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Research Article

Effects of Linopirdine (DuP 996) and X9121 on Age-Related Memory Impairments and on the Cholinergic System

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Linopirdine (DuP 996) has been shown to enhance K^+-stimulated release of acetylcholine from cerebral cortex, striatum, and hippocampus of rats in vitro. X9121 is a structurally different compound identified as having similar release properties. The present experiments compare the effects of linopirdine and X9121 on cognitive deficits in aged rats, and on the pharmacological properties in young rats. For cognitive testing, aged male Fischer-344 rats (24 months old, n = 116) received either vehicle or one of 5 doses of linopirdine or X9121 prior to behavioral testing; young rats (4 months old, n = 13) were controls and received vehicle prior to testing. Place discrimination and repeated acquisition were tested in the water maze, and a variety of sensorimotor tasks were given. Aging impaired performance in all tasks. Linopirdine (0.25, 2.5, and 8.5 mg/kg po [0.64, 7.4, and 25 μmol/kg]) and X9121 (0.85 and 8.5 mg/kg po [2.1 and 24 μmol/kg]) moderately improved place discrimination. None of the doses tested improved repeated acquisition or sensorimotor function. No behavioral indications of toxicity were observed. Acetylcholine release, acetylcholinesterase (AChE) inhibition, and nicotinic and muscarinic binding were measured in vitro in cerebral cortical tissue from young male Wistar rats (2 months old). Both linopirdine and X9121 enhanced K^+-stimulated release from cerebral cortex; X9121 produced greater release with a broader range of active concentrations. Linopirdine weakly inhibited AChE (1,000× weaker than physostigmine) and X9121 did not. Neither drug bound significantly to muscarinic or nicotinic cholinergic receptors. These results support the hypothesis that linopirdine and X9121 have some cognition enhancing properties which may be due to enhancement of stimulation-induced acetylcholine release. These results suggest that linopirdine and X9121 may be useful in treating disorders involving cognitive impairment.

Key Words: linopirdine, X9121, spatial memory, acetylcholine

INTRODUCTION

Linopirdine (DuP 996, Fig. 1) enhances depolarization-induced release of acetylcholine, dopamine, and serotonin, but not norepinephrine, in several brain regions and its releasing action may be mediated by a unique receptor present in the brain

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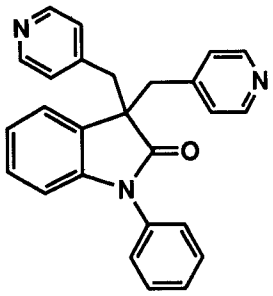
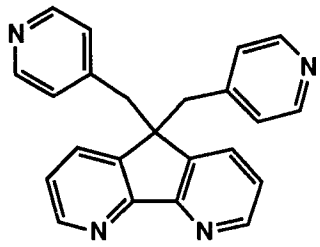


Linopirdine (DuP 996)

3,3-bis(4-pyrindinylmethyl)-1-phenylindolin-2-one



X9121

5,5-bis(4-pyridinylmethyl)-5H-cyclopenta-[2,1-b:3,4-b']dipyridine

Figure 1. Chemical structures and names of linopirdine (DuP 996) and X9121.

[Nickolson et al., 1990; Tam et al., 1991]. X9121, as shown in Figure 1, is a structurally different compound identified as having similar release enhancing affects. Linopirdine reversed deficits in passive avoidance retention consequent to hypoxia-induced amnesia in rats, increased the number of rats that learn to press a lever for food [Cook et al., 1990], improved deficits in T-maze spatial alternation produced by lesions of the nucleus basalis in rats (Lerer, unpublished observation), and reversed impairments in spatial discrimination in the water maze caused by medial septal lesions in young rats [Brioni et al., 1993]. The purpose of the present experiments was to compare the effects of linopirdine and X9121 a newly identified agent [Earl, 1989] in water maze place discrimination in aged rats, repeated acquisition in the water maze in aged rats and K^+-stimulated acetylcholine release.

The present experiment used two discriminations in a water maze to assess the ability of linopirdine and X9121 to reduce age-associated memory impairments in rats. A variable interval probe trial procedure was used, which allowed for repeated measures of spatial memory with no extinction [Markowska et al., 1993]. In the water maze, the rat is motivated to learn the location of the platform in order to escape from the water and learns to reach the platform based on its relation to the spatial cues present in the environment [Morris, 1981]. Aged rats are impaired in performance of this task [Gage et al., 1984; Gallagher and Burwell, 1989].

Certain measures of performance in the water maze can be affected by factors other than spatial memory. Sensory and motor abilities were measured with a battery of tasks to assess the possible beneficial effects of these compounds on sensorimotor skills and to determine the extent to which changes in the discrimination tasks were due to alterations in sensorimotor function rather than improvements in spatial memory.

In addition, the comparative biochemical profiles of linopirdine and X9121 with respect to in vitro K^+-stimulated release of acetylcholine, inhibition of acetylcholinesterase, and muscarinic and nicotinic cholinergic receptor binding were measured, to examine potential pharmacological mechanisms of action of the two drugs.

MATERIALS AND METHODS

Subjects

Studies in this report were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Male Fischer-344 rats, 4 or 24 months old at the start of behavioral testing, were obtained from the NIA colony at Harlan Sprague Dawley (Indianapolis, IN) and were housed in pairs in a colony room with a 12:12 light:dark cycle (lights on at 7 A.M.). Food and water were available ad libitum. All behavioral testing occurred during the light portion of the cycle.

For the biochemical experiments, male Wistar rats (Charles River Inc., Wilmington, MA) weighing 175–200 g (2 months old) were used. These animals were housed for at least 7 days in the animal facility under a 12:12 light:dark cycle (lights on 6 A.M.). Food and water were available ad libitum.

Behavioral Testing

Apparatus

The tank, made of galvanized metal, was 180 cm in diameter and 60 cm high. Water filled the tank to a depth of 35 cm, and was maintained at 24 ± 2°C by an aquarium heater (Visitherm type VTH300, Aquarium Systems, Italy), which was removed prior to testing. White watercolor paint (Rich Art, Rich Art Color

Company, Lodi, NJ) was added to the water to make it opaque. The inside of the tank was painted white. The tank was divided into four quadrants (NE, NW, SE, SW) by two imaginary perpendicular lines crossing in the center of the tank. A tracking system (HVS Image Analysis VP-112, HVS Image, Hampton, England) was used to record data. A camera (Burle Security Products, Lancaster, PA) was mounted 1.4 meters above the surface of the water, and was connected to a computer which controlled the tracking system and stored the data. Light was provided by four 40-watt bulbs, mounted in a circle, 1.2 meters above the surface of the water. The tank was located at one end of a small rectangular room, with a variety of visual cues throughout the room. Visual stimuli were both distal (on the walls of the room) and proximal (located on the rim of the maze). Auditory stimuli from a radio were also present during all behavioral testing.

The platform was made of transparent plastic (Lucite, DuPont, Wilmington, DE), 100 cm^2, with 1-cm diameter holes that provided a gripping surface for the rat. The platform could be raised and lowered by a cable that was located beneath the water and ended outside of the tank. In the raised position, the platform was 1 cm below the surface of the water and available but not visible to the rat for escape from the water. In the lowered position, it was 19 cm below the surface of the water, unavailable for escape.

For shaping and straight swim (see below), two pieces of transparent plastic (Lucite), 100 cm long and 61 cm high, were placed parallel to each other in the tank to form an alley, 15 cm wide, with edges 26 cm above the surface of the water. One end of the alley was placed against the edge of the tank. A third piece of transparent plastic (Lucite), 15 cm wide and 61 cm long, extended 26 cm above the surface of the water and was placed to block the other end of the alley.

Drug administration

The drugs administered were linopirdine (DuP 996) and X9121. Each rat received the same dose of the same drug on each day of testing. Each drug solution had 1 mg of the drug per ml of vehicle (0.06 *N* HCl). A fresh solution of each drug was made each day of testing. Both compounds were synthesized at The DuPont-Merck Pharmaceuticals Company (Wilmington, DE).

Control. Vehicle was given to each control rat throughout behavioral testing.

Linopirdine and X9121. Linopirdine and X9121 were dissolved in 0.06 *N* HCl (pH = 1.2) and administered by gavage in the following doses: 0.085, 0.25, 0.85, 2.5, and 8.5 mg/kg. Testing began 15 min after drug administration and lasted approximately 45 min.

TABLE 1. Summary of Experimental Groups

Group	Age (months)	n	Dose (mg/kg/μmol/kg)	Abbreviation
Young controls	4	13	None	**4MO-CON**
Old controls	24	11	None	**24MO-CON**
Linopirdine (DuP 996)	24	11	.085/.19	**24MO-996(.085/.19)**
	24	11	.25/.62	**24MO-996(.25/.64)**
	24	10	.85/2.2	**24MO-996(.85/2.2)**
	24	10	2.5/7.4	**24MO-996(2.5/7.4)**
	24	11	8.5/25	**24MO-996(8.5/25)**
X9121	24	10	.085/.18	**24MO-9121(.085/.18)**
	24	10	.25/.62	**24MO-9121(.25/.62)**
	24	10	.85/2.1	**24MO-9121(.85/2.1)**
	24	11	2.5/7.2	**24MO-9121(2.5/7.2)**
	24	11	8.5/24	**24MO-9121(8.5/24)**

Design

Each 4-month-old rat was assigned to the young control group and given vehicle before testing. Each 24-month-old rat was randomly assigned to one of 11 groups, one control group and 10 drug groups. The groups for the aged rats differed in the drug and doses administered prior to each session of behavioral testing. All groups and their abbreviations are summarized in Table 1.

Procedure

The general procedure for testing in the water tank was the same for all tasks. The platform was placed in the appropriate location in the tank. Each rat was placed at the start location and allowed to swim to the platform. If the rat did not reach the platform within 60 sec, the experimenter led the rat to the platform by hand. Upon reaching the platform, the rat remained there for 10 sec, and was then removed from the tank and returned to a holding cage for an intertrial interval (ITI) of approximately 2 min. Signs of hypothermia were monitored throughout testing. Testing proceeded in the following order: shaping, straight swim, place discrimination, repeated acquisition, sensorimotor testing. Two days with no testing occurred between straight swim and place discrimination, place discrimination and repeated acquisition, and repeated acquisition and sensorimotor testing.

Shaping. Shaping was designed to teach rats to swim in the water and to climb onto the platform. No

spatial discrimination was involved because the alley was sufficiently narrow that the rat went directly to the platform. The Lucite pieces were placed in the tank to form the alley. The platform was placed 25 cm from the end of the alley opposite the wall of the tank. Black curtains around the tank eliminated the visual cues that were used for place discrimination on subsequent days of testing. Saline was given 15 min before testing to habituate the rat to the method of drug administration.

For each trial, the rat was placed at a specific place in the alley, allowed to reach the platform, and then removed from the tank after 10 sec on the platform. Each of five successive start locations, each farther away from the escape platform, was used twice: (1) on the escape platform, (2) with forepaws on the platform, (3) several cm from the platform, (4) one body length from the platform, and (5) halfway down the alley, approximately 25 cm from the platform.

Straight swim. One session took place on the second day. The apparatus was identical to that used in the shaping procedure. Each rat was placed at the end of the alley against the edge of the tank and was allowed to swim to the platform. Six trials were given. The time taken by the rat to reach the platform was recorded.

Place discrimination. One session took place each day for five consecutive days. Each session had six trials. For each session, the first five trials were platform trials and the sixth was a variable interval probe trial. For all sessions, the platform was located in the SW quadrant, 40 cm from the edge of the tank.

For platform trials, the platform was in the raised position, 1 cm below the water. The rat was lowered by hand into the water near the rim of the tank, facing the center of the tank. The start location was in the center of one of the three quadrants not containing the platform, in a pseudorandom order differing across sessions but consistent within each session.

For each probe trial, the platform remained in the lowered position for a variable time: 10, 30, 20, 40, 10 sec (for sessions 1–5, respectively). At the end of the interval, the platform was raised, making it available to the rat.

Repeated acquisition. One session took place each day for 5 consecutive days. The procedure was identical to that for the place discrimination (i.e., five platform trials and one probe trial per session), except that for each session the platform was placed in a quadrant and at a distance (20, 40, or 60 cm) from the edge of the tank that was different than that used in the previous session. The platform location remained the same within each session.

Measures of performance. The platform trials had three measures of performance. *Swim time* was the time, in seconds, to reach the platform. *Swim distance* was the distance, in centimeters, swam between the start location and the platform. *Heading angle* was the angle between the direction when leaving the edge of the tank, and a straight line drawn from the start location to the platform. For all three measures, lower scores indicated better performance.

The variable interval probe trials had three measures of performance. *Quadrant time* was the percentage of time spent in the quadrant containing the platform. (Quadrant time was not measured on probe trials during repeated acquisition.) *Annulus-40 time* was the percentage of time spent within a circle with a diameter of 40 cm, centered on the location of the platform during the previous platform trials. *Platform crossings* were the number of times the rat crossed the location of the submerged platform. For all three measures, higher scores indicated better performance.

Sensorimotor tasks. Sensorimotor testing was performed for 3 days. Eight sensorimotor tasks were given in the order in which they are listed, on each of the 3 days. The mean scores for each rat on each task was taken across the three daily sessions; these means were used in the data analysis. Drug doses were the same as for place discrimination and repeated acquisition (Table 1). Drug or vehicle was administered to each rat, as described above. A maximum of 120 sec was allowed for the completion of each task.

Initiation of walking. Each rat was placed on a flat, opaque plastic sheet. The time (in seconds) taken to move one body length was recorded.

Turning around in an alley. The wooden alley was 10 cm wide, 25 cm high, and 32 cm long. Each rat was placed 17 cm from the closed end of the alley, facing the closed end. The time (in seconds) to turn around and face the open end of the alley was recorded.

Bridges. A wooden bridge, 48 cm long, was suspended 60 cm above foam padding. At each end of the bridge was a wooden platform, 16 cm × 18 cm. The rat was placed in the center of the bridge. The trial ended when the rat escaped to one of the platforms, fell from the bridge, or when 120 sec had elapsed. Time to escape was the amount of time taken to reach one of the platforms, and was recorded as 120 sec if the rat fell. Time to fall was the time spent on the bridge before falling, and was recorded as 120 sec if the rat escaped to one of the platforms. If the rat remained on the bridge for 120 sec but did not escape or fall, the time to escape and to fall were both recorded as 120 sec. Three flat bridges, 6, 4, and 2 cm

in width, and one round bridge, 2 cm in diameter, were used. The order of testing for each day was: 6 cm, 4 cm, 2 cm, round.

Turning on an inclined grid. Each rat was placed on a wire grid and the grid was gently rotated until the rat was facing downward at a 45° angle. The time for the rat to turn completely around, facing upright, was recorded.

Falling from a wire. Each rat was placed on a horizontal metal wire 2 mm in diameter, hanging by his front paws 60 cm over a foam cushion. The time to fall from the wire was recorded.

Data analysis

All data analyses were conducted with SYSTAT statistical software (SYSTAT, Inc., Evanston, IL).

Place discrimination. For the place discrimination, the mean of each measure of performance from platform trials was taken across Trials 1–5 in each session for each rat, yielding 5 values for each measure for each rat. The single value for each measure of probe trial performance was used in the data analysis.

An age effect was assessed by repeated-measures ANOVA on each measure of performance from control rats (**24MO-CON** vs. **4MO-CON**).

Drug effects on place discrimination performance were analyzed in separate ANOVAs for each drug. A one-way repeated-measures ANOVA was performed on data from place discrimination performance, comparing each drug group to **24MO-CON** rats. The independent variable was the drug condition, the criterion variable was one of the measures of performance, and the repeated measure was the session (Session 1–5, or Sessions 3–5 only). Post hoc ANOVAs compared each single dose with the control condition to determine if specific doses differed from the control.

Repeated acquisition. For repeated acquisition, the mean of each measure of performance from platform trials was taken across Sessions 1–5 for each rat, yielding 5 values for each measure for each rat. Repeated acquisition data was analyzed in a similar fashion for each independent variable and criterion variable. The repeated measure was trials (Trial 1 through Trial 5) for platform trial measures, and sessions for probe trial measures (Sessions 1 through 5).

Sensorimotor skills. The mean score from sensorimotor tasks was taken across the three days of sensorimotor testing; each rat provided one data point for each task. Data were analyzed for each task in ANOVAs identical to those employed to analyze the water maze data (except that repeated measures were not necessary).

Biochemical Assays

Acetylcholine release assay

Tissue preparation and release assays were performed as described previously [Nickolson et al., 1990]. Briefly, male Wistar rats (Charles River Laboratories) were euthanized by decapitation and specific brain areas were immediately dissected. Brain areas dissected were chopped into 0.25×0.25 cm^2 squares using a McIlwain tissue chopper (Brinkmann Instruments, Inc., Westbury, NY). Approximately 100 mg of the slices were transferred to 10 ml of Krebs-Ringer (KR) solution, made up of 116 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM Na_2SO_4, 25 mM $NaHCO_3$, and 11 mM glucose containing 10 nmol choline chloride labeled with 10 μCi of [^{3}H]choline chloride (80 Ci/mM; Du Pont NEN, Boston, MA). The preparation was allowed to incubate for 30 min under an atmosphere of 95% O_2:5% CO_2. After the incubation period, the slices were washed three times with fresh KR buffer and an aliquot of the slices (approximately 10 mg) was transferred to perfusion chambers of a Brandel FS-18 superfusion apparatus (Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). The slices were superfused with oxygenated KR solution at a rate of 0.25 ml/min for 10 min before fractions of the effluent were taken. Hemicholinium-3 (10 μM) was added to the superfusion medium to inhibit reuptake of [^{3}H]choline during the release assay. After the 10-min washout period, fractions were collected in 4-min intervals (1.0 ml fractions) directly into scintillation vials; at the end of experiments, the chambers were emptied into scintillation vials and residual radioactivity was extracted from the slices in 1.0 ml of 0.1N HCl. Scintillation cocktail was subsequently added to the vials which were then assessed for radioactivity in a Packard 1900 TR scintillation counter (Packard Instrument Company, Meriden, CT).

A total of ten fractions were collected from each chamber during an experiment. Stimulated release was elicited by raising the KCl concentration from 5 to 25 mM for a period of 4 min (i.e., the period of one fraction) immediately before fraction 4 (S1) and fraction 8 (S2). Drugs, if added, were introduced at fraction 5 and remained in the superfusion media through fraction 8. Fractional release was calculated by dividing the radioactivity (dpm) found in each fraction by the total radioactivity in the tissue at the start of the experiment and is expressed in percent. S1 is defined as the fractional release found in fraction 4 plus fraction 5 minus the fractional release found in fraction 3 plus fraction 6; S2 is defined as the fractional release

found in fraction 8 plus fraction 9 minus the fractional release found in fraction 7 plus fraction 10.

Acetylcholinesterase assay

Male Wistar rats (Charles River) were sacrificed and decapitated, brains were removed, and the frontoparietal cortex was dissected. Cortical tissue was homogenized at 0°C in 50 volumes of KR medium with eight up-and-down strokes in a teflon-in-glass homogenizer at 800 rpm. The KR medium had been equilibrated with 95% O_2/5% CO_2.

Assessment of acetylcholinesterase activity was performed as described previously [Nickolson et al., 1990]. Incubation was performed in glass scintillation vials. To 1 ml of KR medium, 0.05 ml of 2% (w/v) cortex homogenate and 0.01 ml of saline (0.9% NaCl in H_2O) or solutions containing linopirdine or X9121 at appropriate concentrations were added. Samples were then pre-incubated at room temperature for 30 min. After pre-incubation 0.01 ml of a solution containing 5 nCi [acetyl-^{3}H]-acetylcholine iodide (AChI; specific activity 90 mCi/mmol; Du Pont NEN) and 10^{-4} M unlabeled AChI (final concentration in sample 1 mM) was added. After 0 min (blanks) or 5 min, 0.4 ml of 10 mol acetic acid was added to stop the reaction and convert the formed ^{3}H-acetate into ^{3}H-acetic acid. Subsequently, 10 ml organic-base liquid scintillation fluid (Econofluor-1, Du Pont NEN), containing 20% (w/v) n-amyl alcohol, was added. The vials were then shaken vigorously to extract the ^{3}H-acetic acid into the organic phase and samples were counted in a Packard Tricard liquid scintillation counter (Packard Instrument Company).

Muscarinic receptor binding assay

Male Wistar rats (Charles River) were sacrificed by decapitation. Muscarinic receptor binding was performed as described previously [Nickolson et al., 1990]. Brain membranes were prepared by the method described by Tam [1983]. Whole brains were homogenized for 20 sec in 10 volumes of ice-cold 0.34 M sucrose with a Brinkmann Polytron (setting 8, Brinkmann Instruments, Inc.). The homogenate was centrifuged at 920g for 10 min at 4°C. The supernatant was centrifuged at 47,000g for 20 min. The resulting membrane pellet was washed by resuspension in 50 mM Tris buffer (pH 7.4) and centrifuged at 47,000g for 10 min. This washing step was repeated two more times. The resulting membrane pellet was resuspended in Tris/$MgCl_2$ buffer (100 ml/brain) containing 50 mM Tris HCl and 10 mM $MgCl_2$ (pH 7.4).

Aliquots (0.5 ml) of the membrane preparation were added to 0.5 ml Tris/$MgCl_2$ buffer containing unlabeled drugs and 0.5 nM (−)-[^{3}H]3-quinuclidinyl benzilate [(−)-QNB] (specific activity 33.1 Ci/mmol; Du Pont NEN). Nonspecific binding was measured in the presence of 1 mM atropine. After 45 min of incubation at room temperature, samples were rapidly filtered through Whatman GF/C glass-fiber filters under negative pressure and washed three times with ice-cold Tris $MgCl_2$ buffer (5 ml). The filters were placed in scintillation vials containing 5 ml Liquiscint scintillation fluid, mixed, allowed to stand for 2 h, and then counted in a Packard Tricard liquid scintillation counter (Packard Instrument Company).

Nicotinic receptor binding assay

Tissue was prepared as described above for muscarinic receptor binding and was resuspended in 50 mM Tris HCl buffer at pH 7.4 (50 ml/brain). Nicotinic receptor binding was performed as described previously [Nickolson et al., 1990].

Aliquots (0.5 ml) of the membrane preparation were added to 0.5 ml 50 mM Tris HCl (pH 7.0) buffer containing unlabeled drugs and 10 mM (−)-[^{3}H]nicotine (specific activity 75.7 Ci/mM; Du Pont NEN). Nonspecific binding was measured in the presence of 100 μM (−)-nicotine [Costa and Murphy, 1983]. After 40 min of incubation at room temperature, samples were filtered rapidly through Whatman GF/B glass fiber filters that had been presoaked in 0.1% polyethyleneimine for 2 h and then washed three times with ice-cold Tris buffer (5 ml). The filters were placed in scintillation vials containing 5 ml Liquiscint, mixed, allowed to stand for 2 h, and then counted in a liquid scintillation counter.

Data analysis

Potassium stimulated release of acetylcholine data was analyzed by ANOVA and Duncan's test. Acetylcholinesterase activity, muscarinic and nicotinic binding data was analyzed by Student's t-test. IC50 values were calculated by log-logit transformation with 95% confidence limits based on Fieller's Theorem (Schmidt and Tallarida, personal communication).

RESULTS

Behavioral Measures

Subjects

Of the 138 **24MO** rats ordered for the experiment, 10 were excluded before testing began because of cataracts, tumors, or other signs of poor health. Twelve died during testing; of these, one was a control rat, seven were receiving linopirdine, and four were receiving X9121. The mortality rates are there-

TABLE 2. Place Discrimination, Age Effects: 24MO-CON Compared to 4MO-CON

Measure	F-statistic	$P <$
Swim time	F(1,22) = 35.80	.0005
Swim distance	F(1,22) = 3.68	.068
Heading angle	F(1,22) = 7.97	.010
Annulus-40 time	F(1,22) = 45.71	.0005
Quadrant time	F(1,22) = 36.63	.0005
Platform crossing	F(1,22) = 34.32	.0005

fore: controls, 1/12, 8.3%; linopirdine, 7/60, 11.7%; X9121, 4/56, 7.1%. The mortality of Fischer-344 rats increases markedly at 18 months of age; this number of deaths in 24-month-old Fischer-344 rats is normal. No obvious toxicity was associated with either of the drugs tested.

Place discrimination

Age effects. Aging significantly impaired performance (**24MO-CON** rats performing worse than **4MO-CON** rats) on five measures of performance (Table 2).

Drug effects. For platform trials, the drugs did not alter performance. For probe trials, three doses of linopirdine (0.25, 2.5, and 8.5 mg/kg; 0.64, 7.4, and 25 μmol/kg) and two doses of X9121 (0.85 and 8.5 mg/kg; 2.1 and 24 μmol/kg) produced significant, or nearly significant, improvements when compared singly to **24MO-CON** rats (Table 3). X9121 generally had larger effects than linopirdine, as illustrated by a comparison of effect sizes (the statistic η^2, computed by dividing the sum of squares of effect by the total sum of squares in the ANOVA). For example, for the effect of the 8.5 mg/kg (25 and 24 μmol/kg for linopirdine and X9121, respectively) dose on annulus-40 time tested over all sessions, the η^2 for localization was 0.16; the η^2 for X9121 was 0.29.

A graph for a representative measure of performance, annulus-40 time, is presented for linopirdine (Fig. 2) and X9121 (Fig. 3).

Repeated acquisition

Age effects. Aging significantly impaired performance (**24MO-CON** rats performing worse than **4MO-CON** rats) on all behavioral measures (Table 4).

Drug effects. Linopirdine did not significantly improve performance in any of the conditions tested. A slight improvement occurred in the number of platform crossings for one dose of linopirdine: **24MO-996(2.5/7.4)** vs. **24MO-CON**: platform crossings, F(1,19) = 3.052, P = .097.

X9121 had an overall effect on swim time in an overall ANOVA: all **24MO-CON** and **24MO-9121** rats: swim time, F(5,57) = 2.560, P = .037, but subsequent analyses revealed that this effect was primarily due to differences between drug groups and not to differences between drug groups and control rats. An individual comparison of **24MO-9121(2.5/7.2)** with **24MO-CON** was marginally significant: **24MO-9121(2.5/7.2)** vs. **24MO-CON**: swim time, F(1,20) = 3.103, P = .093; annulus-40 time, F(1,20) = 3.289, P = .085; number of platform crossings; F(1,20) = 3.334, P = .083.

Sensorimotor skills

Age effects. An effect of age (**24MO-CON** rats performing worse than **4MO-CON** rats) was significant on six measures of sensorimotor skills (Table 5).

Drug effects. Neither drug altered sensorimotor skills (statistics not presented). The doses tested were the same as the doses tested in place discrimination and repeated acquisition.

Biochemical Measures

Acetylcholine release assay

Both linopirdine and X9121 enhanced K^+-stimulated release of acetylcholine from rat cerebral cortex in vitro (Fig. 4). Linopirdine enhanced acetylcholine release by approximately 290% at 10 and 100 μM. X9121 enhanced acetylcholine release by 363%, 477% and 328% at 1 mM, 100 μM, 10 μM, respectively. X9121 enhanced acetylcholine to a greater degree and over a broader range of concentrations than linopirdine (Fig. 4).

Acetylcholinesterase inhibition

Linopirdine was a very weak inhibitor of acetylcholinesterase in vitro in rats (Fig. 5). It inhibited acetylcholinesterase with an IC50 value of > 10,000 nM. This is > 100 times weaker than physostigmine which had an IC50 of 91 nM. X9121 had no effect on brain acetylcholinesterase.

Muscarinic and nicotinic cholinergic binding

Neither linopirdine nor X9121 had a significant effect on binding to muscarinic or nicotinic cholinergic receptors in vitro (Figs. 6, 7). Linopirdine and X9121 inhibited ^{3}H-QNB binding to muscarinic receptors with IC50s of >100,000 nM. This compares to an IC50 of 2.7 ± 0.4 nM for atropine. Linopirdine and X9121 inhibited ^{3}H-(−)-nicotine binding to nicotinic receptors with IC50s of >100,000 nM. This compares to an IC50 of 57 ± 16 nM for (−)-nicotine.

TABLE 3. Place Discrimination, Probe Trials: Some Doses of Linopirdine and X9121 Improved Performance[+]

Comparison with **24MO-CON**[a]	Annulus-40 time	Quadrant time	No. platform crossings
Linopirdine (DuP 996)			
All sessions			
24MO-996(.25/.64)	$P = .054$	$P = .091$	NS
24MO-996(2.5/7.4)	$P = .042$	$P = .068$	NS
24MO-996(8.5/25)	$P = .069$	$P = .068$	NS
Sessions 3–5 only			
24MO-996(2.5/7.4)	$P = .042$	$P = .058$	NS
24MO-996(8.5/25)	$P = .058$	$P = .066$	$P = .078$
X9121			
All sessions			
24MO-9121(0.85/2.1)	NS	NS	$P = .038$
24MO-9121(8.5/24)	$P = .010$	$P = .020$	$P = .016$
Sessions 3–5 only			
24MO-9121(8.5/24)	$P = .009$	$P = .036$	$P = .092$

[+]The numbers in parentheses indicate the doses in mg/kg and μmol/kg, respectively.
[a]Results are presented only for those comparisons in which differences at least approached statistical significance. NS = not significant ($P > 0.1$).

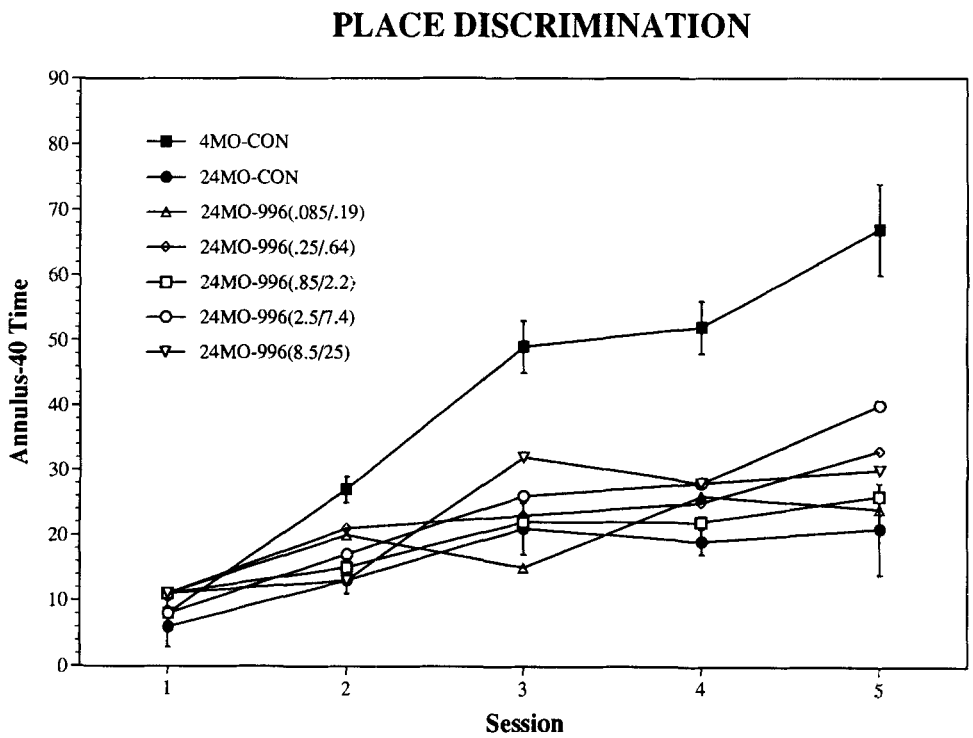


Figure 2. Linopirdine (DuP 996) effects on annulus-40 time in the place discrimination. The symbols represent the mean value; the vertical lines represent standard error of the mean (s.e.m.). The numbers in parentheses are the doses in mg/kg and μmol/kg, respectively. Drugs are given po 30 min before each session.

DISCUSSION

Aging significantly impaired place discrimination, repeated acquisition, and sensorimotor skills. These behavioral tasks assessed two cognitive abilities: spatial memory in the place discrimination and recent memory in repeated acquisition, as well as sensorimotor skills. Young **4MO-CON** rats learned the discriminations quickly and performed well in all

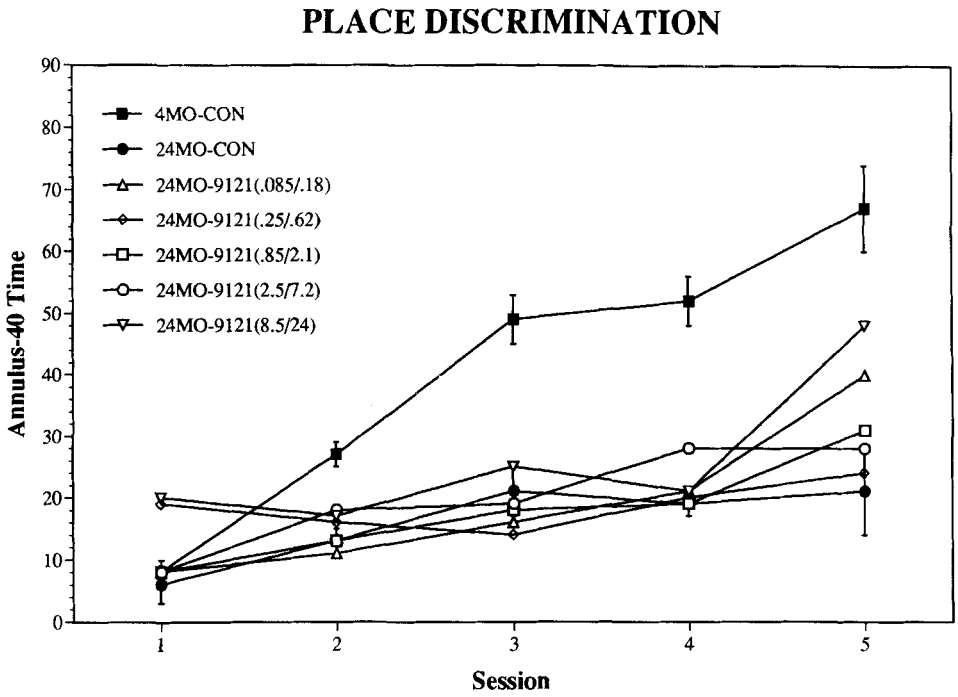


Figure 3. X9121 effects on annulus-40 time in the place discrimination. The symbols represent the mean value; the vertical lines represent standard error of the mean (s.e.m.). The numbers in parentheses are the doses in mg/kg and μmol/kg, respectively. Drugs are given po 30 min before each session.

TABLE 4. Repeated Acquisition, Age Effects: 24MO-CON Compared to 4MO-CON

Measure	F-statistic	$P <$
Swim time	F(1,22) = 24.44	.005
Swim distance[a]	F(4,88) = 7.91	.0005
Heading angle	F(1,22) = 4.58	.045
Annulus-40 time	F(1,22) = 31.60	.0005
Platform crossings	F(1,22) = 20.43	.0005

[a]Age × trial interaction.

TABLE 5. Sensorimotor Skills, Age Effects: 24MO-CON Compared to 4MO-CON

Measure	F-statistic	$P <$
Time to		
Turn in alley	F(1,22) = 5.36	.030
Fall from 6 cm bridge	F(1,22) = 7.48	.012
Fall from 4 cm bridge	F(1,22) = 11.80	.002
Escape from 2 cm bridge	F(1,22) = 7.51	.012
Fall from 2 cm bridge	F(1,22) = 136.87	.0005
Fall from round bridge	F(1,22) = 8.71	.007

tasks. All these tasks were very sensitive to age-related behavioral impairments as indicated by the impaired performance of **24MO-CON** rats as compared to **4MO-CON** rats, and are in accordance with other studies indicating age-related behavioral deficits [Gage et al., 1984; Gallagher and Burwell, 1989]. Consequently, these procedures are appropriate to assess the beneficial effects of potential cognitive enhancers.

Linopirdine and X9121 enhanced place discrimination at several of the doses. The effects were most pronounced in the probe trials at the end of the place discrimination. X9121, as compared to linopirdine, produced greater improvement in place discrimination performance. These results, in conjunction with others showing that these drugs can improve performance in a variety of different tasks [Cook et al., 1990; Brioni et al., 1993], suggest that both of these drugs may have significant potential as cognitive enhancers; X9121 may be more potent than linopirdine.

Repeated acquisition was only marginally affected by the two drugs. In conjunction with the results showing greater effects during the last sessions of place discrimination as compared to the initial sessions, this pattern of results suggests that the two drugs are more effective at increasing the strength of long-term memories, rather than initial acquisition of new information. Asymptotic performance on the place discrimination is usually reached by aged rats

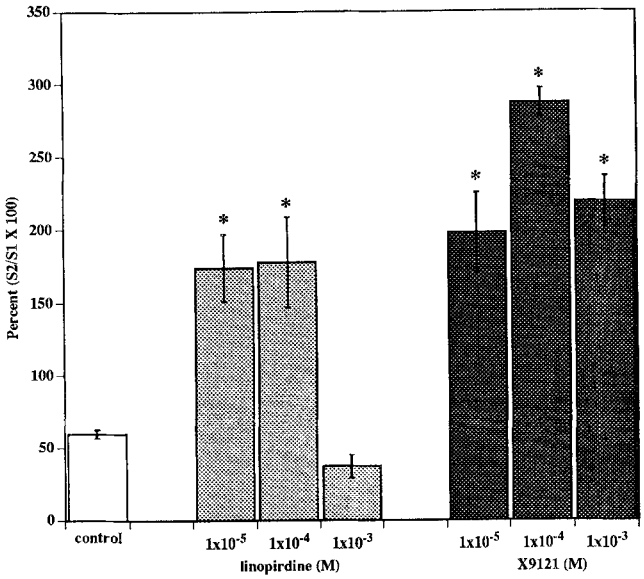


Figure 4. Linopirdine and X9121 effects on 25 mmol K^+-evoked release from rat cerebral cortical slices in vitro. Bars represent the mean value; vertical lines represent the standard error of the mean (s.e.m.). *Significant difference from control, $P<.05$ (ANOVA, post-hoc Duncan test).

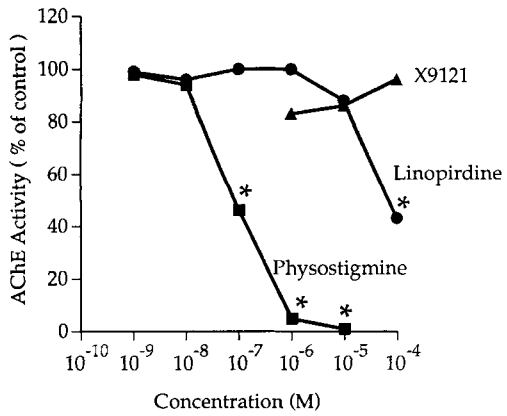


Figure 5. Effects of physostigmine, linopirdine, and X9121 on acetylcholinesterase activity in rat cerebral cortex homogenate. IC50 values for physostigmine, linopirdine, and X9121 are 91 nM, >10,000 nM, and >100,000 nM, respectively. Data represent the means of 4 determinations. *$P<0.001$ (Student's t-test).

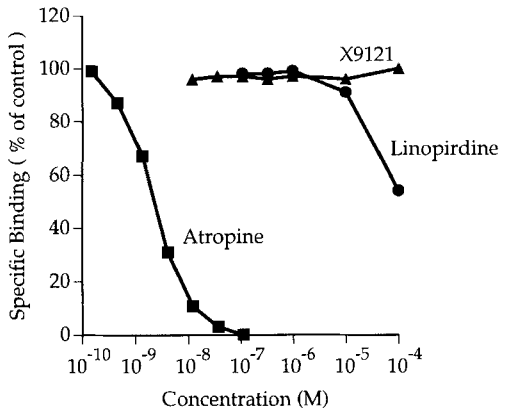


Figure 6. Effects of atropine, linopirdine, and X9121 on [^{3}H](−)-QNB binding to muscarinic cholinergic receptors in rat brain membranes. IC50 values are 2.7 ± 0.4 nM for atropine and >100,000 nM for linopirdine and X9121. Data represent means of 3–4 experiments in duplicate.

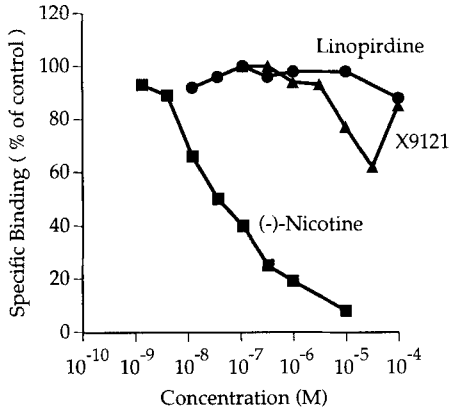


Figure 7. Effects of (−)-nicotine, linopirdine, and X9121 on [^{3}H](−)-nicotine binding to rat forebrain membranes. The IC50 values are 57 ± 16 nM for nicotine and >100,000 nM for linopirdine and X9121. Data represent means of 3–4 experiments in duplicate.

after 9 or 10 sessions (Markowska, personal communication). Further experiments with more sessions of place discrimination could determine if the effects of linopirdine and X9121 become more pronounced with long-term stable memories as testing progresses.

Both linopirdine and X9121 enhanced K^+-stimulated release in rat cerebral cortical tissue in vitro. X9121 produced a greater degree of release and over a broader dose range than linopirdine. This may account for the greater improvement in place discrimination performance seen with X9121. Linopirdine is a weak acetylcholinesterase inhibitor, approximately 1,000 times less active than physostigmine. This weak activity and the fact that X9121 had a greater effect in place discrimination and was not active as an acetylcholinesterase inhibitor makes it extremely unlikely that the behavioral activity of linopirdine is the result of acetylcholinesterase inhibition. Since neither compound bound significantly to either muscarinic or nicotinic cholinergic receptors, it is unlikely that the behavioral effects could be the result of cholinergic agonist activity.

In summary, linopirdine and X9121 had moder-

ate effects in aged rats. The most pronounced effects were the enhancement of long-term reference memory as reflected in the place discrimination. The likely mechanism for this effect is the enhancement of stimulation induced acetylcholine release. These compounds show promise as treatments of cognitive deficits in aged individuals.

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