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Prophylactic Detoxification by Physostigmine and Procyclidine of Diisopropylfluorophosphate Poisoning

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ABSTRACT *: The antidotal, anticonvulsant and neuroprotective effects of physostigmine and procyclidine, the combinational prophylactics for organophosphate poisoning, were evaluated in rats. In comparison with a low protective effect (1.6 fold) of atropine (15 mg/kg) and 2-pralidoxime (30 mg/kg), the traditional antidotes regimen, a marked protection ratio of 7.3 fold was achieved by combinational pretreatment with physostigmine (0.05 mg/kg) and procyclidine (10 mg/kg), which was superior to that (3.5 fold) with pyridostigmine (0.1 mg/kg) and atropine (15 mg/kg). Rats exposed to a high dose (10 mg/kg, 2 X LD_{50}) of diisopropylfluorophosphate showed severe epileptiform seizures on electroencephalography, resulting in necrotic and apoptotic brain injuries in discrete brain regions under histopathological and TUNEL immunohistochemical examinations in 24 hr. Such seizures and excitotoxic brain injuries were fully prevented by pretreatment with physostigmine (0.05 mg/kg) and procyclidine (10 mg/kg), in contrast to a negligible effect of pyridostigmine (0.1 mg/kg) and atropine (15 mg/kg). Taken together, it is proposed that the prophylactics composed of physostigmine and procyclidine could be a promising regimen for the prevention of lethality, seizures and brain injuries induced by organophosphate poisoning.*

Key Words *: Physostigmine, Procyclidine, Organophosphate, Seizures, Brain injuries, Neuroprotection*

I. INTRODUCTION

Organophosphate poisoning induces epileptiform seizures and thereby brain and cardiac injuries, in addition to cholinergic toxicities following inhibition of acetylcholinesterase (Kim *et al.*, 1999; McDonough and Shih, 1993; Shih *et al.*, 1991; Tryphonas and Clement, 1995; Tryphonas *et al.*, 1996). Atropine plus an oxime has been used as the standard treatment of organophosphate poisoning for their great synergistic antidotal efficacy (Dunn and Sidell, 1989). However, such a synergistic effect was not achieved by combinational treatment with atropine and 2-pralidoxime or obidoxime in the poisoning by diisopropylfluorophosphate or pinacolylmethylphosphonofluoridate (soman), since those oximes do not appear to be effective (Berry and Davies, 1970; Inns and Leadbeater, 1983). The ineffectiveness of the oximes might be due to the rapid "aging (dealkylation)" of the organophosphates after phosphorylation of acetylcholinesterase (Berman and Decker, 1986; Talbot *et al.*, 1988).

On the contrary, it is well known that the pretreatment with carbamates greatly improve the efficacy of anticholinergic atropine in reducing the lethality induced by diverse organophosphates including diisopropylfluorophosphate and soman (Berry and Davies, 1970; Dirnhuber *et al.*, 1979; Gordon *et al.*, 1978). Pyridostigmine, a quaternary carbamate, has been used as a prophylactic of organophosphate poisoning according to its high protective potential in combination with atropine (Dunn and Sidell, 1989; Keeler *et al.*, 1991). However, centrally-inactive pyridostigmine was found to be less effective for the successful protection and the rapid recovery after survival than centrally-active physostigmine (Harris *et al.*, 1984; Kim

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et al., 1998).

Furthermore, the combination of pyridostigmine and atropine could not prevent organophosphate-induced seizures leading to brain injuries (Kim *et al.*, 1997), in spite of their potential in the reduction of mortality. It has been reported that organophosphate-induced seizures were triggered by acetylcholine and potentiated by excitatory amino acids accumulated following organophosphate poisoning (Lallement *et al.*, 1991; Mattson, 1989; McDonough and Shih, 1993; Shih *et al.*, 1991). The excitatory amino acids and their receptor agonists (excitotoxins) were proposed to play a causative role in the injury of central nervous tissues (Choi *et al.*, 1987; Garthwaite and Garthwaite, 1989). Recently, we demonstrated that diisopropylfluorophosphate poisoning induced severe epileptiform seizures, and subsequent necrotic and apoptotic brain injuries (Kim *et al.*, 1997, 1999). Also, it was proved that antagonists of excitatory amino acid receptors, especially *N*-methyl-D-aspartate subtype of glutamate receptor, were effective in the prevention of convulsions and neuronal injury induced by soman poisoning (Shih *et al.*, 1991). Moreover, such an effect of anticonvulsants were potentiated by a low dose of anticholinergics (Kim *et al.*, 1997; Shih *et al.*, 1991). Interestingly, procyclidine, an *N*-methyl-D-aspartate antaginist possessing anticholinergic actions (Gao *et al.*, 1998; McDonough and Shih, 1995; Waelbroeck *et al.*, 1990), substantially attenuated diisopropylfluorophosphate-induced convulsions (Kim *et al.*, 1997). Accordingly, it was suggested that such anticonvulsants possessing both anticholinergic and *N*-methyl-D-aspartate-antagonistic actions could be a promising antidote for the prevention of lethality and brain injuries induced by organophosphate poisoning.

In this context, we have investigated the efficacy of combinational antidotes composed of physostigmine and procyclidine, in which a remarkable effectiveness was found in the prevention of diisopropylfluorophosphate-induced lethality in mice (Kim *et al.*, 1998). For further efficacy evaluation of the combinational antidotes as a prophylactic regimen, in comparison with traditional antidotes such as atropine plus 2-pralidoxime and pyridostigmine plus atropine, we investigated the antidotal, anticonvulsant and neuroprotective effects under technical examinations of protection ratio, electroencephalography and terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling (TUNEL) immunohistochemistry, respectively, in rats.

II. MATERIALS AND METHODS

1. Materials

Pyridostigmine bromide, physostigmine sulfate, atropine sulfate, procyclidine hydrochloride, 2-pralidoxime chloride and methyl green were procured from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diisopropylfluorophosphate was from Adrich Chemical Co. (Milwakee, WI, U.S.A.), and diaminobenzidine was from Pierce Co. (Rockford, IL, U.S.A.). *In situ* Apoptosis Detection Kit-Peroxidase (ApopTag TUNEL assay kit) was obtained from Oncor Co. (Gaithersberg, MD, U.S.A.).

2. Animals

Specific pathogen-free Sprague-Dawley female rats (body weight, 200~250 g) were housed in an environmentally-controlled room with temperature of 23± 2°C, relative humidity of 55±5%, a 12-hr light/dark cycle, and feed and water available *ad libitum*.

3. Antidotal efficacy

Protective effect of each combination composed of atropine (15 mg/kg) and 2-pralidoxime (30 mg/kg), pyridostigmine (0.1 mg/kg) and atropine, or physostigmine (0.05 mg/kg) and procyclidine (10 mg/kg) on the lethality of rats poisoned with diisopropylfluorophosphate was expressed as protection ratio (fold of median lethal dose [LD_{50}] in treated group over LD_{50} in control group). Each compound was administered subcutaneously 30 min prior to intraperitoneal injection of diisopropylfluorophosphate (Kim *et al.*, 1998). All the compounds, except diisopropylfluorophosphate which was diluted in distilled water, were dissolved in physiological saline and administered in a volume of 1 ml/kg. All estimates of LD_{50} were based on 24-hr mortalities in each group of rats given 5 increasing dose levels according to the method of Litchfield and Wilcoxon (1949).

4. Anticonvulsant effect

Rats were anesthesized by intraperitoneal injection with pentobarbital sodium (60 mg/kg), and mounted in a stereotaxic frame. A skin incision was made over parietal to occipital cranium, and two holes (2 mm in diameter) were made using a dental drill on the occipital cranium bilaterally 5 mm distant from cental line. Cortical screw electrodes were fixed into the holes, and electric wires attached to the head of crews were collected in a plug socket and anchored to the skull with dental acrylic, and the wound was closed with sutures (McDonough and Shih, 1993). The animals were allowed to recover 7~10 days prior to experimentation. On the day of experiment, the freely-moving animal was connected to NCI digital electroencephalography system (Middleton, WI, U.S.A.), and allowed 1 hr for acclimation to the experimental environments to show baseline recording. After 15-min monitoring of baseline electrocorticogram and behavior, the rat was administered with each combinational antidotes regimen. Thirty min after antidotes injection, the animal was challenged with 10 mg/kg (2 X LD_{50}) of diisoprophylfluorophosphate (Kim *et al.*, 1997). The electrocorticographic activity and behavior of animals were monitored for at least 2 hr after diisoprophylfluorophosphate poisoning (McDonough and Shih, 1993).

5. Neuroprotective effect

Rats were pretreated with each combinational antidotes regimen 30 min before intoxication with 10 mg/kg (2 X LD_{50}) of diisopropylfluorophosphate. Twenty-four hr later, whole brain was removed after fixation by *in situ* intracardial perfusion with 10% neutral formalin solution containing 2 IU heparin/ml under ether anesthesia. For the identification of apoptotic injury, paraffin-embedded brain sections (4 μm in thickness) were stained immunohistochemically using an ApopTag TUNEL assay kit with diaminobenzidine as chromogenic substrate, and counterstained with methyl green (Kim *et al.*, 1999). The degree of neuronal injury and staining pattern of morphologically-dead cells in three brain regions (hippocampus, piriform cortex and thalamus) were examined under a light microscope (Kim *et al.*, 1999).

The experiments performed here were conducted according to the *"Guide Principles in the Use of Animals in Toxicology"* which had been adopted by the Society of Toxicology in 1989.

III. RESULTS

The LD_{50} of diisopropylfluorophosphate in rats, intraperitoneally administered, was determined to be 5.0 mg/kg. Coadministration of atropine (15 mg/kg) and 2-pralidoxime (30 mg/kg) increased the LD_{50} value of diisopropylfluorophosphate by 1.6 fold (Table 1). In contrast, the protection ratio was enhanced to 3.5 fold by combinational pretreatment with pyridostigmine (0.1 mg/kg) and atropine (15 mg/kg). More predominent protection (7.3 fold) was obtained with the combination of physostigmine (0.05 mg/kg) and procyclidine (10 mg/kg).

In spite of pretreatment with atropine and 2-pralidoxime, rats poisoned with a high dose (10 mg/kg, 2 X LD_{50}) of diisopropylfluorophosphate exhibited severe limbic seizures, displaying early (15~90 min) tonic-clonic seizures followed by prolonged mild clonic epilepsy, which led to 100% mortality within 1 hr. Although all the rats pretreated with pyridostigmine and atropine survived the lethal challenge with diisopropylfluorophosphate, they displayed similar pattern of epileptiform seizures (Fig. 1A-1C). Interestingly, the seizure activity induced by diisopropylfluorophosphate poisoning was fully prevented by coadministration of physostigmine and procyclidine (Fig. 1D-1F). Noteworthy, the animals pretreated with physostigmine and procyclidine did not show any considerable toxic signs, in contrast to a long-lasting incoordination in rats received pyridostigmine and atropine.

Diisopropylfluorophosphate-induced limbic seizures in rats pretreated with pyridostigmine and atropine led to a mixed type of brain injuries in discrete brain regions in 24 hr (Fig. 2). Necrotic injury was observed

Table 1. Protective effect of combinational antidotes against diisopropylfluorophosphate poisoning. All the compounds were pretreated subcutaneously 30 min prior to intraperitoneal poisoning with diisopropylfluorophosphate

Treatment (mg/kg)	Protection ratio (95% confidence limits)
Control (saline)	1.0
Atropine (15) + 2-pralidoxime (30)	1.6 (1.3-2.0)
Pyridostigmine (0.1) + atropine (15)	3.5 (2.8-4.3)
Physostigmine (0.05) + procyclidine (10)	7.3 (5.8-9.1)

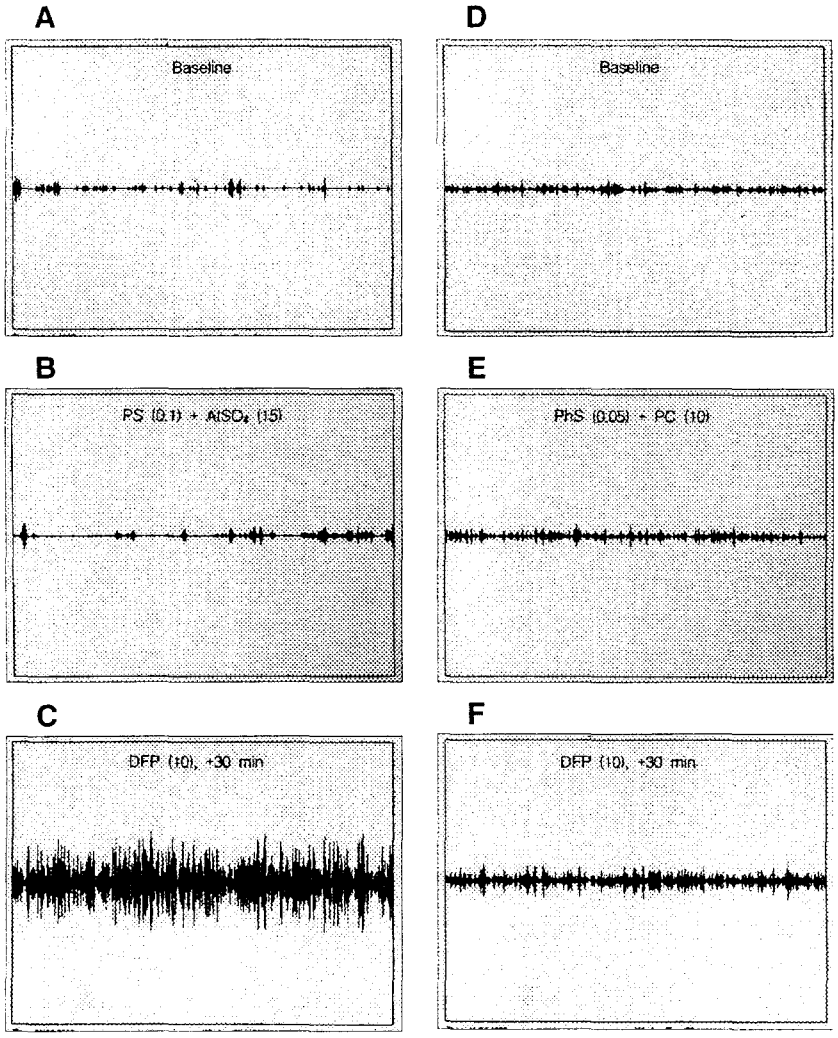

Fig. 1. Effect of combinational pretreatment with pyridostigmine and atropine (A-C) or physostigmine and procyclidine (D-F) on the seizure activity induced by diisopropylfluorophosphate. After baseline recording (A and D), the combinational antidotes, pyridostigmine (0.1 mg/kg) and atropine (15 mg/kg) (B) or physostigmine (0.05 mg/kg) and procyclidine (10 mg/kg) (E) were administered. Thirty min later, the animals were challenged with 10 mg/kg (2 X LD_{50}) of diisopropylfluorophosphate, followed by electrocorticographic minitoring for longer than 30 min (C and F).

predominently in hippocampal pyramidal cell layers and piriform/entorhinal cortices, showing dark degeneration of neuronal cells and malacia of neuropils (Fig. 2A and 2C). In comparison, typical TUNEL-positive cells, indicative of apoptosis, appeared most predominently in thalamic nuclei (Fig. 2B). Such apoptotic cells showed a strong TUNEL-positive staining and a morphological shrinkage, producing a pericellular halo. It is worthy of noting that such necrotic and apototic brain lesions were completely eliminated by pretreatment with physostigmine and procyclidine in all brain regions examined, exhibiting normal features (Fig. 2D-2F).

IV. DISCUSSION

Atropine plus 2-pralidoxime, the traditional combination of antidotes for organophosphate poisoning, was found not to be very effective against diisopropylfluorophosphate (Kim *et al.*, 1998; Table 1) and soman poisoning (Berry and Davies, 1970; Inns and Leadbeater, 1983). In spite of prophylactic treatment, atropine and 2-pralidoxime exerted only 1.6 fold of protection ratio. In contrast, a remarkable protective effect was achieved with the combination of a carbamate and anticholinergics. Thus, the inclusion of prophylactic carbamate rather than 2-pralidoxime was

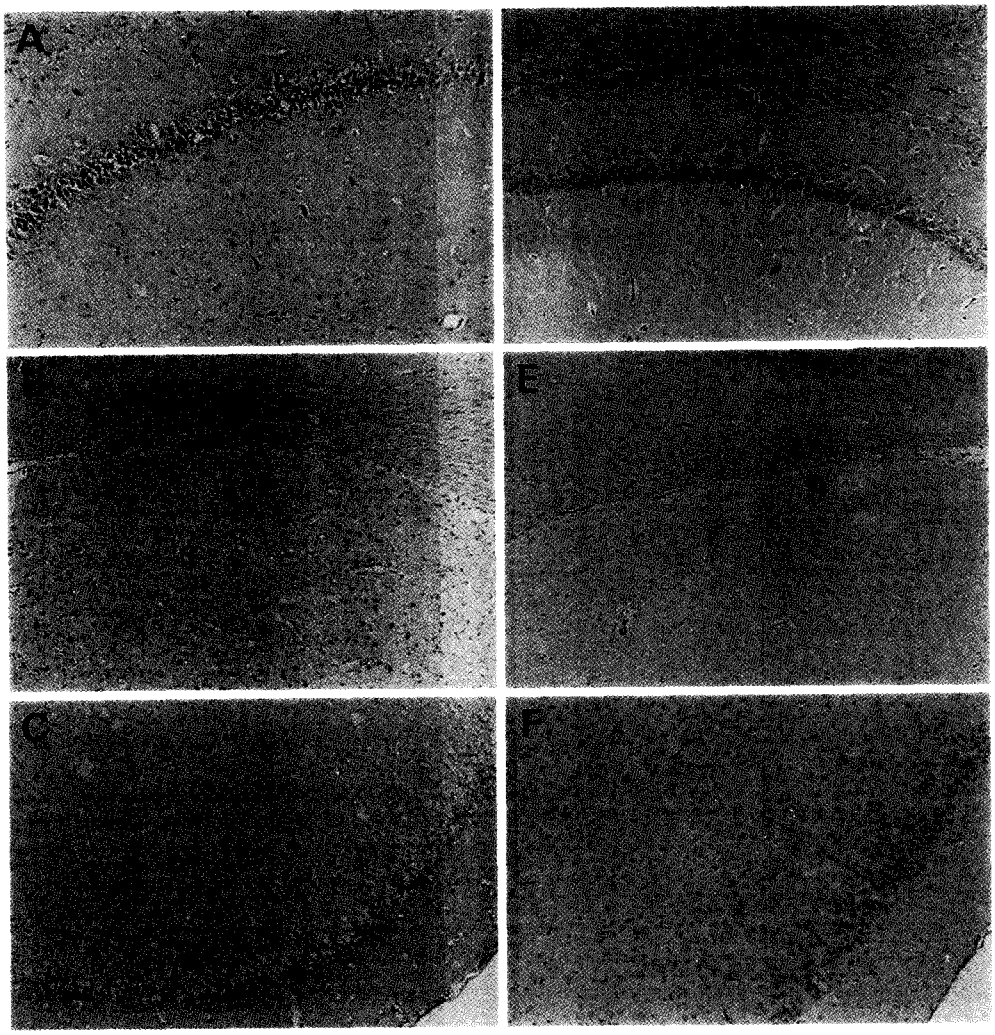

Fig. 2. Effect of combinational pretreatment with pyridostigmine (0.1 mg/kg) and atropine (15 mg/kg) (A-C) or physostigmine (0.05 mg/kg) and procyclidine (10 mg/kg) (D-F) on the injuries of hippocampal (A and D), piriform cortical (B and E) and thalamic (C and F) regions induced by 10 mg/kg (2 X LD_{50}) of diisopropylfluorophosphate. Note the TUNEL-negative, dark degeneration of hippocampal pyramidal neurons and severe malacia of neurophils in hippocampus (A) and piriform cotex (C), and typical TUNEL-positive neurons in thalamic nuclei (B), in contrast to normal features in D-F.

found to be much more efficient in the detoxification of poisoning with diisopropylfluorophosphate and soman, the organophosphates that undergo rapid aging progress during acetylcholinesterase inhibition (Talbot *et al.*, 1988). Moreover, it is of interest to note that the prophylactic regimen composed of physostigmine and procyclidine was much more effective than that of pyridostigmine and atropine.

Pyridostigmine, in combination with atropine and an oxime, has been used as a battlefield pretreatment tablet for nerve-agent poisoning (Dunn and Sidell, 1989). In spite of synergistic action between pyridostigmine and anticholinergics for reducing the mortality, however, the combination of pyridostigmine and atropine did not exhibit anticonvulsant and neuroprotective effects (Fig. 1 and 2) against diisopropylfluorophosphate poisoning. For neuroprotection, diazepam, an agonist of γ-aminobutyric acid receptors, has been used (Clement and Broxup, 1993; Dunn and Sidell, 1989). However, the anticonvulsant diazepam was found to exert insufficient neuroprotective activity, and to enhance the respiratory suppression by organophosphate poisoning, which led the investigators to study on the novel neuroprotective compounds (Lallement *et al.*, 1998; McDonough and Shih, 1993; McDonough *et al.*, 2000; Shih *et al.*, 1991). In this respect, we made the combinational regimen, physostigmine and procyclidine, to achieve high anti-

dotal, anticonvulsant and neuroprotective efficacies, since it had been reported that physostigmine synergistically enhanced the antidotal and anticonvulsant actions of anticholinergics (Dunn and Sidell, 1989; Kim *et al.*, 1998, manuscript in preparation), and procyclidine rapidly blocked the convulsions (Kim *et al.*, 1997; McDonough *et al.*, 2000).

The *N*-methyl-D-aspartate antagonists such as dizocilpine (MK-801) and trihexylphenidyl have been proposed as novel anticonvulsants with a high efficacy in soman poisoning (Lim *et al.*, 1991; McDonough and Shih, 1993; Shih *et al.*, 1991). Also, procyclidine substantially attenuated diisopropylfluorophosphate- and soman-induced seizures (Kim *et al.*, 1997; McDonough *et al.*, 2000; Shih *et al.*, 1997; Fig. 1), and thereby prevented the seizure-related brain injuries (Fig. 2). In these respects, procyclidine, possessing antimuscarinic (Waelbroeck *et al.*, 1990), antinicotinic (Gao *et al.*, 1998) and *N*-methyl-D-aspartate-antagonistic (McDonough and Shih, 1995) actions, may be a promising antidote, based on the findings that blockade of cholinergic systems were important for the maximal prevention of organophosphate-induced convulsions and brain injuries with *N*-methyl-D-aspartate antagonists (De Groot *et al.*, 1990; McDonough and Shih, 1995) or nitric oxide synthase inhibitors (Kim *et al.*, 1997).

Anticholinergics have been used as a posttreatment antidote of organophosphate poisoning to block muscarinic receptors, and thereby prevent cholinergic signs which induce respiratory failure by paralyzing the central respiratory drive and peripheral respiratory muscles and by obstructing the airways (Dunn and Sidell, 1989). In this way, however, the therapeutic effect of anticholinergics may not be displayed fully (Kim *et al.*, 1998). Rather, it might be a better strategy to administer the anticholinergics prophylactically in combination with carbamates, which could overcome the limited effectiveness of carbamates when the standard treatment, atropine and an oxime, was delayed. For example, pyridostigmine pretreatment was reported to reduce the efficacy of atropine and oxime posttreatment against isopropylmethylphosphonofluoridate (sarin) and *O*-ethyl-S-[2-(diisomethylethyl)ethyl]-methylphosphonothiolate (VX) poisoning in rodents (Koplovitz *et al.*, 1992), whereas the protection ratio of the standard regimen was rather greatly enhanced by pyridostigmine when the antidotes were treated prior to sarin challenge (unpublished data). This suggestion could be supported by the idea that the possible adverse effects of carbamates and anticholinergics might be offset by each other (Berry and Davies, 1970; Lim *et al.*, 1991; Philippens *et al.*, 2000). Recently, we confirmed that the influence of procyclidine on the behavioral, learning and memory, and physiologcal performances of rats was attenuated by the combination with physostigmine to some extent, resulting in increased sign-free doses of procyclidine (unpublished data).

Taken together, the prophylactic combinational regimen of physostigmine and procyclidine exerted high antidotal, anticonvulsant and neuroprotective efficacies against diisopropylfluorophosphate poisoning, which were superior to those of traditional antidote regimens, atropine and 2-pralidoxime or pyridostigmine and atropine. Therefore, it is suggested that physostigmine and procyclidine could be promising mate antidotes for the prophylactic detoxification of organophosphate poisoning.

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