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Protection by a transdermal patch containing physostigmine and procyclidine of soman poisoning in dogs

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Abstract

The prophylactic efficacy of a combinational patch system containing physostigmine and procyclidine against soman intoxication was evaluated using dogs. Female beagle dogs (body weights 9–10 kg) were shaved on the abdominal side, attached with a matrix-type patch (7×7 cm) containing 1.5% of physostigmine plus 6% procyclidine for 2 days, and challenged with subcutaneous injection of serial doses (2–10 LD_{50}) of soman. Separately, in combination with the patch attachment, atropine (2 mg/dog) plus 2-pralidoxime (600 mg/dog) or atropine plus 1-[([4-(aminocarbonyl)pyridinio]methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium (HI-6, 500 mg/dog) were injected intramuscularly 1 min after soman poisoning. The LD_{50} value of soman was determined to be 9.1 μg/kg, and high doses (≥1.4 LD_{50}) of soman induced salivation, emesis, defecation and diarrhea, tremors and seizures, and recumbency of dogs, leading to 100% mortality in 24 h. The prophylactic patch, which led to mean 18.5–18.8% inhibition of blood cholinesterase activity by physostigmine and mean 7.9–8.3 ng/ml of blood concentration of procyclidine, exerted a high protection ratio (4.7 LD_{50}), in comparison with relatively-low effects of traditional antidotes, atropine plus 2-pralidoxime (2.5 LD_{50}) and atropine plus HI-6 (2.7 LD_{50}). Noteworthy, a synergistic increase in the protection ratio was achieved by the combination of the patch with atropine plus HI-6 (9 LD_{50}), but not with atropine plus 2-pralidoxime (5 LD_{50}). In addition, the patch system markedly attenuated the cholinergic signs and seizures induced by soman, especially when combined with atropine plus HI-6, leading to elimination of brain injuries and physical incapacitation up to 6 LD_{50} of soman poisoning. Taken together, it is suggested that the patch system containing physostigmine and procyclidine, especially in combination with atropine and HI-6, could be a choice for the quality survival from nerve-agent poisoning.

Keywords: Transdermal patch; Physostigmine; Procyclidine; Soman; Mortality; Brain injury; Incapacitation

1. Introduction

Organophosphorus anticholinesterases induce cholinergic symptoms that may cause acute death, and epileptiform seizures (McDonough and Shih, 1993; Shih et al., 1991a), leading to brain injuries (Kim et al., 1999; McDonough et al., 1989; Tryphonas and Clement, 1995). Traditionally, atropine plus an oxime have been used as the standard treatment of organophosphate poisoning to reduce lethality by blocking cholinergic symptoms (Dunn and Sidell, 1989). However, coadministration of 2-pralidoxime or obidoxime with atropine did not exert synergistic protection against the lethality of soman which undergoes rapid dealkylation after inhibition of cholinesterases (Berman and Decker, 1986; Fleisher and Harris, 1965; Talbot et al., 1988). In comparison, HI-6, in combination with atropine, has been proved to be effective for the detoxification of soman poisoning (Hamilton and Lundy, 1989; Shih et al., 1991b; Koplovitz and Stewart, 1994). In spite of a high protective effect

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of atropine plus HI-6 on the lethality of soman, additional anticonvulsant such as diazepam, an agonist of γ-aminobutyric acid receptors, is required for neuroprotection (Dunn and Sidell, 1989; McDonough et al., 1989). However, diazepam was not found to exert sufficient neuroprotective efficacy, since a long duration after injection of diazepam is necessary for seizure termination (McDonough and Shih, 1997).

Because of low effectiveness of therapeutic antidotes in emergency situations, some prophylactic regimens were proposed as one of the effective measures for survival and neuroprotection from chemical warfare and terrorism. There have been attempts to develop a simple prophylactic regimen containing physostigmine and anticholinergics, such as trihexyphenidyl (Lim et al., 1991) and scopolamine (Lim et al., 1988; Meshulam et al., 1995; Philippens et al., 1996, 2000a; Wetherell, 1994), which exerted synergistic efficacy.

In a previous study, we demonstrated that a combinational prophylactic regimen composed of physostigmine and procyclidine fully prevented rats from lethality, seizures and brain injuries induced by 1.3 LD_{50} of soman (Kim et al., 2002). Recently, it was confirmed that appropriate doses of the combinational prophylactics were found to exhibit complete protection up to 1.6 LD_{50} and partial effect against 2 LD_{50} of soman (Myhrer et al., 2004b). In addition, sustained release of optimized doses, without adverse-effects, of physostigmine and procyclidine from an osmotic minipump, a substitute model of patch system, exerted full protective effects on lethality, seizures and brain injuries of rats exposed to 1.3 LD_{50} of soman (Choi et al., 2004).

Recently, we successfully developed a prototype of matrix-type patch system containing 1.5% physostigmine, 6% procyclidine and 0.5% hydrocortisone. The concentrations of physostigmine and procyclidine were optimized to obtain clinically-available blood concentrations and inhibition rate of blood cholinesterase activity, without considerable skin irritation (Kim et al., 2003; Lee et al., 2003b). In the present study, beagle dogs were transdermally attached with the prototype patch for 2 days prior to challenge with serial dose levels of soman. Effectiveness of the patch, alone or in combination with antidotes atropine plus 2-pralidoxime or atropine plus HI-6, were evaluated based on protection ratio, symptoms, brain injuries and physical incapacitation following soman poisoning.

2. Materials and methods

2.1. Materials

Physostigmine, procyclidine hydrochloride, hydrocortisone, atropine sulfate and 2-pralidoxime chloride were from Sigma Chemical Co. (St. Louis, USA). Soman and 1-[([4-(aminocarbonyl)pyridinio]methoxy)methyl]-2-[(hydroxyimino)methyl] pyridinium (HI-6) were synthesized in Single Small Scale Facility of Agency for Defense Development, Republic of Korea.

For the preparation of a matrix-type patch, physostigmine (1.5%), desalted procyclidine (6%) and hydrocortisone (0.5%) were dissolved in ethylacetate–ethanol and mixed with an acrylic adhesive DURO-TAK® 387-2287/87-287 (National Starch and Chemical Co., Bridgewater, USA). The mixture was dispersed on Scotchpak 1009® polyester film laminate (3M Co., St. Paul, USA), made even with a casting knife to make 160 μm in thickness, dried, and finally attached to an impermeable backing membrane. The concentrations of physostigmine, procyclidine and hydrocortisone was selected based on the balance of blood concentrations clinically available in human (unpublished data) and skin irritation and recrystallization profiles (Kim et al., 2003; Lee et al., 2003b).

For injection, clinical doses of atropine (2 mg/dog), 2-prelidoxime (600 mg/dog) and HI-6 (500 mg/dog), according to field formulas, were dissolved in physiological saline and administered in a volume of 1 ml/dog. Soman was dissolved in 10% isopropyl alcohol to make a stock solution (10 mg/ml) and stored at 4 °C. The stock solution was further diluted in physiological saline immediately before use, and administered in a volume of 1 ml/dog.

2.2. Animals

Female beagle dogs (body weights 9–10 kg) were housed in an environmentally-controlled room with temperature of 23±2 °C, relative humidity of 55±5% and a 12-h light/dark cycle. Feed was restricted to 300 g/day and water was available ad libitum. 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, and the protocol was approved by Institutional Animal Care and Use Committee of Laboratory Animal Research Center, Chungbuk National University, Republic of Korea.

2.3. Antidotal efficacy

In a preliminary study, it was confirmed that the blood concentrations of 0.5–1.5 and 5–10 ng/ml for physostigmine and procyclidine, respectively, 30 min after subcutaneous injection, were effective against 2 LD_{50} of soman (unpublished data). In a follow-up test to obtain profiles of blood concentrations of physostigmine and procyclidine and enzyme inhibition rates, various sizes (6×6, 6×7, 7×7, 7×8 or 8×8 cm/dog) of the combinational patch were attached to the abdominal area of dogs after removal of the hair with an electric clipper and a depilation cream. After 2-day attachment of the patch, blood (8 ml) was collected for the assay of cholinesterase activity and the measurement of blood concentrations of physostigmine and procyclidine. In brief, an aliquot (50 μl) of blood was collected into heparinized capillary tubes, and plasma, after centrifugation, was used for the enzyme assay by a slight modification of the method of Ellman et al. (1961) using butyrylthiocholine as a substrate (Kim et al., 1998; Choi et al., 2004). Separately, diisopropylfluorophosphate (final 50 μM) was added to the remaining blood samples, and serum was collected by centrifugation. Physostigmine was extracted by serial treatment with 0.7% ammonium hydroxide,

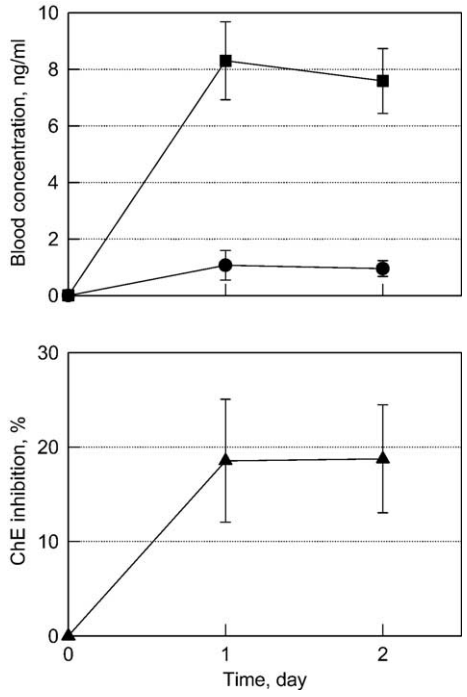

Fig. 1. Time-courses of blood concentrations of physostigmine (●) and procyclidine (■), and inhibition rate (▲) of blood cholinesterase (ChE) activity during 2-day attachment with a patch (7×7 cm) containing 1.5% physostigmine and 6% procyclidine in dogs (body weights 9–10 kg). Values are mean±S.E.M. (n=16).

tert–butyl ether and 0.01 M hydrochloric acid, and then analyzed with a high pressure liquid chromatograph (Waters 2695 XC-D, Milford, USA) using an internal standard *N*,*N*-dimethylcarbamate (Brodie et al., 1987). Procyclidine was extracted with ethyl acetate–hexane (1:3), 1 M hydrochloric acid and 10 M sodium hydroxide plus ethyl acetate–hexane, and then analyzed with a gas chromatograph (Hewlett Packard 6890, Oxford, USA) using trihexyphenidyl as internal standard (Owen et al., 1989).

From the profiles of enzyme inhibition and blood concentrations, the optimal size (7×7 cm/dog) of the combinational patch was selected to achieve approximately 20% inhibition of blood cholinesterase activity by physostigmine and 5–10 ng/kg of blood concentration of procyclidine. The dogs, attached with the combinational patch for 2 days, were subcutaneously challenged with increasing dose levels (2–10 LD_{50}) of soman. Separately, the traditional antidotes composed of atropine (2 mg/dog) plus 2-pralidoxime (600 mg/dog) or atropine plus HI-6 (500 mg/dog), alone or in combination with the patch, were treated intramuscularly 1 min after the soman poisoning. The dogs were monitored for cholinergic symptoms such as salivation, emesis, defecation, diarrhea and tremors as well as seizures and physical incapacitation such as incoordination and recumbency. In addition, time to death was recorded, and the LD_{50} values were estimated on the basis of 24-h mortality. The protective effect of each combination on the lethality of dogs exposed to soman was expressed as protection ratio (folds of LD_{50} in treated group over LD_{50} in control group).

2.4. Neuroprotective effect

The dogs challenged with soman for the investigation of antidotal efficacy were subjected to the evaluation of neuroprotective effect of the patch and therapeutic antidotes. At the time of death or 24 h after soman challenge for survived dogs, whole brain was removed and fixed in 10% neutral buffered-formalin solution. For the evaluation of neural injuries, paraffin-embedded brain sections (4 μm in thickness) were stained with hemathoxylin and eosin. Neuronal death and integrity of neuropils in hippocampus, the most-susceptible region, were examined under a light microscope, and the degree of brain injury was evaluated using 5-point scores based on the approximate percentage of tissue involvement according to the grading system of McDonough et al. (1995) with a slight modification (Kim et al., 1999, 2002); 0, no lesion; 1, minimal (1–10%); 2, mild (11–25%); 3, moderate (26–45%); 4, severe (46–60%); 5, extreme (>60%).

3. Results

3.1. Blood concentrations and enzyme inhibition

Two-day attachment of combinational patch (7×7 cm) containing 1.5% physostigmine and 6% procyclidine led to mean 0.95–1.07 ng/ml of blood concentration of physostigmine and 18.5–18.8% inhibition of enzyme activity, showing stable time-courses (Fig. 1). Also, a similar pattern of profile in blood

Table 1
Effect of antidotes on the incidence of clinical signs and death of dogs following intoxication with soman

Treatment (dose/dog)	Soman (×LD_{50})	Response ratio[a]				Time to death (h)[b]
		Salivation	Tremors	Seizures	Death	
None	1.0	2/2	2/2	1/2	1/2	11.2
	1.4	2/2	2/2	2/2	2/2	7.0±2.1
	2.0	3/3	3/3	3/3	3/3	1.7±1.0
Patch (7×7 cm)	2.0	0/3	0/3	0/3	0/3	–
	3.0	0/3	1/3	0/3	0/3	–
	4.0	0/3	1/3	0/3	0/3	–
	5.0	4/4	4/4	3/4	3/4	8.4±2.5
	6.0	3/3	3/3	3/3	3/3	4.3±1.4
Atropine (2 mg)+ 2-pralidoxime (600 mg)	2.0	1/2	2/2	1/2	0/2	–
	3.0	3/3	3/3	3/3	3/3	6.1±2.3
	4.0	3/3	3/3	3/3	3/3	2.0±0.8
	5.0	3/3	3/3	3/3	3/3	1.3±0.8
Atropine (2 mg)+ HI-6 (500 mg)	2.0	1/2	0/2	0/2	0/2	–
	3.0	3/3	2/3	2/3	2/3	6.0±1.7
	4.0	3/3	3/3	3/3	3/3	5.3±3.8
	5.0	3/3	3/3	3/3	3/3	1.1±0.5
Patch→ Atropine+ 2-pralidoxime	4.0	0/2	1/2	1/2	0/2	–
	5.0	0/2	2/2	1/2	1/2	8.7
	6.0	2/4	4/4	4/4	4/4	6.4±2.8
	8.0	1/2	2/2	2/2	2/2	1.2±0.7
Patch→Atropine +HI-6	6.0	0/3	3/3	0/3	0/3	–
	8.0	0/3	2/3	3/3	0/3	–
	10.0	0/3	3/3	3/3	3/3	4.0±2.2

[a]Values are the number of animals responded/challenged. [b]Values are mean±S.E.M.

Table 2
Protective effects of combinational antidotes against soman poisoning

Treatment (dose/dog)	Protection ratio (×LD_{50})
Patch (7×7 cm)	4.7
Atropine (2 mg)+2-pralidoxime (600 mg)	2.5
Atropine (2 mg)+HI-6 (500 mg)	2.7
Patch→Atropine+2-pralidoxime	5.0
Patch→Atropine+HI-6	9.0

concentration of procyclidine was achieved, ranging from 7.6 to 8.3 ng/ml during 2 days.

3.2. Effect on clinical signs and mortality

In a previous report, the LD_{50} value of soman in dogs was demonstrated to be 9.1 μg/kg, which was also confirmed in beagle dogs in our preliminary study (unpublished data). In the present study, the dogs exposed to 9.1 μg/kg (1 LD_{50}) of soman exhibited severe salivation, emesis, defecation and tremors, leading to seizures and death in 1 out of 2 dogs 11.2 h after soman challenge (Table 1). Higher doses (1.4–2 LD_{50}) of soman induced cholinergic signs and profound convulsions in all dogs, resulting in 100% mortality, in which the time to death was remarkably shortened according to the increase in the exposure level.

In contrast, the dogs attached with the patch containing physostigmine (1.5%) and procyclidine (6%) 2 days before challenge with 2 LD_{50} (18.2 μg/kg) of soman did not show toxic signs. Furthermore, only mild transient tremors were observed in patch-attached dogs after exposure to 3–4 LD_{50} of soman, leading to 100% survival without additional treatment. The patch exerted a partial protection against seizures and mortality of dogs poisoned with 5 LD_{50}, but no effect on 6 LD_{50} of soman, although it markedly delayed the time to death of animals. Thus, the protection ratio was estimated to be 4.7 fold (Table 2).

The traditional antidotes atropine plus 2-pralidoxime, intramuscularly administered 1 min after soman poisoning, prevented dogs from mortality following exposure to 2 LD_{50} of soman, in spite of partial effects on clinical signs. It was found that the efficacy of atropine plus HI-6 was somewhat higher than that of atropine plus 2-pralidoxime; i.e., 100% and 33% of

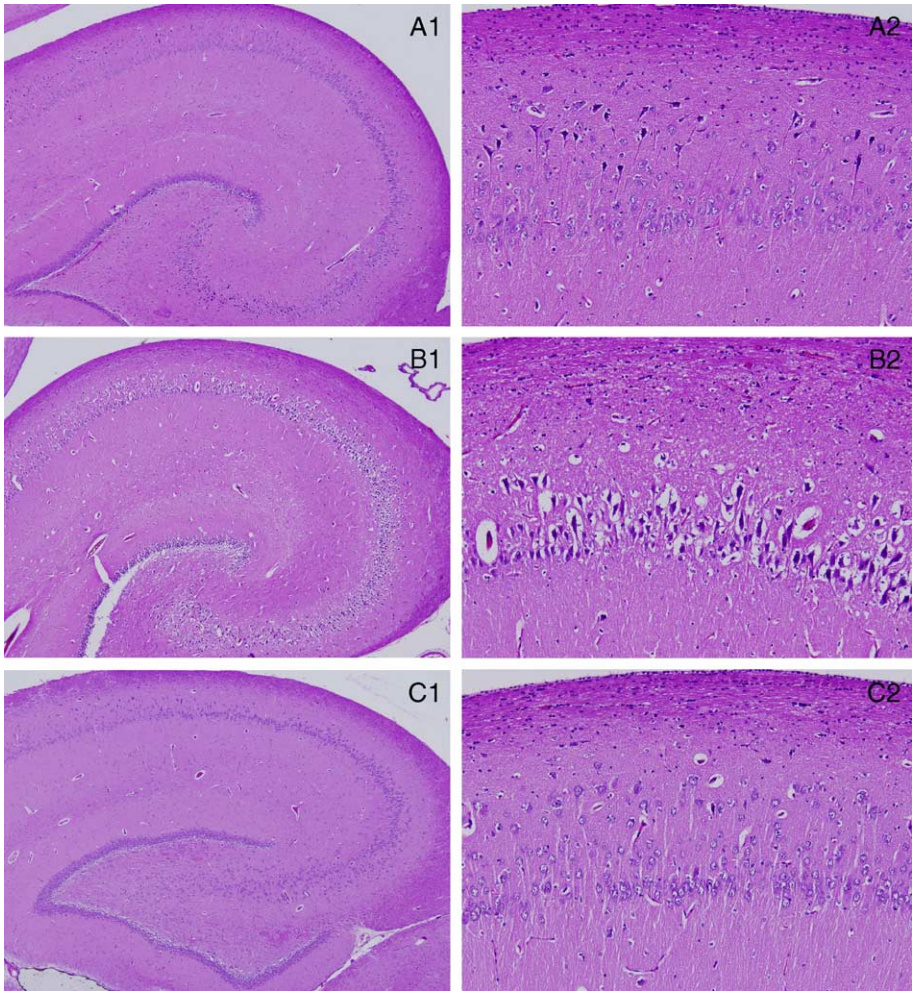

Fig. 2. Effect of combinational antidotes on brain injuries induced by soman. Note the pyramidal neurons exhibiting dark degeneration, leading to pericellular halo, in hippocampus of a dog treated with atropine (2 mg) plus HI-6 (500 mg) following exposure to 4 LD_{50} of soman (B), in contrast to mild injuries in a dog attached with a patch (7×7 cm) (A). After intoxication with 6 LD_{50} of soman, normal features are observed in dogs attached with the patch followed by atropine plus HI-6 (C).

dogs treated with atropine plus HI-6 survived the challenge with 2 and 3 LD_{50} of soman, respectively, in comparison with no protective effect of atropine plus 2-pralidoxime on 3 LD_{50} of soman. The estimated protection ratios were determined to be 2.5 and 2.7 folds for atropine plus 2-pralidoxime and atropine plus HI-6, respectively.

For further investigation on the combinational effects of the prophylactic patch and therapeutic antidotes, the patch-attached dogs were additionally treated with atropine plus 2-pralidoxime or atropine plus HI-6 after soman poisoning. Noteworthy, a substantial synergistic effect was achieved by the combination of patch with atropine plus HI-6, but not with atropine plus 2-pralidoxime. In comparison with slight additional reduction of clinical signs and mortality of patch-attached dogs by combination with atropine plus 2-pralidoxime (5-fold protection ratio), the cholinergic symptoms, seizures and mortality were greatly decreased by combination with atropine plus HI-6, leading to 9 fold of protection ratio.

3.3. Effect on brain injuries

To compare the neuroprotective effects of the patch and therapeutic antidotes, the degrees of brain injury induced by poisoning with 2, 4, 6 or 8 LD_{50} of soman were compared. The dogs underwent epileptiform seizures showed dark shrinkage degeneration of neurons, producing a pericellular halo. Although hippocampal CA1 region was the most susceptible, the neurodegeneration was found to spread to all hippocampal formation (CA1–CA4 and dentate gyrus) in severe cases (Fig. 2B).

In control group, the dogs exposed to 2 LD_{50} of soman exhibited 100% seizures and mortality with mean survival time of 1.7 h, leading to mean score 2.00 of brain lesions (Table 3). In contrast, the patch-attached animals survived the challenge with 2 LD_{50} of soman did not show any brain lesions, and the dogs exposed to 4 LD_{50} of soman exhibited only a minimal neuronal injuries (mean score 0.50) (Fig. 2A). In comparison, antidotes-treated dogs survived 2 LD_{50} of soman exhibited mean scores 1.90 and 1.00 of brain lesions for atropine plus 2-pralidoxime and atropine plus HI-6, respectively. In addition, the dogs exposed to 4 LD_{50} of soman died in 2.0 and 5.3 h, resulting in mean scores 2.00 and 3.25 of brain injuries, in spite of treatment with atropine plus 2-pralidoxime and atropine plus HI-6, respectively. Interestingly, combination of the patch with atropine plus HI-6 fully prevented death and brain injuries of the dogs exposed to 6 LD_{50} of soman (Fig. 2C), although the combination did not exert remarkable neuroprotective effect in dogs challenged with 8 LD_{50} of soman.

Table 3
Effect of antidotes on the score of hippocampal lesions of dogs following intoxication with soman

Treatment (dose/dog)	Soman (×LD_{50})	Mortality response ratio[a]	Time to death or sacrifice (h)[b]	Lesion (score)[b]
Control	2.0	3/3	1.7±1.0	2.00±0.25
Patch (7×7 cm)	2.0	0/3	(24.0)	0.00±0.00
	4.0	0/3	(24.0)	0.50±0.50
	6.0	3/3	4.3±1.4	1.75±0.50
Atropine (2 mg)+ 2-pralidoxime (600 mg)	2.0	0/2	(24.0)	1.90±0.58
	4.0	3/3	2.0±0.8	2.00±0.50
Atropine (2 mg)+ HI-6 (500 mg)	2.0	0/2	(24.0)	1.00±0.00
	4.0	3/3	5.3±3.8	3.25±0.75
Patch→Atropine+ 2-pralidoxime	6.0	4/4	6.4±2.8	1.75±0.50
	8.0	2/2	1.2±0.7	1.00±0.00
Patch→Atropine+ HI-6	6.0	0/3	(24.0)	0.00±0.00
	8.0	0/3	(24.0)	1.67±0.29

[a]Values are the number of animals responded/challenged. [b]Values are mean±S.E.M.

4. Discussion

It is well known that carbamates potentiate antidotal efficacy of anticholinergics against nerve-agents including soman (Berry and Davies, 1970; Dirnhuber et al., 1979; Gordon et al., 1978), and that early treatment with anticholinergics including scopolamine attenuates seizures, leading to brain injuries (Shih et al., 1991a; McDonough and Shih, 1993). Accordingly, there was an attempt to develop a simple prophylactic regimen containing physostigmine and scopolamine (Lim et al., 1988; Meshulam et al., 1995; Philippens et al., 1996, 2000a; Wetherell, 1994). The combinational regimen, in both formulas of osmotic minipumps and transdermal patch, was confirmed to exert high synergistic protective effects against soman poisoning (Meshulam et al., 1995; Wetherell, 1994).

More recently, a combination of physostigmine with procyclidine, an *N*-methyl-D-aspartate receptor antagonist possessing anticholinergic activity, was found to be highly effective in the prevention of lethality, seizures and brain injuries induced by high doses of soman (Kim et al., 2002; Myhrer et al., 2004b). In addition, continuous infusion of physostigmine and procyclidine using osmotic minipumps was confirmed to exert high efficacies (Choi et al., 2004). In this context, we developed a matrix-type patch, containing 1.5% physostigmine, 6% procyclidine and 0.5% hydrocortisone, in which the drug concentrations in the patch were optimized to achieve a balance of approximately 30% inhibition of blood enzyme activity by physostigmine and blood concentration (50–100 ng/ml) of procyclidine clinically available for the treatment of Parkinsonism in human (Dean et al., 1980; Whiteman et al., 1985), and to minimize skin irritancy (Kim et al., 2003; Lee et al., 2003b). The patch exhibited constant blood concentrations of physostigmine and procyclidine over 2 days in hairless guinea pigs and in human (unpublished data). The controlled release of physostigmine and procyclidine from the patch also led to stable profiles of blood concentrations and enzyme-inhibition rate during 2 days in dogs (Fig. 1).

It was reported that the efficacy of carbamates in combination with anticholinergics was very high in monkeys followed by guinea pigs, dogs, rabbits, mice, chickens and rats (Berry and Davies, 1970; Dirnhuber et al., 1979; Gordon et al., 1978). The efficacy of physostigmine (0.1 mg/kg) alone or in combination with low doses (0.3–3.0 mg/kg) of procyclidine in guinea pigs was much higher than that in rats (Kim et al., 2002). In addition,

it was proposed that continuous infusion of physostigmine via osmotic minipumps in primates was much more effective than a bolus injection or continuous infusion in rodents (Lim et al., 1988; Philippens et al., 2000b). In the present study, the combinational patch fully protected seizures and mortality induced by 4 LD_{50} of soman in dogs (Table 1), in contrast to only 1.6 LD_{50} in rats (Myhrer et al., 2004b). Thus, it is expected that a remarkable effect could be achieved with the patch containing physostigmine and procyclidine in primates including human. In a follow-up study, we observed a marked protection with the patch against soman poisoning in Cynomolgus monkeys (unpublished data).

Dogs were more sensitive not only to nerve-agent soman, but also to physostigmine and procyclidine. The LD_{50} value of soman was determined to be 9.1 μg/kg in dogs (Boskovic et al., 1984; the present study), much lower that that (75 μg/kg) in rats (Kim et al., 2002). Also, a low blood concentration (0.95–1.07 ng/ml) of physostigmine in dogs inhibited 18.5–18.8% of enzyme activity (Fig. 1), in comparison with 2.6 ng/ml for 20% inhibition in rats (Choi et al., 2004). In the present study, a small size (7 × 7 cm) of patch was selected to achieve a relatively-low inhibition rate of enzyme activity, compared with adequate enzyme inhibition rate of 20–40% for the effective protection against nerve-agent poisoning (Cook and Kolka, 1992; Dunn and Sidell, 1989; Kim et al., 1998; Wetherell, 1994). It is expected that higher protection ratios (>4.7 fold) could be obtained with a larger size of patch than used in this study (7 × 7 cm), since a positive correlation was found between the degree of enzyme inhibition by carbamates and protective efficacy, in combination with antidotes, against soman lethality, wherein inhibition level as low as 10% provided some protection (Lennox et al., 1985). Moreover, it is believed that a higher efficacy could be achieved by procyclidine in dogs than in rats, as inferred from that only 7.6–8.3 ng/ml of blood concentration of procyclidine, in combination with physostigmine, exerted 4.7 LD_{50} of protection ratio (Table 2), in comparison with 2.24 LD_{50} obtained by 50 ng/ml of blood concentration following injection with 1 mg/kg of procyclidine in rats (Kim et al., 2002).

It was expected that the possible side effects of carbamates and anticholinergics in combinational pretreatment might be offset by each other (Berry and Davies, 1970; Lim et al., 1991; Philippens et al., 1996, 2000a), although somewhat different results were reported (Myhrer et al., 2004a). In addition, sustained release of physostigmine and procyclidine from osmotic minipumps, at doses without adverse effects, was proved to exert high efficacies in rats (Choi et al., 2004). In our previous studies, adverse effects and mortality were markedly attenuated and eliminated in dogs implanted with osmotic minipumps containing both physostigmine and procyclidine, although profound cholinergic symptoms, debility and death were induced by each extremely-high dose of physostigmine or procyclidine (Huang et al., 2003). In the present study, a relatively-small size (7 × 7 cm) of the patch was applied to a dog (9–10 kg) to avoid possible adverse effects, in which the blood concentrations of physostigmine (0.95–1.07 ng/ml) and procyclidine (7.6–8.3 ng/ml) were much lower than sign-free concentrations of physostigmine (2.49–2.57 ng/ml) and procyclidine (357–502 ng/ml) continuously infused via osmotic minipumps (Lee et al., 2003a), indicating that the drugs are well tolerated. Interestingly, very low blood concentration of procyclidine in dogs was obtained compared to that in human (mean 73 ng/ml) by attachment with the same size (7 × 7 cm/body) of the patch, suggestive of a high penetration into human skin (unpublished data). In spite of the relatively-low blood concentration of procyclidine in dogs, the patch was superior to the traditional antidotes in the prevention of dogs from toxic signs, brain injuries and mortality.

On the other hand, the convulsive dogs treated with therapeutic antidotes exhibited physical incapacitation, showing incoordination and recumbency. The protective efficacy of atropine plus HI-6 (2.7 LD_{50}) against soman was somewhat higher than that of atropine plus 2-pralidoxime (2.5 LD_{50}) (Table 2), although there was a marked difference in rats (Shih et al., 1991b; Kim et al., 2002). However, the patch containing physostigmine and procyclidine greatly enhanced the efficacy of atropine plus HI-6 only; that is, cholinergic signs, seizures, brain injuries and mortality of dogs poisoned with high doses (6–8 LD_{50}) of soman were substantially attenuated and eliminated, indicative of synergistic protective effects between physostigmine and atropine against mortality, procyclidine and atropine against cholinergic signs and brain injuries, and procyclidine and HI-6 against mortality and brain injuries (Tables 1 and 3). In contrast, only slight increases in the anticholinergic effect and survival rate were obtained by the combination of the patch and atropine plus 2-pralidoxime. It is of interest to note that the anticonvulsant and neuroprotective effects of the combination of physostigmine, procyclidine, atropine and HI-6 were higher than those of physostigmine, procyclidine, atropine and 2-pralidoxime (Tables 1 and 2), suggestive of a role of HI-6 in the efficacy. Such a beneficial effect of HI-6 in combinational therapy might be due to its potentials to recover neuronal transmission in spite of continued inhibition of acetylcholinesterase (van Helden et al., 1996). Also, superior effects of HI-6 to 2-pralidoxime may be inferred from a beneficial neuroprotective effect of a combination of atropine, HI-6 and prodiazepam, compared to that of atropine, 2-pralidoxime and diazepam in monkeys (Lallement et al., 1997), although central effects of HI-6 are in controversy (Clement, 1992; Lundy and Shih, 1983). In spite of this, the neuronal injury was more severe in dogs treated with atropine plus HI-6 (mean score 3.25) than the animals administered with atropine plus 2-pralidoxime (mean score 2.00) following exposure to 4 LD_{50} of soman (Table 2). However, this may be due to the long survival time (5.3 h) of dogs treated with atropine plus HI-6, suggesting that the duration of seizures governs the degree of neuronal injuries. Such a phenomenon was also observed in the dogs treated with the patch and atropine plus 2-pralidoxime, in which more severe brain lesions were seen in dogs (mean score 1.75) experienced 6.4-h seizures after exposure to 6 LD_{50} of soman than in animals (mean score 1.00) died in 1.2 h after poisoning with a higher dose (8 LD_{50}) of soman (Table 2).

An anticonvulsant diazepam, widely used in field, was found to exert insufficient neuroprotective activity (McDonough and

Shih, 1997), since a long duration after injection of diazepam is necessary for seizure termination, in contrast to a rapid induction of brain injuries (Lallement et al., 1994; McDonough et al., 1995; Myhrer et al., 2004b). Therefore, rapid control of seizures might be a key element for the effective neuroprotection (Kim et al., 1997; Lallement et al., 1998; Myhrer et al., 2003). In the present study, we showed that the combinational prophylactic patch containing physostigmine and procyclidine fully protected dogs against 2 LD_{50} of soman, and that only mild tremors and minimal brain lesions were observed in dogs exposed to 4 LD_{50}, leading to 100% survival without additional treatment. Furthermore, the combination of the patch with atropine plus HI-6 fully prevented death and brain injuries of the dogs exposed to 6 LD_{50} of soman. Taken together, it is suggested that the combinational patch composed of physostigmine and procyclidine, especially in combination with atropine plus HI-6, could be a choice for the quality survival from poisoning with nerve-agents including soman.

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