IN VIVO EFFICACY OF EIGHT NEW BISQUATERNARY K-OXIMES IN COMPARISON TO 2-PAM AND OBIDOXIME AGAINST RAT WITH PARAOXON AND DFP INTOXICATION.

Ph.D Thesis

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Introduction:

Organophosphorus intoxication is due to the irreversible inhibition of acetylcholinesterase, a neurotransmitter enzyme. Oximes reactivators are powerful nucleophilic agents and are well known to reactivate the inhibited/phosphylated acetylcholinesterase. However, no oximes found to be a broad spectrum and effective against different groups of organophosphorus anticholinesterases.

The organophosphorus compounds have a wide variety of applications, hence is a serious threat for occupational hazard, self poisoning, unintentional misuse, terrorists attack and threats of warfare use, not only for army rather civilian targets as well. The organophosphorus anticholinesterases compounds are among the most frequent agents involved in suicidal and accidental intoxication (Buckley et al. 2005a; Eddleston et al. 2008) and food poisoning (Kavalci et al. 2009). The terrorist attack in Motsomoto (Japan) in 1994 and Tokyo subway 1995 with Sarin, an OP nerve agent, caused many deaths and thousands casualties (Okudera et al. 1997; Nagao et al. 1997; Weiner and Hoffman 2004).

The present study was undertaken to find more effective and universal oxime particularly against OPC belongs to pesticide group. The in vivo survival studies were carried out with experimental K oximes which were recently developed and synthesized in the Department of Toxicology, Faculty of Military Health Sciences, Czech Republic by Kamil Kuca and Kamil Musilek (Kuca et al. 2003a & b; Musilek et al. 2005, 2006a, 2006b) and hence named K-oximes. More than 200 structurally different K-oximes have been synthesized since 2003 (Kassa et al. 2008a) but the most promising among them are K-027 which worked well against nerve agents (Kassa et al. 2006; Calic et al. 2006; Musilek et al. 2006a and b etc.) as well as pesticides (Petroianu et al. 2006a & b, 2007a & b; Lorke et al. 2008a, b).Other potentially promising reported K oximes are K 048, K054, K 074. Structurally all the K-oximes are either asymmetrical or symmetrical bispyridinium aldoximes with changes in the position of functional aldoxime as well as in some cases changes in linker chain. The study will help in determining the candidacy of some K-oximes to replace the presently therapeutically available less effective oximes.

Aims and Objectives

The aim of the present study is to;

1 Asses *in vivo* to what degree the eight novel K-oximes, K027, K048, K53, K74, K75, K107, K108 and K113 are effective against rat model for paraoxon and diisopropylfluorophosphate (DFP) intoxication, structurally the two different organophosphorus anticholinesterases.

2 To determine the intrinsic toxicity of experimental oximes in terms of LD_{50} and establish a correlation between intrinsic toxicity and the antidotal efficacy of experimental oximes in respect to paraoxon and DFP intoxication.

3 To establish a relationship between *in vivo* efficacy on rats model with *in vitro* result on human RBC-AChE of the same oximes (Petroianu and Lorke 2008).

4 Since, due to ethical reasons testing of experimental oximes is not possible in human, so establish a hypothesis whether *in vitro* result is sufficient to translate the result for human use or *in vivo* work is indispensable.

5 *In silico* study for predicting the lipophilicity and crossing blood brain property of experimental oximes.

6 Comparison of the efficacy of new oximes against structurally two different organophosphorus anticholinesterases that is paraoxon and DFP.

7 Suggest a mechanism of action of oximes on the basis of Log*P* values, correlation outcome and available literature.

8 Paraoxon and diisopropylfluorophosphate were selected as AChE inhibitor and toxicants because the acute toxicity of paraoxon resembles with the toxicity of nerve agents as well as it is used in organophosphorus pesticides and easily accessible to anyone whether farmers (occupational hazard), misuses (suicides) or terrorists. The second compound is a structural analog of nerve agent, Sarin and is used as an insecticide/pesticide.

Methods

In vivo toxicity determination of the experimental oximes and survival study after intoxication with OPC paraoxon and DFP and subsequent application of oximes were carried out on male Wistar rats (average weight ± SD: 248 g ± 21.). LD_{50} and LD_{01} **of** oxime**s** were determined by using scientific probit-log graph with percent mortality on y-axis and oxime doses on x-axis. The acute toxicity was tested in a step-wise fashion according to the Acute Toxic Class method (Diener and Schlede 1999) largely following the OECD guidelines (2001, no. 423). For the in vivo survival study, half of LD_{01} of oximes were used against three lethal doses of paraoxon (viz. 1 µmol, 3µmol and 5 µmol/rat) and DFP (viz. 6µmol, 10µmol and 14µmol/rat).The experiments were repeated for four times with six rats per group/cycle. All the animals received ip injections at two different anatomical sites. Mortality was recorded at 30min. 1,2,3,4.24 and 48 hours. Statistical analysis was performed on the mortality data of 4 cycles. Mortality data were compared and, for each of the seven time points, the respective hazards ratios (relative risks of death) were estimated using Cox proportional hazards model (Cox 1972). Subsequently, the area under the RR-time curve was determined and pair-wise comparisons (Mann-Whitney U-Test) were performed in order to determine the most protective reactivator.

To determine the physico-chemical properties of new experimental oximes, Log*P* values were calculated by using the PrologP module of the Pallas 3413 software. Details of the algorithm used for calculations are given by Molnar et al. (2004). The program takes into account all lipophilic and hydrophilic fragments of a specific compound and makes minor corrections based on octanol-water partition data as available from the literature.

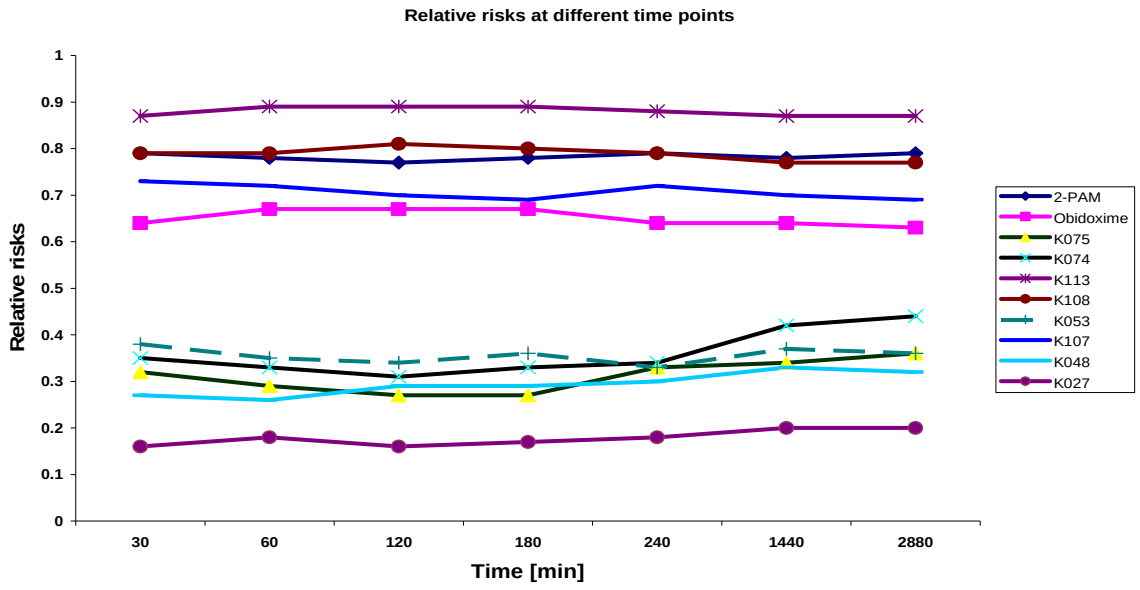
In order to determine the predictive value of in vitro testing (human blood) for *in vivo* (rat), the various *in vitro* and *in vivo* parameters have been correlated in a pair wise manner. The *in vitro* results were obtained from Petroianu and Kalasz 2007; Petroianu and Lorke 2008, and Lorke et al. 2008b.The non parametric Spearman rank correlation coefficient has been employed for data analysis. Only correlations with a rank correlation coefficient $R \geq 0.60$ have been considered.

Result

(a) Relative risk of death after paraoxon exposure

The relative risk (RR) of death at the seven time points (30 minutes, 1, 2, 3, 4, 24 and 48 hours), estimated by Cox analysis in oxime-treated animals, is depicted in Fig. 1. It was compared with untreated animals (RR = 1). Additional injection of all tested oxime-type reactivators reduced mortality significantly ($p \leq 0.01$) as compared to the no-treatment group (G_1; Paraoxon only). Best protection was observed for K-027 (RR = 0.20), which was significantly ($p \leq 0.05$) more effective than all the other tested oximes. Marked reduction in mortality was also achieved by K-48 and the three new bispyridinium oximes, K-048 (RR = 0.32), K-053 (RR = 0.36), K-074 (RR = 0.42), K-075 (RR = 0.35). These four oximes were significantly ($p \leq 0.05$) superior to 2-PAM, obidoxime, K-107, K-108 and K-113, but also significantly ($p \leq 0.05$) less effective than K-27. Protection by obidoxime (RR = 0.64) was significantly ($p \leq 0.05$) superior only to K-113, but significantly ($p \leq 0.05$) inferior to K-027, K-048, K-053, K-074, K-075. Very poor protection was observed by 2-PAM (RR = 0.78), K-107 (RR = 0.70), K-108 (RR = 0.77) and K-113 (RR = 0.87), which were significantly ($p \leq 0.05$) less effective than all other tested oximes, except obidoxime

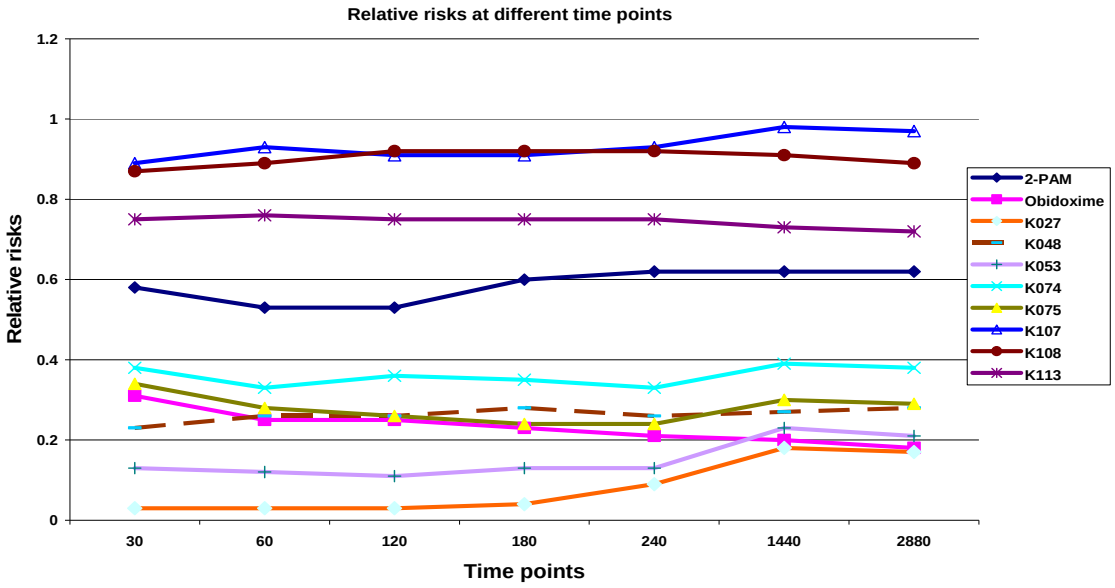
Fig. 1



(b) Relative risk of death after DFP exposure

Best protection was obtained by K027 (R=0.16), which was significantly more effective than all the other tested oximes, except obidoxime, K053, and K-075.Good protection was achieved with obidoxime (RR=0.19), K-053 (RR0.22) and K-075 (RR=0.29). Moderate protection was given by K-74(RR=0.38) but it was more efficacious than 2-PAM, K107, K108 and K113 but significantly less effective than obidoxime, K-053, and K-027. K-048 (RR=0.28) was also significantly superior to 2-PAM, K107, K108 and K113. The two new K-oximes K-107 and K108 did not significantly reduce DFP induced mortality

Fig.2



Calculated Log*P*

All tested oximes are hydrophilic, as indicated by negative Log*P* value. The most hydrophilic substance is obidoxime (Log*P* -3.12) followed by K-027 (Log*P* -2.66), K-048 (Log*P* -2.61), 2-PAM (Log*P* -2.31), K-053 (Log*P* -2.05), K-075 (Log*P* -2.02), K-074 (Log*P* -1.96). K-107, K108 and K-113 are much less hydrophilic (Log*P* > -1)

Correlation between *in vivo* and *in vitro* parameters.

In the present study, the *in vivo* toxicity data was correlated with the *in vitro* toxicity data (Table 1a, 1b,1c). Paraoxon induced toxicity data did not reveal correlation with comparable *in vitro* parameters. Except tan α vs. RR, all the pairs showed moderate to strong statistically significant correlation (Spearman rho) with DFP induced toxicity.

Table 1a

Variable pairs	**Spearman correlation σ**	**Statistical significance (two tailed)**
IC50 of oximes (in vitro) versus LD50 of oximes (in vivo)	0.83	0.001
Tan α (in vitro) versus LD50 of oximes (in vivo)	-0.95	0.000
Tan α (in vitro) versus IC50 of oximes (in vitro)	-0.81	0.005
LogP (in vitro) versus LD50 of oximes (in vivo)	-0.88	0.001
LogP (in vitro) versus IC50 of oximes (in vitro)	-0.94	0.000
LogP (in vitro) versus Tan α (in vitro)	0.77	0.010

Table 1b (Paraoxon)

IC50 of oximes (in vitro) versus cumulative relative risk of death (in vivo)	-0.30	0.405
Tan α (in vitro) versus cumulative relative risk of death (in vivo)	0.37	0.293
LogP (in vitro) versus cumulative relative risk of death (in vivo)	0.52	0.121

Table 1c (DFP)

IC50 of oximes (in vitro) versus cumulative relative risk of death (in vivo)	-0.73	0.017
Tan α (in vitro) versus cumulative relative risk of death (in vivo)	0.58	0.077
LogP (in vitro) versus cumulative relative risk of death (in vivo)	0.89	0.001

Table 1a, 1b, 1c: Synopsis of the correlation results. The first column shows the variable pairs compared the second column the Spearman rank correlation coefficient, while the third column gives the p value for statistical significance. A statistical significance level α of 0.05 has been used.

Conclusion

The following conclusions may be drawn from the present study;

- The gold standard therapeutically available oxime, 2-PAM is least effective.
- The second available oxime which is considered promising for insecticides is not equally effective against structurally different kinds of organophosphorus compounds. In the present study, it was moderately effective against DFP and poor for paraoxon.
- Among the experimental K-oximes, K-027 was found to be superior for both paraoxon and DFP intoxication. This oxime may be a candidate to replace all available oximes.
- Other K-oximes like K-048, K-053, K-075 and K-074 are also better than or equal to established oximes though they are lesser in efficacy than K-027.
- Generally *in vitro* result correlates with *in vivo* data but it is not must. *In vitro* system may not be an alternative to *in vivo* system
- *In vitro* reactivation capacity of human red blood cell RBC-AChE has no predictive value for *in vivo* (rat) efficacy; hence *in vivo* testing on different species of animal is indispensible.
- All the oximes are hydrophilic, suggesting that their mechanism of action is other than on central nervous system.
- The study suggests that oximes protective effect is not only due to AChE reactivation rather other mechanism also involves in addition to AChE reactivation.
- Further *in vivo* studies on other species of animals like guinea pig etc is also needed before translating the *in vivo* animal results for human use.

Publications connected to the Dissertation.

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