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

P. Kumar1, D. Swami1, H. N. Karade2, J. Acharya2, P. C. Jatav1, A. Kumar1 and M. K. Meena1

1 Pharmacology and Toxicology Division, Defence Research & Development Establishment, Jhansi Road, Gwalior, 474002 India.
2 Process Technology Development Division, Defence Research & Development Establishment, Jhansi Road, Gwalior, 474002 India.

**Corresponding author:** Pravin Kumar, Pharmacology and Toxicology Division, Defence Research & Development Establishment, Jhansi Road, Gwalior, 474002 India. Tel: +91-751-2344301, Fax: +91-751-2341148, Email: pravinkumar43@hotmail.com

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**Abstract**

This study reports efficacy of three bis pyridinium derivatives of 2-(hydroxyimino)-N-(pyridin-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.

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**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, bronchoconstriction, 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 muscaranomic receptor stimulation (4), AChE reactivator (oximes) which reactivate OP-inhibited AChE and anti-convulsant 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 nucleophilic 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 reanimate the inhibited AChE, both in brain and serum in vivo.

**Materials and methods**

**Chemicals**

Diisopropylphosphorofluoridate (DFP); 1,1’-(ethane-1,2-diy1) bis(3-(2-hydroxyimino) acetamide) pyridinium dibromide (HNK-102 or 3b); 1,1’-(hexane-1,6diyl) bis(3-(2-hydroxyimino) acetami-
The dose of 0.20 LD₅₀ 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 37°C. The blood samples were centrifuged for 10 min at 2,700 r.p.m.; 100µl serum was collected and stored at -80°C until use. Parallel to it, the whole brain of anesthetized animals was dissected out quickly and stored at -80°C 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 deepened 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°C. 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 LD₅₀, 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.

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

**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 ± 2°C, RH 40-60%) and were provided with pellet diet (Ashirwad brand, Chandigarh, India) and potable water ad libitum.

**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 37°C. The blood samples were centrifuged for 10 min at 2,700 r.p.m.; 100µl serum was collected and stored at -80°C until use. Parallel to it, the whole brain of anesthetized animals was dissected out quickly and stored at -80°C until use.

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Determination of IC<sub>50</sub> 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<sub>50</sub> of DFP (1.0 LD<sub>50</sub> = 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<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> of DFP or below.

Median lethal dose (LD<sub>50</sub>)

Median lethal dose (LD<sub>50</sub>) 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<sub>50</sub> of HNK-102, 0.10 LD<sub>50</sub> of HNK-106 and 0.20 LD<sub>50</sub> 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

**Table 1.** Median lethal dose (LD<sub>50</sub>) of the compounds studies including DFP.

<table>
<thead>
<tr>
<th>Name of the reactivator</th>
<th>Route of administration</th>
<th>Dose (mg/kg)</th>
<th>Mortality (Died/Treated)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP</td>
<td>s.c.</td>
<td>1.95</td>
<td>0/4</td>
<td>3.90 (2.4-3.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.90</td>
<td>2/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.80</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>HNK-102</td>
<td>i.m.</td>
<td>100</td>
<td>0/4</td>
<td>282.84 (173.3-461)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>2/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>HNK-106</td>
<td>i.m.</td>
<td>17.50</td>
<td>0/4</td>
<td>35.00 (21.4-57.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35.00</td>
<td>2/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>70.00</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>HNK-111</td>
<td>i.m.</td>
<td>17.50</td>
<td>0/4</td>
<td>35.00 (21.4-57.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35.00</td>
<td>2/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>70.00</td>
<td>4/4</td>
<td></td>
</tr>
</tbody>
</table>

* 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.
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,

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Atropine ig/kg; i.p.</th>
<th>Oxime (mg/kg; i.m.)</th>
<th>LD$_{50}$ of DFP$^a$ (mg/kg; s.c.)</th>
<th>PI (LD$<em>{50}$ with treatment / LD$</em>{50}$ without treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>-</td>
<td>3.90±0.220</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>10.00</td>
<td>7.07 (3.2-16)</td>
<td>1.810</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>-</td>
<td>6.72 (4.4-10)</td>
<td>1.720</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>10.00</td>
<td>32.7±1.56</td>
<td>8.405</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>10.00</td>
<td>HNK-102 (14.14)</td>
<td>29.62±1.32</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>10.00</td>
<td>HNK-102 (28.28)</td>
<td>50.42±7.35$^a$</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>10.00</td>
<td>HNK-102 (56.56)</td>
<td>81.7±12.52$^a$</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>-</td>
<td>HNK-102 (56.56)</td>
<td>10.07±6.913$^a$</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>10.00</td>
<td>HNK-106 (1.75)</td>
<td>8.46±0.599</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>10.00</td>
<td>HNK-106 (3.50)</td>
<td>42.6±2.600$^a$</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>10.00</td>
<td>HNK-106 (7.00)</td>
<td>66.59±3.47$^a$</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>-</td>
<td>HNK-106 (7.00)</td>
<td>6.72 (2.6-6.1)</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>10.00</td>
<td>HNK-111 (1.75)</td>
<td>9.50±2.430</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>10.00</td>
<td>HNK-111 (3.50)</td>
<td>13.8±1.56$^a$</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>10.00</td>
<td>HNK-111 (7.00)</td>
<td>44.9±5.69$^a$</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>-</td>
<td>HNK-111 (7.00)</td>
<td>4.75 (3.1-7.2)</td>
<td></td>
</tr>
</tbody>
</table>

* 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.

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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.

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.
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<sub>50</sub> to 0.502 LD<sub>50</sub> DFP dose compared to 2-PAM. Only HNK-102 significantly reactivated brain AChE at 0.502 LD<sub>50</sub> 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.

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**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.**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Dose of DFP (s.c.)</th>
<th>AChE activity (µ moles ASChI hydrolyzed/min/gm of wet tissue or 20 µl of serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD&lt;sub&gt;50&lt;/sub&gt; mg/kg</td>
<td>Whole brain</td>
</tr>
<tr>
<td>1.</td>
<td>Control -</td>
<td>4.70±0.47&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.</td>
<td>0.0031 0.0121</td>
<td>ND</td>
</tr>
<tr>
<td>3.</td>
<td>0.0062 0.0242</td>
<td>ND</td>
</tr>
<tr>
<td>4.</td>
<td>0.0125 0.0487</td>
<td>ND</td>
</tr>
<tr>
<td>5.</td>
<td>0.0250 0.0975</td>
<td>ND</td>
</tr>
<tr>
<td>6.</td>
<td>0.0500 0.1950</td>
<td>4.72±0.40</td>
</tr>
<tr>
<td>7.</td>
<td>0.1000 0.3900</td>
<td>3.75±0.71</td>
</tr>
<tr>
<td>8.</td>
<td>0.2000 0.7800</td>
<td>2.29±0.32&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>9.</td>
<td>0.4000 1.5600</td>
<td>1.21±0.23&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>10.</td>
<td>0.8000 3.1200</td>
<td>0.95±0.08&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>11.</td>
<td>2.0000 7.8000</td>
<td>0.26±0.08&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* p<0.05 or below, compared to respective control group. ASChI = Acetylthiocholine iodide. LD<sub>50</sub> of diisopropylphosphorofluoridate (DFP) = 3.90 (2.1-7.1) mg/kg, s.c.

Each value is Mean±SEM of four experiments.

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Figure 5. Sixty minutes post DFP exposure, inhibition of brain AChE activity induced by 0.05, 0.1, 0.2, 0.4 LD<sub>50</sub> of DFP (LD<sub>50</sub> = 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<sub>50</sub> of DFP (LD<sub>50</sub> = 3.9 mg/kg, s.c.) in mice. Each point represents Mean±SEM four experiments.
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 oximes (HNK-102, HNK-106 and HNK-111) were selected to slightly delay appearance of toxic effects of DFP so as to closely mimic actual condition. Administration of FPAH and propylene glycol (ratio 9:1, v/v) has appreciably given better protection (synergistic effect) against OP poisoning and its concentration is greater in blood than in other body tissue when injected subcutaneously. 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. RBCs in serum contain mainly AChE enzyme. The plausible reasons for these findings are:

- The blood enzyme is inhibited (28).
- The plasma 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 HKN-102, HKN-106 and HKN-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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