

ANTIDOTAL EFFICACY OF PYRIDINIUM OXIMES AND CHOLINEACETYLTRANSFERASE INHIBITORS AGAINST ORGANOPHOSPHORUS INTOXICATION IN RODENTS

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Abstract : In an attempt to develop effective antidote against organophosphorus intoxication, some new imidazole-pyridinium mono-oximes, long chain pyridinium mono-oximes and cholineacetyltransferase inhibitors were synthesised. These compounds were evaluated for their *in vivo* therapeutic protection and neuromuscular function studies in rodents. The results indicate that SPK-series oximes may be useful against sarin poisoning without any beneficial effect against VX (O-Ethyl S-2-NN-diisopropylaminoethyl methylphosphonofluoridate) intoxication. The cholineacetyltransferase (ChAT) inhibitors may not be of any help against any of the OP compounds studied in this study.

Key words : organophosphorus poisoning nerve agents sarin
protection studies neuromuscular function antidotes
cholineacetyltransferase inhibitors oximes

INTRODUCTION

Although the highly toxic nature of organophosphorus (OP) compounds has been known for many years (1, 2), there still exist some limitations in the antidotal therapy available against poisoning of these compounds. Currently pyridinium oximes 2-pyridine aldoxime (2-PAM), obidoxime and trimedoxime in combination with atropine (3) are used for the treatment of acute intoxications with OP insecticides or the nerve agents tabun, sarin, soman and VX (O-Ethyl S-2-NN-diisopropylaminoethyl methyl phosphonofluoridate). However, clinical experience has indicated that

oximes are not always very efficient reactivators of acetylcholinesterase (AChE) depending on the type of nerve agent intoxication. An effective therapy by a single oxime to all the known organophosphorus chemical warfare agents is still lacking.

It is well known that the quaternary oximes have constrains for crossing the blood brain barrier and hence produce limited beneficial effect against OP intoxications. We have attempted to overcome this problem by substituting with more lipophilic group and synthesising some new imidazolium-pyridinium mono-oximes having

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bridge with-(p)xylene. Since, over the decades much of the impetus has been directed towards development of choline acetyltransferase (ChAT) inhibitors (4), therefore some styrylpyridine prototype cholineacetyltransferase (ChAT) inhibitors were also prepared. Both the class of compounds were evaluated for their protective effect against OP intoxication in mice and the oximes were examined for their effects on neuromuscular functions in rats.

METHODS

Animals: Male Wistar strain rats (140–180 g) and albino Swiss mice (22–26 g) bred in the Animal Facility of the Defence Research and Development Establishment Gwalior were used. The animals were housed in groups of 3 under 12 hr light/12 hr dark cycle in environmentally controlled rooms ($28 \pm 1^\circ\text{C}$) with a constant humidity. The animals were allowed free access to food (Lipton India pellet diet).

Drugs and Chemicals: The organophosphorus compounds sarin and VX were prepared in the Synthetic Chemistry Division of Defence R & D Establishment, Gwalior and were >98% pure. 1-methyl imidazole, 1, 2-dimethyl imidazole and 1-methyl-2-mercapto imidazole were purchased from Fluka Chemicals and were used without further purification. The substituted imidazolium-pyridinium oxime derivatives bridged by (p)-xylene moiety (Fig. 1) were prepared from the condensation reaction of substituted imidazole with 1-bromo-1'-[(3-hydroxyiminomethyl)-pyridinium]-(p)-xylene bromide in ethanol under reflux; which in turn was

prepared from α , α' -dibromo-(p)-xylene and 3-hydroxyiminomethyl pyridine in acetone under reflux for 8 hours. The compounds prepared were homogeneous on TLC and their structures were consistent with the spectral data. All the oximes were crystalline solids with sharp melting points.

Long chain pyridinium oximes (SPK-3 and SPK-4) were prepared by refluxing corresponding alkyl halides with pyridinium oxime (5, 6). The purity was checked by thin layer chromatography (TLC, cellulose, DS-O, Fluka) with 1 butanol : acetic acid : water (3:1:1) as solvent system and their structures were confirmed by IR, ^1H NMR (taken in DMSO- d_6). Choline acetyltransferase (ChAT) inhibitors were prepared by adopting reported procedures (7) as depicted in Fig. 1.

In vivo protection studies: The protection studies were carried out in male Swiss mice as described earlier (8). The OP agents were injected subcutaneously and atropine sulphate (10 mg/kg) was given intraperitoneally 30 sec later, followed by intramuscular administration of oxime (30 mg/kg) after an additional 30 sec. Cholineacetyltransferase (ChAT) inhibitors were administered (10–30 mg/kg, i.m.) 2–60 min prior to organophosphate challenge and then 24 hr survival of animals was observed. The 24 hr LD_{50} values for sarin and VX were determined by Dixon's up and down method (9) using 6–8 animals at each concentration of the toxicant. The protective index (PI) was calculated using the following formula:

$$PI = \frac{LD_{50} \text{ of organophosphate + antidote}}{LD_{50} \text{ of organophosphate}}$$

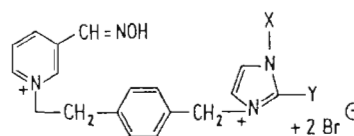
Neuromuscular (NM) function studies: The *in vitro* NM function studies were carried out using phrenic nerve diaphragm preparation of albino rats. The left hemidiaphragm was removed as described by Bulbring (10) and suspended in an organ bath containing Krebs' Ringer solution (composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 24.8 mM NaHCO₃, 11.1 mM glucose, 1.2 mM KH₂PO₄ and 1.2 mM MgSO₄). The phrenic nerve was stimulated with supra maximal voltage (1–10 V) of 0.2 ms duration at 0.2 Hz with a Grass Stimulator (Model S-88). The analysis of over all percentage recovery from VX poisoning was determined from the four tetanic responses as described earlier (11, 12).

The analysis of NM recovery from VX poisoning following oxime administration was carried out in three ways: (i) recovery due to direct oxime action, which was estimated by subtracting the percentage recovery in test D (after washout of oxime) from that in test C (oxime present in the bath), (ii) oxime induced reactivation of acetylcholinesterase, which was estimated by subtracting percentage recovery in test E (after reinhibition by a second dose of VX) from that in test D, and (iii) the persistence of neuromuscular recovery, which was defined as adaptation (11).

RESULTS

Synthesis of compounds: The imidazole-pyridinium mono-oximes were prepared

by standard procedures. The structures of all compounds were established based on IR and ¹HNMR spectra, and their purity was established by thin layer chromatography and microanalyses. The sharp melting point of the compound and the fact that only one oximino compound was formed from the pyridine carbaldoxime, attest to the formation of stable single isomer of 'E'-configuration. This is further supported by IR and ¹HNMR data (7, 13).



Imidazolium-pyridinium oximes bridged with p-xylylene moiety.

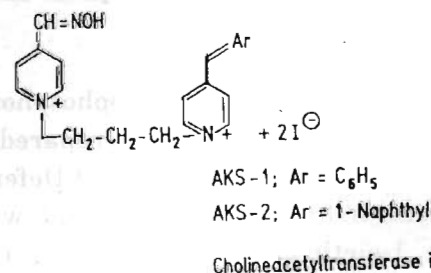
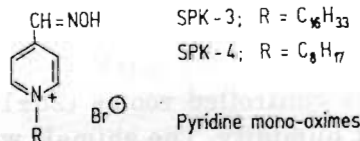


Fig. 1 : Chemical structures of compounds.

Protection index: The 24 hr LD₅₀ of sarin in male mice was found to be 196.74 (169.12–228.88) µg/kg, s.c. and of VX 18 (16.23–21.35) µg/kg, s.c. The results (Table I) demonstrate that atropine, 2-PAM, imidazolium-pyridinium oximes (JA Group) and ChAT inhibitors alone

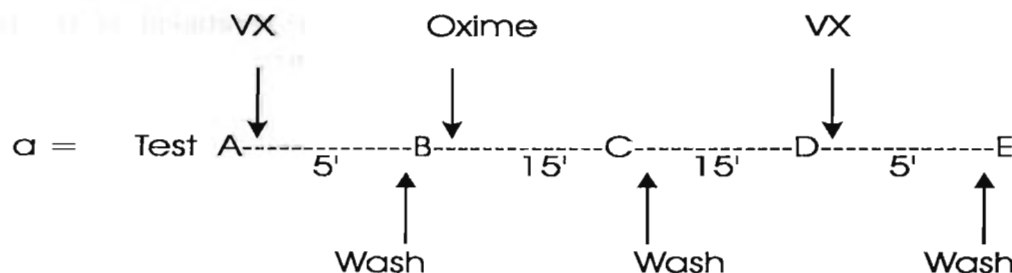
TABLE I: *In vivo* protection studies against sarin and VX intoxication in mice.

S.No.	Treatment	Sarin		VX	
		LD ₅₀ (µg/kg)	Pl	LD ₅₀ (µg/kg)	Pl
1.	None	196.74 (169.12-228.88)	-	18.00 (16.23-21.35)	-
2.	AT	238.98 (205.26-277.75)	1.21	28.54 (24.04-33.88)	1.59
3.	2-PAM	212.81 (182.94-247.57)	1.08	20.21 (17.01-22.15)	1.12
4.	AT+2-PAM	476.43 (409.54-554.24)	2.42	88.04 (74.17-104.52)	4.89
5.	AKS-1	204.67 (172.89-210.97)	1.03	19.20 (17.82-22.31)	1.07
6.	AKS1+AT+2-PAM	534.56 (459.51-621.81)	2.72	74.98 (65.35-74.98)	4.17
7.	JA1	205.13 (189.26-211.72)	1.04	18.89 (16.37-20.96)	1.05
8.	AT+2-PAM+JA1	503.65 (462.27-543.37)	2.56	86.04 (73.91-105.26)	4.78
9.	SPK-3	210.91 (180.34-246.75)	1.06	19.96 (17.10-21.98)	1.10
10.	SPK-4	211.85 (182.12-247.19)	1.07	20.34 (17.98-22.34)	1.13
11.	AT+SPK-3	751.55 (681.24-831.12)	3.82	35.82 (29.81-40.87)	1.99
12.	AT+SPK-4	753.51 (682.37-829.68)	3.83	45.18 (36.24-53.24)	2.51

Atropine sulphate (AT) 10 mg/kg, i.p. 30 sec post OP challenge; Oximes (monopyridinium/bispyridinium) 30 mg/kg, i.m. 1 min post OP; ChAT inhibitors 10-30 mg/kg, i.m. 2-60 min prior to OP; SPK-3: SPK-4: 30 mg/kg i.m.

TABLE II: Effect of oximes on isolated phrenic nerve diaphragm preparation after blockade by VX.

Group	Oximes	%Neuromuscular Transmission ^a (Mean ± S.E.)				
		Test B	Test C	Test D	Test E	Test D-E
1.	None	02.3±0.13	03.1±0.14	04.5±0.15	02.1±0.11	02.4
2.	SPK-3	01.6±0.10	04.5±0.19	68.7±3.81	3.5±0.13	65.2
3.	SPK-4	01.2±0.11	05.3±0.20	71.5±4.12	04.1±0.18	67.4
4.	2-PAM	02.1±0.12	02.8±0.19	74.3±4.01	04.7±1.1	69.6



Test A = Control series of tetanic contractions taken as 100% NM transmission

Test B = Completeness of NM blockade after 1 µM VX

Text C = Recovery in presence of oxime (0.1µM)

Test D = Recovery after a second dose of VX (2 µM); SPK-3, SPK-4 (30 µM); 2-PAM (0.15 µM)

Control experiments were carried out in which the normal Ringer solution was substituted for the oxime.

did not show any significant protection against sarin/VX intoxication in mice. Moreover, atropine alone was found to produce slightly higher (statistically insignificant) protection as compared to ChAT inhibitors and imidazole-pyridinium oximes against sarin and VX poisoning. However, these compounds along with atropine produced statistically significant protection against VX as compared to sarin poisoning. The administration of SPK-series compounds alone did not produce any significant effect on protection index. However, these compounds along with atropine produced better protection against sarin poisoning as compared to VX poisoning. On the other hand, monopyridinium oxime (2-PAM) and its isomeric-N-alkylated derivatives (SPK-3 and SPK-4) along with atropine demonstrated an enhanced protection against sarin and VX poisoning in mice. Further, the antidotal efficacy of 2-PAM was found to be higher (PI = 4.89) against VX as compared with sarin intoxication (PI = 2.42). Administration of atropine and N-alkylated pyridine aldoxime derivatives (SPK-3 and SPK-4) showed a high degree of protection index (PI = 3.82) as compared to 2-PAM (PI = 2.42) in sarin poisoning.

The imidazolium-pyridinium oximes bridged by (-p)-xylene moiety either alone or in combination with atropine sulphate did not produce any beneficial effect against sarin or VX poisoning in mice (data shown only for one compound). The ChAT inhibitors (AKS-1, AKS-2) given 2-60 min before OP challenge followed by atropine and 2-PAM did not give any added beneficial effect (data shown only for one

compound) as compared to atropine and 2-PAM.

Neuromuscular function studies: Neuro-muscular studies demonstrated that VX (1 μ M) 5 min after washout from the organ bath produced a complete tetanic fade on all the four frequencies studied, which could not recover significantly even after repeated washing (Table II). Addition of SPK-3 and SPK-4 (30 μ M) *per se* did not produce any significant effect on NM function for an observation period of 2 hrs. However, the addition of SPK-series oximes (SPK-3 and SPK-4) after VX challenge was able to produce recovery in VX induced tetanic fade. This recovery was observed 15 min after washout from the organ bath. Again after giving a second concentration of VX (2 μ M) after test D, a significant tetanic fade was observed (test E), which could not be recovered after administration of SPK-3 or SPK-4 oximes.

DISCUSSION

The search for an effective antidote is critical to the treatment of OP poisoning especially since variation exists in the effectiveness of current drugs of choice. The ultimate intention is prophylaxis or therapy, rational approaches to the design of antidotes have focused on essentially two ways to counter the toxicity of OP compounds, namely (i) blocking the cholinergic effects of the elevated acetylcholine levels, and (ii) reducing those levels. Since so far no single compound acting in either of these ways has proved completely satisfactory, in practice combination of drugs having complementary modes of action are generally used.

The results of the present study clearly demonstrate that atropine, oximes (2-PAM, SPK-series, imidazole pyridinium) and ChAT inhibitors *per se* did not produce any protection against sarin or VX intoxication in mice. This is in agreement with our earlier studies (6, 12). However, administration of SPK-series oximes alongwith atropine produced better protection against sarin as compared to VX poisoning. This might be probably due to difference in the degree of toxicity of both OP compounds. Among the drugs used to counteract the muscarinic effects of OP poisoning, atropine sulphate is currently the drug of choice because of their pharmacological effectiveness even after repeated administration in man (14). The variation in the protection index observed following administration of 2-PAM or SPK-series oximes against sarin or VX poisoning needs the determination of cholinesterase in blood and various tissues including different areas of brain to relate the degree of cholinesterase reactivation against sarin and VX intoxication. SPK-series oximes alongwith atropine demonstrated a higher degree of protection index as compared to 2-PAM against sarin intoxication. This is also in good agreement with our earlier findings in case of sarin poisoning (6). The prophylactic administration of ChAT inhibitors alongwith atropine and 2-PAM did not produce any additional beneficial effect against either of the OP poisoning. In contrast, *in vivo* protection against soman toxicity by known inhibitors of acetylcholine synthesis has been demonstrated (15). Further, the expected acetylcholine synthetase inhibitors could not be subjected for evaluation to *in vitro* reaction activity against OP inhibited AChE due to poor water

solubility (7) and the solvent dimethyl sulfoxide (DMSO) being inhibitor of enzyme could not be used to make aqueous solution of compounds. On the other hand since no desired efficacy could be achieved by any one of the compounds reported herein, we did not make any attempt to assay cholineacetyltransferase activity as it would not have given any further useful information.

It is very much evident from the data that washing of oximes from the organ bath resulted in higher recovery of NM transmission (69–74%) as compared to recovery in the presence of oximes as discussed earlier (7, 11). In all cases, the recovery was most prominent at lower frequencies of stimulation. A lower concentration of 2-PAM was used in the present study, since earlier studies (16) showed that it produced inhibition of muscular twitch response due to curare like action at higher frequencies. However, SPK-series oximes did not produce NM blockade even at 30 μ M concentration, but resulted in recovery of neuromuscular transmission. The latter effect is beneficial since NM blockade is one of the side effects associated with oxime therapy in the treatment of anticholinesterase poisoning (17). The SPK-3 and SPK-4 produced a better recovery when given alongwith VX as compared to given 15 min post VX challenge. In the present investigation, the neuromuscular recovery may be attributed to oxime induced reactivation of AChE (D%–E%) and not due to either direct oxime action (C%–D%) or due to adaptation (E%) by desensitization of end plates (18).

CONCLUSION

Based on the above findings, it is concluded that SPK-series oximes may be useful against sarin poisoning and not against other OP poison (such as highly toxic compound VX). In addition ChAT inhibitors may not be of any help

against any of the OP compound studied in this study.

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