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In vivo protection of diisopropylphosphorofluoridate (DFP) poisoning by three bis-quaternary 2 -(hydroxyimino) -*N* -(pyridin-3-yl) acetamide derivatives in Swiss mice

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Abstract

This study reports efficacy of three bis pyridinium derivatives of 2-(hydroxyimino)- N-(pyridine-3-yl) acetamide in terms of survival, reactivation of brain and serum acetylcholinesterase (AChE) activity in diisopropylphosphorofluoridate (DFP) intoxicated Swiss albino male mice. LD_{50} of DFP (3.9 mg/kg, s.c.) and new oximes, HNK-102, HNK-106, HNK-111, (282.8, 35.0 and 35.0 mg/kg respectively, i.m.) was determined. Various doses of DFP and oximes as treatment doses with atropine (10 mg/kg, i.p.) were used to determine protection index (PI). For time dependent maximum AChE inhibition, two doses of DFP (0.20 and 2.0 LD_{50}) were chosen. At optimized time i.e. Sixty minutes, IC_{50} value was calculated as 0.249 and 0.017 LD_{50} of brain and serum AChE, respectively. Shift of DFP induced brain AChE IC_{50} curves to right was observed at 0.20 LD_{50} treatment dose of oximes with respect to 2-PAM. These findings propose that new HNK series of oximes are effective antidote, compared to that of 2-PAM *in vivo.*

Key words: Acetylcholinesterase Reactivators, 2-(hydroxyimino)-N-(pyridin-3-yl) acetamide, Organophosphorous, Nerve agents.

Introduction

During recent past there has been a noticeable increase in incidence of organophosphorous nerve agent exposure to military personnel and civilian population. The use of nerve agents in Iraq-Iran war (1) during terrorist attacks by Aum Shinrikyo sect in Japan (2) and the recent sarin attack in Syria 'war' (3) have emphasized the need for developing more effective therapeutic regimen against nerve agent(s) poisoning. It is well documented that the main mechanism involved in organophosphate poisoning is the inhibition of post synaptic membrane embedded acetylcholinesterase (AChE) enzyme. Due to AChE inhibition, neurotransmitter acetylcholine (ACh) accumulates at cholinergic synapses, resulting in over stimulation of both nicotinic and muscarinic receptors. Sign and symptoms of organophosphorous (OP) poisoning includes - constriction of pupil (miosis), increased production of saliva, running nose, increased perspiration, urination, defecation, bronchoconstriction, bronchosecreation, bradycardia, cardiac arrhythmias, tremors and convulsions. The critical effects are paralysis of respiratory muscles and inhibition of respiratory centre. The present therapeutic regime available against OP compounds poisoning involves the use of anticholinergic drugs (atropine) to competitively block excessive muscaranic receptor stimulation (4), AChE reactivator (oximes) which reactivate OP-inhibited AChE and anticonvulsant drugs (diazepam) to control OP induced seizures and convulsions (5-6).

Oximes as cholinesterase reactivator play a major role in antidotal action against OP poisoning (7). They are mainly *mono* and *bis*-pyridinium oximes which undergo nucleophillic attack on phosphorylated esteric site of AChE and reactivate the phosphorylated cholinesterase enzyme (8-9). Pralidoxime (2-PAM), a mono-pyridinium oxime, is a potent reactivator of AChE against some nerve agent poisoning such as soman, sarin, and VX but not against tabun, cyclosarin and VR agent (10). Therefore, several AChE reactivators were developed (HI-6, HLö-7, HGG-12, TMB-4, obidoxime, K-oximes) to improve *in vitro* and *in vivo* antidotal efficacy of oximes against nerve agent intoxication (11-14). But none of these oximes could act as universal reactivator of the inhibited AChE. In this concern, recently we have reported a new series amide conjugated oximes viz. bis-[2-(hydroxyimino)-*N*-(pyridin-3-yl) acetamide] dibromide. The *in vitro* reactivation efficacies of these compounds were evaluated against nerve agent sarin and VX inhibited human erythrocyte ghost AChE. This study led to the identification of three new oximes i.e., HNK-102, HNK-106 and HNK-111 (3a, 3e and 3i respectively) (15) to evaluate further their therapeutic efficacy against OP poisoning. In continuation to our work on antidotes against OP poisoning, the present study is aimed at investigating the protection (in terms of survival) ability of new oximes i.e. HNK-102, HNK-106 and HNK-111 against diisopropylphosphorofluoridate (DFP, nerve agent mimic) poisoning in Swiss albino male mice. The study also addresses reactivation efficacy to reactivate the inhibited AChE, both in brain and serum *in vivo*.

Materials and methods

Chemicals

Diisopropylphosphorofluoridate (DFP); 1,1'-(ethane-1,2-diyl) bis(3-(2-hydroxxyimino) acetamido) pyridinium) dibromide (HNK-102 or 3b); 1,1'-(hexane-1,6diyl) bis(3-(2-hydroxyimino) acetami-

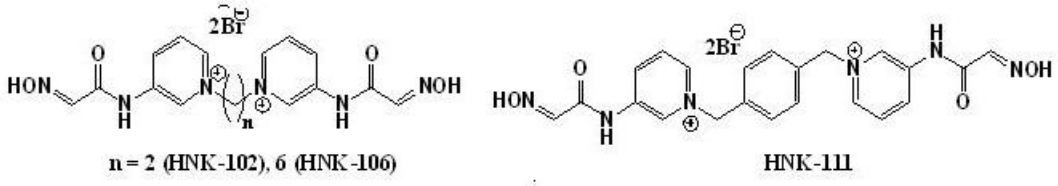

Figure 1. Bis-quaternary 2-(hydroxyimino)-*N*-(pyridin-3-yl) acetamide derivatives.

do) pyridinium) dibromide (HNK-106 or 3f); 1,1'(1,4 Phenylene bis(methylene) bis(3-(2(hydroxyimino) acetamido) pyridinium) dibromide (HNK-111 or 3j) were synthesized in Process Technology Development Division of this Establishment (Figure. 1) (15). The purity of the compounds was checked by thin layer chromatography; characterized by elemental analysis, 1H NMR, mass spectral analysis and were found to be more than 99% pure.

All other chemicals used were of analytical grade. Acetylthiocholiniodide (ASChI), 5, 5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) and atropine sulphate were procured from Sigma Chemicals Co. (St. Louis MO) and Pralidoxime Cl (I.P.) from Kwality Pharma, India.

Animals

Randomized out-bred male Swiss albino mice weighing 25-30 g were used for the study. Steam autoclaved paddy husk used as bedding material in polypropylene cages and four mice housed in each cage. The paddy husk was changed alternate day. The animals were kept in environmentally controlled room (25± 2ºC, RH 40-60%) and were provided with pellet diet (Ashirwad brand, Chandigarh, India) and potable water *ad libitum*. The study was approved by Institutional Animal Ethics Committee (constituted by CPCSEA, Ministry of Environment and Forests, India)

Determination of median lethal dose (LD_{50}), rationale for dose selection and protection index (PI)

Median lethal dose or LD_{50} of DFP (subcutaneous route) and HNK-102, HNK-106 and HNK-111 (intramuscular route) determined following 'moving average' method (16) and expressed as mg/kg of body weight. Freshly prepared mixture solution of normal saline (sodium chloride 0.9% in distilled water) and propylene glycol in a ratio of 9:1, v/v respectively, was used in the entire study as solvent for all injections. Volume of all the injections was kept between 0.1 to 0.2 ml. The treatment dose of atropine sulfate (10 mg/kg, intraperitonial) (17) was kept common with all the oximes treatment. Treatment doses of HNK-102, HNK-106 and HNK-111 were 0.05, 0.10 and 0.20 of their respective LD_{50} values and dose of 2-PAM was 30 mg/kg (i.m.) (17, 18) and all were injected intramuscularly. Protection index (PI) of each oxime with or without atropine treatment was determined against DFP poisoning using the following formula:

Protection Index (PI) = LD_{50} of DFP with treatment / LD_{50} of DFP without treatment.

The animals were observed for mortality up to 24 hours.

The dose of 0.20 LD_{50} of HNK-102, HNK-106 and HNK-111 showed better PI and were selected for *in vivo* determination of AChE enzyme for its inhibition/ reactivation studies.

Estimation of enzyme AChE activity

Activity of enzyme AChE (EC 3.1.1.7) was determined following modified method described by Ellman *et al.,* (19).

Sample collection and storage

The animals were anesthetized with anesthetic ether I.P. (Narsans Pharma, India), the blood from orbital plexus was drawn by heparinised glass micro-capillaries and was allowed to clot at 37ºC. The blood samples were centrifuged for 10 min at 2,700 r.p.m.; 100µl serum was collected and stored at -80º C until use. Parallel to it, the whole brain of anesthetized animals was dissected out quickly and stored at -80ºC until use.

Sample preparation

At the time of assay, the whole brain tissue was thawed, diluted 1:10 in 0.25M sucrose solution and homogenized using vertical homogenizer (REMI Motors, India). Homogenization was done for 100 seconds; however, after every 20 seconds of homogenization, the homogenizer was deepen for 10 seconds into crushed ice for cooling. The homogenates were twice centrifuged (Sigma® Laborzentrifugen model 3–18 k, Germany) at 8,500 x g at 4 ºC for 10 minutes. The supernatant was decanted and the pellet was diluted in 0.35 M sucrose solution for assay. The reaction was started by adding 2.6 ml phosphate buffer pH 8, 100 µl of DTNB (5,5'-Dithio-bis(2-nitrobenzoic acid) in phosphate buffer pH 7.0 and 20 µl of sample and the reaction mixture was incubated for three minutes at 37 ºC. Into the reaction mixture, 20 µl of ASChI, dissolved in phosphate buffer pH 7.0, was added. The blank contained the phosphate buffer in the place of substrate and the enzyme activity was read in kinetic mode (UV VIS Spectrophotometer Specord® 200Analytik Jena AG, Germany) at 412 nm. AChE activity was expressed as µmoles of ASChI hydrolyzed/min/gm of brain tissue and µmoles of ASChI hydrolyzed /min/20µl.

Time optimization for determination of peak AChE inhibition

Atropine (10 mg/kg, i.p.) was injected 15 minutes prior to DFP (7.8 and 0.78 mg/kg, i.p.; equals to 2.0 and 0.2 LD_{50} respectively) exposure. Blood and whole brain were collected at 10, 20, 30, 60 and 240 minutes post exposure. Maximum inhibition of AChE enzyme induced by DFP was found at 60 minutes post exposure. This time period of 60 minutes DFP post exposure was used in further AChE enzyme inhibition/ reactivation studies.

Determination of IC_{50} dose of DFP for AChE in vivo

Animals were injected with doses of 0.0031, 0.00625, 0.0125, 0.025, 0.05, 0.10, 0.20, 0.40 and 0.80 LD_{50} of DFP (1.0 LD_{50}= 3.9 mg/kg). Whole brain and serum samples were collected 60 minutes post exposure and processed accordingly.

In vivo reactivation studies of AChE enzyme

Dose of 0.20 LD_{50} of HNK-102 (56.56 mg/kg), HNK-106 (7.07 mg/kg), HNK-111 (7.07 mg/kg) and 30 mg/kg of 2-PAM was used for the enzyme reactivation studies. All the three injections (DFP, atropine and oxime) were given within duration of 20-25 seconds. The animals of positive control group were injected with DFP and atropine, control group received three injections of same volume of diluent vehicle.

Statistical Analysis

Results are expressed as Mean±SEM. Data were analyzed by one-way ANOVA followed by Dunnett test and Student's t test. $p<0.05$ or less was considered significant.

Results

Gross clinical signs of toxicity

The animals were observed for gross clinical signs and symptoms with or without treatment after DFP exposure. The mice treated with DFP at 2.0 LD_{50} dose showed bouts of convulsions, tremors, seizures and muscle fasciculation, mostly within 5 minutes, culminated in death; however, persisted up to about 15–20 minutes in survived animals. Treatment with atropine with or without oxime could not prevent DFP induced aforesaid clinical signs of toxicity. The animals did not show noticeable signs of toxicity when exposed to 0.20 LD_{50} of DFP or below.

Median lethal dose (LD_{50})

Median lethal dose (LD_{50}) of the compounds studies including DFP is given in Table 1.

In vivo determination of protection index (PI) of the oximes

Treatment either with atropine sulphate or 2-PAM alone offered a marginal protection of 2.05 and 1.72 fold respectively, however combination of both the compounds showed synergistic effect i.e. more than eight fold protection against DFP poisoning in the mice (Table 2). This protection was comparable with 0.05 LD_{50} of HNK-102, 0.10 LD50 of HNK-106 and 0.20 LD_{50} of HNK-111 treatment doses as shown in Table 2. Treatment with HNK-102, HNK-106 or HNK-111 alone protected 2.58, 1.72 and 1.22 fold respectively against DFP poisoning (Table 2). Combination treatment of atropine sulphate with any of the three newly synthesized oximes showed synergistic protective effect in terms of better survival of the mice. All the three treatment doses of the oximes offered significant increase in protection, thus confirmed an ideal dose-response relationship (Table 2). Figure 2 depicts summary of significant findings. *In vivo* maximum protection offered by the oximes can be arrange in following order i.e. HNK-102 > HNK-106 >HNK-111 > 2-PAM. In terms of comparison of protection offered by 2-PAM, can also be arranged like PAM

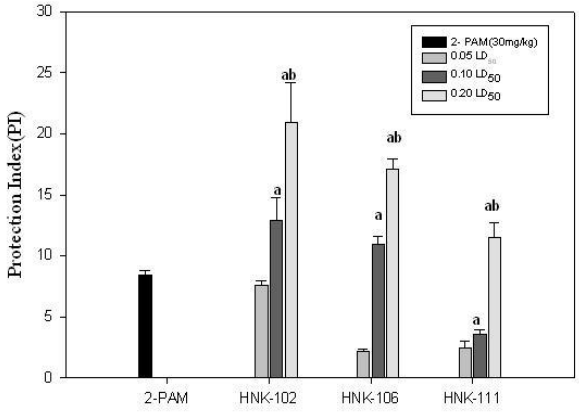

Figure 2. Dose-response curve of the protection offered in terms of PI by HNK-102, HNK-106 and HNK-111 at 0.05, 0.10and 0.20 of their respective LD_{50} doses. PI = LD_{50} of DFP with treatment/LD_{50} of DFP. LD_{50} of HNK-102 = 282.8 mg/kg; LD_{50} of HNK-102 = 35.35 mg/kg; LD_{50} of HNK-111 = 35.00 mg/kg. Values are in Mean±SEM with four animals per group. a $p<0.05$ or less and b $p<0.01$ or less compared to 0.05 and 0.10 LD_{50} treatment of HNK-102, HNK-106, HNK-111 respectively. (HNK-102, $Y = 431.05x + 14.36$, $r^2 = 0.99$; HNK-106, $Y= 55.067x + 5.38$, $r^2 = 0.91$; HNK-111, $Y = 12.87x + 0.5$, $r^2 = 0.97$).

Table 1. Median lethal dose (LD_{50}) of the compounds studies including DFP.

Name of the reactivator	Route of administration	Dose (mg/kg)	Mortality (Died/Treated)	LD_{50} (mg/kg)
DFP	s.c.	1.95	0/4	3.90
		3.90	2/4	(2.4-3.6)
		7.80	4/4	
HNK-102	i.m.	100	0/4	282.84
		200	2/4	(173.3-461)
		400	4/4	
HNK-106	i.m.	17.50	0/4	35.00
		35.00	2/4	(21.4-57.1)
		70.00	4/4	
HNK-111	i.m.	17.50	0/4	35.00
		35.00	2/4	(21.4-57.1)
		70.00	4/4	

* Gad and Weil, 1989. s.c. = Subcutaneous. i.m. = Intramuscular. Values in parentheses are 95% confidence limits. All the compounds were dissolved and diluted in freshly prepared normal saline (90%) + propylene glycol (10%), v/v solution.

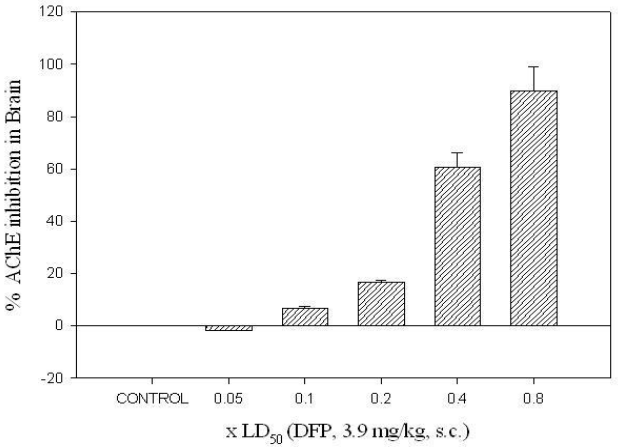

Figure 3. Inhibition of brain AChE induced by 0.05, 0.1, 0.2, 0.4 and 0.8 LD_{50} of DFP corresponding to 0.195, 0.39, 0.78, 1.56 and 3.12 mg/kg respectively, induced 15 min post exposure in mice. Each bar represents Mean±SEM of 4 experiments.

(30 mg/kg or 0.16 LD_{50}) = HNK-102 (14.14 mg/kg or 0.05 LD_{50}) = HNK-106 (3.53 mg/kg or 0.10 LD_{50}) = HNK-111 (7.07 mg/kg or 0.20 LD_{50}) (Figure 2).

In vivo determination of DFP induced inhibition of brain AChE

Percent baseline activities *vis-à-vis* DFP induced inhibition of AChE in brain are given in figure 3. Various doses of DFP i.e. 0.05, 0.1, 0.2, 0.4 and 0.8 LD_{50} correspond to 0.195, 0.39, 0.78, 1.56 and 3.12 mg/kg respectively, inhibited brain AChE following dose-response curve 15 min post exposure. The 0.2 LD_{50} of DFP was able to inhibit brain AChE by 16.2% in 15 min, selected for time-course study (Figure 3). Time intervals taken were 15, 30, 60, 120, 180 min and 16 hours. Maximum inhibition of brain AChE was observed at 60 min post exposure, selected for IC_{50} determination (Figure 4). Significant inhibition of brain AChE was induced by 0.20 LD_{50} of DFP (Table 3). Ten times higher dose of DFP i.e. 2.0 LD_{50} inhibited brain AChE quickly, about 90% brain AChE inhibition was noted which persisted at least up to four hours (data not shown).

In vivo determination of DFP induced inhibition of serum AChE

Inhibition of Serum AChE induced by 0.2 LD_{50} and 2.0 LD_{50} at 30, 60, 120, 180 min and 16 hours time points are estimated (data not shown). Maximum inhibition of serum AChE was noted at 60 min post exposure was selected for IC_{50} determination. Significant inhibition of serum AChE was induced by 0.0125 LD_{50} of DFP (Table 3).

In vivo determination of inhibition concentration (IC_{50}) of AChE

Dose dependent inhibitions of Brain AChE activity at 60 minutes post DFP exposure are shown in figure 5. DFP induced IC_{50} of brain AChE activity calculated as 0.249 LD_{50}, equals to 0.971 mg/kg, s.c. dose. Similarly,

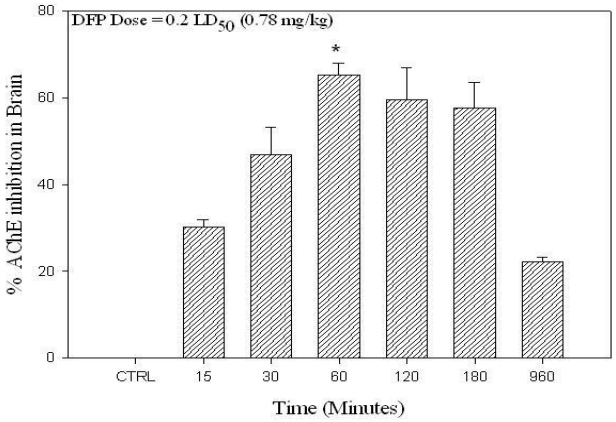

Figure 4. Inhibition of brain AChE induced by 0.2 LD_{50} of DFP (LD_{50}, 3.9mg/kg, s.c.) at various time points in mice. Each bar represents Mean±SEM of 4 experiments. * p<0.05, Compared to 15 min and 960 min post DFP exposure.

Table 2. *In vivo* protection offered by atropine and oximes (2-PAM, HNK-102, HNK-106 and HNK-111) against Diisopropylphosphorofluoridate (DFP) poisoning in Swiss albino male mice.

1	2		3	4
S. No.	Treatment		LD_{50} of DFP# (mg/kg; s.c.)	PI (LD_{50} with treatment / LD_{50} without treatment)
	Atropine mg/kg; i.p.	Oxime (mg/kg; i.m.)		
1.	-	-	3.90±0.220	1.000
2.	10.00	-	7.07 (3.2-16)	1.810
3.	-	2-PAM (30.00)	6.72 (4.4-10)	1.720
4.	10.00	2-PAM (30.00)	32.78±1.56	8.405
5.	10.00	HNK-102 (14.14)	29.62±1.32	7.590
6.	10.00	HNK-102 (28.28)	50.42±7.35*	12.92
7.	10.00	HNK-102 (56.56)	81.72±12.52**	20.95
8.	-	HNK-102 (56.56)	10.07(4.9-13)	2.580
9.	10.00	HNK-106 (1.75)	8.46±0.599	2.170
10.	10.00	HNK-106 (3.50)	42.6±2.600*	10.92
11.	10.00	HNK-106 (7.00)	66.59±3.47**	17.07
12.	-	HNK-106 (7.00)	6.72 (2.6-6.1)	1.720
13.	10.00	HNK-111 (1.75)	9.50±2.430	2.430
14.	10.00	HNK-111 (3.50)	13.81±1.56*	3.540
15.	10.00	HNK-111 (7.00)	44.93±4.59**	11.52
16.	-	HNK-111 (7.00)	4.75 (3.1-7.2)	1.220

* p<0.01 and ** p<0.001 compared to respective lowest treatment doses of the oxime.
# LD_{50} determined following the 'Moving average' method of Gad and Weil, 1989.
Three increasing treatment doses of HNK-102, HNK-106 and HNK-111 are corresponding to their 0.05, 0.10 and 0.20 LD_{50}. Serial numbers 3, 8, 12 and 16 depict protection offered by the oximes without atropine. PI = Protection Index. Values in column 3 are (i) Mean±SEM of four experiments and (ii) in parenthesis are 95% confidence limits.

Table 3. *In vivo* inhibitory effect of various doses of DFP, 60 minutes post exposure, on enzyme AChE activity in brain and serum of Swiss albino male mice.

Sl. No.	Dose of DFP (s.c.)		AChE activity(μ moles ASChI hydrolyzed/min/gm of wet tissue or 20 μl of serum)			
	LD_{50}	mg/kg	Whole brain		Serum	
			Activity	% Inhibition	Activity	% Inhibition
1.	Control	-	4.70±0.47	0	3.97±0.14	0
2.	0.0031	0.0121	ND	ND	2.79±0.02	29.72±0.300
3.	0.0062	0.0242	ND	ND	2.29±0.20	42.31±3.700
4.	0.0125	0.0487	ND	ND	2.02±0.48*	49.11±11.70
5.	0.0250	0.0975	ND	ND	1.63±0.06*	58.94±2.200
6.	0.0500	0.1950	4.72±0.40	1.5±0.10	0.72±0.12*	81.86±14.10
7.	0.1000	0.3900	3.75±0.71	20.21±4.10	0.80±0.03*	79.84±3.400
8.	0.2000	0.7800	2.29±0.32*	51.27±7.30	0.83±0.04*	79.09±3.90
9.	0.4000	1.5600	1.21±0.23*	74.7±14.70	0.26±0.07*	93.45±26.4
10.	0.8000	3.1200	0.95±0.08*	79.78±7.00	0.14±0.03*	96.47±24.0
11.	2.0000	7.8000	0.26±0.08*	94.46±2.90	0.08±0.02*	97.98±24.4

* $p<0.05$ or below, compared to respective control group. AChI = Acetylthiocholine iodide. LD_{50} of diisopropylphosphorofluoridate (DFP) = 3.90 (2.1-7.1) mg/kg, s.c..

Each value is Mean±SEM of four experiments.

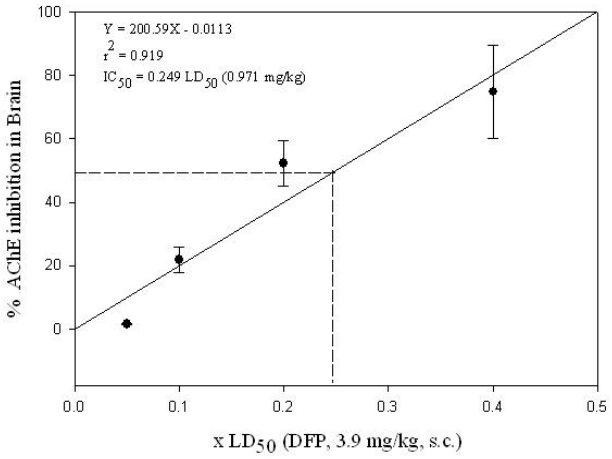

Figure 5. Sixty minutes post DFP exposure, inhibition of brain AChE activity induced by 0.05, 0.1, 0.2, 0.4 LD_{50} (LD_{50}, 3.9 mg/kg, s.c.) in mice. Each point represents Mean±SEM four experiments.

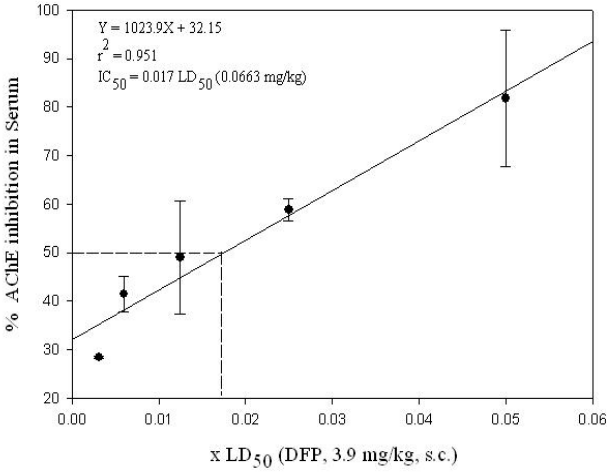

Figure 6. Sixty minutes post DFP exposure, inhibition of serum AChE activity induced by 0.0031, 0.0062, 0.0125, 0.025, 0.05 LD_{50} of DFP (LD_{50}, 3.9 mg/kg, s.c.) in mice. Each point represents Mean±SEM of four experiments.

figure 6 depicts dose dependent inhibitions of serum AChE activity at 60 minutes post DFP exposure. DFP induced IC_{50} of serum AChE activity was found to be 0.017 LD_{50}, which equals to 0.0663 mg/kg, s.c. dose. In other words, to induce 50% inhibition of brain AChE activity compared to that of serum, ca. 15 times more dose of DFP was required. In agreement with brain AChE inhibition, 2.0 LD_{50} DFP inhibited serum AChE completely at initial 10 minutes (data not shown) and it persisted at least, up to 4 hours.

Inhibition concentration (IC_{50}) determination

Various dose dependent AChE inhibitions at 60 minutes post DFP exposure are shown in figures 5 and 6. In whole brain, statistical significant inhibition (~ 51%) was seen with DFP induced IC_{50} calculated as 0.971 mg/kg at 0.25 LD_{50} dose (Figure 5). In serum ~ 50% AChE inhibition (IC_{50}) was noted at 0.0663 mg/kg or 0.017 LD_{50} of DFP dose with a difference of ~16 times compared to that of brain IC_{50} value.

In vivo reactivation of brain AChE by oximes

Estimation of reactivated of brain AChE activity was carried out 60 minutes post DFP exposure with 2-PAM and HNK analogs as presented in figure 7. HNK-102, HNK-106, HNK-111 showed statistically significant reactivation of brain AChE activity from 0.125 LD_{50} to 0.502 LD_{50} DFP dose compared to 2-PAM. Only HNK-102 significantly reactivated brain AChE at 0.502 LD_{50} DFP dose ($p<0.01$)

In vivo reactivation of serum AChE by oximes

Reactivation of serum AChE was carried out parallel to brain AChE estimation, 60 minutes post DFP exposure with 2-PAM and HNK analogues (data not shown). No significant reactivation by oximes was observed at any doses of DFP post 60 minutes exposure.

Discussion

The antidotes of the OP poisoning should have good reactivation efficacy, less toxicity, good binding interaction with the enzyme AChE and it could produce the protection inside the brain region. Monoisonitrosoacetone (MINA, acyl conjugated oxime), being non quaternary in nature can produce the protection inside the brain

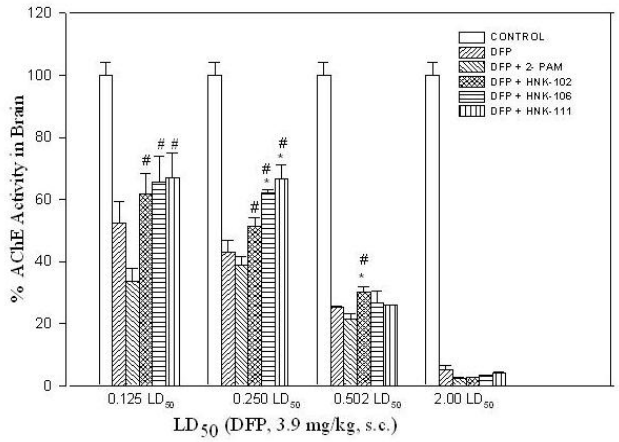

Figure 7. Reactivation of DFP inhibited brain AChE activity by oximes. Doses: DFP, 0.125, 0.250, and 0.502 LD_{50}, estimated 60 min post exposure; oximes, HNK-102, 106 and HNK-111, 0.20 LD_{50}; 2-PAM, 30 mg/kg. DFP LD_{50}, 3.9mg/kg. Each bar represents Mean±SEM of four experiments. * $p < 0.01$, compared to DFP; # $p< 0.01$ compared to DFP + 2-PAM.

region but it was more toxic. Since the HNK oximes showed better *in vitro* reactivation efficacy against nerve agent (sarin and VX) inhibited human erythrocyte ghost AChE, than 2-PAM and obidoxime, therefore three oximes (HNK-102, HNK-106 and HNK-111) were selected for the evaluation of their acute toxicity (LD_{50}) and protection efficacy against DFP (nerve agent mimic) under *in vivo* study. The amide group of the HNK-analogs may provide good binding interactions with the enzyme AChE. The pKa of the HNK oximes (pKa 7.9-8.2) were found in the range of 2- and 4-pyridinium oximes. Therefore, these oximes may have better nucleophilicity to reactivate the OP-inhibited AChE under *in vivo* conditions. Protection index (PI) of these oximes was determined to prove their protection efficiency *in vivo*. The results based on PI have shown that administration of both atropine sulphate and oxime gave better protection (synergistic effect) against OP poisoning *in vivo* (17, 20-21).

Unlike previously reported data on DFP (22-23) in which other solvents (polyethylene glycol, ethanol, normal saline) were used to dilute DFP, in the present study choice of freshly prepared mixture of normal saline and propylene glycol (ratio 9:1, v/v) has appreciably increased the reproducibility of the experiments, especially when higher doses of DFP was used (unpublished data). Subcutaneous route of administration of DFP was selected to slightly delay appearance of toxic effects of DFP so as to closely mimic actual condition. Administration of atropine and oximes was done following intraperitonial and intramuscular routes respectively as being easy and uniform drug absorption (24). In the present study LD_{50} values was determined by 'moving average method' described by Gad and Weil (1989) with the use of minimum 4 animals for each dose. Every drug molecule is associated with its own toxicity at a particular dose, therefore the safety of that drug can be measured through ratio or difference between the median lethal dose to the effective dose or therapeutic dose. In this study, the median lethal dose (LD_{50}) of DFP and three oximes (HNK-102, HNK-106 and HNK-111) were calculated. The PI of the oximes (HNK-102, HNK-106 and HNK-111) were determined and compared with that of standard antidote 2-PAM (dose 30 mg/kg, LD_{50} 180 mg/kg, i.p.) (18). Treatment with alone 2-PAM, HNK-102, HNK-106 & HNK-111 have shown 2.05, 2.58, 1.72, 1.21 fold protection respectively, against DFP poisoning. Like 2-PAM, in combination with atropine synergistic effect has been observed by these three oximes. An ideal dose response relationship curve was established for all the three HNK series of oximes following three treatment doses equal to their 0.05, 0.10 and 0.20 LD_{50} values. *In vivo* results showed that protection offered by these oximes in comparison with 2-PAM with respect to their doses is as follow, HNK-102 > HNK-106 > HNK-111 > 2-PAM.

As OP compounds directly inhibit the brain AChE (at esteretic site) and serum cholinesterase (SChE), therefore, we studied the effect of DFP inhibition and reactivation by oximes (HNK-102, HNK-106 & HNK-111) in whole brain tissue and serum of male mice. The dose and time response curve in brain AChE (Figure 3 and 4) shows that 0.2 LD_{50} dose of DFP causes 16%-30% inhibition at early 15 minutes of DFP exposure. To estimate maximum cholinesterase (AChE) inhibition, time dependent dose response curve was studied at 0.2 LD_{50} (sub lethal dose) and at 2.0 LD_{50} (lethal dose) of DFP in whole brain and serum. Maximum inhibition at sub lethal dose (0.2 LD_{50}) took place at 60 minutes post DFP exposure. Although at 2.0 LD_{50} (lethal dose) with atropine (10 mg/kg, i.p.) caused almost complete AChE inhibition at early 10 minutes with survival of animal up to 4 hours of time response curve study. These results clearly depict that AChE inhibition may not be the sole cause of lethality in OP poisoning. Study by Khan et al. (25) also showed time course and dose response study with sarin (DFP close analog) at sub lethal (0.01 LD_{50}) and lethal dose (1.0 LD_{50}). However, the relationship between the LD_{50} and AChE enzyme inhibition (IC_{50}) has not been reported in a single study. It is necessary to evaluate a particular dose which causes 50% depression in AChE activity so as to establish relation between dose response and AChE inhibition. In this study, we estimated the inhibition of AChE enzyme by 50% (IC_{50}) in whole brain tissue and serum using various doses of DFP (LD_{50}, 3.9 mg/kg. s.c.) by linear curve fitting equation. The IC_{50} was calculated as 0.971 mg/kg and 0.0663 mg/kg in brain and serum, respectively with a difference of ~16 times compared to brain AChE IC_{50} value. The plausible reasons for these findings are firstly, RBCs in serum contain mainly AChE enzyme. The blood is the first subject to be encounter with the OP poisoning and its concentration is greater in blood than in other body tissue when injected subcutaneously. Thus there is a sufficient amount of organophosphate molecules available to get bound to blood tissue which inhibits the enzyme activity completely for longer time at a very low dose (26-27). The blood enzymes act as a buffer for the enzymes in body tissue and cause relatively less inhibition of tissue enzyme until much of the blood enzyme is inhibited (28).

The higher treatment dose of HNK oximes, showed more than ~ 50% reactivation of AChE at IC_{50} dose of DFP compared to that of 2-PAM. In brain, HNK-102 significantly reactivated AChE at sub lethal dose 0.502 LD_{50} DFP dose (1.95 mg/kg; Figure 7). But in case of the serum (data not shown), no significant reactiva-

tion was offered by any of the new oximes including 2-PAM. The reason for these findings could be at higher concentration of DFP in the serum, there is complete inhibition of AChE enzyme in the blood and that could not be efficiently reactivated by the available concentration of the oximes. Further, possibility of unbound or free molecules of DFP, at higher concentration, in circulating blood cannot be ruled out and may re-inhibit the reactivated AChE. The present findings suggested that HNK-102, HNK-106 and HNK-111 have a better therapeutic potential in terms of survival and reactivation of AChE enzyme compared to 2-PAM against DFP poisoning *in vivo*. The detail study of antidotal efficacy of these three newly synthesized oximes against sarin or other nerve agents may unveil more interesting findings.

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