Antiamnesic and Neuroprotective Effects of Donepezil against Learning Impairments Induced in Mice by Exposure to Carbon Monoxide Gas

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ABSTRACT

Donepezil is a potent acetylcholinesterase inhibitor that also interacts with the \( \sigma_1 \) receptor, an intracellular neuromodulatory protein. In the present study, we analyzed the antiamnesic and neuroprotective activities of donepezil in a mouse hypoxia model induced by repetitive CO exposure, comparing donepezil’s pharmacological profile with other cholinesterase inhibitors tacrine, rivastigmine, and galanthamine, and the reference \( \sigma_1 \) agonist igmesine. CO exposure induced, after 7 days, hippocampal neurodegeneration, analyzed by Cresyl violet staining, and behavioral alterations, measured using spontaneous alternation and passive avoidance responses. When injected 20 min before the behavioral tests, i.e., 7 to 8 days after CO, all drugs showed antiamnesic properties. Preadministration of the \( \sigma_1 \) receptor antagonist \( N\,\{\text{-}[2\,-\{(3,4\,-\text{dichlorophenyl})\text{ethyl}\},N\text{-methyl\}-2\,(\text{dimethylamino})\text{ethylamine (BD1047) blocked only the igmesine and donepezil effects. The neuroprotective activity of the drugs was tested by injection 20 min before the first CO exposure (preinsult protection) or by injection 1 h after the last CO exposure (postinsult protection). All drugs alleviated the hypoxia-induced neurodegeneration and behavioral impairments when injected before CO exposure. Preadministration of BD1047 blocked both the igmesine and donepezil effects. However, when injected after CO exposure, only igmesine and donepezil induced effective neuroprotection, and the morphological and behavioral effects were BD1047-sensitive. These results showed that donepezil is a potent antiamnesic and neuroprotective compound against the neurodegeneration induced by excitotoxic insult, and its pharmacological actions as both an acetylcholinesterase inhibitor and \( \sigma_1 \) receptor agonist contribute to its marked efficacy. In particular, the drug is a more potent postin insult protecting agent compared with more selective cholinesterase inhibitors.

Acetylcholinesterase inhibitors inhibit the hydrolysis of acetylcholine and elevate its concentration in the synaptic cleft, provoking an increase of the efficacy of cholinergic neurotransmission. (\( \pm \))-2-[(1-Benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one (donepezil) is a potent acetylcholinesterase inhibitor used for treatment of Alzheimer’s disease. Donepezil raises brain acetylcholine concentration as revealed by in vivo microdialysis studies in rats (Kosasa et al., 1999). Randomized, double-blind, placebo-controlled clinical studies showed that donepezil produces a significant improvement of cognition and global function in patients with mild to moderate Alzheimer’s disease (Rogers and Friedhoff, 1996; Rogers et al., 1998). The compound presents several interesting pharmacological characteristics comparatively with other cholinesterase inhibitors. First, with an IC\(_{50}\) value of 5.7 nM for acetylcholinesterase activity versus 7 \( \mu \)M for butyrylcholinesterase activity, donepezil seems to be a very selective acetylcholinesterase inhibitor (Sugimoto et al., 2000). Second, the compound has high affinity for the \( \sigma_1 \) receptor, with an IC\(_{50}\) of 14.6 nM, i.e., in the same concentration range as its acetylcholinesterase inhibition potency (Kato et al., 1999). The \( \sigma_1 \) receptor is an intracellular protein involved in modulation of intracellular Ca\(^{2+}\) mobilization and neurotransmitter responses (Maurice et al., 1999b). The interaction of donepezil with the \( \sigma_1 \) receptor has been demonstrated at the behavioral level, since the selective \( \sigma_1 \) receptor antagonist \( N\{-[2\,-\{(3,4\,-\text{dichlorophenyl})\text{ethyl}\},N\text{-methyl\}-2\,(\text{dimethylamino})\text{ethylamine (BD1047) or an anti-sense probe targeting the \( \sigma_1 \) receptor blocked the antiamnesic effect of donepezil against dizocilpine-induced learning impairments in mice (Maurice et al., 2006), a behavioral response identifying \( \sigma_1 \) receptor agonist activity (Maurice et al., 1994a). Donepezil and other cholinesterase inhibitors showed neuroprotective effects in both in vivo or in vitro

ABBREVIATIONS: BD1047, \( N\,-\{[2\,-\{(3,4\,-\text{dichlorophenyl})\text{ethyl}\},N\text{-methyl\}-2\,(\text{dimethylamino})\text{ethylamine; CO, carbon monoxide; NMDA, N-methyl-o-aspartate; KW, Kruskal-Wallis.}
models of glutamate neurotoxicity, through a mechanism involving mainly an indirect activation of α1β2- and α1-nicotinic receptors (Takada et al., 2003; Fujiki et al., 2005). However, the putative involvement of the α1 receptor in the neuroprotective activity of donepezil was never examined.

Repetitive exposure to carbon monoxide (CO) gas constitutes an in vivo model of hypoxia. CO exposure induces long-lasting but delayed amnesia in mice that can be measured 1 week after exposure (Nabeshima et al., 1991; Maurice et al., 1994b, 1999a, 2000). The hippocampal cholinergic system seems markedly affected by the hypoxic toxicity involving amino acid excitotoxicity (Nabeshima et al., 1991). In particular, the concentration of acetylcholine and the binding potency of [3H]quinuclidyl benzilate in the frontal cortex and the striatum, but not in the hippocampus, were increased 7 days after CO exposure (Nabeshima et al., 1991). Histological examination of the CA1 region of the hippocampal formation showed a moderate but significant neuronal loss, which was augmented by increasing the severity of the CO exposure (Ishimaru et al., 1991). This model involves the neurotoxic effects of excitatory amino acids. Competitive or noncompetitive NMDA receptor antagonists acting through the glycine or phencyclidine modulatory site prevented the CO-induced amnesia and the concomitant hippocampal neuronal loss (Nabeshima et al., 1991; Ishimaru et al., 1992). On the contrary, the neuroactive steroid pregnenolone sulfate, known to positively modulate the NMDA receptor, worsened the CO-induced neuronal damage and resulting amnesia (Maurice et al., 2000). The CO intoxication model therefore represents a valuable model of excitotoxicity-associated hippocampal damage, which allows evaluating the neuroprotective activity of drugs in vivo.

In this study, we used the repetitive CO exposure model to compare the protective effects of donepezil with a reference σ1 receptor agonist, igmesine, and the acetylcholinesterase inhibitors tacrine, rivastigmine, and galanthamine. Compounds were administered either 7 days after CO exposure, 20 min before the first exposure to CO, or 1 h after the last exposure to differently assess the antiamnesic and neuroprotective activities of the drugs. Cell death in the CA1 hippocampal area was analyzed in Cresyl violet-stained brain sections, and the appearance of the learning deficits were examined in CO-exposed mice using both a short-term and a long-term memory tests.

Materials and Methods

Animals. Male Swiss mice, 1-month old and weighing 28 to 32 g, were purchased from the breeding center of the Faculty of Pharmacy (Montpellier, France). Animals were housed in groups of 20 with access to food and water ad libitum, except during experiments. They were kept in a temperature- and humidity-controlled animal facility on a 12-h light/dark cycle (lights off at 8:00 PM). Behavioral experiments were carried out between 9:00 AM and 2:00 PM in a sound-attenuated and air-regulated experimental room, to which mice were habituated at least 30 min. All animal procedures were conducted in strict adherence to the European Communities Council Directive of 24 November 1986 (86-609/EEC).

Drugs. Donepezil hydrochloride was provided by Eisai Co. Ltd (Tokyo, Japan). (+)-N-Ethyl-2-[(dimethyl-amino)ethyl]-N-methylphenylcarbamate hydrogencarbonate (rivastigmine) was from Novartis (Basel, Switzerland). 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride (tacrine) and (4aS,6R,8aS)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro[3,2-e][2]benzazepine-6-ol hydrobromide (galanthamine) were from Sigma-Aldrich (St-Quentin-Fallavier, France). (+)-N-Cyclopropylmethyl-N-methyl-1,4-diphenyl-1-ethylbut-3-ene-1-yamine hydrochloride (igmesine) was provided by Dr. François J. Roman (Pfizer GRD, Fresnes, France). BD1047 was provided by Dr. Wayne D. Bowen (Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). All drugs were dissolved in physiological saline solution (0.9%) injected intraperitoneally (i.p.) in a volume of 100 µl per 20 g of body weight. Doses refer to the salt form. For antagonism studies, the σ1 receptor antagonist was administered 10 min before the experimental drugs.

Control animals received only one administration of saline solution, since extensive previous studies have shown no differences in behavioral responses after one or two i.p. injections of saline (data not shown).

Exposure to CO Gas. Exposure to CO was carried out as described previously (Ishimaru et al., 1991, 1992; Nabeshima et al., 1991; Maurice et al., 1994b, 1999a, 2000). Mice were placed in a transparent plastic vessel (3-cm radius, 10 cm high), with a pipe feeding into it. CO gas was disseminated at the rate of 125 ml/min, and mice were exposed until they began gasping, i.e., between 30 and 40 s. Animals were exposed three times, with 1 h between each exposure. They were kept on a hot plate (Silab, Montpellier, France) immediately after the first exposure and up to 2 h after the third to maintain their body temperature at 38°C and to avoid the hypothermia induced by CO, which lessens the damages induced by hypoxia (Ishimaru et al., 1991).

The antiamnesic effects of the cholinesterase inhibitors (donepezil, tacrine, rivastigmine, and galanthamine) or σ1 receptor agonist (igmesine) were examined by pretest injections 7 days after exposure to CO. The neuroprotective effects of each compound were examined using 1 injections made 20 min before the first exposure to CO, with animals tested after 7 days, or 2 injections made 1 h after the last exposure to CO, with animals tested on day 7.

Spontaneous Alternation Performances. The spatial working memory was examined using the spontaneous alternation behavior in the Y-maze (Maurice et al., 1994a,b). The maze was made of black painted wood. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converged at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries, including possible returns into the same arm, were checked visually. An alternation was defined as entries into all three arms on consecutive occasions. The number of maximum alternations was therefore the total number of arm entries minus two, and the percentage of alternation was calculated as (actual alternations/maximum alternations) × 100. Neither the exposure to CO nor the treatments used in the study affected the exploratory activity in the test, and the numbers of arm entries were in the 27 to 34 range for all groups.

Step-Through-Type Passive Avoidance Response. The apparatus consisted of an illuminated compartment with white polyvinylchloride walls (15 × 20 × 15 cm high), a darkened compartment with black polyvinylchloride walls (15 × 20 × 15 cm high), and a grid floor. A guillotine door separated each compartment. A 60-W lamp positioned 40 cm above the apparatus lit the white compartment during the experimental period. Scrambled foot shocks (0.3 mA for 3 s) were delivered to the grid floor using a shock generator scrambler (Lafayette Instruments, Lafayette, MA). The guillotine door was initially closed during the training session. Each mouse was placed into the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment and placed all its paws on the grid floor, the door was gently closed and the scrambled foot shock was delivered for 3 s. The step-through latency and the number of vocalizations were recorded. The number of vocalizations did not differ among groups, indicating that shock sensitivity was identical. The retention test was carried out 24 h after training. Each mouse was placed again into the white compartment. After 5 s, the...
door was raised. The step-through latency was recorded up to 300 s. If animals entered the darkened compartment, the escape latency, i.e., the time spent to return into the white compartment, was also measured up to 300 s.

**Histology.** Each mouse was anesthetized with sodium pentobarbital (100 mg/kg i.p.) and quickly transcardially perfused with 50 ml of phosphate-buffered saline solution (PBS), pH 7.4, followed by 50 ml of PBS containing 4% paraformaldehyde (w/v). Brains were removed and kept overnight in the fixative solution. They were cut in coronal sections (60 µm thickness) using a vibratome (Leica VT1000 S; Leica, Wetzlar, Germany). Serial sections were selected to include the hippocampal formation and placed in gelatin-coated glass strip. Sections were stained with 0.2% Cresyl violet reagent (Sigma-Aldrich), then dehydrated with graded ethanol, treated with toluene, and mounted with DePeX medium (BDH Laboratory Supplies, Poole, Dorset, UK). Examination of the CA1 area was performed using a light microscope (Dialux 22; Leitz, Wetzlar, Germany), with slices digitalized through a CCD camera (Sony XC-77CE; Sony, Paris, France).

**Fig. 1.** Anti-amnesic effects of igmesine (A–C) and donepezil (D–F) in CO-exposed mice. Mice were exposed three consecutive times to CO (125 ml/min, 30–40 s) at 38°C. After 7 days, animals were examined for spontaneous alternation performances (A and D). On day 8 after exposure, animals were trained for passive avoidance task, and retention was examined on day 9, in terms of step-through latency (B and E) and escape latency (C and F). Igmesine (0.3–3 mg/kg i.p.), donepezil (0.12–1 mg/kg i.p.), and/or BD1047 (0.5–1 mg/kg i.p.) were administered 20 min before the test. The number of animals per group was n = 8–11. Kruskal-Wallis analysis of variance: KW = 83.46, p < 0.0001 in A; KW = 46.20, p < 0.0001 in B; KW = 52.07, p < 0.0001 in C; KW = 46.20, p < 0.0001 in D; KW = 52.07, p < 0.0001 in E; KW = 52.07, p < 0.0001 in F. *, p < 0.05; **, p < 0.01 versus the vehicle-treated non-CO-exposed group; #, p < 0.05; ##, p < 0.01 versus the vehicle-treated CO-exposed group; o, p < 0.05; oo, p < 0.01 versus the igmesine (1 mg/kg)- or donepezil (0.5 mg/kg)-treated CO-exposed group; Dunn’s test.
France) with the NIH Image version 1.63 software to easily process CA1 measurement and pyramidal cells counts. Data are expressed as mean of six slices of CA1 pyramidal cells per millimeter for each group, according to previously reported method (Nabeshima et al., 1991; Ishimaru et al., 1992; Maurice et al., 2000).

**Statistical Analyses.** Y-maze test data were expressed as mean ± S.E.M. Passive avoidance latencies did not show a normal distribution because a cut-off time was set. They were thus represented as median and interquartile range. Drug doses for antagonism studies were decided a priori based on previous studies (see, for instance, Maurice et al., 2006) and extensive literature in the field. Experiments were thus designed to include dose-response and antagonisms studies in the same set. All behavioral data were therefore analyzed using the Kruskal-Wallis nonparametric analysis of variance (KW values) for coherence and group comparisons being made with Dunn’s nonparametric multiple comparisons test. The level of statistical significance was \( p < 0.05 \).

**Results**

**Antiamnesic Effects of Igmesine, Donepezil, and Acetylcholinesterase Inhibitors against CO-Induced Learning Impairments.** The first series of experiments examined the antiamnesic effects of the drugs. Mice were exposed to CO gas, and the learning deficits were measured after 7 to 9 days using the spontaneous alternation performance, an index of working memory, and the step-through passive avoidance test for long-term memory. CO-exposed mice developed after 1 week significant memory impairments that could be observed as alternation deficits in the Y-maze (Fig. 1, A and D) and decrease in step-through latency (Fig. 1, B and E) and concomitant increase in escape latency (Fig. 1, C and F) in the passive avoidance test. The reference \( \sigma_1 \) receptor agonist igmesine was tested in the 0.3 to 3 mg/kg dose range and allowed a complete reversion of the CO-induced deficits at the dose of 1 mg/kg (Fig. 1A) and for both passive avoidance parameters (Fig. 1, B and C). Pretreatment with the \( \sigma_1 \) receptor antagonist BD1047 (0.5–3 mg/kg) dose-dependently blocked the beneficial effect of igmesine but was ineffective by itself on CO-induced deficits (Fig. 1, A and C).

Donepezil, administered 20 min before the test, also allowed a significant reversion of the CO-induced deficits in alternation, with a maximum effect at the dose of 0.5 mg/kg (Fig. 1D) and for both passive avoidance parameters (Fig. 1, E and F). The beneficial effect of donepezil was blocked in a dose-dependent manner by pretreatment with BD1047 (Fig. 1, D–F).

The cholinesterase inhibitors tacrine, rivastigmine, and galanthamine were tested at the doses of 0.3 and 1 mg/kg i.p. All compounds significantly reversed the CO-induced alternation deficits (Fig. 2A), step-through latency deficit (Fig. 2B), and increase in escape latency (Fig. 2C). The drug effects were, however, not affected by a BD1047 (3 mg/kg) pretreatment, indicating the lack of involvement of the \( \sigma_1 \) receptor in their antiamnesic potency.

**Neuroprotective Effects of Igmesine, Donepezil, and Acetylcholinesterase Inhibitors against CO-Induced Learning Impairments: Preinsult Protection.** Drugs were administered 20 min before the first exposure to CO, and the learning deficits were measured after 7 to 9 days using the spontaneous alternation and step-through passive avoidance test. Igmesine, at a 0.3 to 3 mg/kg dose range, allowed a complete blockade of the CO-induced deficits in alternation at the highest doses tested (Fig. 3A) and for both passive avoidance parameters (Fig. 3, B and C).
C). Pretreatment with BD1047 (0.5–3 mg/kg) dose-dependently blocked the beneficial effect of igmesine, with the $\sigma_1$ receptor antagonist itself ineffective on CO-induced deficits (Fig. 3, A–C).

Donepezil (0.12 to 1 mg/kg) also allowed a significant prevention of the CO-induced deficits in alternation (Fig. 1D) and for both passive avoidance parameters (Fig. 3, E and F), with a maximum effect at the dose of 0.5 mg/kg. The beneficial effect of donepezil against CO-induced alternation deficits was sensitive to the pretreatment with BD1047 but varied depending on the test and parameter. A significant but partial reversion was measured for alternation performances (Fig. 3D). A complete blockade was observed for the step-through latency (Fig. 3E). A nonsignificant attenuation was observed for escape latency (Fig. 3F).

The cholinesterase inhibitors tacrine, rivastigmine, and galanthamine were tested at the doses of 0.3 and 1 mg/kg i.p. All compounds significantly reversed the CO-induced alternation deficits (Fig. 4A), step-through latency deficit (Fig. 4B), and increase in escape latency (Fig. 4C). The drug effects

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**Fig. 3.** Preinsult neuroprotective effects of igmesine (A–C) and donepezil (D–F) in CO-exposed mice. Mice were administered with igmesine (0.5–3 mg/kg i.p.), donepezil (0.12–1 mg/kg i.p.), and/or BD1047 (0.5–3 mg/kg i.p.) 20 min before being exposed three consecutive times to CO (125 ml/min, 30–40 s) at 38°C. After 7 days, animals were examined for spontaneous alternation performances (A and D). On day 8 after exposure, animals were trained for passive avoidance task, and retention was examined on day 9, in terms of step-through latency (B and E) and escape latency (C and F). The number of animals per group was $n = 8–11$. KW = 54.97, $p < 0.0001$ in A; KW = 40.23, $p < 0.0001$ in B; KW = 42.56, $p < 0.0001$ in C; KW = 42.01, $p < 0.0001$ in D; KW = 32.32, $p < 0.0001$ in E; KW = 25.58, $p < 0.001$ in F, *, $p < 0.05$, **, $p < 0.01$ versus the vehicle-treated non-CO-exposed group; #, $p < 0.05$, ##, $p < 0.01$ versus the vehicle-treated CO-exposed group; o, $p < 0.05$, oo, $p < 0.01$ versus the igmesine (1 mg/kg)- or donepezil (0.5 mg/kg)-treated CO-exposed group; Dunn's test.
were, however, not affected by a BD1047 (3 mg/kg) pretreatment.

Loss of pyramidal cells in the CA1 hippocampal subfield (Fig. 5A) was measured using Cresyl violet staining 7 days after CO exposure. Drugs were administered 20 min before the first CO exposure. Stained cell quantification revealed a significant decrease induced by CO exposure (Fig. 5, C and I), accompanied with cell swelling and dispersion compared with control mice (Fig. 5B). The BD1047 (1 mg/kg) was without effect (Fig. 5, D and I). Igmesine (1 mg/kg) and donepezil (0.5 mg/kg) treatments blocked the hypoxia-induced cell loss (Fig. 5, E, G, and I). The igmesine effect was significantly different from that of the vehicle-treatment group (p < 0.05).
antagonized by the BD1047 pretreatment (Fig. 5, F and I). The donepezil effect was attenuated by BD1047 (50% cell loss), but the effect remained nonsignificant (Fig. 5I). Tacrine (1 mg/kg; Fig. 6, C and G), or rivastigmine (1 mg/kg; Fig. 6, D and G), failed to show neuroprotection against neurodegeneration induced by CO exposure (Fig. 6, B and G). Galanthamine (1 mg/kg) showed a slight and significant neuroprotective effect (Fig. 6, E and G). This effect of galanthamine was unaffected by the BD1047 pretreatment (Fig. 6, F and G).

**Neuroprotective Effects of Igmesine, Donepezil, and Acetylcholinesterase Inhibitors against CO-Induced Learning Impairments: Postinsult Protection.** Drugs were administered 1 h after the last exposure to CO, and the learning deficits were measured after 7 to 9 days using the spontaneous alternation and step-through passive avoidance test. Igmesine, at a 0.3 to 3 mg/kg dose range, produced a significant attenuation of the CO-induced deficits in alternation at the highest doses tested (Fig. 7A) and for both passive avoidance parameters (Fig. 7, B and C). Pretreatment with BD1047 (0.5–3 mg/kg) dose-dependently blocked the beneficial effect of igmesine in terms of alternation performance and step-through latency (Fig. 7, A and B). However, the σ1 receptor antagonist was ineffective by itself on CO-induced deficits (Fig. 7, A–C).

Donepezil (0.12 to 1 mg/kg) also allowed a significant but partial prevention of the CO-induced deficits in alternation (Fig. 7D). The step-through latency deficits in CO-exposed mice was attenuated but without reaching significance, in contrast to the escape latency parameter (Fig. 7, E and F). The beneficial effect of donepezil against CO-induced alternation deficits was blocked by pretreatment with BD1047, since animals treated with the highest dose of BD1047 plus donepezil (0.5 mg/kg) performed at a similar level as vehicle-treated CO-exposed mice (Fig. 7, D–F).

The cholinesterase inhibitors tacrine, rivastigmine, and galanthamine were tested at the doses of 0.3 and 1 mg/kg i.p. Only rivastigmine and galanthamine showed significant improvement on the CO-induced alternation deficits, effects that were insensitive to BD1047 (Fig. 8A). A tendency toward improvement of the step-through latency and escape latency deficits in CO-exposed mice was observed for the same drugs, but no differences reached statistical significance. The BD1047 treatment was without any effect in any group (Fig. 8, B and C).

Histological examination of the CA1 pyramidal cell layer showed cell loss in CO-exposed mice (Fig. 9, B and H) compared with control mice (Fig. 9, A and H). Post-treatment with BD1047 (1 mg/kg) failed to have any protective effect (Fig. 9, C and H). Igmesine (1 mg/kg) and donepezil (0.5 mg/kg) protected the CA1 area against hypoxic insult (Fig. 9, D, F, and H), and these beneficial effects were blocked by BD1047 treatment (Fig. 9, E, G, and H). The other cholinesterase inhibitors tested, tacrine (1 mg/kg; Fig. 10, C and F), rivastigmine (1 mg/kg; Fig. 10, D and F), or galanthamine (1 mg/kg; Fig. 10, E and F), failed to protect CA1 area against neurodegeneration induced by CO exposure (Fig. 10, B and F) compared with control mice (Fig. 10, A and F).

**Discussion**

In the present study, we examined the antiamnesic and neuroprotective effects of donepezil and other cholinesterase
inhibitors against amnesia induced in mice by severe hypoxia. We aimed at determining whether an interaction with the $\sigma_1$ receptor is involved in donepezil's antiamnesic and neuroprotective effects and contributes to its behavioral effects. Indeed, with a 14.6 nM affinity for the $\sigma_1$ receptor and an IC$_{50}$ of 5.7 nM for inhibition of acetylcholinesterase activity, the drug is equipotent for these two targets (Kato et al., 1999). Other cholinesterase inhibitors are more selective cholinomimetics and present only low affinity for the $\sigma_1$ receptor. For instance, tacrine shows an affinity of 6 $\mu$M for the $\sigma_1$ receptor (Kato et al., 1999) compared with an IC$_{50}$ of 77 nM for the inhibition of acetylcholinesterase activity (Ogura et al., 2000). Repetitive exposure to CO induces in mice a severe hypoxic insult to the hippocampus, leading to an intense excitotoxic neurodegenerative process with synaptic and cellular losses in the CA1–3 pyramidal layers (Ishimaru et al., 1991, 1992; Nabeshima et al., 1991; Maurice et al., 1994b, 1999a). Within 1 week after exposure, animals gradually develop learning deficits that correlate with these histological changes. Moreover, the model could be used to analyze either antiamnesic effect of drugs, administered at the time point of the behavioral examination, or neuroprotective effect, with drugs being administered during or immediately after the exposure to CO (Maurice et al., 1994b, 2000). The results of the study were summarized in Table 1.

The first part of the present report examined the antiamnesic effects of the drugs in CO-exposed mice. Examination of working memory and contextual long-term memory, using the spontaneous alternation and passive avoidance behavior, respectively, showed highly significant deficits for all parameters examined. All drugs tested alleviated the deficits within the 0.1 to 1 mg/kg dose range, confirming that cholinomimetics as well as $\sigma_1$ receptor agonists are effective against amnesia induced by excitotoxic insults. This was described previously for acetylcholinesterase inhibitors in several models, including amino acid-induced lesions (Dokla et al., 1989) and for selective $\sigma_1$ receptor agonists (Maurice et al., 1994b, 1999a). The new observation is that donepezil's antiamnesic effects were blocked by BD1047, a reference $\sigma_1$ receptor antagonist. This observation demonstrates that these antiamnesic effects therefore involve both its cholinomimetic and $\sigma_1$ agonist properties. Indeed, donepezil, as well as tacrine, galanthamine, and rivastigmine, potentiate extra-cellular levels of acetylcholine in the brain, particularly within the hippocampus, as a result of the inhibition of acetylcholinesterase activity (Kosasa et al., 1999). However, in a
recent report, we reported that the antiamnesic effects of donepezil against dizocilpine-induced learning impairments were blocked not only by BD1047 but also by an antisense probe treatment down-regulating the $\sigma_1$ receptor expression within the cortex and hippocampus (Maurice et al., 2006). Therefore, the interaction of donepezil with the $\sigma_1$ receptor is effective in vivo and contributes to its antiamnesic effects. Our present results extended this observation in a pathological model of amnesia. Moreover, the inactivation of the $\sigma_1$ receptor led to a complete blockade of donepezil’s effects, suggesting that cholinergic and $\sigma_1$ systems are inter-related, and mixed activity does not lead to simple additive effects. This phenomenon has also been observed with drugs acting nonselectively as direct muscarinic agonists and $\sigma_1$ receptor agonists (J. Espallergues, P. Lapalud, A. Vamvakides, and T. Maurice, unpublished observation). The $\sigma_1$ receptor is present within presynaptic neurons, where it may facilitate acetylcholine release (Junien et al., 1991) and within the postsynaptic neurons, where it potently modulates intracellular second messenger cascades, particularly facilitating
concentration in the synaptic cleft, and on intracellular pathways via activation of the $\sigma_1$ receptor. The physiological consequences of such biphasic action on synaptic transmission have to be examined particularly using electrophysiological approaches.

The second aspect of the present study was to examine the neuroprotective efficacy of donepezil in comparison with igmesine and other cholinesterase inhibitors (Table 1). Drugs were administered either before the first exposure or 1 h after the last exposure to CO. Histological damage and behavioral deficits were observed after 1 week. It is hypothesized that the first procedure allows analysis of the drug effects on the acute neurotoxic insult, known to be dependent on excessive glutamate release, whereas the second procedure focuses on the drug effects on the chronic neurodegeneration processes, mainly mediated by long-term oxidative stress in the damage tissue (Nishizawa, 2001). Results show that all drugs are effective neuroprotective agents when they are administered before CO exposure. The CO-induced behavioral deficits and cell loss in the CA1 hippocampal layer are blocked by the drug treatments. The most important result of the present study is the observation that donepezil's effects are antagonized by BD1047 in a similar manner as observed for igmesine, suggesting that the drug behaves more likely as a $\sigma_1$ receptor agonist than as a cholinomimetic. When drugs were administered 1 h after the last exposure to CO, igmesine remained effective at the morphological and behavioral levels. Donepezil effects were more moderate at the behavioral level but remained significant. Tacrine, galanthamine, and rivastigmine showed moderate behavioral efficacy, reaching significance only for the spontaneous alternation parameter with the two last compounds and no improvement on the morphological measure. Donepezil effects were again sensitive to BD1047, suggesting the importance of the $\sigma_1$ receptor in the neuroprotective effect of the compound.

The neuroprotective activity of cholinomimetic drugs has been extensively described using mainly in vitro models. Donepezil, tacrine, galanthamine, neostigmine, pyridostigmine, and metrifonate protect rat cortical neurons against glutamate neurotoxicity and apoptosis (Takada et al., 2003). This effect seems mediated in part via up-regulation of $\alpha_7$- and $\alpha_2$-nicotinic receptors (Kume et al., 2005). Moreover, the neuroprotective activity of cholinesterase inhibitors was also examined in vivo using focal ischemia models in rats. Pre-treatment with a single oral dose of donepezil (12 mg/kg) 2 h before permanent middle cerebral artery occlusion attenuated the infarction volume (Fujiki et al., 2005; Geerts et al., 2005). Moreover, a postischemic and chronic treatment with methanesulfonyl fluoride (1 mg/kg at 24 and 48 h after occlusion and 0.3 mg/kg once a day during 4 weeks) attenuated the motor (body swing bias) and cognitive (passive avoidance) abilities and biochemical parameters (choline acetyltransferase immunoreactivity) of the animals (Borlongan et al., 2005). On the other hand, $\sigma_1$ receptor agonists have also been reported to possess neuroprotective effects (for review, see Maurice et al., 1999b). In particular, $\sigma_1$ receptor ligands protected rat hippocampal and cortical neurons against glutamate or NMDA exposure in vitro (Pauwels et al., 1992) or against transient focal or permanent global ischemia in vivo (O’Neill et al., 1995; Harukuni et al., 2000). Interestingly, $\sigma_1$ receptor agonists including igmesine significantly protected...
rat brain neurons against a hypoxic/hypoglycemic insult more efficiently than toxicity induced by direct application of NMDA (Lockhart et al., 1995; Nakazawa et al., 1998), suggesting that \( \sigma_1 \) receptors exert a potent neuroprotective effect via the regulation of excitatory amino acids release from presynaptic terminals rather than via an action on the postsynaptic neurons.

We observed here a differential involvement of cholinergic systems that seemed effective mainly in the pretreatment procedure in contrast to the \( \sigma_1 \) receptor, which mediated neuroprotection in both the pre- and post-treatment procedures. The neuroprotection induced by cholinomimetic drugs highly effective in the early phase of the glutamate-induced toxicity is likely to involve the \( \alpha_7 \)-nicotinic receptors activation that leads to neuroprotection via the \( \Ca^{2+} \)-dependent phosphatidylinositol 3-kinase pathway. Indeed, nicotine protected neurons by activating phosphatidylinositol 3-kinase, which activated Akt and up-regulated Bcl-2 (Kihara et al., 2000), and pretreatment with donepezil 24 h before glutamate protected rat cortical neurons in a manner sensitive to the \( \alpha_7 \)-nicotinic receptor antagonist methyllycaconitine (Takada et al., 2003). Moreover, galanthamine has been shown to not only inhibit acetylcholinesterase activity but also allosterically modulate both \( \alpha_7 \) and \( \alpha_4 \beta_2 \)-nicotinic acetylcholine receptors (Pereira et al., 1993, 1994). This last effect is involved in the neuroprotective activity of galanthamine, as shown against glutamate and amyloid \( \beta \)-peptide toxicities (Kihara et al., 2001, 2004), which therefore exerts a more nicotine-like neuroprotective action than an indirect enhancement of cholinergic systems. This difference of mechanism may explain the ability of galanthamine to protect against CO-induced neuronal damage more significantly than tacrine or rivastigmine.

The neuroprotection induced by activation of the \( \sigma_1 \) receptor by igmesine or donepezil is effective on both the early and late phase of the neurotoxicity and putatively through different mechanisms. Acutely, \( \sigma_1 \) receptor ligands, by regulating \( \Ca^{2+} \)-mobilizations from endoplasmic reticulum pools (Hayashi et al., 2000), may exert a presynaptic effect by regulating glutamate release. This effect has been demonstrated in monoaminergic neurons (Gonzalez-Alvear and Werling, 1995). During the delayed phase of the toxicity, administration of \( \sigma_1 \) receptor may allow a long-term trophic effect. Indeed, the \( \sigma_1 \) receptor, when activated, targets the lipid-storing subcompartments of the endoplasmic reticulum (Hayashi and Su, 2004a) and is colocalized with cholesterol and neutral lipids. The \( \sigma_1 \) receptor forms detergent-insoluble lipid microdomains on the endoplasmic reticulum subcompartments and translocates to plasma membrane.
lipid rafts, where it allows changes in the lipid components and membrane reconstitution therefore affecting the functions of proteins residing in plasma membrane lipid rafts including tropic factor receptors and tyrosine kinases. Specifically, it was recently described that $\sigma_1$ receptors modulate mitogen-activated protein kinase activation induced by tropic factors, neuritogenesis, and oligodendrocyte differentiation (Hayashi and Su, 2004b; Takebayashi et al., 2004). The mechanism and long-

**TABLE 1**

Effect of acetylcholine inhibitors and $\sigma_1$ receptor ligands against CO-induced learning impairments in mice

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Test</th>
<th>Learning and Memory</th>
<th>Morphological Examination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Active Dose (or Dose Range Tested)</td>
<td>BD1047 Sensitivity</td>
</tr>
<tr>
<td>Anti-amnesic effect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Igesmine SA, PA 0.3–3 mg/kg</td>
<td>+ + (SA), + (PA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donepezil SA, PA 0.25–1 mg/kg</td>
<td>+ + (PA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tacrine SA, PA 1 mg/kg</td>
<td>–</td>
<td></td>
<td></td>
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<tr>
<td>Rivastigmine SA, PA 0.3–1 mg/kg</td>
<td>–</td>
<td></td>
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<tr>
<td>Galanthamine SA, PA 1 mg/kg</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preinsult neuroprotective effect</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Igesmine SA, PA 1–3 mg/kg</td>
<td>+ +</td>
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<tr>
<td>Donepezil SA, PA 0.25–1 mg/kg</td>
<td>+ +</td>
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<tr>
<td>Tacrine SA, PA 0.3–1 mg/kg</td>
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<tr>
<td>Rivastigmine SA, PA 0.3–1 mg/kg</td>
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<tr>
<td>Galanthamine SA, PA 1 mg/kg</td>
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<tr>
<td>Postinsult neuroprotective effect</td>
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<tr>
<td>Igesmine SA, PA 0.3–1 mg/kg</td>
<td>+ + (PA)</td>
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<tr>
<td>Donepezil SA 0.25–1 mg/kg</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tacrine SA, PA 0.5–1 mg/kg</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rivastigmine SA 1 mg/kg</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galanthamine SA 1 mg/kg</td>
<td>–</td>
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</tr>
</tbody>
</table>

SA, spontaneous alternation; PA, passive avoidance; –, ineffective; +, partially effective; ++, fully effective.

$F_{1,5} = 3.40, p < 0.05$. **, $p < 0.01$, +, $p < 0.05$ versus no CO + Veh group, Dunnett’s test.
term validity of this $\sigma_1$ receptor-mediated neuroprotective activity deserves further extensive examination. Moreover, as observed in the antiamnesic effect, the neuroprotective effect of donepezil is largely sensitive to inactivation of the $\sigma_1$ receptor. This observation strongly suggests that mixed cholinomimetic and $\sigma_1$ compounds present a very unique pharmacological action that could not be reduced to the simple addition of to primary pharmacological effects. Activation of the $\sigma_1$ receptor intracellularly results in modulation of Ca$^{2+}$ mobilization and activation of transduction pathways, such as activation of phospholipase C/protein kinase C, and therefore, to shared effects in the morphological and behavioral impairments induced in mice by severe hypoxia as a result of repetitive CO exposure.

The pharmacological actions of donepezil as both an acetylcholinesterase inhibitor and $\sigma_1$ receptor agonist contribute to its marked efficacy. In particular, the drug is a more potent inhibitor of acetylcholinesterase than of butyrylcholinesterase and a more potent $\sigma_1$ receptor agonist. Moreover, in the same experimental conditions, the drug is a more potent inhibitor of central-type acetylcholinesterase than of peripheral-type acetylcholinesterase and a more potent $\sigma_1$ receptor agonist. Donepezil under the maintenance of normal body temperature.

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