precision 20 mg pellets, Formula A/I, Lancaster, NH) and all doors were opened so that animals could freely enter the arms and find a food pellet at the end of each arm. Each daily session was terminated when all eight baited arms were visited and all eight food pellets were consumed. Animals were then submitted to the DNMTP rule training. Each trial consisted of a study phase (two forced runs) followed by a test phase (two choice runs). During the study phase, one arm of the maze was opened (forced run) and the animal was allowed to travel down the arm in order to collect a food pellet. Each animal was given two consecutive forced runs in two different open arms. Once the animal returned to the central platform of the maze after the second forced run, two doors, one giving access to the first arm that had been previously visited during the first forced run and one giving access to an adjacent nonvisited arm, were opened simultaneously (first choice run). When one arm had been chosen and the animal returned to the central platform, the next pair of doors opened. This next pair consisted of the second arm visited in the study phase and an adjacent novel arm. On both choice runs, the animal was reinforced only when it entered the arm that had not been previously visited during the study phase (nonmatching to place). Incorrect choices were neither rewarded nor punished.

Sequences of forced and choice runs were selected in a pseudorandom manner. Forced and choice arms were counterbalanced for left and right positions, thereby resulting in a choice accuracy of 50% for animals utilizing an egocentric strategy (ie always choosing the arm on their left or always choosing the arm on their right). Daily sessions consisted of eight trials (16 choices), with each trial separated by a 45-s intertrial interval. Animals were trained until they reached a criterion of at least 70% correct responses on 2 consecutive days. A total of 54 aged animals that reached this criterion were subsequently used for delay testing. This criterion was necessary to ensure that any decrease in performance during the delay testing phase (see below) was the consequence of forgetting information instead of an error due to misunderstanding the rule or an inability to apply this rule. No treatment was given during the training period.

After mastering the DNMTP rule, animals were tested according to the same protocol with the addition of a delay between study and test phases. For each trial, upon returning to the central platform after the second forced run, mice were confined on the central platform of the maze for 0 or 180 s. Animals then completed the test phase as previously described. Daily sessions consisted of eight trials (four trials/delay) separated by a 45-s intertrial interval. The delay was presented in a randomized order. Mice were tested in this delay paradigm for 3 consecutive days before drug testing to allow for adaptation to this new procedure.

Ascending dose-responses were then established for each animal (ie the same animal was treated daily, with an increasing dose of test compound). Drug testing was conducted for 4 consecutive days followed by 2 days

washout with no training. On the seventh day, animals received saline injection for baseline measurement. SIB-1553A, donepezil, or saline was administered 20 min before the beginning of each testing session. The effect on working memory accuracy was assessed by comparing performance on drug days vs baseline days.

Ascending dose studies require animals to be injected with increasing doses of compound. As results from these studies may be potentially confounded by prior drug experience, the most efficacious doses of both SIB-1553A and donepezil as determined in the ascending dose-response study were re-evaluated in an independent repeated 'best dose' study. In this study, drug-naïve aged mice were tested at delays of 0, 90, and 180 s over 3 consecutive days of drug or saline treatment. These mice had been previously trained to master the DNMTTP rule but had no previous experience with delays. In addition to confirming drug effects on working memory after delays, this test also allowed for the determination of drug effects on the ability to adapt to novel experimental conditions. It has been shown that, in contrast to young animals, aged mice previously trained to the DNMTTP rule perform poorly on the first day of testing even at 0 s delay when novel long delays are introduced (Bontempi et al, 2001). Daily sessions consisted of nine trials (three trials/delay) separated by 45-s intertrial intervals. The three delays were presented in a mixed order after the forced runs. Working memory performance was averaged using all 3 days of testing (total of nine trials/delay). As in the ascending dose study, SIB-1553A, donepezil, or saline was administered 20 min before each daily session.

Tolerability Studies

Motor coordination (rotarod performance). Evaluation of motor coordination was conducted on a standard mouse rotarod (Omnitech Rotarod, Columbus, OH) that measures the time a mouse remains on a rotating rod. The rubber rotating rod (diameter 3 cm, length 11 cm) was raised 35 cm above the bottom of the rotarod enclosure and rotated at a fixed speed of 20 rpm. Two photocell beams at the bottom of the enclosure automatically measured the latency to fall.

Mice were pretrained on the rotarod 1 to 3 h prior to testing. Training consisted of placing a mouse on the rotating rod and measuring latency to fall, up to a maximum of 120 s. Mice that did not remain on the rod for 120 s by the end of the third trial were excluded from the study. Training trials were separated by 60 s.

For drug testing, mice were distributed across treatment groups on the basis of the number of trials required to reach the 120 s criterion during training. Mice were tested three times on the rotarod during drug testing: 5 min preinjection, 3 and 17 min postinjection. Mice were returned to their home cages between trials. The time from the placement onto the rod to falling off the rod (latency to fall in seconds) served as the measure of performance. The benzodiazepine agonist diazepam was used as a positive control.

Locomotor activity. Locomotor activity was assessed in photocell activity cages (San Diego Instruments, San Diego, CA), consisting of a standard plastic rodent cage (24 × 45.5 cm) surrounded by a stainless-steel frame.

Locomotor activity frames consisted of seven infrared photocell beams located across the long axis of the frame and raised 2 cm above the floor and 5.5 cm apart. The number of cage crosses (crossovers, ie consecutive interruptions of one beam followed immediately by interruption of an adjacent beam) was recorded by a computer system during consecutive 5-min intervals and used as a measure of spontaneous locomotion. Locomotor activity was evaluated in animals naive to the test apparatus. The animals were injected with test compound or vehicle and placed in the photocell activity cages 5 min after injection. Locomotor activity was recorded for a 10 min period.

Seizure threshold and lethal doses. Seizure threshold doses and lethal doses were determined by administering varying doses of compounds to groups of two to seven animals and rating the occurrence of seizures and death per group.

Statistical Analysis

Data were analyzed by Student's *t*-test or analysis of variance (ANOVA) using one- or two-factor analysis with repeated measures when applicable followed by *post hoc* comparisons using Dunnett's or Fisher's PLSD multiple comparison tests (SigmaStat Software, Jandel, San Rafael, CA). Percentage data (eg spontaneous alternation) followed a normal distribution and therefore were not normalized prior to the statistical analysis. Scores or data that did not follow a normal distribution were analyzed by nonparametric tests including the Mann-Whitney *U*-test or Kruskal-Wallis, followed by Dunnett's test as appropriate. Comparisons between drug-treated and vehicle-treated groups for all data were made using raw performance scores. Values of $p < 0.05$ were considered significant.

RESULTS

Cognitive Studies

Spontaneous alternation in scopolamine-treated mice. As illustrated in Figure 1a (left panel), the percentage of spontaneous alternation was significantly reduced in scopolamine-treated mice as compared to vehicle-treated mice (40.95 ± 5.37 vs $67.86 \pm 2.44\%$, respectively) ($t(38) = 4.98$; $p < 0.001$). Injections of SIB-1553A reversed this deficit in a dose-dependent manner ($F(4,52) = 5.86$; $p = 0.001$), with a significant effect observed at doses of 73 and 110 $\mu\text{mol/kg}$ ($p < 0.05$). SIB-1553A also induced a dose-dependent increase in choice latency ($F(4,52) = 6.98$; $p < 0.001$), with a significant increase at doses $\geq 110 \mu\text{mol/kg}$ ($p < 0.05$, Figure 1a, right panel).

Similarly, administration of nicotine or donepezil attenuated the scopolamine-induced decrease in spontaneous alternation (Scopolamine effect: ($t(12) = 4.40$; $p < 0.001$; nicotine effect: $F(6,73) = 2.25$; $p = 0.05$) (Figure 1b, left panel); (Scopolamine effect: ($t(13) = 3.53$; $p < 0.005$; donepezil effect: $F(5,55) = 3.34$; $p < 0.01$) (Figure 1c, left panel), with a significant effect observed at doses of 1.25 $\mu\text{mol/kg}$ for nicotine ($p < 0.05$) and 2.40 and 7.21 $\mu\text{mol/kg}$ for donepezil ($p < 0.05$). SIB-1553A was as efficacious as donepezil and nicotine in reversing the scopolamine effect in mice. Nicotine increased choice latency ($F(6,73) = 16.58$;

Figure 1 Acute effects of SIB-1553A (a), nicotine (b), and donepezil (c) on spontaneous alternation behavior in young scopolamine-treated mice. Scopolamine (3.9 μmol/kg, s.c.) was injected 20 min prior to testing in the T-maze and was followed by s.c. injection of SIB-1553A, nicotine, donepezil, or saline (0) 1 min later. Data are presented as % alternation (left panels) and choice latency in seconds (right panels) measured over eight trials (mean ± SEM, $n = 7$ –25/group). Black bars represent scopolamine-treated groups whereas white bars represent the saline-treated group. # $p < 0.05$ vs saline/saline(0)-treated mice, Student's t -test; * $p < 0.05$, vs scopolamine/saline(0)-treated mice, one-way ANOVA followed by Fisher's PLSD multiple comparison test.

$p < 0.001$) at doses ≥ 5 μmol/kg ($p < 0.05$, Figure 1b, right panel). Donepezil also induced a dose-dependent increase in choice latency ($F(5,55) = 8.04$; $p < 0.001$), with significant increase in choice latency at doses ≥ 14.42 μmol/kg ($p < 0.05$) (Figure 1c, right panel).

Pretreatment with mecamylamine, a noncompetitive nAChR-channel blocker, prevented the effect of SIB-1553A on scopolamine-treated mice ($F(1,32) = 11.47$; $p < 0.002$, Figure 2). Although mecamylamine tended to decrease the percentage of spontaneous alternation on its own, this effect was not statistically significant ($p > 0.07$) and cannot entirely account for the blockade of the SIB-1553A effect on scopolamine-treated mice. Neither SIB-1553A nor mecamylamine affected choice latencies at doses tested (data not shown).

Spontaneous alternation in aged mice. As illustrated in Figure 3a, the percentage of spontaneous alternation was

significantly reduced in aged control mice as compared to young mice (42.9 ± 4.76 vs $72.6 \pm 3.27\%$, respectively) ($t(19) = 5.33$; $p < 0.001$). Acute injection of SIB-1553A reversed this deficit in a dose-dependent manner ($F(7,59) = 2.24$; $p < 0.04$). SIB-1553A increased the percentage of spontaneous alternation, with effects observed at both low doses (1.14 – 2.28 μmol/kg, $p < 0.05$) and a high dose (36.50 μmol/kg, $p < 0.05$). SIB-1553A also induced a dose-dependent increase in choice latency ($F(7,59) = 6.70$; $p < 0.001$) with a significant increase in latency at doses ≥ 18.24 μmol/kg (data not shown).

Nicotine did not significantly attenuate the spontaneous alternation deficit observed in aged mice at the doses examined ($F(5,70) = 1.08$; $p = 0.40$, NS) (Figure 3b), although there was an overall trend toward an increase in spontaneous alternation, with the dose of 1.88 μmol/kg being the most efficacious dose examined. The 3.76 μmol/kg dose of nicotine, which was ineffective in reversing the

Figure 2 Effect of pretreatment with mecamylamine on SIB-1553A-induced enhancement of spontaneous alternation in scopolamine-treated mice. All mice were administered scopolamine (3.9 $\mu\text{mol/kg}$, s.c.) followed immediately by saline or mecamylamine (1.47 $\mu\text{mol/kg}$, s.c.), and then by s.c. injection of SIB-1553A (73 $\mu\text{mol/kg}$) 5 min later. Young mice were tested in the T-maze 20 min after the injection of SIB-1553A. Data are presented as % alternation measured over eight trials (mean $\pm$ SEM, $n = 8-12$ /group). # $p < 0.05$ vs saline/saline-treated mice, Student's t -test; * $p < 0.05$ vs saline/SIB-1553A-treated mice, two-way ANOVA followed by Fisher's PLSD multiple comparison test.

working memory deficit, significantly increased choice latency ($F(5,70) = 10.96$; $p < 0.001$) (data not shown). Nicotine's dose-limiting side effects (including motor-depressant effect) prevented the evaluation of higher doses.

Injection of donepezil significantly attenuated the spontaneous alternation deficit observed in aged mice in a dose-dependent manner ($F(5,60) = 2.47$; $p < 0.05$), with a significant effect observed at a dose of 0.48 $\mu\text{mol/kg}$ ($p < 0.03$, Figure 3c). Donepezil also induced a dose-dependent increase in choice latency ($F(5,60) = 5.52$; $p < 0.01$), with a significant increase in latency at doses $\geq 0.96 \mu\text{mol/kg}$ (data not shown).

DNMTP procedure in aged mice. Accuracy of young and aged mice injected with saline differed significantly ($F(1,16) = 15.08$; $p < 0.001$). (Figure 4). Performance decreased as a function of delay (0 vs 180 s) ($F(1,16) = 89.40$; $p < 0.001$), with both groups being affected similarly by the delay (group $\times$ delay interaction: $F(1,16) = 0.09$, NS) (Figure 4a-d). In aged mice, SIB-1553A dose-dependently enhanced performance ($F(6,56) = 2.32$; $p < 0.05$) as a function of delay (group $\times$ delay interaction: $F(6,56) = 7.63$; $p < 0.001$). *Post hoc* analysis revealed that doses of 1.83, 3.65, and 9.12 $\mu\text{mol/kg}$ significantly increased the percentage of correct choices in aged mice at the 180 s delay ($p < 0.03$, $p < 0.04$ and $p < 0.001$, respectively) with the maximal performance enhancement observed at the 9.12 $\mu\text{mol/kg}$ dose (Figure 4b). On the other hand, SIB-1553A did not affect performance at the 0 s delay ($p > 0.34$) (Figure 4a). No significant effect on time required by the animal to complete each of the 8 daily trials was observed at any of the doses tested. The 9.12 $\mu\text{mol/kg}$ dose ('best dose') was subsequently used to confirm the effect of SIB-1553A in naive, aged mice previously trained to criterion on the DNMTTP task (0 s delay), but never exposed to long delay intervals. SIB-1553A was tested at 0, 90, and 180 s delay for 3 consecutive days (Figure 5a). A two-way repeated ANOVA revealed a significant effect of treatment ($F(1,12) = 8.73$;

Figure 3 Acute effects of SIB-1553A (a), nicotine (b), and donepezil (c) on spontaneous alternation behavior in aged mice. SIB-1553A, nicotine, donepezil, or saline (0) was administered s.c. 20 min prior to testing in the T-maze. Data are presented as % alternation measured over eight trials (mean $\pm$ SEM, $n = 7-14$ /group). Black bars represent the aged animals. Young animals (white bars) were administered saline s.c. 20 min prior to testing. *** $p < 0.001$ vs young mice, Student's t -test; * $p < 0.05$ vs aged saline(0)-treated mice, one-way ANOVA followed by Fisher's PLSD multiple comparison test.

$p < 0.02$) with no group $\times$ delay interaction ($F(2,24) = 0.21$; NS), reflecting a significant improvement at all delays ($p < 0.02$). Performance decreased in both saline- and SIB-1553A-treated mice as a function of delay ($F(2,24) = 23.48$; $p < 0.001$). Working memory performance returned to baseline levels when animals were retested 24 h after administration of SIB-1553A ($F(1,12) < 1$, NS, Figure 5c).

Figure 4 Effects of SIB-1553A and donepezil on DNMTTP performance in aged mice as measured in the eight-arm radial maze. Mice were tested 20 min after s.c. administration of (a–b) SIB-1553A or (c–d) donepezil. Ascending dose responses were established for each animal (ie the same animal was repeatedly treated with saline and ascending doses of the test compounds). Drug testing was conducted for 4 days followed by 2 days washout and then a baseline day. On baseline days, animals were injected with saline. Data are presented as % correct responses (mean $\pm$ SEM) at 0 s and 180 s delay. ** $p < 0.01$ young vs aged saline-treated group, *post hoc* Dunnett's test; # $p < 0.001$ vs respective performance at 0 s delay, Dunnett's test; * $p < 0.05$ vs aged saline (0)-treated mice at 180 s delay, two-way ANOVA with repeated measures followed by Dunnett's test ($n = 9$ –15/group).

As illustrated in Figure 4d, donepezil administered to a different set of aged mice also increased the number of correct responses (group effect: $F(5,59) = 2.46$; $p < 0.05$) in a delay-dependent manner (group $\times$ delay interaction: $F(5,59) = 3.42$; $p < 0.009$). *Post hoc* analysis indicated that donepezil dose-dependently increased performance at the 180 s delay only with the doses of 0.24 and 0.96 $\mu\text{mol/kg}$ ($p < 0.009$ and $p < 0.02$ respectively), being the most effective doses. Maximal performance improvement was observed at the dose of 0.24 $\mu\text{mol/kg}$. No significant effect on time required by the animal to complete each of the eight daily trials was observed at any of the doses tested. The 0.24 $\mu\text{mol/kg}$ dose ('best dose') of donepezil was subsequently tested at 0, 90, and 180 s delay for 3 consecutive days in naive mice (Figure 5b). Just like SIB-1553A, donepezil improved performance at all delays (group: $F(1,14) = 4.67$; $p < 0.05$; delay: $F(2,28) = 13.25$; $p < 0.001$; group $\times$ delay interaction ($F(2,28) = 0.30$; NS). Working memory performance returned to baseline levels when animals were retested 24 h after administration of donepezil ($F(1,12) < 1$, NS, Figure 5d).

Tolerability Studies

Locomotor activity in aged mice. Administration of SIB-1553A and nicotine to aged mice induced a dose-dependent decrease in locomotor activity (SIB-1553A: $F(5,53) = 5.86$; $p < 0.001$; nicotine: $F(4,35) = 3.88$; $p < 0.05$) (Figure 6) with a

greater than 50% decrease of locomotor activity at doses of 73 and 7.5 $\mu\text{mol/kg}$, respectively.

Rotarod performance. SIB-1553A significantly impaired motor coordination of young mice at the highest dose tested (146 $\mu\text{mol/kg}$) with no significant effect at doses of 18.24 and 73 $\mu\text{mol/kg}$ (Two-way ANOVA: treatment effect $F(3,36) = 6.39$; $p = 0.001$) (Figure 7a). Similarly, nicotine impaired rotarod performance at a dose of 18.6 $\mu\text{mol/kg}$, with no significant effect at doses of 3.12 and 12.5 $\mu\text{mol/kg}$ (Two-way ANOVA: treatment $F(3,36) = 2.78$; $p < 0.01$). SIB-1553A was approximately 1/8 as potent as nicotine in inducing a similar level of motor incoordination (146 vs 18.6 $\mu\text{mol/kg}$, respectively). As expected, diazepam impaired rotarod performance in young mice. The effect was dose-dependent, was observed 3 min after injection, and lasted more than 17 min (Two-way ANOVA: treatment effect $F(3,36) = 32.0$; $p < 0.001$; time effect: $F(2,72) = 52.7$; $p < 0.001$; time $\times$ treatment $F(6,72) = 19.5$; $p < 0.001$) (Figure 7b).

Seizure thresholds, minimum lethal dose (MLD), and anticonvulsant activity. The average seizure threshold dose for SIB-1553A in aged mice was 426 $\mu\text{mol/kg}$, which was approximately 19 times higher than that of nicotine (22 $\mu\text{mol/kg}$). Seizures produced by SIB-1553A and nicotine were similar in appearance and were characterized by full body contraction with tremor and muscle spasm, appearing

Figure 5 Repeated 'best dose' effect of SIB-1553A and donepezil in naive aged mice as measured in the eight-arm radial maze. Mice were injected for 3 consecutive days with saline or the selected best dose: (a) $9.12 \mu\text{mol/kg}$ for SIB-1553A and (b) $0.24 \mu\text{mol/kg}$ for donepezil. The best dose was selected based on the dose eliciting the highest level of performance in the ascending dose–response study. Animals were tested in the DNMTS procedure 20 min after s.c. administration. Data are presented as the average of % correct response over 3 days of testing (mean $\pm$ SEM, $n = 7\text{--}8/\text{group}$). A two-way ANOVA revealed that SIB-1553A and donepezil produced an overall main effect on working memory performance. Performance returned to baseline levels when animals were injected with saline and retested 24 h after administration of SIB-1553A (c) or donepezil (d).

Figure 6 Dose-dependent decrease of locomotor activity induced by SIB-1553A (filled triangle) and nicotine (open circle) in aged C57BL/6 mice. Data are presented as a percentage of total crossovers from vehicle-treated group (control group) over a 10-min period beginning 5 min after administration of the compounds (mean $\pm$ SEM, $n = 6\text{--}14/\text{group}$). * $p < 0.05$ vs control, one-way ANOVA followed by Dunnett's test.

within 1 to 5 min of administration. Although high doses of SIB-1553A did produce seizures, subconvulsant doses of the compound did not have proconvulsant actions when combined with metrazole or when tested in the electroshock model in mouse (data not shown).

The MLD for SIB-1553A was $730 \mu\text{mol/kg}$ for aged mice, a dose approximately three-fold higher than

nicotine ($250 \mu\text{mol/kg}$) and 20-fold higher than donepezil ($36 \mu\text{mol/kg}$). The same SIB-1553A and donepezil MLDs were observed in young adult mice of the same strain whereas nicotine MLDs were 250 and $187 \mu\text{mol/kg}$ in aged and young mice, respectively (Table 1).

DISCUSSION

The present study was designed to assess whether the subtype-selective nAChR ligand SIB-1553A could enhance cognitive performance in mice with a more desirable safety profile as compared to the nonselective prototypic nAChR ligand nicotine. SIB-1553A significantly improved working memory performance in both scopolamine-challenged and aged mice, with an efficacy greater than or equal to nicotine, and with an improved safety profile. The cognitive enhancing effects of SIB-1553A were qualitatively similar to that of the clinically used AChE inhibitor donepezil.

As previously reported (Stone *et al*, 1991; Ragozzino *et al*, 1994; Hiramatsu and Inoue, 2000), scopolamine severely impaired spontaneous alternation in young mice. SIB-1553A was able to reverse this working memory deficit in a dose-dependent manner, and to a similar degree as nicotine. The dose–response curve was an inverted-U shape, which is a common feature of memory enhancing agents. Spontaneous alternation behavior was used as a rapid cognitive task to evaluate working memory. Although increased

Figure 7 (a) Dose–response relation of SIB-1553A- (filled triangle), nicotine- (open circle) and diazepam (filled square)-induced motor dysfunction in young mice. Motor coordination was measured as latency to fall from a rotating rod. Mice were tested at 3 min post-administration. Data are presented as a percentage of vehicle-treated group (control group) (mean $\pm$ SEM, $n = 10/\text{group}$). * $p < 0.05$ vs control, two-way ANOVA with repeated measures followed by Dunnett's test. (b) Time course of motor dysfunction induced by SIB-1553A (146 $\mu\text{mol/kg}$, filled triangle), nicotine (18.6 $\mu\text{mol/kg}$, open circle), and diazepam (10.5 $\mu\text{mol/kg}$, filled square) after s.c. administration in young C57BL/6 mice. Motor coordination (eg latency to fall from a rotating rod) was recorded before (time 0), 3 and 17 min after administration. Data are presented as a percentage of vehicle-treated group (control group) (mean $\pm$ SEM, $n = 10/\text{group}$). Statistical significance is not shown for the clarity of the graph.

alternation could result from memory improvement, similar effects could also be attributed to nonmnemonic processes such as motor-response strategies. However, at the dose of 73 $\mu\text{mol/kg}$, SIB-1553A improved spontaneous alternation without affecting choice latencies, thus making an effect on general motor activity or exploratory motivation unlikely. Furthermore, in order to improve alternation over successive forced visits in the T-maze, the animal must keep track of the sequence of arms visited, making this protocol likely to involve memory rather than nonmnemonic processes.

The effect of SIB-1553A against scopolamine was antagonized by mecamylamine, a noncompetitive ion-channel blocker. Although an effect of mecamylamine on ion channels other than nAChRs cannot be completely ruled out, this suggests that the cognitive enhancement produced by SIB-1553A is, at least in part, mediated by the activation

of nAChRs. This is consistent with numerous reports confirming that nAChR agonists can improve cognitive performance in experimental animals (Levin and Simon, 1998; Eid and Rose, 1999; Rezvani and Levin, 2001). Interestingly, SIB-1553A has been shown to have predominant agonist properties at β_4 - but not α_7 -containing nAChR subtypes (Menzaghi *et al*, 1998). This may suggest that particular subunits of nAChRs, such as β_4 , may be involved in cognitive processes, and that a subset of nAChRs modulates cognitive processes. Alternatively, distinct groups of nAChRs may modulate different cognitive processes or the cognitive improvement induced by nAChR ligands may result from a combination of blockade and activation of different nAChRs.

The construct validity of scopolamine administration to model the deficits observed in AD is questionable since it involves postsynaptic blockade of cholinergic function rather than presynaptic degeneration of cholinergic neurons, as seen in patients with AD (Cullen *et al*, 1997). However, this pharmacological model is a useful method to evaluate the functional effects of compounds on ACh release. The present data suggest that SIB-1553A may have induced ACh release by activating presynaptic nAChRs to a degree sufficient enough to displace scopolamine from postsynaptic muscarinic sites and to restore working memory function. This is consistent with the proposed role of presynaptic nAChRs in modulating neurotransmitter release (Kaiser and Wonnacott, 1998) and with the finding that SIB-1553A is a potent releaser of cortical and hippocampal ACh *in vivo* (Lloyd *et al*, 1998; Bontempi *et al*, 2001). The ability of SIB-1553A to enhance cholinergic function is also consistent with the present results showing that SIB-1553A is equally efficacious to the AChE inhibitor donepezil in reversing the cognitive deficits induced by scopolamine. Alternatively, the cognitive improvements induced by SIB-1553A may also result from enhanced activation of postsynaptic nAChRs sufficient to overcome the effects of decreased muscarinic tone.

Of greater interest, SIB-1553A was also able to reverse the deficit in spontaneous alternation exhibited by aged mice. SIB-1553A was as efficacious as donepezil and more efficacious than nicotine in this model. Together, these data show that nAChR activation and AChE inhibition produce comparable cognitive improvements in rodents in the absence of an imposed cholinergic deficit. Overall, regardless of the cholinergic agent used, the doses required to improve cognitive function in aged mice were lower than the doses required to reverse scopolamine-induced deficits. This may be explained by the fact that less release of ACh is required to restore a naturally occurring aging deficit as compared to the scopolamine pharmacological challenge where a large amount of muscarinic receptors have been blocked. Alternatively, the SIB-1553A-induced release of other neurotransmitters in addition to ACh, such as catecholamines, may also play a role in reversing aged-related working memory dysfunction (Lloyd *et al*, 1998; Giacobini *et al*, 1996). The improvement of spontaneous alternation in aged mice was seen after administration of both low (1.14 $\mu\text{mol/kg}$) and high doses (36.5 $\mu\text{mol/kg}$) of SIB-1553A. This dose effect was not observed with nicotine, which may be related to its different receptor profile. At different doses, SIB-1553A may activate different popula-

Table 1 Summary of the Pharmacological Properties of SIB-1553A and Nicotine in Young (8–12 Weeks Old) and Aged (24–26 Months Old) C57BL/6 Mice

<i>In vivo</i> assay	SIB-1553A		Nicotine	
	ED _{min} or max dose tested (μ mol/kg)	Effect (% change from control or total animals affected)	ED _{min} or max dose tested (μ mol/kg)	Effect (% change from control or total animals affected)
<i>Working memory (spontaneous alternation)</i>				
Scopolamine model	73	+45%	1.25	+38%
Aging model	1.14	+41%	1.875 NS	+26%
<i>Acute toxicity/physiological tests</i>				
Motor coordination (rotarod 3 min)—young	146	–33%	18.6	–47%
Locomotor activity—aged	73	–64%	7.5	–57%
Lethal dose—aged	730	5/6	250	2/2
Lethal dose—young	730	2/5	187	3/3
Seizure threshold—aged	426	N/A	22	N/A

Since the maximal changes in parameters were indeterminate, comparison between nicotine and SIB-1553A were made using ED_{min} instead of ED₅₀. ED_{min} was defined as the minimum dose of the drug that produced a statistically significant response. In some instances, ED_{min} corresponded to the minimum dose tested. Whenever possible, comparisons between the effects of SIB-1553A and nicotine were made at equi-efficacious doses. N/A, not applicable; NS, not statistically significant.

tions of nAChRs located either in the same or different brain regions. Both caudate and hippocampal lesions have been reported to impair spontaneous alternation, suggesting that these brain regions contribute to this behavior (Gross *et al*, 1965; Packard *et al*, 1989). The deficit in spontaneous alternation observed in aged mice could indicate hypofunction of one or both of these systems, which may be targeted by SIB-1553A.

The cognitive effects of SIB-1553A and donepezil were confirmed by an increase in performance in the DNMTF task, a more challenging test of working memory as compared to spontaneous alternation. SIB-1553A was as efficacious as donepezil at reversing age-dependent deficits in this test. Both doses of donepezil and SIB-1553A differed from those needed to reverse the spontaneous alternation deficit in aged mice, suggesting that the memory systems underlying performance in the two tests were, at least in part, different (White and RJ McDonald, 2002). As previously reported, SIB-1553A improved performance at all delays in the repeated best dose study, suggesting that SIB-1553A could affect attentional processes as well as working memory (Bontempi *et al*, 2001).

As summarized in Table 1, SIB-1553A was less potent than nicotine in inducing side effects in mice. These effects are not species dependent as SIB-1553A is also better tolerated in rats, dogs, and monkeys as compared to nicotine (unpublished observations). It is unlikely that pharmacokinetic differences contribute to the apparent reduction in potency of SIB-1553A relative to nicotine, as pharmacokinetic studies have shown that SIB-1553A has a half-life similar to that of nicotine (unpublished observations). Overall, the safety pharmacology studies conducted in aged mice suggest that the cognitive enhancing effects of SIB-1553A occurred at doses 12 to 640-fold lower than those associated with behavioral, autonomic or physiological changes.

Although the improved tolerability profile of SIB-1553A relative to nicotine in rodents is not necessarily predictive of an improved safety profile in humans, it is notable that

Phase I safety testing in 40- to 70-year-old healthy volunteers indicated that SIB-1553A was well tolerated in single- and multiple-dose studies (Lloyd *et al*, 1999). Using quantitative EEG measurements, SIB-1553A reduced alpha band activity and enhanced theta band activity, an effect commonly reported with drugs that enhance cognitive performance (Gevins *et al*, 2002; Schuck *et al*, 2002). Accordingly, in these Phase I studies, aspects of working memory performance (eg delayed word recall and delayed word recognition) were improved (Lloyd *et al*, 1999). These data suggest that the improved efficacy and tolerability profile of SIB-1553A relative to nicotine in rodents may translate into improved cognitive function in humans.

In conclusion, the present study confirms the importance of nAChR subtypes in modulating cognitive processes, and suggests that activation of nAChR subtypes by selective nAChR ligands may be a viable approach to increase cholinergic activity and enhance cognitive performance.

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