Interdiscip Toxicol. 2016; **Vol. 9**(3–4): 101–105.
doi: 10.1515/intox-2016-0013

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interdisciplinary toxicology

ORIGINAL ARTICLE

Protection studies of new *bis* quaternary 2-(hydroxyimino)-*N*-(pyridin-3yl) acetamide derivatives (HNK-series) oximes against acute poisoning by dichlorvos (DDVP) in Swiss albino mice

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ITX093416A04 • Received: 16 June 2016 • Revised: 10 December 2016 • Accepted: 17 December 2016

ABSTRACT

The available antidotal therapy against acute poisoning by organophosphates involves the use of atropine alone or in combination with one of the oximes, e.g. 2-PAM, Obidoxime, TMB-4 or HI-6. Each of these oximes has some limitation, raising the question of the universal antidotal efficacy against poisoning by all OPs/nerve agents. In the present study, newly synthesized *bis* quaternary 2-(hydroxyimino)-*N*-(pyridin-3yl) acetamide derivatives (HNK-series) oximes were evaluated for their antidotal efficacy against DDVP intoxicated Swiss mice, in terms of the Protection Index (PI) and AChE reactivation in brain and serum. The inhibition concentration (IC_{50}) was determined in brain and serum after optimizing the time point for maximum inhibition (60 min post DDVP exposure). AChE reactivation efficacy of the HNK series was evaluated at IC_{50} and compared with 2-PAM. HNK-102 showed a ~2 times better Protection Index (PI) as compared to 2-PAM against DDVP toxicity. IC_{50} at 60 min DDVP post exposure was found to be approximately one fifth and one half of the LD_{50} dose for brain and serum AChE, respectively. Out of three HNK oximes, HNK-102 & 106 at 0.20 LD_{50} dose significantly reactivated DDVP intoxicated brain AChE ($p<0.05$) as compared to 2-PAM at double IC_{50} dose of DDVP. In light of double PI and higher AChE reactivation, HNK 102 was found to be a better oxime than 2-PAM in the treatment of acute poisoning by DDVP.

KEY WORDS: DDVP; HNK Oximes; AChE reactivation; 2-PAM

Introduction

Every year around 30% of crop yield in developing countries is affected by pest attack (Das, 2013). Facing a similar situation in an agricultural country like India, the use of pesticide has become indispensable. Since 1961, Dichlorvos or DDVP has been used widely as an insecticide and miticide to control internal and external parasites in livestock and domestic animals, to control insects in houses, and also for crop protection (IARC 1991). Dichlorvos (2, 2-dichlorovinyl dimethyl phosphate) has a molecular formula $C_4H_7Cl_2O_4P$ and molecular weight 220.98 g. It is known by its several trade names like DDVP, Dedevap, Nogos, Nuvan, phosvit or Vapona7 and is one of the most commonly used organophosphate pesticides in developing countries (Binukumar & Gill, 2010). Like other organophosphorous compounds (OPs), it causes neurotoxicity due to its irreversible inhibitory action on acetylcholinesterase (AChE). Acetylcholine (ACh) is accumulated at synapses due to inhibition of this enzyme, with resulting persistent depolarization at the postsynaptic receptor (Ricter & Corcoran, 1997). Signs and symptoms include lacrimation, perspiration, miosis, vomiting, diarrhea, bronchial secretion, and bradycardia, caused by disturbed cholinergic transmission in the parasympathetic autonomic nervous system. DDVP also acts on the central nervous system causing drowsiness, convulsions, headache, coma and depression

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of the respiratory center in the brain. Studies on human volunteers have shown that DDVP is rapidly hydrolyzed by esterase present in liver and blood; hence lethality in humans due to its exposure has been rarely reported (Ricter & Corcoran, 1997).

The antidotal therapy available against acute poisoning of DDVP involves the use of atropine and diazepam in combination or alone (Chedi & Aliyu, 2010). As reported, the antidotal efficacy of diphenhydramine (antihistaminic drug) is also effective when given after OPs (dichlorvos) in mice (Faris & Mohammad, 1996). Further studies showed a higher Protection Index (PI) of atropine and 2-PAM when given in combination against DDVP poisoning (Kumar *et al*. 2008). But the issues like inability of 2-PAM to cross the blood brain barrier, intrinsic toxicity of Obidoxime, TMB-4 (Clement, 1981), instability of HI-6 (Aas, 2003) and protection efficiency of these oximes against all OP compounds raised the question on their universal antidotal efficacy.

In search of a more efficacious and broad spectrum oxime, a new HNK series (bis-pyridinium acetamide derivatives) oximes has been synthesized in this establishment. Having shown better protection in terms of the Protection Index and AChE reactivation against acute poisoning of DFP (Kumar *et al*. 2014) and Sarin (Swami *et al*., 2016), the present study was carried out to evaluate the efficacy of HNK series of oximes against acute DDVP poisoning.

Material and methods

Reagents

Dichlorvos (2, 2-dichlorovinyl dimethyl phosphate) with trade name Nuvan® was obtained from the local market (Gwalior, India); Oximes: 1,1'-(ethane-1,2-diyl) bis (3-(2-(hydroxyimino) acetamido) pyridinium) dibromide (HNK-102); 1,1'-(hexane-1,6-diyl) bis (3-(2-(hydroxyimino) acetamido) pyridinium) dibromide (HNK-106); 1,1'-(1,4-phenylene bis (methylene)) bis(3-(2-(hydroxyimino) acetamido) pyridinium) dibromide (HNK-111) (Figure 1) were synthesized in our establishment with >98% purity (GC and NMR). Acetylthiocholiniodide (ASChI), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and atropine sulphate were purchased from Sigma Chemicals Co. (St. Louis Mo, Darmstadt, Germany) and pralidoxime chloride (I.P.) was purchased from Kwality Pharma (Amritsar, India).

Dilution of the oximes and DDVP (76% m/v) was made in freshly prepared solution of normal saline and propylene glycol (9:1, v/v) throughout the experiment. Concentrations of the injectable solutions were prepared in such a way as to keep the volume in the range 0.1–0.2 ml.

Experimental animals

Randomly outbred male Swiss mice weighing 25–30 g were obtained from the Animal House, DRDE, Gwalior, India. Randomized mice were housed in polypropylene cages with dust free steam autoclaved paddy husk and were kept in an environmentally controlled room (Temp 25±2 °C, RH 40–60%). For all the experiments, four mice per dose were used. The study was approved by the statutory committee constituted by the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Cell, Ministry of Environment, Forests and Climatic Change, Government of India, New Delhi.

Sample preparation

The animals were anesthetized using anesthetic ether I.P. (Narsans Pharma, India) and the blood samples were collected through micro capillaries by puncturing the orbital plexus and were allowed to clot at 37 °C for serum separation. Subsequently, whole brain was collected from the exposed mice and stored at −80 °C until use. At the time of assay, the whole brain tissue was thawed and homogenized using a vertical homogenizer (REMI Motors, Mumbai India) in 1:10 (w/v) 0.25 M sucrose solution maintained at 4 °C. The supernatant of the homogenates was twice centrifuged (Sigma® Laborzentrifugen model 3–18 k, Germany) at 8,500 × *g* at 4 °C for 10 min. The supernatant was decanted and the pellet was diluted in 0.35 M sucrose solution for assay. Processed blood and tissue samples were analyzed using the modified Ellman method (Ellman *et al*., 1961) and AChE activity was expressed as µmoles of ASChI hydrolyzed/min/g of brain tissue and µmoles of ASChI hydrolyzed/min/10µl of serum.

Median lethal dose (LD_{50}) and protection index (PI) determination

Median lethal dose (LD_{50}) in the present study was determined following the 'moving average' method (Gad and Weil, 1989) and expressed as mg/kg of body weight. Atropine sulfate at 10 mg/kg, i.p· (Kumar *et al*., 2014) and all HNK oximes (HNK-102, HNK-106 and HNK-111) were used at a dose rate of 0.20 LD_{50} intramuscularly. DDVP was

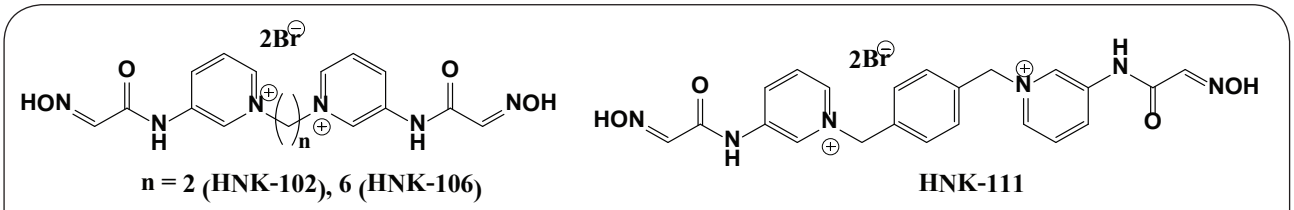

Figure 1. Bis-quaternary 2-(hydroxyimino)-*N*-(pyridine-3-yl)acetamide derivatives.

administered by subcutaneous route at different doses. The dose of 2-PAM was kept at 30 mg/kg throughout the experiment (Kumar *et al*., 2014; Marrs, 1991). In view of the synergistic effect by combination therapy of atropine and 2-PAM (9 fold protection) against DFP poisoning in the mice (Faris & Mohammad, 1996), HNK oximes were used in combination with atropine against DDVP poisoning. Protection Index (PI) of each oxime with or without atropine treatment was determined against DDVP poisoning using the formula: Protection Index (PI) = LD_{50} of DDVP with treatment / LD_{50} of DDVP

Estimation of maximum AChE inhibition at different time points

Various doses of DDVP, i.e. 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 LD_{50}, were injected subcutaneously to mice to find out the approximate 50% inhibition at 15 min DDVP post exposure. Atropine (10 mg/kg, i.p.) was injected 5 min prior to DDVP administration at 0.8 and 1.6 LD_{50} doses to enable the survival of the animals. The dose of DDVP inhibiting approximately 50% AChE (~0.2 LD_{50}) was further used for a time course study to find out the time of maximum inhibition of serum and brain AChE, and accordingly samples were collected at 30, 60, 120, 240 and 960 min DDVP post exposure. After optimization of time for maximum AChE inhibition, various log doses of DDVP, viz. 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 $LD_{50,}$ were administered subcutaneously to find out the IC_{50} at 60 min post DDVP exposure. This time period (60 min) of DDVP post exposure was used in onward AChE enzyme inhibition/reactivation studies.

Determination of AChE enzyme IC_{50} and IC_{50} shift value

IC_{50} value was determined in samples using injectable doses of 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 LD_{50} of DDVP (except for serum) and AChE inhibition was established in whole brain and serum samples at 60 min DDVP post exposure. Using log values of IC_{50} (0.5,1 and 2 IC_{50} corresponding to 0.1, 0.2 and 0.4 LD_{50}), AChE enzyme reactivation was determined at 0.20 LD_{50} dose of oximes and 30 mg/kg dose of 2-PAM (i.m.) *in vivo*. The animals in the untreated group were injected DDVP in the same volume of solvent as used for the treated groups.

Statistical analysis

Results were expressed as mean±SEM. All data were analyzed by one-way ANOVA followed by Student´s t test and Dunnett test. $p<0.05$ or $p<0.01$ was considered significant.

Results

Protection index (PI) of the oximes

In the present study, treatment with 2-PAM (30 mg/kg, i.p.), HNK-102, HNK-106, and HNK-111 (0.20 LD_{50} of each) along with atropine showed 3.41, 5.7, 2.1, 2.5 fold protection, respectively, against DDVP poisoning in mice. Of all the oximes used (2-PAM, HNK-102, HNK-106, HNK-111), HNK-102 offered the highest protection, which was found to be approximately 2 fold of that with 2-PAM (Table 1).

LD_{50} was determined following the 'Moving Average' method of Gad and Weil (1989)[12]. The treatment doses of HNK-102, HNK-106 and HNK-111 are corresponding to their 0.20 LD_{50}. Protection Index (PI) = LD_{50} with treatment/ LD_{50}. Values in column 3 are (i) Mean±SEM with 4 animals in each experiment; (ii) in parenthesis: 95% confidence limits.

Determination of DDVP induced maximum AChE inhibition in brain and serum sample

From control groups, baseline data of AChE activities for brain and serum were estimated (data not shown). Of different doses of DDVP (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 LD50), 0.2 LD_{50} was found to inhibit approximately ~50% AChE at 15 min post DDVP exposure (Figure 2). Further, this 0.2 LD_{50} dose (corresponding to 2.828 mg/kg) was

Table 1. *In vivo* protection offered by atropine and oximes (2-PAM, HNK-102, HNK-106 and HNK-111) against DDVP.

S.No	Treatment: Atropine (mg/kg, ip)	Treatment: Oxime (mg/kg, im)	LD_{50} DDVP (mg/kg, sc)	No. of Experiments (n)	PI
1.	–	–	14.14	4	1
2.	10.00	–	20.0	1	1.41
3.	10.00	PAM (30.00)	48.23±8.33 (3.13–510.6)	2	3.41
4.	10.00	HNK–102 (56.56)	81.49±18.5 (21.5–689.6)	3	5.76
5.	10.00	HNK–106 (7.00)	30.17±5.17 (6.99–89.32)	3	2.13
6.	10.00	HNK–111 (7.00)	35.35±0.0 (16.21–77.11)	3	2.5

LD_{50} determined following the 'Moving Average' method of Gad and Weil (1989). The treatment doses of HNK-102, HNK-106 and HNK-111 are corresponding to their 0.20 LD_{50}. Values in column 3 are (i) Mean±SEM with 4 animals in each experiment (ii) in parenthesis are the 95% confidence limits.

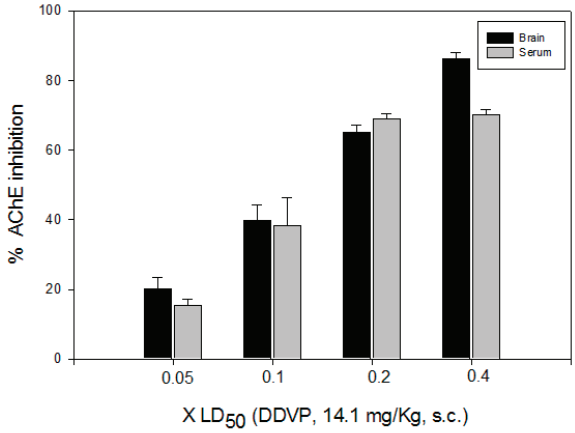

Figure 2. Inhibition of brain and serum AChE induced by 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 LD_{50} of DDVP 15 min post exposure in mice. Each point represents mean±SEM (n=4).

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used to study time dependent maximum AChE inhibition (data not shown). Maximum brain AChE inhibition equal to ca. 66.6% was observed at 60 min post DDVP exposure.

Determination of DDVP induced *in vivo* AChE inhibition concentration, IC_{50} value

Dose dependent AChE inhibition at 60 min post DDVP exposure was estimated using 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 LD_{50} doses (Figure 3). IC_{50} value for brain and serum AChE was calculated to be 3.05 mg/kg and 9.43 mg/kg, corresponding to 0.217 and 0.66 LD_{50} of DDVP, respectively.

In vivo AChE reactivation study and IC_{50} shift value

Considering the control value as baseline, percentage activity of serum AChE and brain AChE (data not shown) evaluated at 60 min post DDVP exposure are depicted in Figure 4. At 0.217 (IC_{50} value) and 0.434 LD_{50} doses of DDVP, significant AChE reactivation of ca. ~20% (p<0.01) from HNK-102 and HNK-106 was observed as compared to 2-PAM. In serum none of the oximes showed significant reactivation against any dose of DDVP (data not shown).

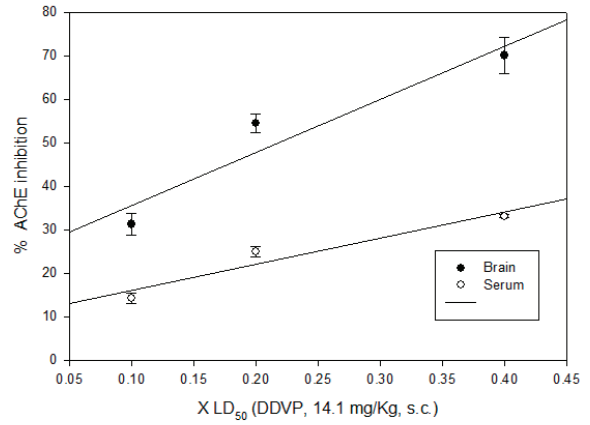

Figure 3. DDVP IC_{50} for brain and serum AChE activity at 60 min post exposure in mice. Each point represents mean ± SEM (n=4). Brain: Y= 122.2X + 23.39; r^2= 0.91; IC_{50} = 0.217 LD50 (3.05 mg/kg). Serum: Y= 59.8 X + 10.0; r^2= 0.93; IC_{50} = 0.66 LD_{50} (9.431 mg/kg)

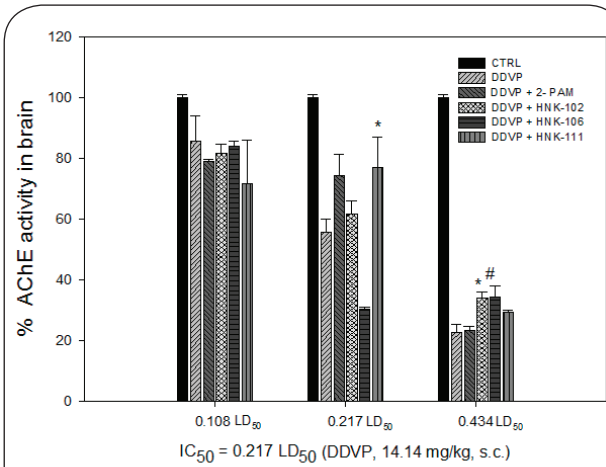

Figure 4. Reactivation of DDVP inhibited brain AChE activity by oximes at 0.108 LD_{50} (0.5 IC_{50}), 0.217 LD_{50} (IC_{50}) and 0.434 LD_{50} (2 IC_{50}) doses 60 minutes post exposure. Doses: HNK oximes 0.2 LD_{50}; 2- PAM 30 mg/kg; DDVP LD_{50} 14.14 mg/kg. Each bar represents mean ± SEM (n=4). *p<0.001, compared to HNK-106; #p<0.01 0r less, compared to DDVP and 2-PAM.

Discussion

For many years pesticides have been used to increase the crop production but their indiscriminate use has adversely affected the environment and human health. According to earlier reports, there are few studies available concerning the usage of pesticides and their hazard for humans. The continuous use of these compounds in fields as a spray or in other forms results in headache (most common symptom), muscle cramps, cough and nausea among the farmers. In order to study the acute and delayed toxicity of organophosphate poisoning in primates and non- primates, various studies have been undertaken both in the presence and absence of therapeutic regimens. Previous studies reported the efficacy of obidoxime and 2-PAM in combination with atropine in terms of AChE reactivation and shift in LD_{50} post OP exposure (Natoff and Reiff, 1970). Though obidoxime proved to be an effective antidote against DDVP and other insecticides showing >22.7 protection, but produces hepatotoxic effects in poisoned patients (Marrs, 1991), hence its usage as a regular antidote for OP poisoning has been questionable. Keeping all above points in view, in the present study we evaluated the antidotal efficacy of new bis-pyridinium acetamide derivatives (HNK oximes) against DDVP toxicity in mice.

Using our earlier reported data of median lethal dose and the dose response curve of HNK oximes against DFP (Kumar *et al*., 2014) and Sarin (Swami *et al*., 2016), in the present study also the 0.20 LD_{50} treatment dose of oximes was used. As per the Protection Index, an approx. two times better protection was offered by HNK-102 at 0.20 LD_{50} dose against DDVP poisoning compared to 2-PAM *in vivo*. The protection showed by HNK-102 supports the studies stating that higher doses of oximes increase the protection ratio when used against OP compounds (Skovira *et al*., 2010). The greater protection offered by HNK-102 clearly depicts its better efficacy in terms of the Protection Index (PI) as compared to 2-PAM.

The message of the well established data is that in OP poisoning primarily cholinesterase is the enzyme being inhibited. To study the effect of DDVP, time–dose dependent AChE enzyme inhibition was determined in mouse brain and serum samples. Using the 0.174 LD_{50} dose at various time points, maximum AChE inhibition was determined to optimize the time period. At this optimized time (60 minutes), 50% AChE inhibition (IC_{50}) was assessed and the IC_{50} value of inhibited brain AChE enzyme was found to be approx. 5 times lower than LD_{50} values of DDVP (Figure 3). Further, the serum IC_{50} value was only 2 times lower than LD_{50} of DDVP. A plausible explanation of these findings may be the highest presence of AChE enzyme in brain followed by serum, muscles and intestine and a higher penetration of irreversible OP compounds in

brain via the blood brain barrier (Li *et al*., 2002). Thus 50% AChE inhibition in brain is caused at a much lower dose (0.217 LD_{50}) compared to serum. As shown, the calculated IC_{50} value is ~ 5 times lower than the LD_{50} dose, and thus undoubtedly nonlethal to the exposed animals. These data invariably support the notion that other factors may also be responsible for AChE inhibition post OP poisoning (Pope, 1999) or some other targets may be involved in DDVP/OP toxicity. These may concern muscarinic receptors in brain, neuropathy target esterase and acylpeptide hydrolases (less pronounced in inhibition) (Jett *et al*., 1991), with most of them sensitive to OPCs toxicity.

Oximes possess nucleophilic characteristics in dephosphorylation of OP intoxicated AChE enzyme at esteratic site. Their potency to dephosphorylate AChE enzyme (AChE reactivation) does however vary among oximes. Our study is of prime importance in showing AChE reactivation efficacy of oximes by plotting shift of log IC_{50} doses *in vivo.* Having shown the superiority of HNK oximes over 2-PAM, in terms of AChE reactivation against DFP poisoning, here we are reporting the AChE reactivation efficacy of HNK series against DDVP poisoning. As shown in Figure 4, HNK-111 at a sublethal dose (IC_{50}), i.e. 0.217 LD_{50}, show ca. 25% AChE reactivity in brain AChE. At the same time, at 0.434 LD_{50} ($2 \times IC_{50}$), HNK-102 was found superior to 2-PAM, with an about 10% higher AChE activity *in vivo*. This better reactivation observed at higher IC_{50} dose (0.434 LD_{50}) of DDVP by HNK-102 may be attributed to two promising characteristics of HNK series of oximes. First, the presence of amide conjugate (with pyridinium ring), which provides greater efficiency in entering the brain region compared to 2-PAM (mono-pyridinium oxime, less permeability). Second, the dissociation constant (pKa) of these oximes which determine the formation of active ion is comparatively low (pKa 7–8), i.e. within the range of 2 and 4-pyridinium oximes. This results in the formation of active ion compounds which may readily react with phosphorylated AChE enzyme and undergo reactivation. These facts may contribute to make HNK-102 a better reactivator in comparison to 2-PAM at physiological pH7.4, however variation in efficacy of different HNK oximes may occur because of some structural differences.

In view of the higher Protection Index (PI) and higher brain AChE reactivation as compared to 2-PAM, HNK 102 may be an effective future antidote as a substitute of 2-PAM in DDVP and other organophosphate poisoning. We have established the superior efficacy of HNK 102 over PAM chloride against DFP and Sarin toxicity. Protection studies of these oximes against nerve agents like Tabun, Soman and other organophosphates are further needed to evaluate their efficacy as universal and better antidotes for clinical use in comparison to the existing 2-PAM.

Acknowledgements

The authors thank Dr.Lokendra Singh, Director, Defence Research and Development Establishment, Jhansi Road, Gwalior, India for his encouragement and for providing the necessary facilities.

Conflict of interest. *The authors declare that there are no conflicts of interest.*

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