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Comparison of DuP 996, With Physostigmine, THA and 3,4-DAP on Hypoxia-Induced Amnesia in Rats

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DENOBLE, V. J., K. F. DENOBLE, K. R. SPENCER, L. C. JOHNSON, L. COOK, M. J. MYERS AND R. M. SCRIBNER. *Comparison of DuP 996, with physostigmine, THA and 3,4-DAP on hypoxia-induced amnesia in rats.* PHARMACOL BIOCHEM BEHAV **36**(4) 957–961, 1990.—DuP 996, 3,3-bis(4-pyrindinylmethyl)-1-phenylindolin-2-one, physostigmine (PH), tetrahydroaminoacridine (THA) and 3,4-diaminopyridine (3,4-DAP) were compared for their ability to protect against hypoxia-induced performance deficits in a passive avoidance (PA) task. The ability to retain PA response was found to decrease as the oxygen concentration decreased with the largest retention deficit occurring at 6.5% oxygen. DuP 996 (0.01–0.1 mg/kg SC), 3,4-DAP (0.1–10.0 mg/kg SC), THA (0.3–5.0 mg/kg SC) and PH (0.001–0.1 mg/kg SC) administered one minute after PA training produced dose-dependent increases in retention latencies following exposure to 6.5% oxygen. In comparing each compound for side effects, DuP 996 induced tremor and mortality at 10 and 40 mg/kg SC, respectively, and PH at 0.3 and 0.8 mg/kg SC, respectively. With PH the 0.3 mg/kg SC dose also produced hypersalivation and a decrease in lift strength. THA produced tremor and mortality at 6.0 and 40 mg/kg SC, respectively, and 3,4-DAP at 50 and 200 mg/kg SC, respectively. 3,4-DAP also produced chromodacryorrhea and hypersalivation at 50 mg/kg SC. Dividing the dose necessary to produce mortality by the highest effective dose active in the hypoxia test yielded a safety ratio for DuP 996 of 400, for 3,4-DAP 20, for PH 8, and for THA 8, showing a greater safety margin for DuP 996 than the other cholinergic agents. These results suggest that DuP 996 may be of use in the treatment of diseases associated with cognitive impairment and may have a greater safety margin than other cholinergic agents.

DuP 996 Physostigmine Tetrahydroaminoacridine 3,4-Diaminopyridine Amnesia Hypoxia Safety ratios Rats

ALZHEIMER'S DISEASE (AD) and senile dementia are generally characterized by a reduction of cortical cholinergic activity. This has led to an experimental focus on the basal forebrain cholinergic system as the major source of neurochemical and neuroanatomical substrates mediating age-related memory loss. As a result a number of drugs that increased brain cholinergic activity have been used clinically to treat various neurological disorders resulting in cognitive impairment (14). Attempts to increase acetylcholine (ACh) synthesis by treatment with ACh precursor choline or lecithin have not been very successful. More recent research focused upon the use of muscarinic agonists, or cholinesterase inhibitors (AChE) such as physostigmine (PH) and tetrahydroaminoacridine (THA), both of which increase ACh by inhibiting its metabolic degradation after release. Recent reports of limited success in reducing the cognitive deficits and symptoms of dementia in AD by treatment with AChE inhibitors such as PH (7,8) and THA (17,19) have renewed interest in the search for other cholinomimetic agents that have similar mechanisms of action. However, the potential therapeutic application of AChE inhibitors is limited by several factors. The most predominant one being side effects resulting from chronic postsynaptic stimulation.

As an alternative to inhibiting the AChE activity resulting in increased ACh with abnormal enzymatic degeneration, a search was undertaken to identify ACh release enhancers. A novel compound, 3,3 - bis(4-pyridinylmethyl) - 1 - phenylindolin - 2 - one (DuP 996) has been shown to enhance stimulated ACh release from rat cortical, hippocampal and striatal slices in vitro and from the cortex of awake freely moving rats in vivo without changes in AChE activity (15). In the present experiment a direct comparison of DuP 996 with PH, THA and 3,4-DAP on hypoxia-induced passive avoidance (PA) amnesia was made with reference to a side effect safety profile.

METHOD

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Kingston, NY) weighing 150–180 g were used. The animals were housed four per cage (26.0 W × 45.0 L × 20.0 H cm) with free access to food and water. They were maintained on a 12-hr light/dark cycle (lights on from 0600 to 1800 hr) at a room temperature of 22 ± 1°C with a relative humidity of 50 ± 10%. In each experiment rats were used once.

Apparatus

The experimental sessions were conducted in a two-compartment PA box. One compartment, made of clear plastic with a perforated clear plastic floor, measured 21(L) × 24.5(H) × 17(W) cm and was illuminated with a 60-watt incandescent light bulb placed 36 cm above the floor of the PA box. The other compartment, made of black plastic, measured 30.5(L) × 20.3(H) × 21.5(W) cm with a floor made of 4 mm stainless steel rods spaced 1.2 cm apart. A Coulbourn Instruments Grid Floor Shocker was connected to the steel rods providing a scrambled footshock. The two compartments were separated by a solenoid-operated slide door (Lafayette Instrument Co., Lafayette, IN). A Coulbourn Instruments Electronic Counter, activated by the opening or closing of the slide door, recorded acquisition and retention latencies. These latencies were defined as the time, in seconds, it took an animal to enter (all four paws on the grid floor) the dark compartment.

For memory disruption, rats were exposed to an hypoxic environment for 30 min immediately prior to PA training. The hypoxia chamber was constructed of clear plastic, measured 32.5(L) × 22.5(H) × 23(W) cm, and was continuously perfused with a gas mixture of oxygen and nitrogen. The flow rate was adjusted such that the gas turnover in the chamber was 15 liters per min. To determine the effects of different oxygen concentrations on PA retention, the rats were exposed to gas mixtures containing different percentages of oxygen (21%, 10%, 9%, 8%, 7% or 6.5%) supplemented with nitrogen for 30 min prior to PA training. Oxygen concentrations were continuously monitored in the hypoxia chamber with an oxygen sensor (Sensitron, Inc., Reading, PA).

Passive Avoidance Training

PA training began by placing the rat into the clear compartment of the two-compartment PA box. Following a 10-sec delay, the slide door was raised, providing access to the dark compartment. Once the rat moved completely into the dark compartment, the slide door was lowered and, after a 10-sec delay, a 1.5 mA inescapable shock was applied to the grid floor for 3 sec. This was followed by an additional 10-sec period at the end of which the rat received another 3-sec shock (1.5 mA). The rats were immediately removed from the dark compartment after receiving the second shock, injected with vehicle or test compound and returned to their home cage. Rats not entering the dark compartment within 90 sec were removed from the study. Of the animals tested, 3% were removed from the study for not entering the dark compartment within the allotted time (90 sec) during acquisition training.

A retention test was given 4 hr later. It proceeded in the same manner as the training session except no shock was applied to the grid floor when the rats entered the dark compartment. During the retention test, the rats were provided access to the dark compartment for 300 sec.

To determine if active doses increased entry latencies by altering the rats' ability to enter the dark chamber during the retention test, the highest effective dose of each compound was administered to separate groups of nonshocked rats. Entry latencies were measured 4 hr later.

Side Effect Profile

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 125–150 g were used. Rats were fasted 17 hr overnight and then administered randomized, coded doses of test compound or vehicle control subcutaneously and were scored by a trained observer at 0.25, 0.5, 1, 2, 4, 6 and 24 hr after drug administration. Each rat was scored for hypersalivation (excessive salivation, wetting the fur around the mouth), tremor (whole-body shaking), chromodacryorrhea (reddish-brown tears), block of the grip strength and lift reflexes (ability to grip a string with both forepaws and lift the hindpaws to the string). Doses were gradually increased until mortality was observed in two rats within each dose group. All doses were evaluated in 10 rats with each rat being used only once.

Drug Preparation and Administration

DuP 996 was synthesized at DuPont, PH (Sigma Chemical Co., St. Louis, MO), THA (Aldrich Chemical Co., Milwaukee, WI) and 3,4-DAP (Aldrich Chemical Co., Milwaukee, WI) were dissolved in 0.85% w/v saline and administered (SC) in a volume of 1 ml/kg of body weight one min after PA training. All doses were calculated as the free base.

Data Analysis

Overall significance was calculated using the Kruskall-Wallis one-way analysis of variance (ANOVA). Post hoc, median retention latencies were compared for vehicle controls and each treated group with a Mann-Whitney U-test. Data for different doses were accumulated over several days with both nonhypoxic and hypoxic vehicle-treated control groups included with each day's testing. Interquartile ranges were calculated and are included in the figure captions.

RESULTS

The median latency to enter the dark chamber following hypoxia decreased as the oxygen concentration in the hypoxia chamber decreased (Fig. 1). That is at 21%, 10% and 9% oxygen the latencies were at a maximum 300 sec. However, the retention latencies of rats after being exposed to 8, 7 and 6.5% oxygen for 30 min before PA training were reduced to 167, 130 and 43 sec, respectively. At the 6.5% oxygen level all animals survived and rats entered the dark chamber during the training session within 40 sec after the slide door opened. At a lower oxygen concentration (6%), however, some mortality occurred, and some surviving animals were motor impaired and failed to enter the dark chamber during the training session.

Posttraining administration with DuP 996 attenuated the hypoxia-induced amnesia in the rats with retention latencies for the groups dosed with 0.01, 0.03, 0.05 and 0.1 mg/kg SC differing statistically from vehicle-treated rats. The dose of 0.01 mg/kg SC was the peak effective dose (PED) producing a 378% increase in retention latency above vehicle control levels (Fig. 2). Similar dose-dependent increases in retention latencies were seen with the nonspecific ACh release enhancer 3,4-DAP (0.1 to 20.0 mg/kg SC) with the PED of 0.3 mg/kg SC producing a 356% increase in the median retention latency (Fig. 3). At the highest dose tested (20.0 mg/kg SC) ataxia and hypoactivity were observed during the training session suggesting that the increased latencies may be a result of nonspecific drug effects. Physostigmine also protected against the hypoxia-induced amnesia. At doses of 0.001 to 0.1 mg/kg SC there was a significant increase in entry latencies during retention testing. The PEDs (0.001 and 0.03 mg/kg SC) produced a 339% increase in retention latencies compared to control values (Fig. 4). An increase in entry latency after hypoxia treatment was also observed in animals dosed with THA at doses ranging from 0.3 to 7.0 mg/kg SC. The peak effect dose (5.0 mg/kg) produced a 415% increase in entry latency (Fig. 5). With DuP 996, 3,4-DAP

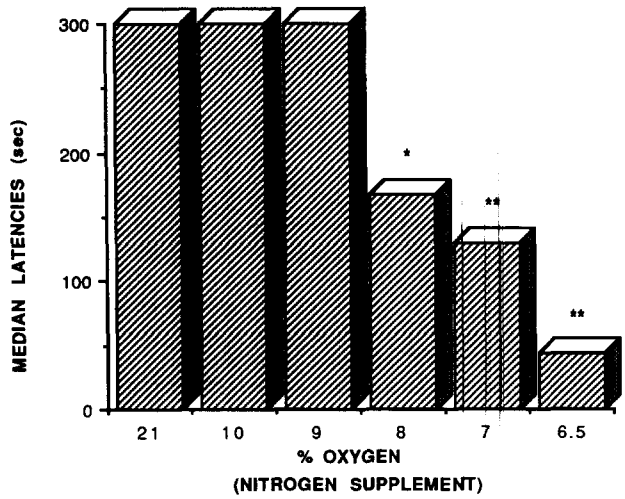

FIG. 1. The median passive avoidance (PA) retention latencies of rats is shown as a function of percent oxygen. Rats were exposed for 30 min to various concentrations of oxygen immediately prior to PA training (normal air contains 21% oxygen). PA retention was measured 4 hr later. Each bar represents the median retention latency (sec) obtained for a minimum of 15 rats with a maximum 300-sec cutoff. The interquartile range of the retention latencies (Q1 and Q3) obtained from the groups exposed to 21 to 6.5% oxygen were 300 and 300 sec for 21, 10 and 9% oxygen, and 60 and 182 sec for 8%; 12 and 158 sec for 7%, and 4 and 63 sec for the 6.5% group. *Significantly different from 21% oxygen, $p<0.05$ (Mann-Whitney U-test). **Significantly different from 21% oxygen, $p<0.025$ (Mann-Whitney U-test).

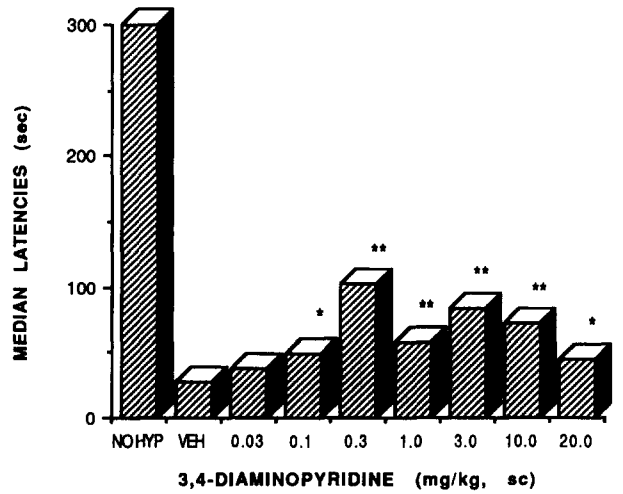

FIG. 3. Effect of 3,4-diaminopyridine on passive avoidance retention latencies following 30-min exposure to a 6.5% oxygen environment. Each bar represents the median retention latencies (sec) obtained for a minimum of 12 rats with a maximum 300-sec cutoff. The interquartile range of the retention latencies (Q1 and Q3) for the vehicle-treated group and proceeding up through the 20.0 mg/kg SC dose were: 8 and 46, 31 and 102, 36 and 153, 44 and 108, 51 and 120, 73 and 137, 40 and 124, and 21 and 143 sec, respectively. *Significantly different from vehicle, $p<0.05$ (Mann-Whitney U-test). **Significantly different from vehicle, $p<0.025$ (Mann-Whitney U-test).

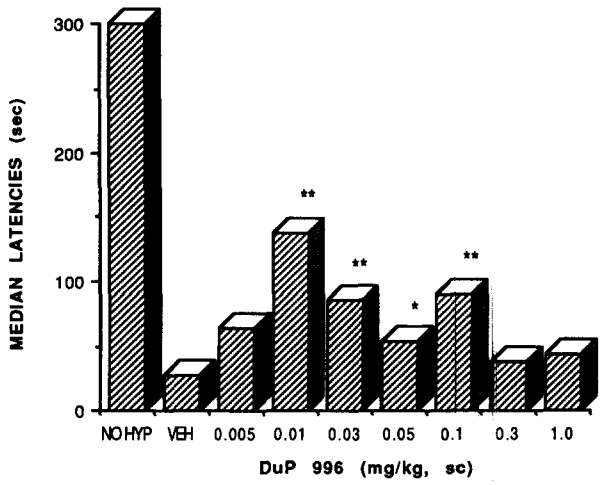

FIG. 2. Effect of DuP 996 on passive avoidance retention latencies following 30-min exposure to a 6.5% oxygen environment. Each bar represents the median retention latencies (sec) obtained for a minimum of 12 rats with a maximum 300-sec cutoff. The interquartile range of the retention latencies (Q1 and Q3) for the vehicle-treated group and proceeding up through the 1.0 mg/kg SC dose were: 6 and 52, 9 and 90, 75 and 193, 43 and 137, 37 and 125, 86 and 162, 10 and 49, and 15 and 61 sec, respectively. *Significantly different from vehicle, $p<0.05$ (Mann-Whitney U-test). **Significantly different from vehicle, $p<0.025$ (Mann-Whitney U-test).

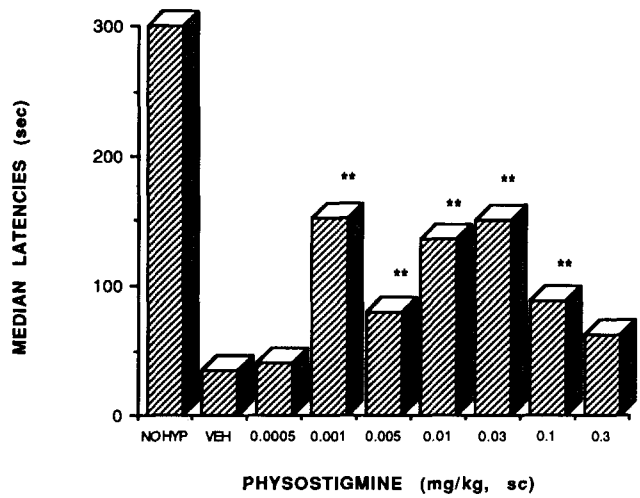

FIG. 4. Effect of physostigmine on passive avoidance retention latencies following 30-min exposure to a 6.5% oxygen environment. Each bar represents the median retention latencies (sec) obtained for a minimum of 12 rats with a maximum 300-sec cutoff. The interquartile range of the retention latencies (Q1 and Q3) for the vehicle-treated group and proceeding up through the 0.3 mg/kg SC dose were: 11 and 61, 9 and 34, 107 and 230, 64 and 128, 110 and 192, 65 and 204, 71 and 139, and 4 and 73 sec, respectively. *Significantly different from vehicle, $p<0.05$ (Mann-Whitney U-test). **Significantly different from vehicle, $p<0.025$ (Mann-Whitney U-test).

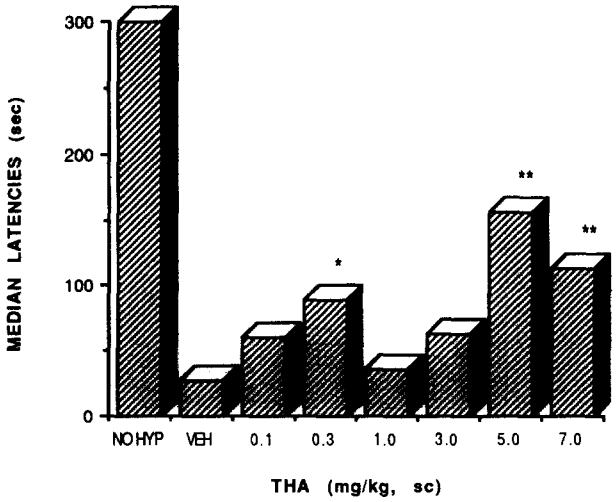

FIG. 5. Effect of THA on passive avoidance retention latencies following 30-min exposure to a 6.5% oxygen environment. Each bar represents the median retention latencies (sec) obtained for a minimum of 12 rats with a maximum 300-sec cutoff. The interquartile range of the retention latencies (Q1 and Q3) for the vehicle-treated group proceeding up through the 7.0 mg/kg SC dose were: 8 and 50, 3 and 28, 21 and 102, 3 and 35, 30 and 109, 75 and 217, and 50 and 190 sec, respectively. *Significantly different from vehicle, $p<0.05$ (Mann-Whitney U-test). **Significantly different from vehicle, $p<0.025$ (Mann-Whitney U-test).

and PH the entry latencies first increased, then decreased, as the dose was increased producing an inverted U-shaped dose-response curve. However, the dose effect function for THA was bimodal (Fig. 5).

The increased latencies with DuP 996 and PH were not a result of nonspecific effects of the compounds on behavior in general (e.g., producing ataxia or nyctophobia), since the nonshocked control animals injected with the highest dose of each compound entered the dark chamber during the retention test with median latencies of less than 18 sec (DuP 996 and PH). However, the highest dose of THA (7 mg/kg SC) and 3,4-DAP (20.0 mg/kg SC) did alter retention latencies in the nonshocked controls (median 125 sec and 146 sec, respectively) suggesting a nonspecific drug effect at these doses. However, the next lowest dose of each compound did not have these effects, and the median latencies were 5 sec and 13 sec for THA and 3,4-DAP, respectively.

In the side effect profile tests DuP 996 induced tremor at 10 mg/kg SC in rats. As the dose was increased, DuP 996 did not induce hypersalivation, chromodacryorrhea or alter lift grip strength. However, mortality was observed at 40 mg/kg SC. With 3,4-DAP, tremor, chromodacryorrhea, and hypersalivation were observed at 50 mg/kg SC, a decrease in lift strength at 25 mg/kg SC and mortality at 200 mg/kg SC. Physostigmine induced tremor at 0.3 mg/kg SC which was accompanied by hypersalivation and a decrease in lift strength and produced mortality at 0.8 mg/kg SC. THA at 6.0 mg/kg SC produced tremor and at 10 mg/kg, all other acute toxicity signs were observed. At a dose of 40 mg/kg SC mortality was observed. With all test compounds, mortality was found within the first hour after compound administration. Using the minimum dose that produced mortality and dividing it by the maximum or minimum effective dose in the hypoxia test revealed safety ratios for DuP 996 of 400 and 4000; 3,4-DAP 20 and 2000; PH 8 and 800; THA 8 and 133 (Table 1).

DISCUSSION

The main result from the present research is that DuP 996, a compound that enhances the depolarization-induced release of ACh, dopamine (DA) and serotonin (5-HT) from rat cortex, hippocampus and caudate nucleus in vitro and ACh from rat cortex in vivo (15), protects against hypoxia-induced amnesia of a PA response. Previous investigators have shown that exposure to hypoxic conditions before and/or after PA training impairs retention in rats when memory is tested either 4 or 24 hr later (1, 10, 16, 18). In the present experiment retention was tested 4 hr after training and hypoxia had similar effects to those previously reported with longer retention intervals. Specifically, in the present study, exposure to hypoxia produced a profound amnesia for the PA response with the median entry latency varying directly as a function of the chamber's oxygen concentration.

Posttraining treatment with DuP 996 (0.01–0.1 mg/kg SC), 3,4-DAP (0.1–10.0 mg/kg SC), PH (0.001–0.1 mg/kg SC) and THA (0.3–5.0 mg/kg SC) prevented the hypoxia-induced PA retention deficit. While the effect of cholinergic drugs on hypoxia-induced behavioral deficits (tight rope test scores) has been documented (12), this is the first report of cholinergically acting drugs preventing an hypoxia-induced amnesia of a single trial PA test. Although the mechanism by which DuP 996, PH, THA and 3,4-DAP exert their effects against hypoxia is not known, it is well

TABLE 1

CHOLINERGIC TOXICITY SIGNS IN RATS TREATED WITH TEST COMPOUNDS

	Minimum Effective Dose (MED)[1] mg/kg SC						Safety Ratio Range*	
Compound	Hypersalivation	Lift	Grip	Chromodacryorrhea	Tremor	Mortality	A	B
DuP 996	N.O.	N.O.	N.O.	N.O.	10.0	40.0	400–4000	
3,4-DAP	50.0	25.0	N.O.	50.0	50.0	200.0	20–2000	
PH	0.3	0.3	N.O.	N.O.	0.3	0.8	8–800	
THA	10.0	10.0	10.0	10.0	6.0	40.0	8–133	

[1]Each dose was evaluated in 10 rats.

MED = minimum dose at which overt symptomatology was observed in at least two rats.

*Safety ratio was calculated by dividing the dose of each compound that produced mortality by the maximum effective dose (column A) or the minimum effective dose (column B) that had significant effects in protecting against the hypoxia-induced PA deficit.

N.O. — not observed.

documented that hypoxia induces alterations in neurotransmitter function which are directly dependent upon oxygen for synthesis. For example, norepinephrine, dopamine and epinephrine are synthesized from the combination of tyrosine and oxygen, and serotonin is synthesized from tryptophan and oxygen. Tyrosine hydroxylation is impaired when the oxygen concentration is reduced in brain striatal synaptosome preparations, suggesting that the availability of oxygen can regulate the synthesis of catecholamines in brain (4–6). While exposure to hypoxia does not represent a model of AD or dementia, exposure to hypoxia may selectively effect neurons that store neurotransmitters that utilize oxygen for synthesis. It would appear that increasing activity in the cholinergic nervous system is sufficient to ameliorate, at least in part, the consequence of hypoxia on behavior.

Considerable literature supports the role of the central cholinergic system as being involved in mediating cognitive processes (14). For example, the anticholinergic drug scopolamine impairs learning and memory, but the quaternary scopolamine methylbromide, which is active peripherally, has no effects on performance (11). Conversely, the AChE inhibitor PH can reverse scopolamine-induced deficits (2), enhance suboptimal performance in young and aged primates (3) and improve performance of rats with nucleus basalis lesions (13). DuP 996 is a novel compound in that it is a potent enhancer of stimulation-induced release of ACh in vitro and in vivo (15), but it does not increase the baseline level of ACh release. DuP 996 is active in tests where PH, THA and 3,4-DAP are also active; however, a comparison of a side effect profile of DuP 996 and these other cholinergic compounds showed that DuP 996 has a wider safety ratio between doses producing cholinergic side effects and doses effective in protecting against hypoxia-induced amnesia. The present data suggest that DuP 996, a compound that enhances stimulated release of ACh, may be useful in the treatment of memory disruption induced by hypofunction of the cholinergic nervous system.

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