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Antagonism of anticholinesterase (DFP) toxicity by donepezil plus scopolamine: a preliminary study☆

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Abstract

Studies in animals exploring the antagonism of the cholinesterase inhibitors soman and sarin have shown that pretreatment with low doses of the centrally acting cholinesterase inhibitor, physostigmine, alone or in conjunction with the centrally acting anticholinergic agent, scopolamine, is effective against their lethality and toxicity. The current study evaluated the effects of pretreatment with the oral anticholinesterase agent, donepezil (Aricept, 2.0 mg/kg), used to treat Alzheimer's disease, with and without scopolamine in decreasing the hypothermic, hypokinetic, and diarrhea-inducing effects of the irreversible long-acting cholinesterase inhibitor diisopropyl fluorophosphate (DFP, 1.0 mg/kg) in adult Flinders sensitive line (FSL) male rats. Donepezil alone and donepezil plus scopolamine (0.1 mg/kg) to a greater extent antagonized the decrease in temperature, hypoactivity, and induction of diarrhea due to DFP observed at 4 h after its administration. However, donepezil alone induced hypothermia at 1 and 2 h after treatment. Therefore, these preliminary findings are encouraging, but many additional studies are needed to establish the effectiveness of donepezil as a prophylactic agent against irreversible cholinesterase inhibition by DFP.

Keywords: Diisopropyl fluorophosphate, DFP; Donepezil; Acetylcholine; Scopolamine; Cholinesterase inhibitors; Flinders rats; Hypothermia; Hypoactivity; Diarrhea; Sarin; Soman

1. Introduction

Organophosphorus cholinesterase inhibitors [i.e., diisopropyl fluorophosphate (DFP), sarin, soman, VX, and diazinon] exert their effects by inhibiting acetylcholinesterase (AChE), the major enzyme in the metabolism of acetylcholine (ACh) in the central nervous system and in the skeletal and smooth muscle (Taylor, 1996). Subsequent increases in ACh lead to central and peripheral muscarinic and nicotinic receptor stimulation, as well as effects on NMDA and other neuroactive neurotransmitter systems (Solberg and Belkin, 1997). Cholinergic hyperstimulation may cause lassitude, lethargy, seizures, salivation, nausea, weakness, bronchoconstriction, vomiting, muscle paralysis, respiratory paralysis, diarrhea, hypotension, hypertension, bradycardia, miosis, and ultimately death (Heath, 1961; Taylor, 1996).

The conventional pharmacological treatment of AChE inhibitor poisoning consists of administration of an anticholinergic (i.e., antimuscarinic) agent, atropine sulfate, and the adjunctive use of a cholinesterase reactivator such as pralidoxime (Heath, 1961; Taylor, 1996; Volans, 1996). However, a number of animal studies have suggested that more centrally effective anticholinergic agents (i.e., scopolamine, trihexyphenidyl, and benztropine) are more effective than atropine on a milligram per kilogram basis or a molar basis in preventing AChE inhibitor (i.e., sarin, soman, and physostigmine) induced effects (Anderson et al., 1991, 1994; Janowsky, 2002; Janowsky et al., 1985, 1986; Lallement et al., 2001; Leadbeater et al., 1985).

Beside the utilization of anticholinergic agents and the use of AChE reactivators, another strategy used to treat irreversible AChE inhibitor toxicity has been to pretreat animals with relatively low, nonlethal doses of reversible AChE inhibitors. These agents, once in place and bound to the AChE molecule, block the subsequent binding of long-

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lasting, irreversible AChE inhibitors. Enthusiasm existed for pretreatment with pyridostigmine, a peripherally acting oral carbamate AChE inhibitor used as a prophylaxis against AChE inhibitor toxicity. Based on early reports that pyridostigmine had some protective effects against organophosphate nerve agents (Dirnhuber et al., 1979; French et al., 1979; Walday et al., 1993; Xia et al., 1981), many allied soldiers in the Persian Gulf War were given this peripherally acting anticholinesterase agent (Keeler et al., 1991). However, the ability of pyridostigmine to antagonize AChE toxicity has subsequently been shown to be limited (Lallement et al., 2002a; Leadbeater et al., 1985; Miller et al., 1993). Indeed, there is some evidence that the central effects of irreversible AChE inhibitors may actually be potentiated by pretreatment with pyridostigmine (Lallement et al., 2001).

More recently, animal studies utilizing subchronic administration of the reversible, short acting and centrally active AChE inhibitor, physostigmine, given as a pretreatment for soman lethality and toxicity in guinea pigs and other rodents, have proved promising (Anderson et al., 1991; Harris et al., 1991; Lim et al., 1988, 1991; Meshulam et al., 1995; Miller et al., 1993). This was found to be especially true when this pretreatment was colinked with pretreatment with a centrally acting antimuscarinic receptor blocking agent (i.e., trihexyphenidyl, scopolamine) (Lim et al., 1988; Meshulam et al., 1995; Philippens et al., 2000). Indeed, pretreatment with physostigmine plus scopolamine or physostigmine plus trihexyphenidyl has been able to cause complete survival, without convulsions or loss of consciousness, in nonhuman primates, when given preceding soman doses that otherwise would have been lethal (Von Bredow et al., 1991). Furthermore, in comparison studies, physostigmine was found to be more effective than pyridostigmine in protecting against the detrimental effects of soman and sarin (Leadbeater et al., 1985; Miller et al., 1993) and other organophosphates (Deshponde et al., 1986; Solana et al., 1990). More recently huperzine, a novel AChE inhibitor that is being considered as a treatment for Alzheimer's disease and myasthenia gravis, was found to be extremely effective as a pretreatment against soman-induced convulsions (Lallement et al., 2002a,b).

Although much animal evidence suggests that physostigmine is a useful pretreatment for AChE toxicity, there are several drawbacks to its use. Most notably, physostigmine has a relatively short half-life. Therefore, it must be given relatively frequently and in relatively high oral doses, transdermally, or as an ongoing infusion to maintain adequate blood levels. Also, physostigmine has been reported to have relatively severe side effects, including nausea, vomiting, and diarrhea, which led to frequent trial dropouts when it was tested as a treatment for Alzheimer's disease (Coelho and Birks, 2001).

In the last decade, several relatively long acting, orally administered, centrally effective AChE inhibitor agents with relatively minor peripheral side effects have been marketed for the purpose of alleviating the signs of Alzheimer's disease (e.g., Wolfson et al., 2002). The rationale for using these agents is the slowing of the memory decrement associated with Alzheimer's disease, presumably due to increased central ACh. These AChE inhibitors include donepezil, rivastigmine, and metrifonate (Clegg et al., 2002; Inglis, 2002; Morris et al., 1998; Rosler, 2002; Wolfson et al., 2002). For the current study, we postulated that these agents, like physostigmine, might be able to block the effects of subsequently administered toxic doses of AChE inhibitors. The objective of the current research was to determine whether pretreatment with donepezil (Aricept) might serve to protect against the effects of the irreversible AChE inhibitor DFP. We hypothesized that pretreatment with donepezil alone would protect against the hypothermic, behavioral-inhibiting, and diarrhea-inducing effects of DFP. Furthermore, we predicted that donepezil, in combination with scopolamine, would protect to a greater extent than donepezil alone or scopolamine alone.

2. Materials and methods

2.1. Animals

In this experiment, male Flinders sensitive line (FSL) rats, developed at Flinders University in Australia, were utilized. The FSL rats were developed from Sprague–Dawley rats by selectively breeding for hypothermic and other responses following administration of the organophosphate, DFP. The FSL rat exhibits greater hypothermic responses to DFP as well as other directly acting muscarinic receptor agonists (i.e., oxotremorine) and has a greater number of hippocampal and striatal muscarinic receptors than the control Flinders resistant line (FRL) or Sprague–Dawley rats (Daws and Overstreet, 1999; Overstreet et al., 1979, 1984, 1996; Rezvani et al., 1994). FSL rats were utilized in the current experiment because they are relatively more sensitive to central and peripheral cholinergic stimulation. The FSL rats used were approximately 100 days old at the beginning of the study. They were selected from breeding colonies maintained at the University of North Carolina at Chapel Hill. They were housed in groups of three to five in polycarbonate cages under standard housing conditions (22 °C, 50% humidity) and a 12:12-h light/dark cycle (lights on from 0700 to 1900).

2.2. Drugs

DFP was obtained from Sigma (St. Louis, MO). It was dissolved in peanut oil at a concentration of 1.0 mg/ml and injected intramuscularly at a dose of 1.0 mg/kg. This dose was selected because it was the dose used to selectively breed the FSL rats (e.g., Overstreet, 1993; Overstreet et al., 1979; Russell et al., 1982) and was expected therefore to elicit substantial effects (hypothermia, hypoactivity, and diarrhea) in the FSL rats without resulting in lethality.

Donepezil (5.0 mg) tablets were obtained and were crushed and suspended in isotonic saline at a concentration of 2.0 mg/ml. Donepezil was injected intraperitoneally at a dose of 2.0 mg/kg. This 2.0 mg/kg dose of donepezil was selected on the basis of preliminary studies showing a modest hypothermic effect and protection against the more severe hypothermic effects of DFP. A dose of 1.0 mg/kg donepezil induced smaller hypothermic effects but did not protect against the more intense effects of DFP. Other ingredients in donepezil tablets included talc, polyethylene glycol, hydoxypropyl methylcellulose, and titanium dioxide. Scopolamine hydrochloride was dissolved in saline vehicle at a concentration of 0.1 mg/ml and injected intraperitoneally at a dose of 0.1 mg/kg. This comparatively low dose of scopolamine was selected on the basis of previous studies showing central ACh antagonism (Sipos et al., 1999). This dose was expected to have little or no effect on temperature regulation itself but to block the hypothermic effects of the AChE inhibitors.

2.3. Design

Eight experimental groups consisting of 8–11 FSL rats each were used. Each rat received a pretreatment injection (or two injections) followed 30 min later by an injection of DFP or DFP vehicle (peanut oil). Experimental groups were as follows: (1) vehicle (i.e., saline) followed 30 min later by vehicle; (2) 2.0 mg/kg donepezil followed by vehicle; (3) vehicle followed by 1.0 mg/kg DFP, (4) 2.0 mg/kg donepezil followed by 1.0 mg/kg DFP, (5) 0.1 mg/kg scopolamine followed by vehicle, (6) 0.1 mg/kg scopolamine followed by 1.0 mg/kg DFP; (7) 0.1 mg/kg scopolamine and 2.0 mg/kg donepezil followed by vehicle, and (8) 0.1 mg/kg scopolamine and 2.0 mg/kg donepezil followed by 1.0 mg/kg DFP.

2.4. Procedure

Baseline temperatures were recorded about 60 min prior to the first injection using a thermistor probe connected to a telethermometer (Physiotemp, Clifton, NJ). The FSL rats were injected according to the above design. Temperatures were taken again by rectal thermistor probe at 1, 2, 4, and 6 h following DFP or DFP vehicle (peanut oil) injection. The presence or absence of diarrhea at each time point was also recorded. Diarrhea is a frequent sign in animals exposed to AChE inhibitors and is probably a reasonable index of peripheral cholinergic overstimulation. Approximately 5 min after the 4-h post-DFP recording of temperature, the rats were placed in an open field apparatus [60 × 60 cm having 16 squares (15 × 15 cm)] and line crossings were recorded for 2 min.

2.5. Statistical analysis

The temperature data (decrease in °C from baseline recorded 60 min before the first injection) were initially subjected to a two-way mixed ANOVA, with treatment as the independent factor and time as the related factor. When this analysis revealed a significant interaction between treatment and time, subsequent one-way ANOVAs of the treatment effects were carried out at each time point. When significant ANOVAs were found, subsequent Tukey's protected *t* tests were carried out to determine which pairs of groups differed. A one-way ANOVA and follow-up Tukey's tests were also conducted to determine the effects of the treatments on locomotor activity at the 4-h time point. The incidence of diarrhea at the 4-h time point was analyzed by Fisher exact probability tests.

3. Results

Table 1 demonstrates that donepezil and donepezil plus scopolamine significantly decreased the hypothermic, hypokinetic, and diarrhea-inducing effects of DFP 4 h after its administration. The differences among the treatments on hypothermia were highly significant ($F = 22.7$, $P < .0001$). The decrease produced by DFP was threefold greater than that produced by vehicle. Scopolamine alone did not prevent the decrease in temperature produced by DFP, but donepezil and donepezil + scopolamine pretreatment significantly prevented the hypothermia induced by DFP. Group differences were confirmed by the use of Tukey's protected *t* tests.

Table 1
Effects of pretreatment with donepezil and donepezil/scopolamine on DFP-induced temperature and activity decreases and diarrhea induction 4 h after DFP administration

Pretreatment	Treatment	Temperature decreases (°C)[a]	Line crossings per 2 min	Diarrhea (% positive)
Vehicle	Vehicle	−0.84 ± 0.11	30.4 ± 3.5	0
Donepezil	Vehicle	−0.66 ± 0.13	25.5 ± 4.6	0
Scopolamine/ donepezil	Vehicle	−0.56 ± 0.21	35.9 ± 6.0	0
Scopolamine	Vehicle	−0.86 ± 0.12	44.8 ± 4.1 *	0
Vehicle	DFP	−3.46 ± 0.29 *	7.3 ± 1.5 *	87 [+]
Scopolamine	DFP	−3.00 ± 0.41 *	16.4 ± 4.3 *	72 [+]
Donepezil	DFP	−1.42 ± 0.19 [#]	23.5 ± 4.6 [#]	24
Scopolamine/ donepezil	DFP	−0.63 ± 0.15 [#]	31.9 ± 2.6 [#]	0 [£]
F value		22.7, $P < .001$	9.99, $P < .001$	

Donepezil = 2.0 mg/kg ip, DFP = 1.0 mg/kg im, and scopolamine = 0.1 mg/kg ip.

[a] Temperature baselines varied from 37.3–37.8 °C and there were no significant group differences.

* Significantly different from vehicle/vehicle group, $P < .01$, according to Tukey's test.

[+] Significantly different from vehicle/vehicle group, $P < .01$, according to Fisher's Test.

[#] Significantly different from vehicle/DFP group, $P < .01$, according to Tukey's test.

[£] Significantly different from vehicle/DFP group, $P < .01$, according to Fisher's Test.

A similar pattern was observed for open field line crossings ($F = 9.99$, $P < .001$). The reduction in activity induced by DFP was counteracted by pretreatment with donepezil and donepezil + scopolamine but not by scopolamine alone. It should also be emphasized that scopolamine given prior to the vehicle produced a significant elevation of line crossings (Table 1), indicating that this low dose is still behaviorally activating at 4 h after injection (see Sipos et al., 1999).

The data on the incidence of diarrhea were comparable to the findings for hypothermia and hypoactivity. Only the groups given vehicle + DFP and scopolamine + DFP exhibited substantial diarrhea. These results suggest that the donepezil pretreatments were counteracting all of the effects of DFP that were measured.

Fig. 1 illustrates the time-dependent nature of the antagonism of the hypothermic effects of DFP by donepezil in FSL rats. An overall two-way ANOVA indicated that there were highly significant time and treatment effects as well as a significant Time × Treatment interaction. Because of the significant interaction effect, further analyses focused on the changes at each time point.

Even as early as 1 h after the DFP injection, there were significant differences in temperature ($F = 8.07$ $P < .001$). At the 1-h time point, donepezil itself induced a decrease in temperature that was greater than that induced by DFP at that time but less than the maximum decrease induced by DFP. When the DFP was added, a slightly greater hypothermic effect was observed. Scopolamine was able to completely block the hypothermia induced by donepezil or DFP at 1 h, but not the combination (Fig. 1).

At 2 h after DFP administration, a different pattern emerged. Although there was still a significant difference among groups ($F = 7.61$, $P < .001$), the greatest changes in temperature were seen in the groups treated with DFP only or donepezil plus DFP. Scopolamine pretreatment now only partially protected against the hypothermic effects induced by DFP and donepezil plus DFP.

The results at 4 h, described above, were similar to those at 6 h (Fig. 1). Once again, there were significant group differences ($F = 17.07$, $P < .001$). At the 4-h time point, only the groups treated only with DFP or with scopolamine plus DFP exhibited significant reductions in temperature. All groups treated with donepezil, regardless of whether they were cotreated with scopolamine or subsequently treated with DFP, were not significantly different from the control group given two injections of vehicle (Fig. 1). Thus, donepezil exhibits early hypothermia itself but protects against the late-developing hypothermia induced by DFP. In contrast, scopolamine pretreatment protects against the early hypothermia induced by donepezil or DFP but does not counteract the late-developing hypothermia induced by DFP. The most effective treatment is the combination of donepezil and scopolamine.

4. Discussion

These data demonstrate that donepezil, especially when used in combination with scopolamine, reduces the hypothermic, diarrhea-inducing, and behavioral inhibiting effects of DFP. Because donepezil has substantial short-term inhibitory effects of its own on AChE, it would appear that short-term (i.e., 1 h) antagonism by scopolamine of both donepezil and DFP's effects is advantageous. Thus, donepezil, a

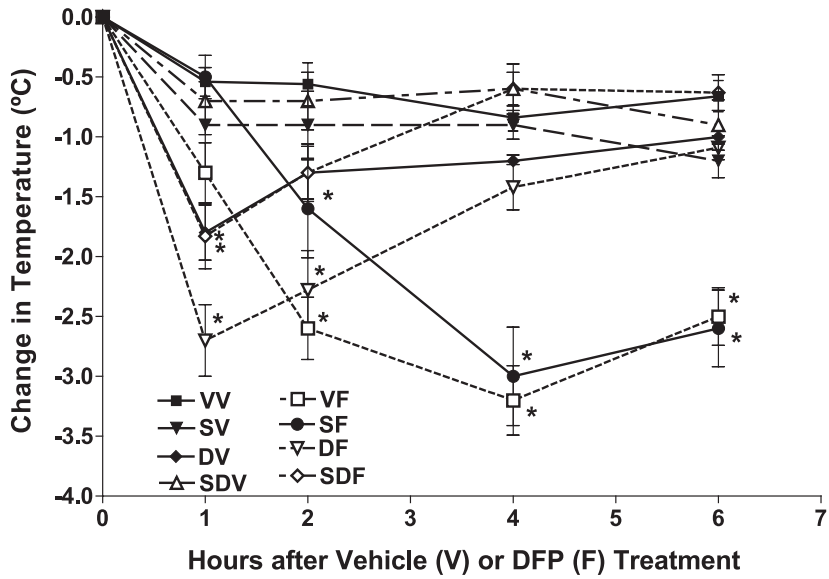

Fig. 1. Changes in temperature induced by DFP following pretreatment with donepezil or scopolamine alone and their combination. Rats were pretreated with vehicle, scopolamine (0.1 mg/kg), and/or donepezil (2 mg/kg) 30 min prior to being treated with vehicle or DFP. Temperatures were then recorded 1, 2, 4, and 6 h later and related to previously recorded baselines. The values represent the mean ± S.E.M. change in temperature (°C) for 8–11 rats. Group codes: VV = vehicle–vehicle; SV = scopolamine–vehicle; DV = donepezil–vehicle; SDV = scopolamine + donepezil–vehicle; DF = donepezil–DFP; SDF = scopolamine + donepezil–DFP; VF = vehicle–DFP; and SF = scopolamine–DFP. * Significantly different, $P < .01$, from VV group according to Tukey's test.

centrally acting anti-AChE agent used in the treatment of Alzheimer's disease, has parallels in effectiveness to physostigmine, another centrally active anti-AChE agent (Lim et al., 1988, 1991; Philippens et al., 2000). However, unlike physostigmine, which can have many unwanted side effects such as lethargy, nausea, vomiting, and diarrhea at clinically used doses (Coelho and Birks, 2001), donepezil has been used in treating Alzheimer's patients with relatively few (i.e., 11%) side effects (Inglis 2002; Pratt et al., 2002).

It is important to note that the current study did not use lethal or supralethal doses of DFP, nor were doses used that were high enough to elicit seizures. Thus, it is not certain that pretreatment with a combination of donepezil plus scopolamine can prevent death or seizures, as does physostigmine plus scopolamine. However, it is likely that death and seizures arising from AChE inhibitor poisoning, like hypothermia, hypoactivity, and diarrhea, also involve cholinergic mechanisms (see Lennox et al., 1992, Muggleton et al., 2003; Wetherell, 1994). Therefore, one would predict that donepezil plus scopolamine pretreatment would also protect against the lethal effects of other AChE inhibitors, as does physostigmine. There is evidence that other systems, particularly the glutamatergic systems, are recruited during the progression of toxicity to soman (Solberg and Belkin, 1997) and that seizures once initiated tend to resistant to the effects of anticholinergic agents (McDonough et al., 1987). Importantly, there is recent evidence that donepezil can inhibit the cellular toxicity induced by glutamate (Takada et al., 2003), which as noted above has been implicated as a secondary mechanism underlying AChE inhibitor toxicity (Lallement et al., 1999; Solberg and Belkin, 1997).

Although this preliminary study provided encouraging results, there are limitations to abstracting these data to potential effects in humans. All AChE inhibitors do not behave in the same way (Lynch et al., 1986; Sivam et al., 1984). Thus, the positive result for pretreatment of one AChE inhibitor may not apply to others. In particular, the blockade of the effects of DFP by donepezil plus scopolamine does not guarantee that this combination treatment will have the same beneficial effects on other potent organophosphorus anti-AChE agents such as soman, sarin, and VX. Our data do not conclusively show that donepezil or scopolamine exerts effects on diarrhea, movement, and temperature via central mechanisms. Protecting the periphery with a quaternary anticholinergic agent, such as methyl scopolamine or propantheline that do not enter the brain, in one experimental group and giving centrally acting scopolamine in another, followed by donepezil, would allow dissection of whether scopolamine protects against DFP's various effects by a central or a peripheral mechanism. Moreover, the protective effects of donepezil could be compared with those of neostigmine, a peripherally acting anti-AChE agent, to confirm whether the effects of donepezil are centrally mediated.

Centrally acting anticholinergic agents, such as scopolamine, may have unique side effects of their own, including disorientation, confusion, and hallucinations, and such effects may limit their potential use (Brown and Stoudemire, 1998). Cholinesterase inhibitors used as pretreatments can also have effects such as anergy, lethargy, and fatigue as well as nausea, vomiting, and diarrhea (Janowsky et al., 1987; Pratt et al., 2002). Thus, careful selection of drug doses will be necessary before these findings can be translated into parallel studies of human subjects.

Although the present findings have established the ability of a combination of donepezil and scopolamine to counteract several effects of DFP, they are still very preliminary in nature. Further studies are necessary. Studies utilizing lethal or seizure-inducing doses of DFP should be investigated in animal models. Also, studies in which donepezil and related agents are given following rather than preceding exposure to DFP should occur, particularly since donepezil has been found to counteract glutamate toxicity (Takada et al., 2003). The effects of subchronic/chronic pretreatment, such as have been used recently by others (Lallement et al., 2002a,b; Philippens et al., 2000), should be studied as well. These studies could employ the strategy of gradually escalating the dose of donepezil. The ability of donepezil to antagonize lethal and other effects of AChE inhibitors other than DFP (e.g., soman, sarin, VX) also needs to be determined, since different AChE inhibitors are different chemical compounds with different effects in spite of having the common ability to inhibit AChE (Lynch et al., 1986; Sivam et al., 1984). Finally, studies substituting other AChE inhibitor agents used in treating Alzheimer's disease, such as rivastigmine and metrifonate, for donepezil are indicated.

Although our experiments are very preliminary and can only be said to be related to several of the nonlethal effects of DFP, the possibility that donepezil may also antagonize other effects of DFP and other AChE inhibitors is likely. However, to apply the above results clinically, certain drawbacks would need to be overcome. Thus, scopolamine as a co-pretreatment given at the time of cholinesterase inhibitor exposure can cause hallucinations, disorientation, and confusion (Brown and Stoudemire, 1998). Most significantly, donepezil, as now given, appears to exert an early additive effect along with the effects of DFP on hypothermia at least, although later blocking DFP's more intensive effects. Whether such additive effects would remain after chronic pretreatment with donepezil is an open question inviting further studies.

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