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 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, cytotoxicity

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 attacks¹. 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 OPC 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 cytotoxicity, genotoxicity⁶ and LD₅₀ in comparison to other oximes. But the only drawback is its instability in aqueous solution⁷. 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 Diisopropylphosphorofluoridate (DFP) and sarin poisoning⁸, 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 LD₅₀ determination, the toxicity of these compounds (HNK series) was compared by determining LD₅₀ 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)⁹. Pralidoxime chloride (IP) obtained from Kwalita Pharma, India. Dulbecco MEM media, FBS and standard 3-(4,5-dimethylthiazole)-
2,5-diphenyltetrazolium bromide (MTT) reagent were procured from Sigma Chemical Co. (St. Louis Mo).

![Structure of Bis-quaternary 2-(hydroxyimino)-N-(pyridin-3-yl) acetamide derivatives.](image)

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

2.2 Cell Lines

Three malignant Human cell lines: Human cervical 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 CO₂ 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, Roskilde, Denmark) at a density of Hela (2 × 10⁴), Hep-G2 (1 × 10⁴) and HEK-293 (1 × 10⁴) 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 sulphoxide. 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 (CC₅₀) 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 °C, 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 LD₅₀ Determination

Median lethal dose (LD₅₀) of oxime was determined following ‘moving average’ method and expressed as mg/Kg of body weight (Gad and Weil)¹¹. For LD₅₀ determination, scales of doses were taken in logitogarhythm with four animals for each dose. The LD₅₀ 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 Weil¹¹.

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 injections⁸. 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’s 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 mg/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/mL concentration 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.
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% (CC50) of the cells was determined from the plots of viability of cells by MTT assay. In Hela cell lines CC50 of 2-PAM (4.15 mg/mL) and HNK-102 (> 5mg/mL) was comparable and marginally higher than CC50 of HNK-106 (3.9 mg/mL) and HNK-111 (3.9 mg/mL). In Hep G2 cells CC50 of 2-PAM, HNK-102, HNK-106 was found to be 4.0 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 CC50 of HNK 102 data was extrapolated. In HEK-293 cells CC50 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 cytotoxicity in 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 LD50 dose of oxime exposure. The mice treated with 1.0 LD50 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

LD50 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 (LD50 = 35.35 mg/Kg)8. HNK-102 was least toxic having shown 282.8 mg/Kg LD50 values6, 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 LD50 value. HNK-102, HNK-106 and HNK-111 showed LD50 as 1131.3 mg/Kg, 1600 mg/Kg, > 565 mg/Kg, respectively.
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. Moreover the delay in the treatment with oxime therapy also leads to inefficient medical treatment against OP poisoning. 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. 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 CC50 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 CC50 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 CC50 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, 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 LD50 and the mouse is one of the species used in these studies. 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 among all the oximes. The rapid absorption of the drug via intramuscular route showed the least toxicity (282.8 mg/Kg) in albino mice (Table 1). The results showed that HNK-102 via i.m. and i.p. 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. Likewise, in order to evaluate HNK-102 oxime via i.v. route, lethal toxicity was determined. Hence, the results also showed that HNK-102 is equivalent safe with 2-PAM, thus, favorable compound given via i.v. and i.p. route.

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 LD50 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 LD50 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.

**REFERENCES**


| Table1. Acute oral and parenteral median lethal dose or LD50 (mg/Kg) of oximes in Swiss male mice |
|---------------|--------------------|----------------|----------------|
| Route         | 2-PAM              | HNK-102        | HNK-106        | HNK-111        |
| Intramuscular | 180±2              | 282.8±8        | 35.35±8        | 35.35±8        |
| Intraperitoneal| 141.4              | 407            | 28.28±8        | 14.14±8        |
| Oral          | 2520               | 1131.3±7       | 1600±7         | >565±7         |
| Intravenous   | 90                 | 70.71±7        | 7.93±7         | 3.53±7         |

LD50 determined following the ‘Moving average’ method of Gad and Weil, 1989. Animals were observed 14 days for mortality.
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CONFLICT OF INTEREST
The author(s) declare(s) that there are no conflicts of interest.

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