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Comparative Toxicity of *Bis*-pyridinium Acetamide Derivatives in Human Cell Lines and their Acute Toxicity in Swiss Albino Mice

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ABSTRACT

Having established the antidotal efficacy of 2-(hydroxyimino)-N-(pyridin-3-yl)acetamide (HNK oximes) against Diisopropylphosphorofluoridate (DFP) and sarin poisoning. Toxicity of HNK series and 2-PAM oximes on Human cell lines and Swiss male mice i.e. *in vitro* and *in vivo* to reported. Toxicity of the oximes was investigated in Hela, Hep G2 and HEK 293 cell lines and compared with most commonly used 2-PAM. Median lethal doses (LD_{50}) of the oximes (2-PAM, HNK-102, HNK-106, and HNK-111) were also determined following intramuscular, intraperitoneal, intravenous and oral routes of administration. All tested oximes showed no cytotoxic effect on all three cell lines in concentrations up to 0.05 mg/mL. At higher dose (0.5 mg/mL), HNK-102 found to be less toxic thus safer than 2-PAM and other oximes in all the three cell lines. In corroboration with *in vitro* finding, HNK-102 was found to be least toxic compared to other oximes via intra-peritoneal and intravenous routes of administration. Also, HNK-102 was found to be unequivocally safer compared to that of 2-PAM through i.m. and i.p. routes. For all tested oximes, toxicity following oral route, was found to be lower compared to injections, signifying that these are safer and convenient compounds for administration. These finding also suggested that HNK-102 is safer and better lead as an antidote compared to 2-PAM, against OP intoxicants.

Keywords: HNK oximes, 2-PAM, exposure route, median lethal dose, cytotoxicty

1. INTRODUCTION

Organophosphorous compounds (OPCs) are the group of phosphorus containing organic derivatives which have been largely used as pesticides and insecticides in agricultural including chemical warfare agents (CWA) in terrorists attacks[1]. OPC causes fatality in acute poisoning and also have long term complications due to their inhibitory action on acetylcholinesterase enzyme (AChE)[2]. For the management of OP poisoning, available therapeutic regimen includes atropine (anti-muscaranic), oximes (AChE reactivator) and diazepam (anti- convulsant)[3-5]. Pralidoxime (2-PAM), obidoxime and HI-6 oximes are currently used against acute poisoning of OP/ nerve agent but their unequal efficacy against the entire nerve agent raises the ambiguity on the broad antidotal efficacy of these oximes. Thus, search for a new, efficacious, least toxic AChE reactivator oxime is the standing goal of the researcher since long run.

For the development of a new compound and its usage as a drug, its toxicology study is the basic requirement. The pharmacological profiles of oximes have been widely studied but there is dearth of studies available concerning to preclinical toxicology. The toxicity of HI-6 is least in reference to cytotoxicty, genotoxicity[6] and LD_{50} in comparison to other oximes. But the only drawback is its instability in aqueous solution[7]. Hence, in search of an efficacious, least toxic and stable AChE reactivator, new HNK series oximes (*bis*-pyridinium derivatives of 2-(hydroxyimino)-N-(pyridin-3-yl) acetamide) have been synthesised in this establishment.

Having shown better *in vivo* antidotal efficacy against Diisopropylphosphoroflouridate (DFP) and sarin poisoning[8, 9], the present study aims to investigate *in vitro* cytotoxicity study and *in vivo* acute toxicity of HNK series oximes in comparison to 2-PAM. Different Human cell lines were used (Hela, HepG2, and HEK 293) for cytotoxicity studies. For LD_{50} determination, the toxicity of these compounds (HNK series) was compared by determining LD_{50} using intramuscular (i.m.), intra-peritoneal (i.p.), oral (p.o.) and intravenous (i.v.) routes of administration in Swiss albino male mice.

2. MATERIALS AND METHODS

2.1 Chemicals

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-(hydroxyimino) acetamido) pyridinium) dibromide (HNK-111) (as shown in Fig. 1) were synthesised in this establishment with > 98 per cent purity (NMR)[10]. Pralidoxime chloride (IP) obtained from Kwality Pharma, India. Dulbecco MEM media, FBS and standard 3-(4,5-dimethylthiazole)-

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2,5diphenyltetraazolium bromide (MTT) reagent were procured from Sigma Chemicals Co. (St. Louis Mo).

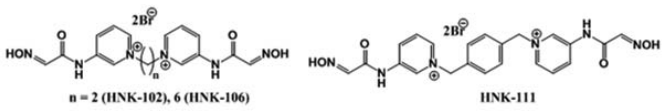

Figure 1. Structure of *Bis*-quaternary 2-(hydroxyimino)-*N*-(pyridin-3-yl) acetamide derivatives.

2.2 Cell Lines

Three malignant Human cell lines: Human cervix adenocarcinoma (Hela, epithelial), Human Caucasian hepatocyte carcinoma (Hep G2, epithelial), Human embryonic Kidney (HEK 293, epithelial) were obtained from national centre for cell sciences (NCCS), Pune, India. Cells were maintained in DMEM (high glucose) supplemented with 10 per cent heat inactivated Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine at 37 °C in a humidified atmosphere containing 5 per cent CO_2 and 95 per cent air in incubator.

2.3 Cell Viability Assay

The concentration of oximes that is toxic to the cells was determined from the plots of viability of cells by MTT assay. Cells were dispensed in 96-well flat bottomed micro titer plates (NUNC, Rosklide, Denmark) at a density of Hela (2×10^5), Hep-G2 (1×10^5) and HEK- 293 (1×10^5) cells / well. Cells were incubated with broad range of oxime concentration starting from 5 mg/mL to 0.5 mg/mL with tenfold dilution. After 24 h of incubation, 0.5 mg/mL MTT in DMEM medium was added to the cells and incubated for 4 h. The resulting formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured using an ELISA reader (Chameleon Multi-detection Platform, Finland) at a wavelength of 570 nm. Each experiment was done in quadruplicate and concentration of the oximes that is 50 per cent toxic to the cell (CC_{50}) was determined by regression analysis.

2.4 Animals

Randomised out-bred male Swiss albino mice (Animal House, DRDE, Gwalior, India) weighing 25 g - 30 g were used for the study. Four mice were housed in each cage. Dust free steam autoclaved paddy husk was used as bedding material. The cages were maintained in environmentally controlled room (25 ± 2 ºC, RH 40 % - 60 %). For all the experiments, maximum four mice per dose were used. The 3Rs (Replace, Reduce and Refine) of animal's ethics were strictly followed. Prior approval was obtained for use of animals by Institutional Animal Ethics Committee (IAEC); a statutory committee constituted by 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.

2.5 LD_{50} Determination

Median lethal dose (LD_{50}) of oxime was determined following 'moving average' method and expressed as mg/Kg of body weight (Gad and Weil)[11]. For LD_{50} determination, scales of doses were taken in logarithm with four animals for each dose. The LD_{50} value was then calculated using formula: $Log\ m = log\ D + df.$ where m = median effective dose, D = lowest dose, f = table value from Gad and Weil[11].

All the oximes were diluted in freshly prepared mixture solution of normal saline (sodium chloride 0.9 per cent in distilled water) and propylene glycol in a ratio of 9:1, v/v, respectively and used in the entire study as solvent for all injections[8]. Intramuscular administration of oximes was carried out in lateral thigh muscles of mice at angle of 45°.

Oral administration (gavage) was done using a 20-gauge oral feeding cannula (Harvard Apparatus). Oximes were administered intravenously by pre dilating the tail vein in warm water (40 °C - 45 °C) for 5 min - 7 min using 27 gauge needles.

Intraperitoneal injections were given using 24 gauge needles into the peritoneal cavity of the mice. Volume of all injections was kept between 0.1 mL - 0.2 mL. The animals were observed for ensuing mortality up to 14 days, post exposure of oximes.

2.6 Statistical Analysis

Results are expressed as mean±SEM. Data were analysed by one-way ANOVA followed by Dunnett test and Student't' test. Level of $p < 0.05$ was considered significant.

3. RESULTS

3.1 Cytotoxicity Assay

Cytotoxicity assay was done to establish the toxicity and maximum tolerated dose of HNK series of oximes as compared to 2-PAM. Cells were treated with wide range of doses of oximes (5 mg/mL to 0.5 ng/mL) except HNK-102 which was not soluble beyond 0.5mg/mL in aqueous media. In Hela cells, at 5 mg/mL concentration 2-PAM, HNK-106 and HNK-111 showed 50 ± 7 %, 45 ± 7 % and 45 ± 3 % cell survival respectively as compared to control. At 0.5 mg/mL HNK-106(69 ± 1.2 % cell survival) and HNK-111(71 ± 4 % cell survival) induced significant toxicity as compared to 2-PAM (90 ± 2 %), while HNK-102 (83 ± 4 %) is equally safe as 2-PAM. A dose response cytotoxic effect of oximes in Hela cells were determined by MTT assay. Cells were plated on to a 96 well and treated with varying concentration of oximes for 24 h as shown in Fig. 2. In Hep G2 cells at 5mg/mLconcentration HNK-106 showed highest toxicity with 28±4% cell survival followed by 2-PAM (40 ± 4 %) and HNK-111(56 ± 3 %). At 0.5mg/mL HNK-102 (93 ± 3 %), offered least toxicity as compared to 2-PAM (79 ± 8 %), HNK-106 (63 ± 2 %) and HNK-111(71 ± 7 %). A dose response cytotoxic effect of oximes in Hep-G2 cells were determined by MTT assay. Cells were plated on to a 96 well and treated with varying concentration of oximes for 24 h as shown in Fig. 3. In HEK-293 cells at 5 mg/mL HNK-111 offered maximum cell protection with 71 ± 1 % cell survival as compared to HNK-106 (64 ± 1 %) and 2-PAM (59 ± 2 %). At 0.5 mg/mL PAM (100 ± 4 %) and HNK-102(122 ± 2 %) are equally safe as compared to HNK-106 (64±3.3 %) and HNK-111(84 ± 3 %). A dose response cytotoxic effect of oximes in HEK293 cells

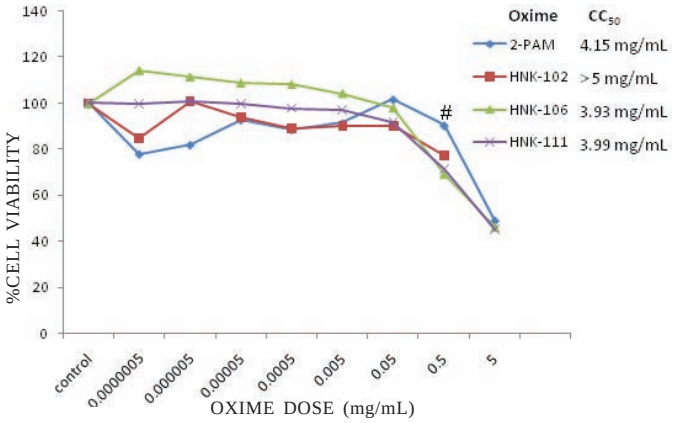

Figure 2. Cytotoxicty of oximes: The 50 % cytotoxic concentration (CC_{50}) was calculated by regression analysis. 2-PAM; Y = -26.31x + 159.2; r^2 = 0.901; CC_{50} = 4.15 mg/mL; HNK-102, Y = -12.66x + 115.2; r^2 = 1; CC_{50} = >5 mg/mL; HNK-106, Y = - 20.41x + 130.4; r^2 = 0.948; CC_{50} = 3.93 mg/mL; HNK-111, Y = - 17.56x + 120.2; r^2 = 0.934; CC_{50} = 3.99 mg/mL. Data are the mean ± SEM of four independent experiments each in quadruplicate. 2-PAM showed 90 % viability at 0.5 mg/ml dose; #p < 0.01 compared to HNK-106 and HNK-111.

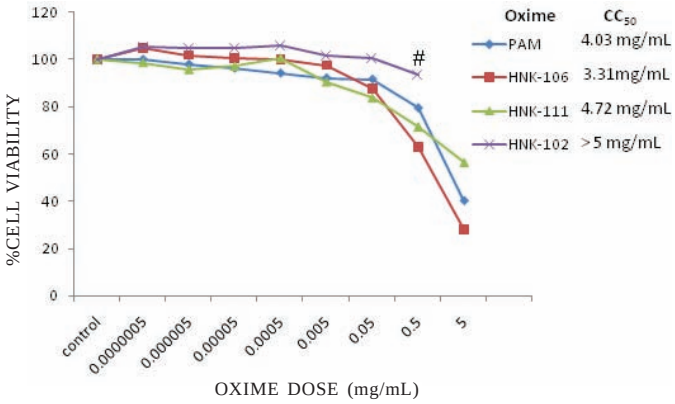

Figure 3. Cytotoxicty of oximes: The 50 % cytotoxic concentration (CC_{50}) was calculated by regression analysis. 2-PAM, Y = - 16.83x + 117.9; r^2 = 0.784; CC_{50} = 4.03 mg/mL; HNK-106, Y = - 23.34x + 127.4, r^2 = 0.944; CC_{50} = 3.316 mg/mL; HNK-111, Y = - 11.43x + 104.0, r^2 = 0.973; CC_{50} = 4.72 mg/mL; HNK-102, Y = - 4.148x + 106.8, r^2= 0.854, CC_{50} > 5 mg/mL. HNK-102 showed 93 % viability at 0.5 mg/ml doses; #p<0.001compared to 2-PAM.

were determined by MTT assay. Cells were plated on to a 96 well and treated with varying concentration of oximes for 24 h as shown in Fig. 4. At 5 mg/mL HNK-111 offered least or comparable toxicity to 2-PAM, while At 0.5 mg/mL HNK-102 was found to be better than other oximes. The concentration of oximes that is toxic to 50 % (CC_{50}) of the cells was determined from the plots of viability of cells by MTT assay. In Hela cell lines CC_{50} of 2-PAM (4.15 mg/mL) and HNK-102 (> 5mg/mL) was comparable and marginally higher than CC_{50} of HNK-106 (3.9 mg/mL) and HNK-111 (3.9 mg/mL). In Hep G2 cells CC_{50} of 2-PAM, HNK-102, HNK-106 was found to be 4.0 mg/mL,

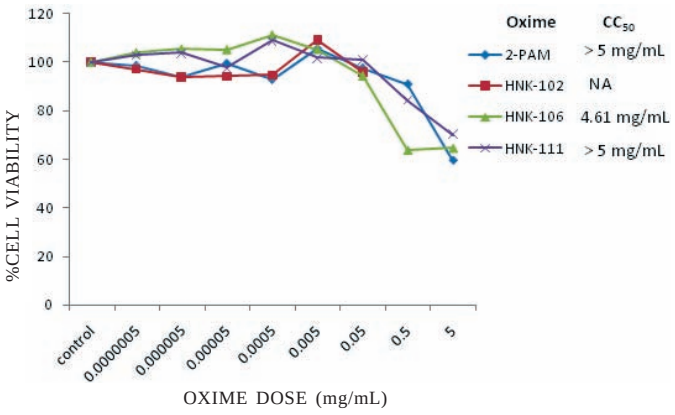

Figure 4. Cytotoxicty of oximes: The 50 % cytotoxic concentration (CC_{50}) was calculated by regression analysis. 2-PAM, Y = - 14.55x + 124.8, r^2 = 0.859; CC_{50} > 5 mg/mL; HNK-106, Y = -15.13x + 119.9, r^2 = 0.871; CC_{50} = 4.61 mg/mL; HNK-111, Y = - 15.45x + 131.5, r^2 = 0.9987, CC_{50} > 5 mg/mL.

> 5 mg/mL, 3.3 mg/mL, and 4.7 mg/mL, respectively. Since HNK 102 was soluble up to 0.5 mg/mL for calculation of CC_{50} of HNK 102 data was extrapolated. In HEK-293 cells CC_{50} was calculated as: 2-PAM (> 5 mg/mL), HNK-106 (4.61 mg/mL) and HNK-111(> 5 mg/mL) respectively. Parallel to this, HNK-102 oxime did not show any cytotoxicityin HEK 293 cells at 0.5 mg/mL dose and lower. Further phase contrast microscopic observation of cell treated with same doses of oximes, also showed dose-dependent morphological alterations. Similar to cell viability assays at 5mg/mL of oxime treatment, cells showed the visible change in the cell morphology with relative reduction in cell size and condensation of cellular material (as shown in Figs. 5 (a), (b), (c)). Lower doses of oxime did not bring any change in cellular morphology.

3.2 Signs of Toxicity by Oximes

The clinical signs and symptoms were observed in Swiss mice following 1.0 LD_{50} dose of oxime exposure. The mice treated with 1.0 LD_{50} dose of oxime showed bout of convulsions, tremors, dyspnea and were culminated in death. The animals did not show noticeable signs of toxicity when exposed to sub lethal doses of oximes.

3.3 *In Vivo* Toxicity of Oximes through Different Routes

LD_{50} values of oximes (mg/Kg) through different route are given in Table 1. Through intramuscular route HNK-106 and HNK-111 was found to be most toxic (LD_{50} = 35.35 mg/Kg)[8]. HNK-102 was least toxic having shown 282.8 mg/Kg LD_{50} values[8], compared to 2-PAM (180 mg/Kg)[12]. Similarly, HNK-102 showed least toxicity (407 mg/Kg) through intraperitonial route, compared to 2-PAM, HNK-106 and HNK-111(141.14 mg/Kg, 28.28 mg/Kg, 14.14 mg/Kg).When given orally, 2-PAM was found least toxic among all the oximes, having 2520 mg/Kg LD_{50} value. HNK-102, HNK-106 and HNK-111 showed LD_{50} as 1131.3 mg/Kg, 1600 mg/Kg, > 565 mg/Kg, respectively.

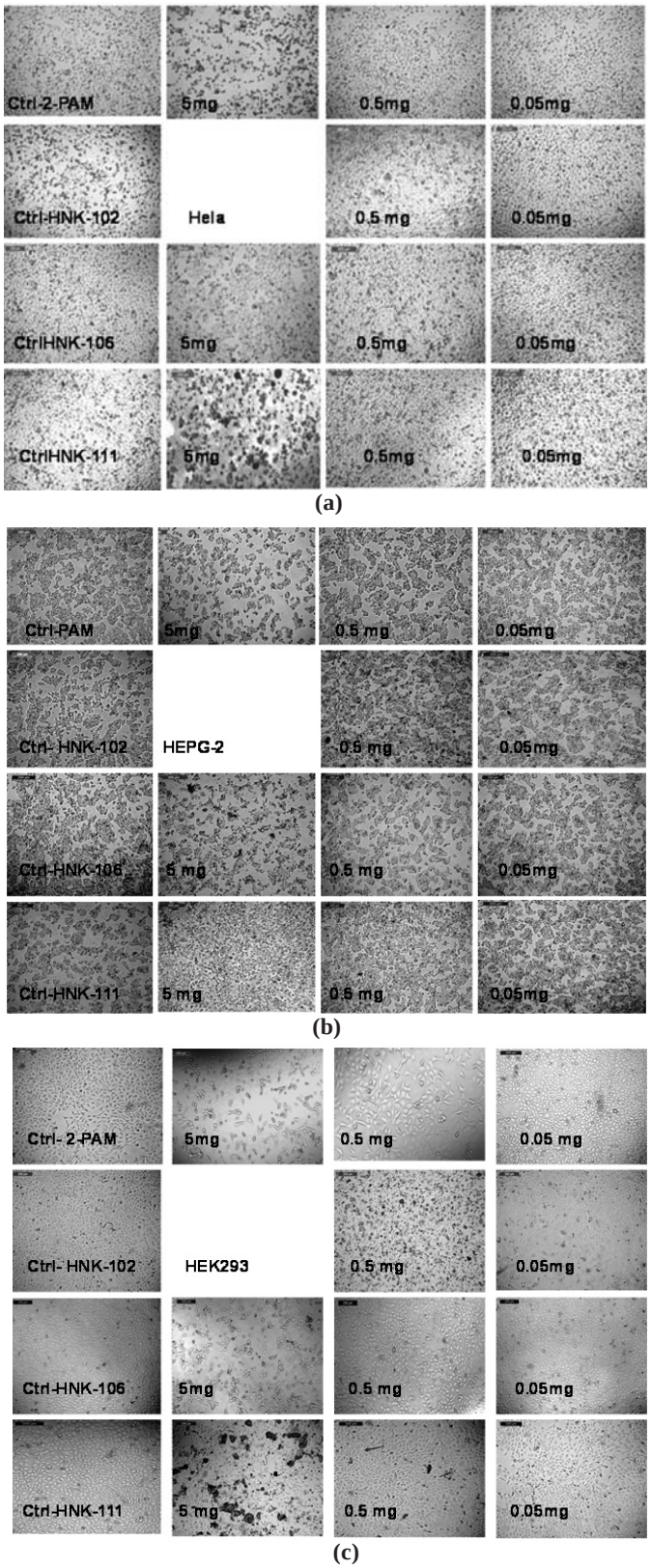

Figure 5. Cell morphology: phase contrast microscopic observation of cells treated with different doses of oximes, showing dose-dependent morphological alterations. Cells appeared shrunken at dose of 5 mg/mL of oximes (2-PAM, HNK-106 and HNK-111). No change in the morphology was observed at 0.5 mg/mL dose or lower; comparable to control group. (a) Hela (b) Hep G2 (c) HEK 293.

4. DISCUSSION

The choice of antidotal treatment against acute poisoning of OP compounds is a serious problem and to protect the exposed victims of acute poisoning is a big challenge for the researchers. The veterans are trying to counteract the OP poisoning either by increasing the efficacy of antidotes (post exposure treatment) or by developing a more effective Acetylcholinesterase (AChE) enzyme reactivator (oximes). But the failure of oximes against wide OP compounds is due to absence of optimal concentration of oximes in the body, post OP exposure[13]. Moreover the delay in the treatment with oxime therapy also leads to inefficient medical treatment against OP poisoning[14]. Along with aforesaid issues, problems like stability, intrinsic toxicity, solubility and broad spectrum efficacy against all OPs urge the need for a better AChE reactivator in a therapeutic regimen. In the light of this view, the novel *bis*-pyridinium acetamide derivatives (HNK oximes) were synthesised and their antidotal efficacy was evaluated against acute poisoning of DFP and sarin[8,9]. Showing better efficacy in terms of protection and AChE reactivation, further oximes were evaluated for their toxicity study in comparison to established 2-PAM oxime. Herein, the study reports the toxicity of new HNK series oximes using *in vitro* and *in vivo* approach in different human cell lines and Swiss male mice.

Evaluation of the inherent toxicity of the oximes is one of the major prerequisite for the development of newly synthesised oximes as a drug. *In vitro* toxicity of oximes was analysed in three human cell lines using broad range of concentration from 5 mg/mL to 0.5 ng/mL to observe the maximum tolerated dose and CC_{50} of HNK series of oximes, in comparison to 2-PAM. Three cell lines were chosen for this study to represent a broad range of origin of tissues. HEK 293, HepG2 and Hela cell lines were arose from cervical tissue, liver and kidney respectively. Generally, for studying the basal cytotoxicity such as cell growth and viability, epithelial cell lines Hela are used. Presence of enzymes relevant to xenobiotic metabolism makes HepG2 cell line a suitable model to study oxime efficacy. The selection of HEK 293 cells in oxime toxicity referred due to the presence of Acetylcholine receptors on these cells. The oxime having direct effect on these receptors in OP toxicity, hence these cells can be better subject to study the dose dependent oxime toxicity. Determination of maximum tolerated dose and CC_{50} of the drug candidate will give better idea for its therapeutic index. Observing maximum safe dose of oximes in all three cell line suggest that HNK-102 and 2-PAM are equally good or HNK-102 is better than 2-PAM. As far as CC_{50} of the compounds are concerned, again HNK-102 was found to be better than other oximes included in the study and comparable to 2-PAM. Similar observation were found when cells were analysed morphologically. We also validated our finding using trypan blue exclusion method for few experiments and observation was well corroborated with MTT assay suggesting membrane integrity of these cells. In our previous study HNK-102 was found to be better AChE reactivator as compared to 2-PAM[8], but less toxicity of HNK-102 makes it better candidate drug in comparison to 2-PAM.

The acute toxicity determination of any compound plays an important role in the designing of drug and its toxicological

evaluation. The promising approach for the determination of acute toxicity in mammals is by using single dose of a chemical wherein lethality can be determined within 24 h. The lethality is expressed as LD_{50} and the mouse is one of the species used in these studies[15]. Toxicity of a compound involves absorption, distribution, metabolism of chemical and reaction with the targeted organ within the mammalian body. Hence, different exposure routes can have different bioavailability of a chemical which results in variable toxic effect within the same species.

Our study reports the acute toxicity of HNK series oximes and 2-PAM using different route of exposure in Swiss male albino mice (Table 1). The results showed that HNK-102 via intramuscular route showed the least toxicity (282.8 mg/Kg) among all the oximes. The rapid absorption of the drug via the muscles helps in easy entry of the drug into the circulation which can there by show the immediate effect. Also in the reported studies, HNK-102 showed far better Protection Index compared to 2-PAM against DFP and sarin poisoning via intramuscular route. Hence the choice of this drug via i.m. route is favaourable[8,9]. Similarly in case of intraperitoneal route, HNK-102 showed the least toxicity. In comparison to 2-PAM ca. 3 times lower toxicity was shown by HNK-102 via intraperitoneal route. Likewise via intravenous route, HNK-102 and 2-PAM was seen lesser toxic (90 mg/Kg and 70.71 mg/Kg, respectively) compared to HNK-106 and HNK-111. The large difference between the toxicity of HNK-102, HNK-106 and HNK-111 via i.p. and i.m. route underlay to the structural difference. This toxicity and structural difference can be explained on the following characteristics:

(i) The presence of quaternary nitrogen
(ii) The number of quaternary nitrogen
(iii) The number of oxime moiety and its position to the quaternary nitrogen atom and
(iv) The nature of the linker. As shown, HNK-102 contains two carbons as a linker between the pyridine rings whereas HNK-106 and HNK-111 have 6 and 1 carbon linker chain respectively.

Also, HNK-111 contains three pyridine ring compared to other oximes. The previous reports say that toxicity of alkenes, alkynes and -cyclo compounds are greater than alkanes group[16]. Hence, the presence of more alkyl and quaternary nitrogen group contributes to the higher toxicity by HNK-106 and HNK-111.

As reported elsewhere, the bioavailability of the drug via intravenous and intraperitoneal route is almost similar[16]. Our *in vitro* data is satisfactorily correlating with data obtain by i.p. and i.v. route of administration. Being a small laboratory animal, the favourable choice of drug administration in mice is intra-peritoneal. But in actual condition of OP exposure among Humans, 2-PAM, 30 mg/Kg (1 g - 2 g) is administered by i.v. therapy over 15 min - 30 min, repeated in 1 h if necessary[17]. Likewise, in order to evaluate HNK-102 oxime via i.v. route, lethal toxicity was determined. Herein, the results also showed that HNK-102 is equivalent safe with 2-PAM, thus, favorable compound given via i.v. and i.p. route.

Table1. Acute oral and parenteral median lethal dose or LD_{50} (mg/Kg) of oximes in Swiss male mice

Route	2-PAM	HNK-102	HNK-106	HNK-111
Intramuscular	180[12]	282.8[8]	35.35[8]	35.35[8]
Intraperitoneal	141.4	407	28.28	14.14
Oral	2520	1131.3	1600	>565
Intravenous	90	70.71	7.93	3.53

LD_{50} determined following the 'Moving average' method of Gad and Weil, 1989. Animals were observed 14 days for mortality.

Although, metabolism or biotransformation both occur inside the body after oral or injection route, but there is difference in the rate of their occurrence. Through oral exposure, the chemical has to undergo first pass metabolism where it counter digestive enzymes, carried further into liver through portal vein and finally available for circulation in the body. As a consequence, the metabolised product (mostly the active chemical) from the liver, reached into the circulatory system in a very small amount. Whereas alternative routes like intramuscular and intravenous bypass the liver metabolism (first pass effect), results in the circulation of larger amount of compound in the body system. Henceforth, the drugs undergoing first pass metabolism and biotransformation in stomach can decrease the concentration of actual compound, leading to the difference of toxicity between oral and injection route. In our study, we compared LD_{50} of HNK oximes with standard oxime, 2-PAM. The results showed HNK-102, HNK-106 and HNK-111 offered acute toxicity 1130 mg/Kg, 1600 mg/Kg and > 565 mg/Kg, respectively. Similarly, 2-PAM showed 2520mg/Kg of LD_{50} via oral (gavage) route. The plausible explanation to these findings is that the toxicity of the metabolites from the first pass effect if greater than their parent compound, the oral toxicity will be higher than the injection route. However, the results showed that all the reported oximes have lower oral toxicity compared to other routes of exposure. This clearly indicates that the HNK series oximes are safe and convenient for administration (comparable to 2-PAM) to be given via oral route against OP exposure. Moreover, the difference in lethal toxicity among HNK oximes and standard 2-PAM in oral route cannot only be attributed through the toxic metabolite of HNK oximes in the stomach. Gender (male and female Swiss mice) and species strain should also be considered in comparing the toxicity between these oximes. More studies in future related to the changes in liver and kidney markers, may depict the clear picture of their toxicity profile.

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CONFLICT OF INTEREST

The author(s) declare(s) that there are no conflicts of interest.

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