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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Contents

List of tables, figures and schemes

List of tables

List of figures

List of Schemes

ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
bw	Body weight
BuChE	Butyryl or plasma cholinesterase
CaE	Carboxylesterase
ChE	Cholinesterase
95%CI	95% Confidence Interval
Cum RR	Cumulative relative risk
CVX	Chiness VX (nerve agent)
DFP	Diisopropylfluorophosphate
FMHS	Faculty of Medicine and Health Sciences
Hb	Hemoglobin
IC	Inhibitory concentration
i.m.	Intra muscular
i.p.	Intra peritoneal
K	Binding constant
Kg/bw	Kilogram per body weight
LD	Lethal dose
mg/rat	milligram per rat
µM	Micro molar
µmol	micromole
mmol	millimole
nM	Nanomolar
OP	Organophosphorus
OPC	Organophosphorus compound

POX	Paraoxon-ethyl
2-PAM	Pralidoxime
RBC	Red blood cells
RR	Relative risk of death
SD	Standard deviation
Tan α	Tangent alpha

Chapter 1: Introduction and Background

Introduction

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.

Organophosphates are mainly used for civilian purposes like pesticides or acaricides etc. but their acute toxicity is comparable to the organophosphonates, developed for military purposes.

There are different types of cholinesterases in the human body, which differ in the location in tissues, substrate affinity, and physiological function. The principle ones are acetylcholinesterase (EC 3.1.1.7, AChE) found in the nervous system and also present in the outer membrane of red blood cells and plasma cholinesterases (EC 3.1.1.8, BuChE).The acute toxicity of OPC is primarily due to inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) which belongs to serine esterase.

Organophosphorus intoxication is due to the inhibition of acetylcholinesterase, a neurotransmitter enzyme. Oximes reactivators are powerful nucleophilic agents and are well known to reactivate the inhibited/phosphylated acetylcholinesterase but even after fifty years of the discovery of first oxime, no oximes found to be a broad spectrum and effective against different groups of organophosphorus anticholinesterases. Although the present study is concerned with new potential oximes, but for the better understanding, all three components are being discussed here that is

A. Enzyme inhibitor (OPC),

B. Acetyl cholinesterase and

C. Oximes.

Background

A1.1: Organophosphorous anticholinesterase compounds

Organophosphorus anticholinesterase compounds are esters, amides or thiol derivatives of phosphoric, phosphonic, phosphinic acids, and phosphorothioic or phosphonothioic acids. The phosphonic acids derivatives are more toxic than the phosphoric acids (Inchem.org) whose oxygen atom can be substituted by sulphur or nitrogen atoms (Bosak 2006). In other words it is an organic compound that contains phosphorus as an integral part of the molecule and formed by the reaction of alcohol and phosphoric/phosphonic/phosphinic acids. The first synthesized organophosphorus compound was a mono ester named tetraethyl pyrophosphate (TEPP). The compound was synthesized in the early 1800 by Moschnine (Antonijevic and Stojiljkovic 2007; Petroianu 2008). The process was first published in 1854 by de Clermont (Kenneth et al. 2008).The organophosphorus compounds (OPC) were primarily synthesized for crop protection against insect pests. During 1934 – 1944, Schrader's team in Germany synthesized approximately 2000 OPCs and in 1944 two well known organophosphorus insecticides Paraoxon and Parathion were synthesized and reported (Antonijevic and Stojiljkovic 2007). Since then hundreds of new OPC have been produced as insecticides, pesticides, acaricides or nematocides etc.

Anticholinesterase compounds have been used as weapons in Africa since the XIX century (Delfino et al. 2009). During Second World War extremely toxic anticholinesterase OPCs were produced under the direction of Nazi's regimen but fortunately, were not used in the war. These extremely toxic OPC were called G-agents, OP-nerve agents and warfare chemicals. The first among those compounds was Tabun, developed in 1936, followed by Sarin in 1938, Soman in 1944, Cyclosarin 1949, and VX in 1957.During the same period Russian scientists developed the so called Russian VX known as VX (VR). Another structural analogue of VX is Chinese VX (CVX). In the late 1980 and

early 1990, chemists in Russia produced several new agents called Novichok-5 and Novichok-7.

The use of OP nerve agent in war was observed for the first time in 1983-1984 between Iran-Iraq war when Iraqi troops used Tabun in Majnoon Island in Iran and the Sarin was used in Halabjeh massacre in 1988 (Foroutan 1984). Then terrorist attack in Motsomoto (Japan) in 1994 and Tokyo subway 1995 with Sarin caused many deaths and thousands casualties (Okudera et al. 1997; Nagao et al. 1997; Weiner and Hoffman 2004).

A1.2: General structure of an OP

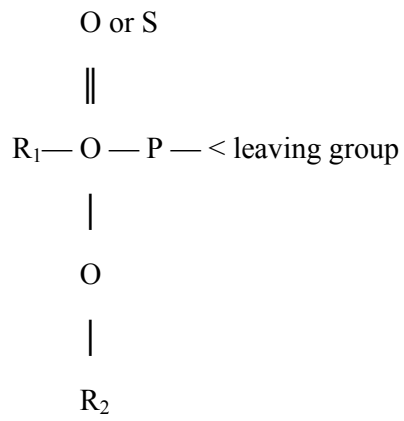
The basic structure of an organophosphorus compound consists of the following;

a. A central phosphorus atom (P).

b. P is double bonded to either oxygen or sulphur.

c. A leaving group which is specific to the individual organophosphorus. It is a labile acyl residue (halide,cyano,phenol, or thio group)

d. R1 and R2 groups which are ethyl or methyl, alkyl, akoxy, alkylthio or amino group.



General structure of an organophosphorus compound

A1.3: Mechanism of Toxicity

There are various groups of organophosphorus compounds which are structurally and toxicologically different. However, a common mechanism of action is found in all different and diversified groups of OPC which is irreversible inhibition of acetylcholinesterase (EC 3.1.1.7) with the active centre serine hydroxyl group (Marrs 1993; Fukuto 1990; Mileson et al.1998; Pope 1999; Reiner 2001; Reiner et al. 2007; Delfino et al. 2009).

In all cases the initial step of inhibition requires that OPC compound should be in the oxon (P=O). If there is P=S, then at first it will transform in O form (oxon) in the body that is in vivo and then exhibit the anticholinesterase action. There are four stages of interaction of OP oxon with AChE (Johnson et al. 2000).

Reaction 1: In the first step, a Michaelis complex forms due to reaction between enzyme and OP oxon. A specific serine in enzyme is phosphylated with loss of the leaving group.

Reaction 2: Step 2 is a progressive reaction leading to the formation of a reasonably stable covalent bond between OP compound and enzyme with consequent inhibition of catalytic activity. In this step organophosphorylation of the enzyme (esterase) takes place.

Reaction 3: Reaction may occur spontaneously but slowly at a rate that is dependent both on the nature of the attached group and on the enzyme protein. We say it is dephosphorylation step.

Reaction 4: Fourth step is the process of ageing. The ageing phenomenon is the time dependent loss of ability of the phophorylated enzyme to be reactivated by nucleophilic agents like oxime. The mechanism consists of cleavage of one R group and formation of a charged mono substituted phosphoric acid residue.

Since enzyme acetyl cholinesterase (AChE) is responsible for terminating the action of neurotransmitter acetylcholine (ACh) at nerve synapse, the inhibition

of enzyme results in the accumulation of acetylcholine at cholinergic synapses and stimulates both nicotinic receptors which are found at neuromuscular junctions and at autonomic ganglia (sympathetic and parasympathetic) and muscarinic receptors which are found on organs innervated by parasympathetic nerve endings. In addition, both nicotinic and muscarinic receptors are over stimulated in the central nervous system (Gupta 2004). Death usually occurs due to respiratory failure, cardiovascular collapse and/or generalized seizure (Bajgar 2004; Eddleston et al. 2006, 2008).

A1.4: Structural diversity and differences in toxicity

There are over 100 different synthesized OPC though a generalized structure is same as mentioned earlier. Each OPC has a unique individual structural profile for toxicity and behavior, ranging from extremely toxic nerve agents to a moderate or little toxic. From a chemistry perspective the family of organophosphorus compounds comprises organophosphate, organophosphonates and organophosphinates and each of them is further divided into sub and sub groups depending upon the R1 (ethyl, methyl) and R2 (alkyl, amide etc.) of general structure and the number of covalent bonds between P and C. For instance if no P-C bond exists in the molecule then the compound is an organophosphate e.g. TEPP. It has one P-C bond in the molecule. Organophosphinates have two P-C bonds in the molecule (Petroianu 2007).

A1.5: Potential targets of OPC exposure

Over the last 100 years, the use of organophosphorus compounds has dramatically increased with new applications still being developed. They are used as insecticides, pesticides, helminthcides, acaricides, and nematocides and to lesser extent herbicides and fungicides (Sultatos 1994). OP pesticides are used for public health purposes to control disease vectors. In addition to insecticides and pesticides, it is also being used in flame retardants, polymer additives, compounds with pharmacological and chemotherapeutical effects, radio diagnostic agents, metal extraction agents, corrosion inhibitor, Emulsifier,

antistatic agents, ligand modifier of catalyst etc. (Gupta 2006). However, not all the organophosphorus is anticholinesterases.

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). Acute organic insecticides poisoning is a major health problem all over the world particularly in developing countries, where organophosphates are the most common suicidal poison with high mortality and morbidity (Cherian et al. 2005). Organophosphorus compounds accounts for several hundreds of thousands of death worldwide every year and even greater number of casualties (Karalliedde and Senanayake 1999). According to Eddleston et al. 2008 organophosphorus pesticide self poisoning is important chemical problem in the rural regions of the developing world and kills an estimated 200,000 people every year. Another worldwide mortality studies report mortality rates from 3-25%. (Kenneth et al. 2008). Food contamination by organophosphate in human mostly occurs in farmers and agriculture workers (Littefield 2005).

The risk of asymmetric and terrorist use of extremely toxic Ops has been increased due to the easy access of the compounds. The compounds can be synthesized easily and are readily available Now the target of such chemical attacks by terrorist groups is not only the armed forces but the civilian population as well (Gordon et al. 2005). The terrorist attack in Japan in 1994 and 1995 by a religious group is well known which killed many civilians. In 2005, 15 victims were poisoned after accidently ingesting ethion (OPC) contaminated food in a social ceremony in Magrawa, India (Kenneth et al. 2008)

Despite of intensive endeavors by the International community, culminating in the chemical weapons convention that came into force in 1997, highly toxic organophosphorus war fare agents are stockpiled by different countries and pose a potential threat to military force (Worek et al. 2007).The organization for the prohibition of weapons has reported the existence of thousands of tons of OPs nerve agents (Greenfield et al.2002).It is also believed that allied troops has been

exposed to sarin during the gulf war in1991 (Abu-Qare and Abou-Donia 2002; Abdel-Rahman et al.2002).

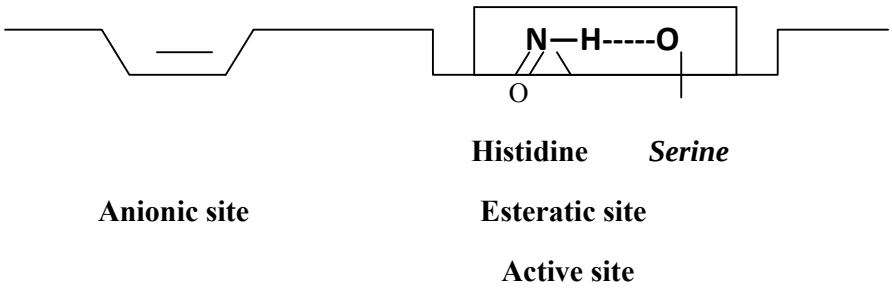
A1.6: Treatment strategies.

The standard medical treatment of organophosphorus poisoning is atropine + oxime + benzodiazepines (e.g. diazepam).Clinically atropine relieves muscarinic signs and symptoms and oxime (pralidoxime/obidoxime/HI6 etc.) is supposed to shorten the duration of the respiratory muscle paralysis by cholinesterase reactivation. Benzodiazepines are used to control OP seizures. Pretreatment with pyridostigmine along with regular therapy is recommended in case of war fare attack. Petroianu 2007 described the treatment as AFLOP that is atropine + fluid + oxygen + pralidoxime (oxime).

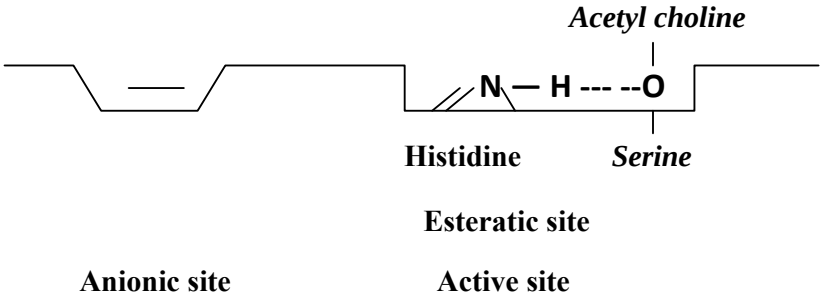
If we look at the literature, it may be concluded that standard treatments are not satisfactory in all cases with every organophosphorus poison rather considered disappointing by many scientists and clinicians researchers (Peter and Cherian 2000; Eddleston et al. 2002; Buckley et al. 2005b).

B1.1:Acetylcholinesterases (E.C.3.1.1.7)

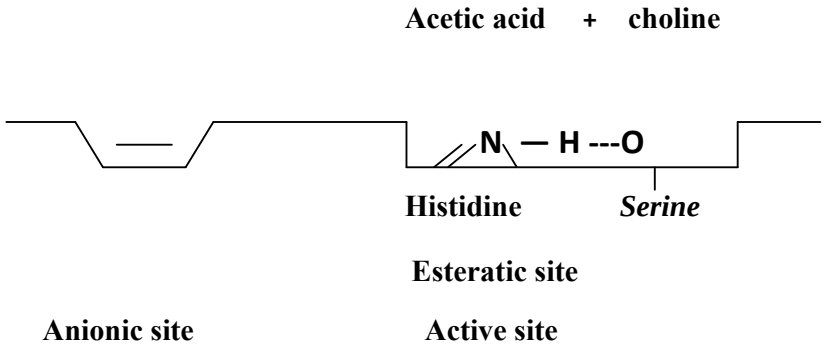
Acetylcholinesterase is a globular protein. Its physiological substrate is acetylcholine. The biological role of AChE is the termination of nerve impulse transmissions at cholinergic synapses within the nervous system by the rapid hydrolysis of the neurotransmitter Acetylcholine into choline and acetic acid (Schumacher et al. 1986). Acetylcholinesterase contains one catalytic centre which itself is composed of two compartments; the esteratic subsite and anionic subsite. The esteratic subsite contains the catalytic machinery of the enzyme with Serine, histidine and glutamic acid, a catalytic triad. The role of anionic site is to orient the charged part of the substrate that enters the active sit. The second anionic site of AChE, also called peripheral anionic site is located at the active centre gorge entry (Patocka et al. 2005).



Scheme A .Schematic diagram of acetylcholinesterase showing the active site and anionic site.

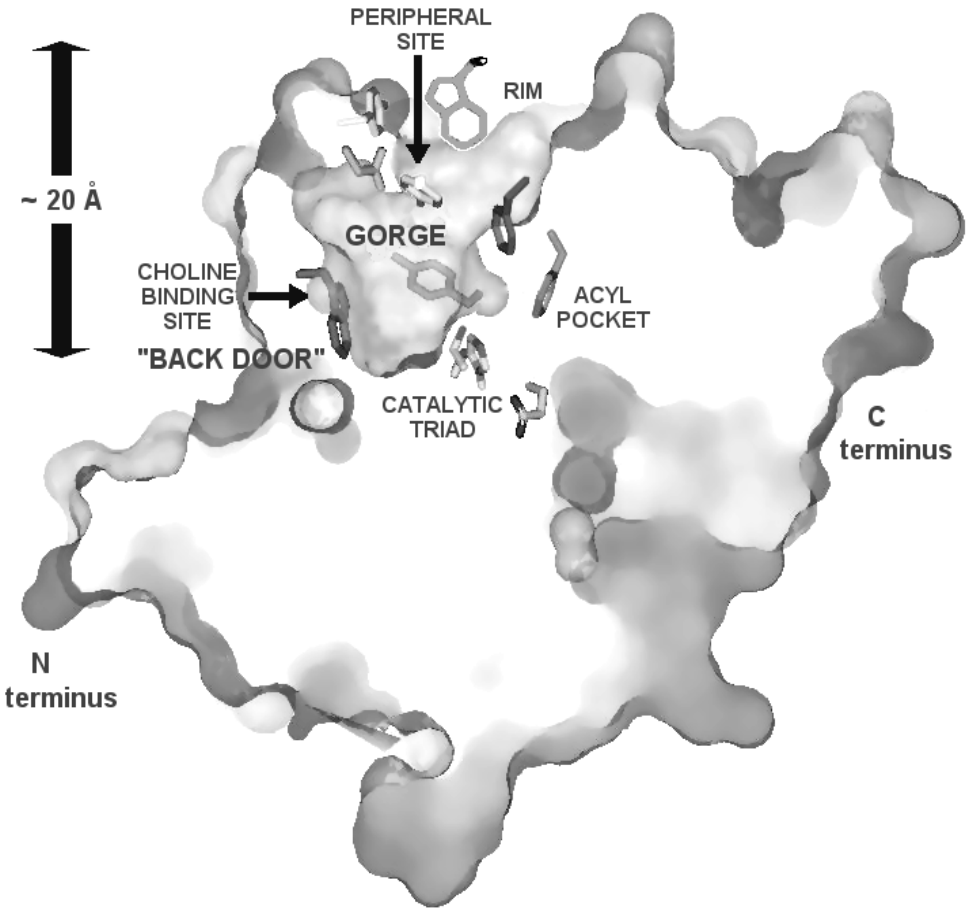


Scheme B: Schematic diagram of *acetylated* acetylcholinesterase.



Scheme C: Schematic diagram showing hydrolysis of acetylcholine into acetic acid and choline at esteratic site of acetylcholinesterase and AChE is regenerated.

According to three dimensional structures, the active centre of AChE is in the centre of the molecule, accessible through a narrow gorge lined with water molecules. The catalytic triad (serine, histidine and glutamic acid), a choline binding pocket, and an acyl binding pocket form the active site. AChE has an allosteric site close to the rim of the gorge. The allosteric site is catalytically inactive. However, reversible binding of the substrates or other ligands to that site affects catalysis in the active site.



Scheme D. Schematic drawing of the AChE molecule (prepared by Zoran Radic, UCSD, USA)

C1.1: OXIMES

A. Oximes are the nitrogen containing organic compounds derived from ketones or aldehydes by condensing them with hydroxylamine. Those derived from aldehydes is called aldoxime and those from ketones is called ketoxime. General formula of an oxime is RCH=NOH.

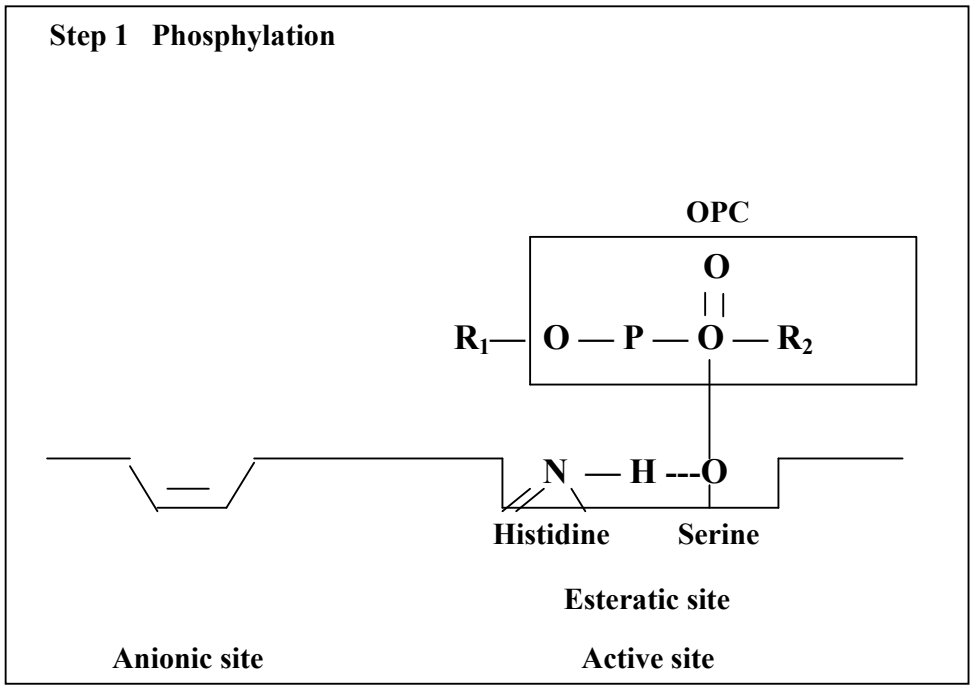
B. Organophosphorus antidote oximes are strong nucleophilic oxime type compounds, comprised oxime moiety (RCH=NOH) attached to a quaternary nitrogen pyridinium ring or imidazolium ring or quinuclidinium ring or other modified structure with basic oxime moiety to enhance the nucleophilicity. However, therapeutically available oximes for OPC antidote are only pyridinium aldoximes (2-PAM, Obidoxime, Trimedoxime, and HI6).

C1.2: Working principle.

The concept for oxime use in OP poisoning as proposed by the Wilson in 1956 for the first oxime pralidoxime was "organophosphorus poisoned acetylcholinesterase is not completely dead instead the poisoned enzyme retains the catalytic ability to transfer its blocking organophosphorus group away from its enzymes active site and on to pralidoxime (Alston 2005).

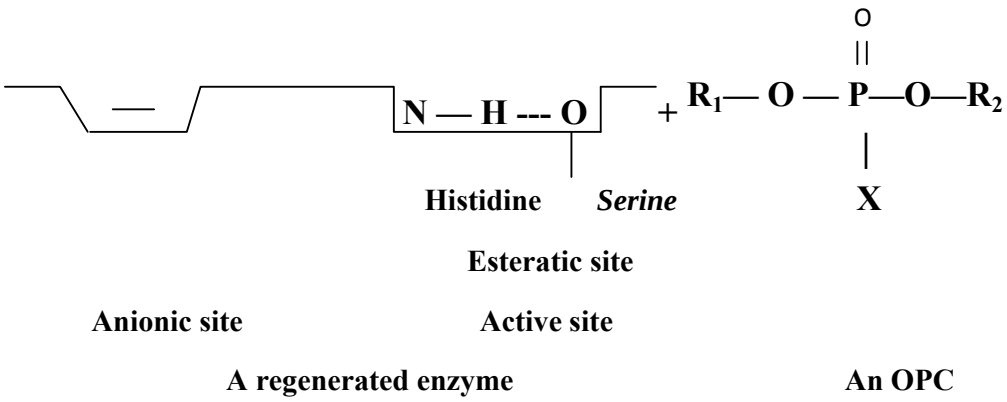
Oximes are the reversible ligands and the binding to cholinesterase causes inhibition of the enzyme activity as well (Primozic et al.2004). However, binding of oximes to the enzymes also protects cholinesterase from phosphylation by OPC and reactivation of inhibited AChE by removal of phosphyl moiety from the AChE active site serine is considered to be the primary mechanism of action for oximes (Antonijevic and Stojiljkovic 2007). AChE has two binding sites for reversible ligands, the catalytic site (acylation site) and the peripheral allosteric site. The allosteric site is catalytically inactive. Oximes bind to the cholinesterase either at the catalytic site or at the allosteric site or at both sites of the enzymes (Primozic et al.2004).When the reversible

inhibitiors binds to the active site, the protection is due to direct competition between the organophosphorus compound and reversible inhibitor. Binding of a reversible inhibitor to the allosteric site induces indirect protection of the active site Oximes reactivate phosphylated cholinesterase by displacing the phosphoryl moiety from the enzyme by virtue of their high affinity for the enzyme and their powerful nucleophilicity. As generally visualized, the oxime is oriented proximally to exert a nucleophilic attack on the phosphorus of the enzyme-inhibitor (OP) complex. Intermediate in the reactivation is a complex between the phosphylated enzyme and the reactivators. The enzyme-inhibitor (OP)-oxime complex is then split off, leaving the regenerated enzyme. However, phosphylated oxime formed during the reactivation process might be potent inhibtors of cholinesterases, which could cause re-inhibition of the previously reactivated enzyme (Antonijevic and Stojiljkovic 2007). A schematic explanation of the mechanism is as follows;

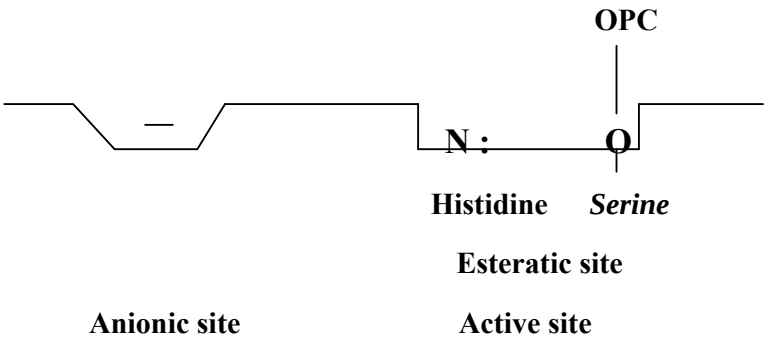


Scheme E: Phosphylation of AChE with an organophosphate at esteratic site, instead of acetyl choline which is a physiological substrate.

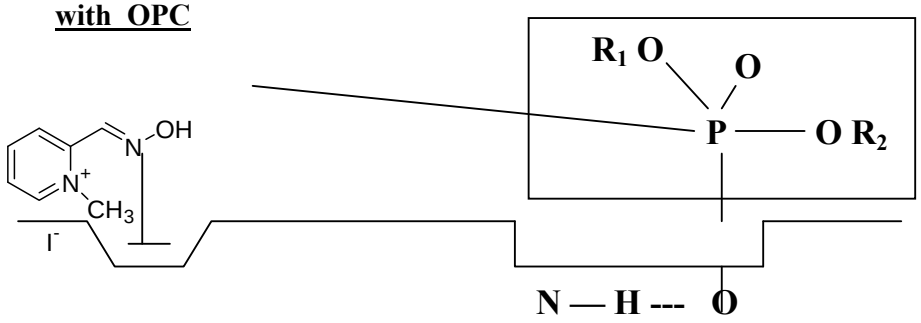
Step 2 (a) Spontaneous hydrolysis and regeneration of enzyme.



Step 2 (b) or enzyme may be permanently catalytically inactive (aged enzyme)



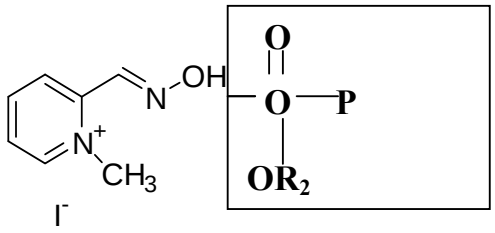
Step 2 (c) An oxime binds at anionic site along with the nucleophilic attraction with OPC



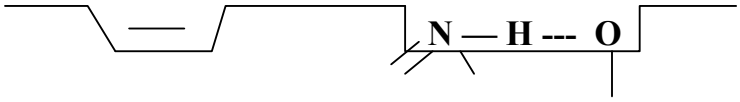
Histidine ***Serine***

Esteratic site

Step 3 Regeneration of AChE and formation of phosphyl oxime



A phosphyl oxime

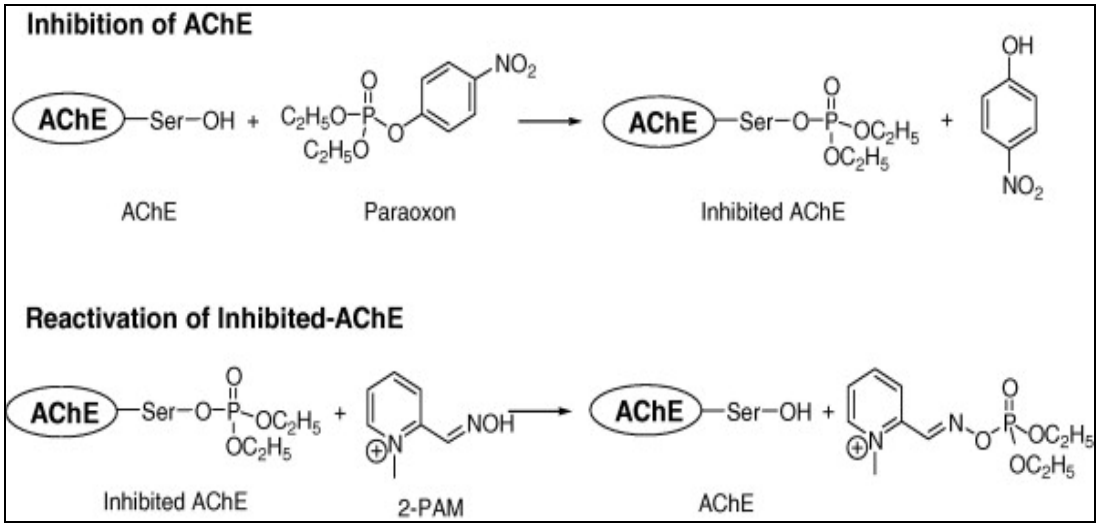


Histidine ***Serine***

Esteratic site

Anionic site **Active site**

A regenerated enzyme



Scheme F: Phosphorylation by an OPC paraoxon and dephosphorylation by an oxime Pralidoxime

C1.3: Oxime development

The search for oxime based reactivators dates back to the early 1950s, starting with hydroxylamine and hydroxamic acids (Hobbiger, 1963). Later on, ketoximes and aldoximes were investigated. The development of specific antidotes originally aimed to regenerate AChE inhibited by nerve agents has been based on the presumption that successful regeneration of inhibited enzymes could be achieved by the nucleophilic attack of a compound structurally similar to the chemical structure of ACh, means a presence of a quaternary ammonia group in a molecule of reactivator. Namely, Pralidoxime (PAM-2), the first pyridinium aldoxime could reactivate the phosphylated oxime about a million times faster than hydroxylamine **(**Bismuth et al. 1992**)**. 2-PAM is a mono pyridinium oxime. Further research resulted in the development of bis pyridinium oxime like Trimedoxime (TMB-4) in 1957, Methoxime (MMC4) in 1959, BI6, Obidoxime (LuH-6) in 1964, HI-6 in 1967 and HLo-7 in 1986. More than 1500 compounds have been investigated so far with different structural modifications and theme but only the above few have been studied for human use and are therapeutically available. But no one is universally broad spectrum oxime. Among them, Pralidome is the least efficacious, Obidoxime is

considered effective against most of the OPC pesticides and HI-6 is against OPC nerve agents but it does not work against soman. Numerous other oximes based reactivators, in part bearing imidazolium and quinuclidinium groups, have been synthesized in the laboratories in Croatia (Primozic et al. 2004), USA (Bedford et al. 1986), Israel (Amitai et al. 1995) and most recently in Czech Republic as K series oximes (Kassa et al. 2006; Musilek et al. 2006a and b).

A brief description of the therapeutically available and the oximes that remained dominant among all the oximes in experimental work is being given here;

C1.3.1: Pralidoxime

The first practically available oxime reactivator was Pralidoxime prepared almost simultaneously by Wilson and Ginsburg (1955) in USA and Childs et al. (1955**)** in UK. It was 2-hydroxyiminomethyl-1-methypyridinium iodide. It is a mono pyridinium aldoxime. By 1956, the scientific public was introduced to the use of this oxime and for the first time it was used against parathion poisoning in Japan (Namba and Hiraki 1958). There are four salt of pralidoxime namely chloride (PAM-2 Cl), methiodide, methysulphate and mesylate (P2S) which mostly differ in their stability and solubility. PAM-2 Cl is mostly used all over the world where as P2S is used in UK.PAM-2 is very efficient in reactivating of AChE inhibited with Sarin or VX (Nozaki and Aikawa 1995) but was not successful in reactivation with the tabun or soman inhibited enzyme (Koplovitz and Stewart 1994). Similarly, it is not equally effective against structurally different kinds of OPC pesticides.

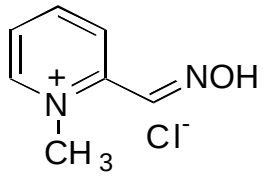


Figure 1a: **Pralidoxime**

(2-hydroxy-imino-methyl-methylpyridinium chloride)

C1.3.2: Trimedoxime (TMB-4)

TMB-4 Cl_2 was synthesized in USA in 1957 (Poziomek et al. 1958). It is the only of the major bispyridinium oximes with a propylene bridge between the two pyridinium rings. This bis pyridinium derivative was clearly superior that PAM-2 particularly in tabun poisoning.

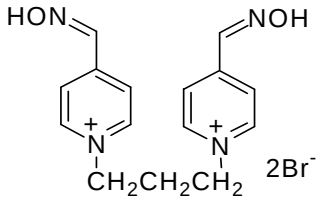


Figure 1b: Trimedoxime (TMB-4)

(1, 3-bis (4-hydroxyiminomethylpyridinium)-propane dibromide)

C1.3.3: Obidoxime (LüH-6, Toxogonin)

LüH-6 Cl_2 was named in honour of A Lüttringhaus and I Hagedorn, who synthesized it in Germany and introduced in medical practice in 1964 (Luettringhaus and Hagedorn 1964).The new oxime showed an immediately a significant potential as an antidote in poisoning with OPCs and upto now, it is one of the most active reactivators for against OP pesticides (Erdmann and Engelhard 1964; Worek et al. 1996).It is a bis pyriridinium aldoxime. Chemically it is 1, 3-Bis (4-hydroxyiminomethyl-1-pyridinio)-2-oxapropane dichloride. LüH-6 was more effective than TMB-4as an antidote against tabun and not effective against soman like the previous two.

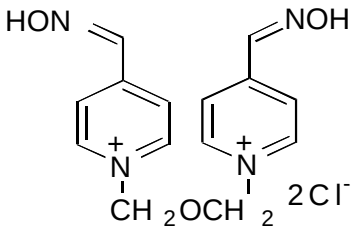


Figure 1c: **Obidoxime (LuH-6)**

1, 1-(Oxy-dimethylene) bis(4-formyl-pyridinium) dioxime dichloride

C1.3.4: HI-6

This oxime was synthesized by Ilse Hagedorn and named HI-6 after her initials (Hagedorn et al. 1969; Stojiljkovic et al. 2001).It is asymmetric bispyridinium oximes which is considered to be superior to other oximes for protection against OP nerve agents including soman however, this oxime could not reactivate tabun inhibited AChE (Clement 1982; Cetkovic et al.1984).Chemically it is [1-(2-hydoxyiminomethyl-1-pyridinio)-3-(4-carbamoyl-1-pyridinio)-2-oxapropanedichloride]. The intrinsic toxicity of HI-6 is lowest among the other oximes with LD_{50} values 781 mg/kg body weight or 2071 µmol/kg in rats (Clement 1981; Rousseaux and Dua 1989).

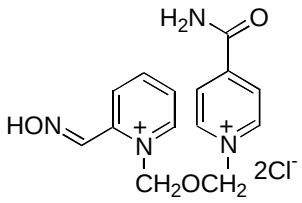


Figure 1d: **HI-6**

(1-2-hydroxyiminomethyl-1-pyridino-3-4-carbamoyl-1-pyridino-2-oxapropane dichloride)

C1.3.5: HLö-6

The next and latest important "Hagedorn oxime" was HLö-6 synthesized in Germany by Isle Hagedorn and Marianne Löffler in 1986. Chemically this oxime is 1-(4-(aminocarbonyl)pyridinio)methoxy)methyl)-2,4-bis((hydroxyimino) methyl) pyridinium diiodide. The new oxime is effective against any of four major nerve agents (Worek et al. 1994, 1995) as well as the enzyme inhibited by cyclosarin (Lundy et al. 1992).The LD_{50} of HLö-6 is 2.5 times less than HI-6. On the scale of efficacy, HLö-6 turned out to be more effective than HI-6 against tabun and VX poisoning and less effective against sarin, soman and cyclosarin (Eyer et al. 1992; Lundy et al. 1992).

C1.3.6: Other oximes

During the last two decades thousands of new oximes were synthesized in different parts of the world. Only in croatia more than 200 new oximes were synthesized and tested (Primozic et al. 2004).Similarly new oximes are being developed in USA, UK, Israel and other countries. Then Kamil Kuca group in the Department of Toxicology, Faculty of Military Health Sciences, Czech Republic are busy in synthesizing K-series of oximes with different structural molecule.

C1.4:Critical view of the use of oximes

Clinical view on the value of oximes as adjuncts in the therapy of OPCs poisoning of human remains divided. It has been argued that oximes are unnecessary when intoxication is not severe (Johnson et al. 2000). Clinical experience with oxime was reported disappointing by some clinicians and researchers (Peter and Cherian 2000; Eddleston et al. 2002; Buckley et al. 2005b). Peter et al. 2006 in a meta analysis stated that oximes give either null effect or produce possible harm.

In fact there are some limitations with oxime therapy. An oxime may be effective against specific organophosphorus anticholinesterase and ineffective for others. For instance obidoxime is good for most of the OPC used in

pesticides but not all structurally different OPCs. Hence there would be very limited basis for choosing an effective oxime for unknown OPC exposure. Secondly, AChE inhibited by OPC anticholinesterase undergoes process of ageing and the oximes do not work on aged enzyme that is permanently inactive AChE. Different OPC has different time period or half for ageing ranging from few minutes to many days. Dosing and time of treatment also plays vital role for successful oxime therapy. In short, there are many factors that influence oxime efficacy, in the earlier mentioned negative reports might not have considered all the factors for success or failure of oxime therapy. Among many factors, following are the few points which play role in successful oxime therapy, in addition to the choice of oximes.

1 Inhibitory potential of OPC and its toxikokinetics.

2 Ageing kinetics of inhibited AChE.

3 Reactivating property and potency of oximes and its pharmacokinetics.

4 Correct dosing and evaluation for the persistent need of oxime therapy.

5 Correct timing that is whether oxime may have been started too late or discontinued too early.

C1.5: Experimental K series of Oximes.

The experimental K-oximes were developed 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. The oximes were basically targeted for tabun and other OP nerve agent (Kassa et al. 2006) but tests were extended for pesticides induced AChE inhibition and found promising potential. Now the goal of the work has been extended to see the broad spectrum efficacy in 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. Structures of some of the notable K-oximes are given below.

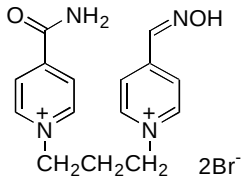


Figure 1e: (**K027)**

1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)–propane dibromide

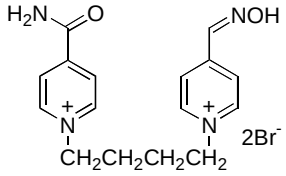


Figure 1f: **(K048)**

1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)–butane dibromide

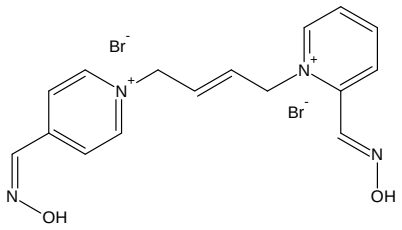


Figure 1g: **(K-053)**

(E)-1-(-hydroxyiminomethylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide

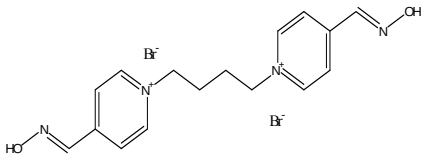


Figure 1h: **(K-074)**

1, 4-bis (4-hydroxyiminomethylpyridinium) butane dibromide

The first group of these included K-27 and K-48, two bisquaternary asymmetric pyridinium aldoximes with only one functional aldoxime group in position four of the pyridine ring. Both K-27 and K-48 have been shown to possess low toxicity (Calic et al. 2006) and to have the ability to protect AChE *in vitro* from inhibition by paraoxon, methyl paraoxon, diisopropylflourophosphate (DFP), tabun and VX (Kassa et al. 2007a & b; Petroianu et al. 2006a & b; Petroianu et al. 2007b & c; Petroianu & Kalasz 2007; Petroianu & Lorke 2008; Lorke et al. 2007; Lorke et al. 2008d). In addition, after intoxication with the same OPCs, their *in vivo* life-preserving properties are superior to those of the conventional oximes 2-PAM, obidoxime, trimedoxime, methoxime and HI-6. (Calic et al. 2006; Petroianu et al. 2006b; Petroianu et al. 2007b & c; Lorke et al. 2008a,d; Nurulain et al. 2009). This makes them promising substance for the therapy of poisoning with a wide variety of OPCs.

A second group of K-oximes consists of bispyridinium oximes with two aldoxime groups, either in position two and four (K-53) or twice in position four (K-74, K-75) of the pyridine rings (Musilek et al. 2005, 2007a & b). K-74 and K-75 were shown to be effective in reactivating AChE inhibited by tabun *in vitro* (Musilek et al. 2007b) and *in vivo* (Kassa and Humlicek 2008).

A third group of K-oximes containing a xylene linker has recently been synthesized (Hrabinova et al. 2006; Musilek et al. 2007b). These bisquaternary symmetric pyridinium aldoximes with two functional aldoxime groups at position two (K-107, K-108) or four (K-113, K-114) effectively reactivate tabun-, cyclosarin- and DFP-inhibited AChE *in vitro* (Hrabinova et al. 2006; Musilek et al. 2007b).

In addition, these bispyridinium oximes with a xylene linker are also very potent *in vitro* reactivators of human red blood cell (RBC) AChE inhibited by paraoxon, the active metabolite of the pesticide parathion (Petroianu and Kalasz 2007). If the shift of the IC_{50} is taken as a measure for reactivation potency the effect of K-107, K-108, K-113 and K-114 is about 20 times stronger than that of the oximes clinically available (2-PAM and obidoxime) and of the well-characterized new oximes (K-27 and K-48). However, oximes containing a xylene linker also have a very high intrinsic AChE inhibitory activity. When administered alone, they strongly inhibit RBC AChE themselves, which is suggestive of high toxicity. Whereas the established (pralidoxime, obidoxime, trimedoxime, methoxime) and the new oximes (K-27 and K-48) have a relatively low intrinsic AChE inhibitory activity, indicating low toxicity, the intrinsic AChE-inhibitory activity of K-107, K-108, K-113, K-114 is about 50 times higher, suggesting that they may be much more toxic *in vivo* (Petroianu and Kalasz 2007).

The present study will help in determining the candidacy of some K-oximes to replace the presently therapeutically available less effective oximes particularly against OPC belongs to pesticide groups. The correlation study between *in vitro* and *in vivo* results will emphasize the importance of in vivo tests in rodents as

alternately the oximes can not be trialed on human volunteers. The intrinsic toxicity of oximes and octanol-partition coefficient, Log*P* determination will help in describing the physico-chemical properties of tested oximes and then to predict a possible mechanism of action of the experimental oximes. The two OPC selected are structurally different. Paraoxon is used as acute toxicity model, a commonly used pesticide OPC, extremely toxic and its toxicity resembles the toxicity of deadly OPC nerve agent. DFP is an structural analog of sarin, an OPC nerve agent but this compound belongs to insecticides group of organophosphorus compound.

Chapter 2: 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 an insecticide/pesticide.

Chapter 3: Materials and Methods

3.1 Materials

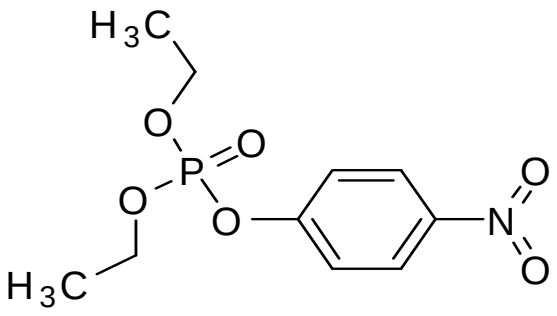
3.1.1: Experimental animals:

During the entire experiment, the Guiding principles in the care of and use of Laboratory Animals have been observed. All studies were performed with the approval of the FMHS Animal Research Ethics Committee (AE/05/56).

The original stock Wistar rats were purchased from Harlan Laboratories (Harlan Laboratories, Oxon, England). The animals used in the present studies were bred at our own Animal Facility from the original stock. Adult male rats (average weight ± SD: 248 g ± 21; 95% confidence interval: 246 g – 250 g) were housed in polypropylene cages (43 x 22.5 x 20.5 cm^3; six rats/cage) in climate- and access-controlled rooms (23 ± 1°C; 50 ± 4% humidity). The day/night cycle was 12h/12h. Food and water were available ad libitum. The food was standard maintenance diet for rats purchased from Emirates Feed Factory (Abu Dhabi)

3.1.2: Chemicals.

1.Paraoxon (ethyl) (Molecular weight 275.20): Paraoxon was purchased from Sigma-Aldrich Chemie (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), It is an ester of phosphoric acid and is a breakdown product of parathion. It is 10 times more toxic than the parent compound. Its linear formula is $O_2NC_6H_4OP(O)$ $(OC_2H_5)O_2$. Synonyms are O,O-Diethyl O-(4-nitrophenyl) Phosphate, Diethyl *p* -nitrophenyl phosphate , O, O'Diaethyl-p-nitrophenylphosphat, Phosphoric acid, diethyl 4-nitrophenyl ester etc. Paraoxon is extremely toxic compounds. Its parent compound parathion is placed in Class Ia by WHO's toxicity classification. Class Ia includes all extremely hazardous compounds. According to PAN (Pesticide Action Network) acute toxicity description, it is extremely toxic compounds. The acute toxicity of paraoxon in rats is analog to the acute toxicity in human. Structure is shown below.

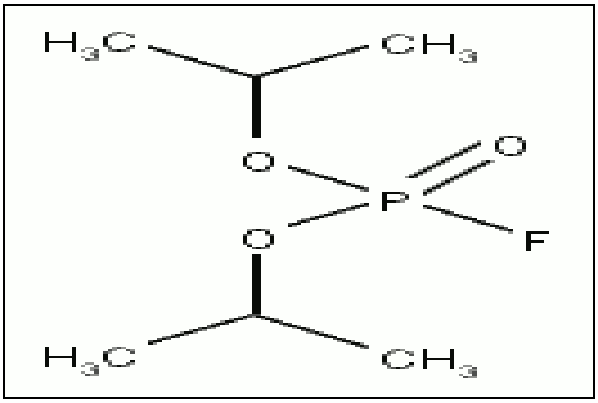


Paraoxon

2.Diisopropylflourophosphate (DFP) (Molecular weight 184.15) was purchased from Sigma-Aldrich Chemie (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Its linear formula is $[(CH_3)_2CHO$. Synonyms are DFP, DIFP, Diisopropyl phosphorofluoridate, Phosphoric acid diisopropyl ester fluoride, Difluorophate, Diflupyl, Diflurphate, Dyflos, Dyphlos, Fluropryl, Fluostigmine, isofluorophate, isofluorphate, Neoglaucit, PF-3, PF3, T-1703, TL 466, and others.

Diisopropylfluorophosphate (DFP) is structurally different from paraoxon: Whereas paraoxon contains a nitrophenyl group and two ethyl groups bound to the three oxygen atoms of the phosphate molecule, DFP is a monofluorophosphate and a structural analog of sarin. However, DFP is an organophosphate, since the fluorophosphate molecule is substituted by two isopropyl groups bound to two oxygen atoms by ester bonds. In contrast, sarin is an organophosphonate, because the fluorophosphate is substituted by one methyl group, which is directly bound to the phosphorus atom, and one isopropyl group, which is bound to an oxygen atom (Fig. 2). DFP is a neuropathic compound, capable of inducing organophosphate-induced delayed neuropathy (OPIDN) in susceptible animals (Carrington & Abou-Donia 1985; Glynn 2006; Qian et al. 2007). In military research, due to its physical and chemical similarities and comparatively low toxicity, it is used as a simulant of G-agents (GA, GB, GD, GF). Diisopropyl fluorophosphate has been used in ophthalmology as a miotic

agent in treatment of chronic glaucoma, as a miotic in veterinary medicine, and as an experimental agent in neuroscience.



Diisopropylfluorophosphate

3.Pralidoxime chloride

2-PAM, Molecular weight 172.6, is an oxime type acetylcholinesterase reactivator discovered in 1957 as an antidote for organophosphorus poisoning. It is a monopyridinium aldoxime with the functional group at position two.

The compound was purchased from Sigma-Aldrich Chemie (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Its linear formula is $C_7H_9N_2O \cdot Cl$. Chemical Name; 2-formyl-1 methylpyridinium chloride oxime (pyridine-2-aldoxime methochloride). Also referred to as 2-PAM Chloride, 2-[(hydroxyimino)methyl]-1-methylpyridinium chloride,2-Pyridinealdoxime methochloride, 1-Methyl-2-aldoximinopyridinium chloride.

Pralidoxime Chloride occurs as an odorless, white, nonhygroscopic, crystalline powder which is soluble in water to the extent of 1 g in less than 1 mL. Stable in air, it melts between 215°C and 225°C, with decomposition.

The principal action of pralidoxime is to reactivate cholinesterase (mainly outside of the central nervous system) which has been inactivated by

phosphorylation due to an organophosphate or related compound. Clinically, it is used as an adjunct to atropine in organophosphate intoxication. Structure is shown in figure 1a.

4.Obidoxime chloride

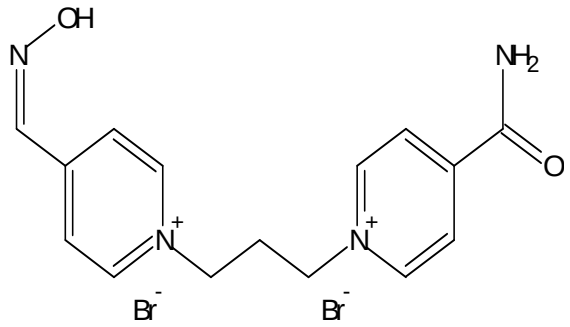
Its molecular weight is 359.21. It was purchased from Fluka Chemical AG (Buchs, Switzerland). It was discovered in 1959 as antidote for organophosphorus poisoning. Obidoxime is a bisquaternary symmetric pyridinium aldoxime with the functional groups at position four of the pyridine rings. Pralidoxime and obidoxime are oximes which are currently used clinically. Its linear formula is $C_{14}H_{16}N_4O_3C_{l2.}$ Synonyms are ,1'-(oxydimethylene)bis(pyridinium-4-carbaldoxime) dichloride, bis(4-formylpyridiniomethyl) ether dioxime, bis(isonicotinaldoxime 1-methyl) ether dichloride, efosin, LueH6, LuH6, obidoxime hydrochloride, toksobidin, toxobidin, toxogonin, toxogonine, toxogonin dichloride. The principal action is same as pralidoxime chloride. Structure is shown in figure 1e

5. K-oximes.

The eight K-oximes (K-27, K-48, K-53, K-74, K-75, K-107, K-108 and K-113) were synthesized in the Department of Toxicology at the Faculty of Military Health Sciences (University of Defence, Hradec Kralove, Czech Republic) by Kamil Kuca, Kamil Musilek and co workers. The compounds were obtained through the courtesy of Dr Kamil Kuca and Dr Kamil Musilek. A brief description of these compounds has been given in introduction. The experimental K-oximes are bisquaternary symmetric (K-74, K-75, K-107, K-108, K-113) or asymmetric (K-27, K-48, K-53) pyridinium aldoximes with the functional aldoxime group at position two (K-107, K-108), four (K-27, K-48, K-74, K-75, K-113) or both (K-53) of the pyridine rings. Whereas K-27 and K-48 only have one functional aldoxime group, the other K-oximes have two. In addition, K-107, K-108 and K-113 contain a xylene linker.

i) **K027 (Molecular weight 446.16)**.

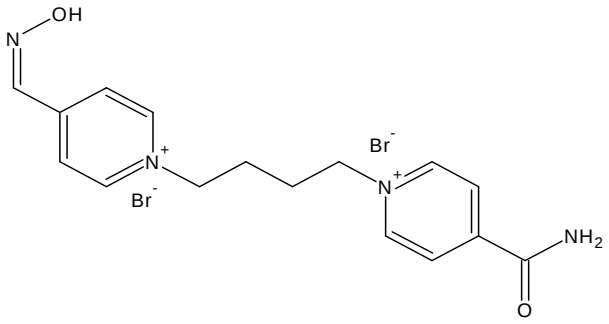
Chemical name is 1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium) propane dibromide). Compound is synthesized and reported by Kuca et al. (2003a)



K027

ii) K048 (Molecular weight 460.16)

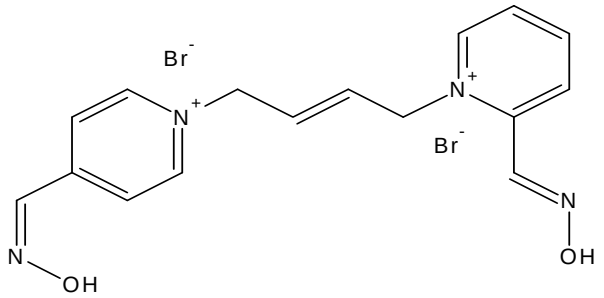
Chemical name is 1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium) butane dibromide.Compound was synthesized and reported by Kuca et al. (2003b)



K048

iii) K053 (Molecular weight 458.15)

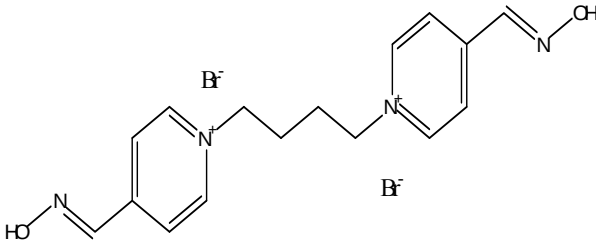
Chemical name is (E)-1-(-hydroxyiminomethylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide. The compound is synthesized and reported by Musilek et al. 2007a



K053

iv) K074 (Molecular weight 460.16)

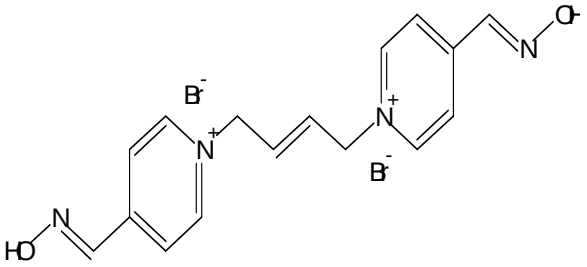
1,4-bis(4-hydroxyiminomethylpyridinium)butane dibromide . Compound is synthesized and reported by Kuca et al. (2005a).



K074

v) K075 (Molecular weight 458.15)

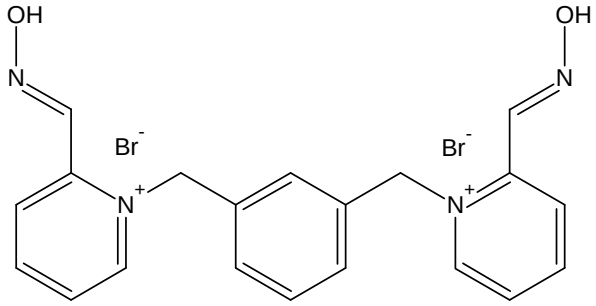
(*E*)-1,4-bis(4-hydroxyiminomethylpyridinium)but-2-en dibromide. Compound is reported by Kuca et al. (2005b).



K075

vi) K107 (Molecular weight 508.23)

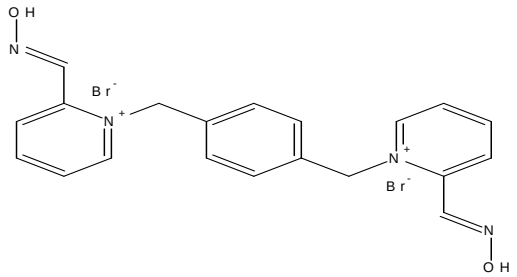
Chemical name is 2,2'-bis(hydroxyiminomethyl)-1,1'-(1,3-phenylene-dimethyl)-bispyridinium dibromide. The compound was synthesized and reported by Musilek et al. 2005.



K107

vii) K108 (Molecular weight 508.23)

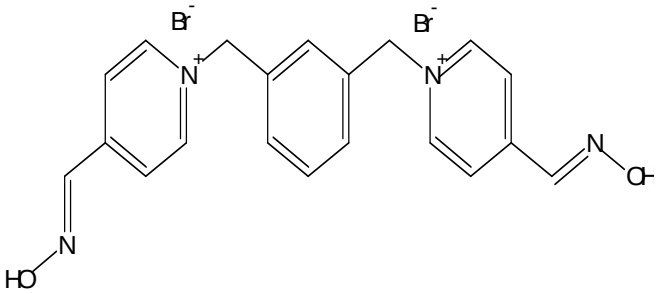
Chemical name is 2,2'-bis(hydroxyiminomethyl)-1,1'-(1,4-phenylene-dimethyl)-bispyridinium dibromide. The compound was synthesized and reported by Musilek et al. 2005.



K108

viii) K113 (Molecular weight 508.21)

Chemical name is 4,4'-bis(hydroxyiminomethyl)-1,1'-(1,2-phenylene-dimethyl)-bispyridinium dibromide. The compound was synthesized and reported by Musilek et al. 2005.



K113

3.1.3: Other materials

Other materials for the experiment include sodium chloride which was purchased from Sigma and one ml/CC sterile disposable syringes (Jung Rim Medical Industrial Co., Ltd. Korea) were obtained from m/s Al Nawras Supplies, Dubai. The water used in the experiment was obtained from Millipore, USA. Probit – Logit graph paper was obtained through the courtesy of Prof. Arshad Azmi, Karachi University.

3.1.4: Softwares;

The SPSS 15.0 (SPSS Inc. Chicago, Illinois) software package was used for all statistical evaluation. LD50 plus v1.0 software (freeware) prepared by Mario H. Vargas (2000) of Instituto Nacional de Enfermedades Respiratorias Mexico was used for LD_{50} determination (rechecking) of oximes. Correlation graphs and some other graphs were drawn with MS Excel 2003. *In silico* study that is Log*P* values calculation was done by using the PrologP module of the Pallas 3413 software (CompuDrug Inc., Sedona, AZ, USA)

3.2 Methods

3.2.1: Chemical preparations

Paraoxon and DFP stock solution (100 mmol/L) was prepared in dry acetone. Working solution for i.p. application was prepared *ex tempore* by diluting stock solution with saline shortly before application. All the oximes dose was prepared in saline before i.p. application. The dosage of oximes and OPC were according to table 1 and section 2.2.3.

3.2.2: LD_{50} determination of oximes

LD_{50} and LD_{01} 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 each oxime tested, one fixed random dose was injected i.p. to six male wistar rats. The starting doses were selected according to their in vitro intrinsic AChE inhibitory activity (Petroianu and Kalasz 2007; Lorke et al. 2008d) and other literature resources. The animals were monitored for 48 hours. Mortality was recorded at 4, 24 and 48 hours. Mortality was found to be same at all three observation time points. According to the outcome (number of dead animals), testing continued at the next higher or lower fixed dose, until an adequate assessment could be made. The method entailed testing at three to five step-wise doses. Mortality as a function of oxime dose was depicted on scientific probit-

log graph paper and LD_{01} and LD_{50} were obtained graphically. In addition, LD values were also determined using an Excel based ED50plus v1.0 software programme, developed by Mario H Vargas, Instituto Nacional de Enfermedades Respiratorias, Mexico. This essentially yielded the same result as obtained graphically.

3.2.3: Choice of oxime dosage:

Half of LD_{01} (Lorke et al., 2008d) was considered a quantity well tolerated by the experimental animals, and therefore the following oxime dosages were administered (table 1): (1) reference group, only paraoxon / DFP exposure.

(2) 2-PAM: 50 µmol = 8.63 mg (= 33.5 mg/ kg average body weight);

(3) Obidoxime: 50 µmol = 18.0 mg (= 69.6 mg/ kg average body weight);

(4) K-27: 50 µmol = 22.3 mg (= 86.5 mg/ kg average body weight).

(5) K-48: 50 µmol = 23.0 mg (= 89.2 mg/ kg average body weight);

(6) K-53: 5 µmol = 2.3 mg (= 8.9 mg/ kg average body weight);

(7) K-74: 5 µmol = 2.3 mg (= 8.9 mg/ kg average body weight);

(8) K-75: 5 µmol = 2.3 mg (= 8.9 mg/ kg average body weight);

(9) K-107: 0.3 µmol = 0.15 mg (= 0.59 mg/ kg average body weight);

(10) K-108: 0.1 µmol = 51 µg (= 0.20 mg/ kg average body weight);

(11) K-113: 1 µmol = 0.51 mg (= 2.0 mg/ kg average body weight)

Compound	Mol. wt.	Injected dose (µmol/rat)	Injected dose (mg/rat)	Injected dose (mg/kg average body weight)	LD_{50}/LD_{01} [µmol/rat] (Lork et al. 2008c)
2-PAM	172.6	50	8.36	33.7	180/117
Obidoxime	359.21	50	17.96	72.4	132/107
K-27	446.16	125	55.77	225	350/250
K-48	460.16	50	23.01	92.8	140/110
K-53	458.15	5	2.29	9.2	21/13
K-74	460.16	5	2.30	9.2	28/13
K-75	458.15	5	2.29	9.2	51/13
K-107	508.23	0.3	0.15	0.6	1.4/0.6
K-108	508.23	0.1	0.05	0.2	0.7/0.2
K-113	508.21	1	0.51	2.1	4/2.4

Table 1: Dosage of oximes administered to protect from paraoxon-induced mortality. Listed are two oximes that are clinically available, pralidoxime (2-PAM) and obidoxime, and experimental K-oximes (K-27, K-48, K-53, K-74, K-75, K-107, K-108 and K-113). Values are given in µmol/animal (column three), mg/animal (column four) and in mg/kg average body weight (column five). Column six lists the LD_{50} (first figure) and LD_{01} (second figure) values in µmol/animal. The injected dose is approximately half the LD_{01}.

3.2.4: Paraoxon exposure

In the experimental groups, animals received i.p. injections of paraoxon, either in a dosage of 1 µmol= 272 µg (1.09 mg/kg average body weight ≈ LD_{75}), 3 µmol= 816 µg (3.28 mg/kg average body weight), or 5 µmol = 1.36 mg (5.46 mg/kg average body weight). All substances were diluted in 500 µl saline solution. For each dosage, there were 11 groups of rats, the experiments were repeated four times (4 cycles; 6 rats/cycle). The first group (paraoxon) was given paraoxon i.p. alone. Groups 2-11 received a paraoxon injection and, thereafter (within one minute), i.p. injections of an oxime-type reactivator (diluted in 500 µl saline solution).

Groups for Paraoxon

Group 1(G_1): 1µmol POX (≈ LD_{75})

Group 2(G_2): lµmol POX + 50 µmol of 2-PAM

Group 3(G3): 1 µmol POX + 50 µmol of obidoxime

Group 4(G_4): 1 µmol POX + 125 µmol of K-027

Group(G_5): 1µmol POX + 50 µmol of K048

Group6(G_6): 1µmol POX + 5 µmol of K53

Group7(G_7): 1µmol POX + 5 µmol of.K74

Group8(G_8): 1µmol POX + 5 µmol of K75

Group9(G_9): 1µmol POX + 0.3 µmol of K107

Group10 (G_{10}): 1 µmol POX + 0.1µmol of K108

Group 11(G_{11}): 1 µmol POX + 1 µmol of K113

The above groups were repeated with two supra lethal doses that is 3 and 5 µmol paraoxon.

3.2.5: DFP exposure

In the experimental groups, animals received i.p. injections of DFP either in a dosage of 6 µmol= 1104.8 µg (4.40 mg/kg average body weight ≈ LD_{80}), 10 µmol= 1841.40µg (7.36 mg/kg average body weight), or 14 µmol = 2578 µmol (2.58 mg/kg average body weight). All substances were diluted in 500 µl saline solution. For each dosage, there were 11 groups of rats; the experiments were repeated four times (4 cycles; 6 rats/cycle). The first group (DFP) was given DFP i.p. alone. Groups 2-11 received a DFP injection and, thereafter (within one minute), i.p. injections of an oxime-type reactivator (diluted in 500 µl saline solution).

Groups for DFP

Group1 (G_1): 6µmol DFP (≈LD_{80})
Group2(G_2): 6µmol DFP + 50 µmol of 2-PAM
Group3(G_3): 6µmol DFP + 50 µmol of obidoxime

Group4 (G_4): 6 µmol DFP + 50 µmol of K-027

Group5 (G_5): 6µmol DFP + 50 µmol of K048
Group6(G_6): 6 µmol DFP + 5 µmol of K53

Group7 (G_7): 6 µmol DFP + 5 µmol of.K74

Group8 (G_8): 6µmol DFP + 5 µmol of K75

Group9 (G_9): 6µmol DFP + 0.3 µmol of K107
Group10(G_{10}): 6 µmol DFP + 0.1µmol of K108

Group11(G_{11}): 6 µmol DFP + 1 µmol of K113

Only K 027 was used at much lower dose (50 µmol) that is one fifth of LD_{01} because toxicity of K027 turned out to be much lower than that of the other tested substances and this dosage had been administered with promising result in several previous studies (Petroianu et al. 2006a & b, 2007 b & c Lorke et al.

2007, 2008a & d). The above groups were repeated with two supra lethal doses that is 10 and 14 µmol.

3.2.5 Data recording

The animals were monitored for 48 hours and mortality was recorded at 30 minutes, 1, 2, 3, 4, 24 and 48 hours. There were 10 control groups, consisting of 6 rats each, which received only the oxime but no paraoxon or DFP injections .Data were also noted for all these groups.

3.2.6 In Silico study (Log*P*)

The concept of partition of substances between oil and water has apparently been introduced over a century ago by Berthelot (Buchwald and Bodor 1998). The Octanol-water partition coefficient (Log*P*) has first been shown to yield correlation with biological activities by Hansch and Fujita (1964) and Leo et al. (1971). Log*P* values have been calculated 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. The Log*P* value of a substance is most relevant for neutral substances and is also useful as a general reference point to help compare overall hydrophobicity trends of compounds.

3.2.7 Correlation study

In order to determine the predictive value of in vitro testings (human blood) for *in vivo* (rat), the various *in vitro* and *in vivo* parameters (table 21) have been correlated in a pairwise manner. The non parametric Spearman rank correlation coefficient has been employed for data analysis. This approach is robust to departures from the assumption that X and Y are normally distributed and/or linearly related as well as to outlying (atypical) observations (Crawford 2006). Only correlations with a rank correlation coefficient $R \geq 0.60$ have been considered.

3.2.8 Statistical analysis:

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). Both paraoxon dose (5 and 10 µmol/rat, respectively, with 1 µmol the reference category) and group, i.e. type of reactivator (with group 1, i.e. no reactivator, as the reference category) were treated as categorical variables.

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.

The non parametric Spearman rank correlation coefficient has been employed for data analysis in correlation study. MS Excel was used to draw scatter plot.

No Bonferroni correction for multiple comparisons was applied and an $\alpha \leq 0.05$ was considered significant. The SPSS 15.0 (SPSS Inc. Chicago, Illinois) software package was used for all statistical evaluation.

Chapter 4: Results

There are four major sections of the study;

1 Intrinsic toxicity of oximes in terms of LD determination.
2 Efficacy of oximes with structurally two different organophosphorus compounds.
3 Prediction of lipophilicity/hydrophilicity for tested oximes.
4 Correlation between in vitro and in vivo data of tested oximes

4.1 LD_{50} and LD_{01} determination.

Lethal dose at which 50% and 1 % of the tested animals died (LD_{50} and LD_{01}) was determined for the 10 tested oximes by probit-log graph and software. The values are depicted in table 2. Out of all tested oximes, K027 was the least toxic, having an LD_{50} of 350 µmol/rat corresponding to 612 mg/kg average body weight. The other lesser LD_{50} values for the tested oximes were 2-PAM, 180 µmol/rat corresponding to 121 mg kg-1 average body weight; K048 140 µmol/rat corresponding to 246 mg kg-1 average body weight; Obidoxime, 132 µmol/rat corresponding to 177 mg kg-1 average body weight. The mid range toxic oximes were K075, K074 and K053. Their LD_{50} values were K075, 51 µmol/rat corresponding to 91 mg kg-1 average body weight. K074, 28 µmol/rat corresponding to 50 mg kg-1 average body weight and K53, 21 µmol/rat corresponding to 37 mg kg-1 average body weight. The most toxic oximes were K107, K108 and K113. The LD_{50} values for these oximes were 1.4.17 and 4 µmol/rat corresponding to 3, 1.4 and 8 mg kg-1 average body weight respectively. They were more than 2 orders of magnitude more toxic than K027

The LD_{01} of 2-PAM, Obidoxime, K027, K048, K053, K074, K075 ,K107, K108 and K113 were 117 µmol, 107 µmol, 250 µmol, 110 µmol, 13 µmol, 13µmol, 13 µmol, 0.6 µmol, 0.2 µmol, 2.4µmol per rat respectively. Their corresponding mg kg-1 average body weight was 80,143,437,193,23,23,1,0.4 and 5 respectively.

OXIMES	LD_{50}	LD_{01}
2-PAM	180µmol/rat (121mg/kg average bw)	117µmol/rat (80mg/kg average bw)
OBIDOXIME	132µmol/rat (177mg/kg average bw)	107µmol/rat (143 mg/kg average bw)
K-O27	350µmol/rat (612mg/kg average bw)	250µmol/rat (437mg/kg average bw)
K-048	140µmol/rat (246mg/kg average bw)	110µmol/rat (193mg/kg average bw)
K-053	21µmol/rat (37mg/kg average bw)	13µmol/rat (23 mg/kg average bw)
K-074	28µmol/rat (50 mg/kg average bw)	13µmol/rat (23 mg/kg average bw)
K-075	51µmol/rat (91 mg/kg average bw)	13µmol/rat (23 mg/kg average bw)
K-107	1.45µmol/rat (3 mg/kg average bw)	0.65µmol/rat (1 mg/kg average bw)
K-108	0.72µmol/rat (1.46mg/kg average bw)	0.2µmol/rat (0.4 mg/kg average bw)
K-113	4µmol/rat (8.1mg/kg average bw)	2.4µmol/rat (5 mg/kg average bw)

Table 2: The toxicity data for both established oximes (2-PAM and Obidoxime) and experimental K-oximes. First column shows LD_{50} values and second column LD_{01}.The first row in each box represent the dose in µmol per rat and second row reveals the dose in mg per kg average body weight (bw). The least toxicity was noted with K027 followed by established 2-PAM and Obidoime. The highest intrinsic oxime toxicity was shown by K108.

4.2 Mortality data

Table 3 and 4 corroborates the mortality data at different observation time points after intoxication with different doses of Paraoxon (table 3) and diisopropylfluorophosphate (table 4) and then treatment with half of LD_{01} of oximes. The number of experimental animals dying depended both upon the dosage and the subsequent treatment. In contrast, the mortality rate of control that had only received equitoxic doses of oximes but no paraoxon or DFP, was 0% that is all rats survived.

Table 5 show the result of mortality in mean percentage with standard deviation, 95% confidence interval and cumulative survival after the treatment with 1µmol/rat of paraoxon (≈LD_{70}). K075 showed the best protection with little difference from K027 (end point mortality is same in both treatment that is 17% ± 1) among all tested established and experimental oximes. The least end point protection was provided by K113 (50 %± 0) and K107 (46% ± 28 mortality). Protection by two established oximes was comparable to K053, K074, K108 and K048.

Table 6 show the result of mortality in mean percentage with standard deviation, 95% confidence interval and cumulative survival after the treatment with 5µmol/rat of paraoxon (≈LD_{100}).2-PAM, K108, K113 were not effective antidote (100/96 % mortality). K027 showed the best protection with little difference from K048 among all tested established and experimental oximes (50/54 % mortality).Except K107, all the remaining oximes showed mortality with progression of time and their end point mortality was higher than earlier time.

Table 7 show the result of mortality in mean percentage with standard deviation, 95% confidence interval and cumulative survival after the treatment with supra lethal i.e. 10µmol/rat of paraoxon.2-PAM and Obidoxime, the two established and clinically available oximes did not protect the animal (100% mortality). Among experimental k-oximes, K107, K108 and K113 were failed to protect animal (100% mortality). K027 showed the best protection (38% ± 16 end point

mortality), followed by K075 (63% ± 8), K048 (63% ± 16), K053 (67% ±13) and K074 (88% ± 16).In most cases, mortality rate was almost same at all time points.

Conclusively, K027 found to be the best protective oximes among all ten tested oximes at lethal and supra lethal doses of paraoxon. Moreover, the two established oximes were failed to protect the animals at supra lethal dose.

Table 8 show the result of mortality in mean percentage with standard deviation, 95% confidence interval and cumulative survival after the treatment with 6µmol/rat of DFP (≈LD_{85}). Obidoxime was found to be the best among all tested oximes at this dose with end point mortality 29%±25 followed by K027 and K048 (42±22 and 42±16 respectively). However, the high standard deviation in obidoxime result reflects that it is not superior to K027 and K048. K053 and K075 were moderately effective (end point mortality 54±8 and 58±16 respectively).In all cases, mortality increased with interval of time that is proportional to time points.

Table 9 show the result of mortality in mean percentage with standard deviation, 95% confidence interval and cumulative survival after the treatment with 10µmol/rat of DFP (≈LD_{100}). Obidoxime was found to be the best among all tested oximes at this dose with end point mortality 58%±22 followed by K027 and K048 (63±9 and 79±8 respectively). 2-PAM and K107 were ineffective (end point mortality 92 % ±16 and 100% ±0 respectively).The percent mortality among the other groups was in between 70-80%.

Table 10 show the result of mortality in mean percentage with standard deviation, 95% confidence interval and cumulative survival after the treatment with 14µmol/rat of DFP. The pattern of result was same as in earlier two doses except that 2-PAM, K107, K108 and K113 were ineffective (100% mortality) and K074 yielded 96 %± 9 mortality.

Groups (G)	30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1:POX	15/24/24	16/24/24	17/24/24	17/24/24	17/24/24	17/24/24	17/24/24
G2:POX+2-PAM	2/23/24	2/24/24	3/24/24	4/24/24	6/24/24	6/4/24	7/24/24
G3: POX+OBIDOXIME	3/13/24	5/15/24	6/16/24	7/16/24	7/16/24	8/18/24	8/18/24
G4:POX+K027	1/4/5	1/5/7	1/5/7	1/7/7	1/10/7	3/12/9	4/12/9
G5:POX+K048	2/5/10	3/5/10	4/7/11	5/8/11	5/9/14	7/13/15	7/13/15
G6:POX+K053	4/7/13	4/7/13	5/7/15	5/8/16	5/8/16	5/14/16	5/17/16
G7:POX + K074	1/6/15	1/6/16	2/6/16	3/7/17	5/8/17	8/16/19	8/17/21
G8:POX + K075	0/10/10	0/10/10	1/10/10	1/10/11	4/15/11	4/18/12	4/19/15
G9:POX + K107	4/18/24	5/18/24	6/18/24	6/18/24	10/18/24	11/18/24	11/18/24
G10:POX+ K108	3/23/24	4/23/24	7/23/24	7/23/24	7/23/24	7/23/24	7/23/24
G11:POX +K113	8/23/24	4/23/24	7/23/24	7/23/24	7/23/24	7/23/24	7/23/24

Table 3: Mortality data (out 0f 24) of paraoxon treatment alone and with oximes. The first figure shows the result of 1µmol POX treatment, second figure represents 5µmol results and third figure is the result of 10 µmol Pox treatment.The dose of oximes was half of their LD_{01}.

Groups (G)	30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1:DFP	17/23/24	20/24/24	21/24/24	21/24/24	21/24/24	21/24/24	21/24/24
G2:DFP+PAM	3/14/20	6/14/21	10/14/22	17/17/22	17/20/24	19/22/24	19/22/24
G3: DFP+OBIDOXIME	4/9/7	4/11/11	4/11/11	5/11/11	5/11/12	6/12/14	7/14/16
G4:DFP+K027	2/2/0	2/2/1	4/2/1	5/3/3	6/8/6	11/15/15	13/15/17
G5:POX+K048	1/5/9	2/12/9	5/12/10	7/14/12	7/14/12	10/19/18	10/19/19
G6:DFP+K053	2/2/4	2/4/4	4/4/5	5/7/6	6/9/8	11/18/15	13/20/18
G7:DFP + K074	1/9/14	4/10/14	9/12/14	9/14/15	9/15/16	18/18/21	20/18/23
G8:DFP+ K075	3/8/11	3/8/12	4/9/12	5/9/13	5/10/15	13/15/21	14/17/21
G9:DFP+ K107	14/19/23	21/21/24	21/21/24	22/21/24	22/23/24	22/24/24	22/24/24
G10:DFP+ K108	13/19/24	19/20/24	22/21/24	23/21/24	23/21/24	23/21/24	23/21/24
G11:DFP+K113	11/17/20	15/20/22	17/20/22	19/20/22	19/20/24	20/20/24	21/21/24

Table 4: Mortality data (out of 24) of DFP treatment alone and with oximes. The first figure shows the result of 6µmol DFP treatment, second figure represents 10µmol results and third figure is the result of 14 µmol DFP treatment.The dose of oximes was half of their LD_{01}.

Groups		30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1:	Mean%±SD	63±9	67±13	71±16	71±16	71±16	71±16	71±16
POX	95% CI	49-76	45-88	46-96	46-96	46-96	46-96	46-96
G2:	Mean%±SD	8±9	8±9	13±8	17±0	25±9	25±9	29±16
POX+2-PAM	95% CI	0-24	0-24	0-26	0-0	10-40	10-40	4-54
G3:	Mean%±SD	0±0	0±0	4±8	4±8	17±19	17±19	17±19
POX+K075	95% CI	0-0	0-0	0-18	0-18	0-47	0-47	0-47
G4:	Mean%±SD	17±0	17±0	21±8	21±8	21±8	21±8	21±8
POX+K053	95% CI	0-0	0-0	8-34	8-34	8-34	8-34	8-34
G5:	Mean%±SD	4±8	4±8	8±10	13±8	21±16	33±24	33±24
POX + K074	95% CI	0-18	0-18	0-24	0-26	0-46	0-71	0-71
G6:	Mean%±SD	33±13	42±10	46±8	50±0	50±0	50±0	50±0
POX+K113	95% CI	12-55	26-57	32-59	0-0	0-0	0-0	0-0
G7:	Mean%±SD	13±16	17±24	29±25	29±25	29±25	29±25	29±25
POX+K108	95% CI	0-38	0-54	0-69	0-69	0-69	0-69	0-69
G8:	Mean%±SD	17±13	21±16	25±9	25±9	42±22	46±28	46±28
POX+K107	95% CI	0-38	0-46	10-40	10-40	7-76	1-91	1-91
G9:	Mean%±SD	4±8	4±8	4±8	4±8	4±8	13±16	17±19
POX+K027	95% CI	0-18	0-18	0-18	0-18	0-18	0-38	0-47
G10:	Mean%±SD	8±16	12±16	17±13	21±16	21±16	29±21	29±21
POX+K048	95% CI	0-34	0-37	0-38	0-46	0-46	0-62	0-62
G11:	Mean%±SD	13±8	21±16	25±16	29±21	29±21	33±13	33±13
POX+Obidoxime	95% CI	0-26	0-46	0-51	0-62	0-62	12-55	12-55

Table 5: Mortality data in percentage with standard deviation and 95% CI after ip injection of ***1 µmol*** paraoxon and then within minute i.p. injection of oxime in respective group.

Groups		30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1:POX	Mean%±SD	100±0	100±0	100±0	100±0	100±0	100±0	100±0
	95% CI	0-0	0-0	0-0	0-0	0-0	0-0	0-0
G2:	Mean%±SD	96±8	100±0	100±0	100±0	100±0	100±0	100±0
POX+2-PAM	95% CI	82-109	0-0	0-0	0-0	0-0	0-0	0-0
G3:	Mean%±SD	42±10	42±10	42±10	42±10	63±8	75±21	79±16
POX+K075	95% CI	26-57	26-57	26-57	26-57	49-76	41-109	54-104
G4:	Mean%±SD	29±8	29±8	29±8	33±13	33±13	58±22	71±16
POX+K053	95% CI	16-42	16-42	16-42	12-55	12-55	24-93	46-96
G5:	Mean%±SD	25±9	25±9	25±9	29±16	33±13	67±0	71±8
POX + K074	95% CI	10-40	10-40	10-40	4-54	12-55	0-0	58-84
G6:	Mean%±SD	96±8	96±8	96±8	96±8	96±8	96±8	96±8
POX+K113	95% CI	82-109	82-109	82-109	82-109	82-109	82-109	82-109
G7:	Mean%±SD	96±8	96±8	96±8	96±8	96±8	96±8	96±8
POX+K108	95% CI	82-109	82-109	82-109	82-109	82-109	82-109	82-109
G8:	Mean%±SD	75±9	75±9	75±9	75±9	75±9	75±9	75±9
POX+K107	95% CI	60-90	60-90	60-90	60-90	60-90	60-90	60-90
G9:	Mean%±SD	17±13	21±16	21±16	29±8	42±10	50±14	50±14
POX+K027	95% CI	0-38	0-46	0-46	16-42	26-57	28-72	28-72
G10:	Mean%±SD	21±21	21±21	29±16	33±13	38±16	54±16	54±16
POX+K048	95% CI	0-54	0-54	4-54	12-55	12-63	28-80	28-80
G11:	Mean%±SD	54±8	63±16	67±19	67±19	67±19	75±21	75±21
POX+Obidoxime	95% CI	41-68	37-88	36-97	36-97	36-97	41-109	41-109

Table 6: Mortality data in percentage with standard deviation and 95% CI after ip injection of ***5 µmol*** paraoxon and then within minute i.p. injection of oxime in respective groups.

Groups		30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1: POX	Mean%±SD 95% CI	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0
G2: POX+2-PAM	Mean%±SD 95% CI	96±8 82-109	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0
G3: POX+K075	Mean%±SD 95% CI	42±21 7-76	42±21 7-76	42±21 7-76	46±16 20-72	46±16 20-72	50±14 28-72	63±9 49-76
G4: POX+K053	Mean%±SD 95% CI	54±21 21-87	54±21 21-87	63±21 29-96	67±13 45-88	67±13 45-88	67±13 45-88	67±13 45-88
G5: POX + K074	Mean%±SD 95% CI	63±16 37-87	67±13 45-88	67±13 45-88	71±16 46-96	71±16 46-96	79±21 46-112	88±16 62-112
G6: POX+K113	Mean%±SD 95% CI	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0
G7: POX+K108	Mean%±SD 95% CI	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0
G8: POX+K107	Mean%±SD 95% CI	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0
G9: POX+K027	Mean%±SD 95% CI	21±16 0-46	29±8 16-42	29±8 16-42	29±8 16-42	29±8 16-42	38±16 12-63	38±16 12-63
G10: POX+K048	Mean%±SD 95% CI	42±21 7-76	42±21 7-76	46±16 20-72	46±16 20-72	58±22 24-93	63±16 37-88	63±16 37-88
G11: POX+Obidoxim	Mean%±SD 95% CI	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0	100±0 0-0

Table 7: Mortality data in percentage with standard deviation and 95% CI after ip injection of ***10 µmol paraoxon*** and then within minute i.p. injection of oxime in respective group.

Groups		30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1:DFP	Mean%±SD	71±8	83±0	88±9	88±9	88±9	88±9	88±9
	95% CI	58-84	0-0	74-101	74-101	74-101	74-101	74-101
G2: DFP+PAM	Mean%±SD	13±16	25±17	42±10	71±16	71±16	79±8	79±8
	95% CI	0-38	0-51	26-57	46-96	46-96	66-92	66-92
G3: DFP+OBIX	Mean%±SD	17±19	17±19	17±19	21±25	21±25	25±21	29±25
	95% CI	0-47	0-47	0-47	0-60	0-60	0-59	0-69
G4: DFP+K075	Mean%±SD	13±16	13±16	17±13	21±16	21±16	54±8	58±16
	95% CI	0-37	0-37	0-38	0-46	0-46	41-68	32-85
G5: DFP + K074	Mean%±SD	4±9	17±13	38±16	38±16	38±16	75±21	83±24
	95% CI	0-18	0-38	12-63	12-63	12-63	41-109	46-121
G6: DFP+K113	Mean%±SD	46±16	63±16	71±16	79±8	79±8	83±13	88±16
	95% CI	20-72	37-88	46-96	66-91	66-91	62-105	62-113
G7: POX+K108	Mean%±SD	54±16	79±21	92±10	96±9	96±9	96±9	96±9
	95% CI	28-80	46-112	76-107	76-107	76-107	76-107	76-107
G8: DFP+K053	Mean%±SD	9±10	9±10	17±13	21±8	25±9	46±8	54±8
	95% CI	0-24	0-24	0-38	8-34	10-40	32-59	41-68
G9: DFP+K107	Mean%±SD	58±10	88±16	88±16	92±16	92±16	92±16	92±16
	95% CI	43-74	62-113	62-113	65-118	65-118	65-118	65-118
G10: DFP+K48	Mean%±SD	4±8	9±10	21±16	29±21	29±21	42±16	42±16
	95% CI	0-18	0-24	0-46	0-62	0-62	15-68	15-68
G11: DFP+K027	Mean%±SD	0±0	0±0	0±0	0±0	9±10	38±16	42±22
	95% CI	-	-	-	-	0-24	12-63	7-76

Table 8 Mortality data in percentage with standard deviation and 95% CI after ip injection of ***6 µmol DFP*** and then within minute i.p. injection of oxime in respective group.

Groups		30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1: DFP	Mean%±SD 95% CI	96±9 82-109	100±0 ---	100±0 ---	100±0 ---	100±0 ---	100±0 ---	100±0 ---
G2: DFP+PAM	Mean%±SD 95% CI	58±10 43 - 74	58±10 43 - 74	58±10 43 - 74	71±8 58 - 84	83±13 62- 105	92±16 65- 18	92±16 65- 118
G3: DFP+OBIX	Mean%±SD 95% CI	38±16 12 - 63	46±16 20 - 72	46±16 20- 2	46±16 20-72	46±16 20 - 72	50±14 28- 72	58±22 24 - 93
G4: DFP+K075	Mean%±SD 95% CI	33±24 0 - 71	33±24 0 - 71	38±21 4 - 71	38±21 4 - 71	42±17 14 - 69	63±9 49- 76	71±16 46 - 96
G5: DFP+ K074	Mean%±SD 95% CI	38±21 4 - 71	42±17 14 - 69	50±14 28 - 72	58±22 24 - 93	63±16 37 - 88	75±9 60- 90	75±9 60 - 90
G6: DFP+K113	Mean%±SD 95% CI	71±25 31-110	83±23 46 - 121	83±23 46- 121	83±23 46-121	83±23 46- 121	83±23 46-21	88±16 62-113
G7: POX+K108	Mean%±SD 95% CI	79±21 46-112	83±24 46-121	88±16 62-113	88±16 62-113	88±16 62-113	88±16 62-13	88±16 62-113
G8: DFP+K053	Mean%±SD 95% CI	9±10 0-24	17±13 0-38	17±13 0-38	29±8 16-42	38±16 12-63	75±9 60-90	83±13 62-105
G9: DFP+K107	Mean%±SD 95% CI	79±8 66-92	88±9 74-101	88±9 74-101	88±9 74-101	96±9 82-109	100±0 ---	100±0 ---
G10: DFP+K48	Mean%±SD 95% CI	21±15 0-46	50±0 0-0	50±0 0-0	58±16 32-85	63±16 37-88	79±8 66-92	79±8 66-92
G11: DFP+K027	Mean%±SD 95% CI	8±16 0-35	8±16 0-35	8±16 0-35	13±16 0-38	34±24 0-71	63±9 49-76	63±9 49-76

Table 9: Mortality data in percentage with standard deviation and 95% CI after ip injection of ***10 µmol DFP*** and then within minute i.p. injection of oxime in respective group

Groups		30min	1 hr	2 hrs	3 hrs	4 hrs	24hrs	48hrs
G1: DFP	Mean%±SD 95% CI	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -
G2: DFP+PAM	Mean%±SD 95% CI	83±13 62-105	87±9 74-101	92±10 76-107	92±10 76-107	100±0 -	100±0 -	100±0 -
G3: DFP+OBIX	Mean%±SD 95% CI	29±16 4-54	29±16 4-54	46±8 32-59	46±8 32-59	50±14 28-72	58±21 24-92	67±19 36-97
G4: DFP+K075	Mean%±SD 95% CI	46±28 1-91	50±27 7-93	50±27 7-93	54±28 9-99	63±21 29-96	88±16 62-113	88±16 62-113
G5: DFP+ K074	Mean%±SD 95% CI	58±29 12-104	58±29 12-104	58±29 12-104	63±25 23-102	67±16 29-104	87±9 74-101	96±9 82-109
G6: DFP+K113	Mean%±SD 95% CI	84±19 53-114	92±17 65-118	92±17 65-118	92±17 65-118	100±0 -	100±0 -	100±0 -
G7: POX+K108	Mean%±SD 95% CI	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -
G8: DFP+K053	Mean%±SD 95% CI	17±13 0.38	17±13 0-38	21±16 0-46	25±17 0-51	33±13 12-55	63±25 23-102	75±21 41-109
G9: DFP+K107	Mean%±SD 95% CI	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -	100±0 -
G10: DFP+K027	Mean%±SD 95% CI	0±0 --	4±9 0-18	4±9 0-18	13±16 0-38	25±21 0-59	63±16 37-88	71±25 31-110
G11: DFP+K48	Mean%±SD 95% CI	38±16 12-63	38±16 12-63	42±22 7-76	50±20 19-81	50±20 19-81	75±17 48-101	79±21 46-112

Table 10: Mortality data in percentage with standard deviation and 95% CI after ip injection of ***14µmol DFP*** and then within minute i.p. injection of oxime in respective group.

4.3.1 Survival analysis

Survival analysis was performed by determining cumulative survival time in minutes, Kaplan Meir survival analysis and relative risk of death (RR) by Cox regression. The later two methods were applied by statistical software SPSS 15.0. The different methods of analysis were used to get a robust conclusion for the efficacy of oximes. Table 11 and 12 and figure 2 and 3 represents the survival analysis of the data.

4.3.1.1 Cumulative survival

Table 11 shows the cumulative survival time in minutes for seven time point observation with three different doses of paraoxon. The second column shows the cumulative survival time for 1µmol paraoxon dose, second column 5 and third column 10 µmol. According to the table K107, K108 and K113 are not effective in comparison to all tested oximes. The two standard oximes were effective only at lower challenged doses of Paraoxon and completely ineffective at higher dose. The five experimental K-oximes (K027, K048, K053, K074 and K075) were found to be effective at all challenged doses. Among all tested oximes, K027 was superior to all at all challenged doses.

Table 12 shows the cumulative survival time in minutes for seven time point observation with three different doses of DFP. The second column shows the cumulative survival time for 6µmol, second column 10 µmol and third column 14 µmol DFP dose. According to the table K107, K108 and K113 are not effective in comparison to all tested oximes. The two standard oximes were effective only at lower challenged doses of Paraoxon and completely ineffective at higher dose. The five experimental K-oximes (K027, K048, K053, K074 and K075) were found to be effective at all challenged doses. Among all tested oximes, K027 was superior to all at all challenged doses.

Compound	1 µmol/rat	5 µmol/rat	10 µmol/rat
Paraoxon	5063±2734 712-9413	0±0 ---	0±0 ---
2-PAM	12,735±2110 9377-16093	8±15 0-31	0±0 ---
Obidoxime	11,640±2378 7856-15424	4410±3727 0-10,341	0±0 ---
K-27	14,880±2833 10,372-19,388	8963±2107 5609-12,316	10,935±2648 6721-15149
K-48	13,013±3084 8105-17,920	8265±2653 4044-12,486	6690±2609 2538-10842
K-53	13,695±1410 11,451-15,939	6510±2597 2378-10,642	5820±2401 2000-9640
K-74	11,835±3820 5757-17,913	5955±667 4894-7016	3038±2940 1640-7715
K-75	14,550±3153 9533-19,567	4365±2812 0-8840	7650±1481 5293-10,006
K-107	9623±4629 2156-17,088	4320±1663 1674-6966	0±0 ---
K-108	12,293±4289 5468-19,116	720±1440 0-3011	0±0 ---
K-113	8700±65 8597-8803	720±1440 0-3011	0±0 ---

Table 11: Cumulative survival time in minutes with seven time points and three progressive doses i.e. 1, 5 and 10 µmol/rat of paraoxon. The first row in each box shows the cumulative survival in minutes and second row shows the 95% confidence interval of the data. The message from this table is same as yielded in earlier mortality tables with individual time data that is K027 is the best protective oxime and standard established oximes failed to protect at higher dose.

Compound	6 µmol/rat	10 µmol/rat	14 µmol/rat
DFP	2160±1440 0-4451	8±15 0-31	0±0 ---
2-PAM	4028±1417 1773-6282	1785±2732 0-6132	112±9 0-270
Obidoxime	12,690±3963 6383-18,997	7995±2967 3274-12,716	6705±3222 1578-1132
K-27	10,950±2936 6278-15,622	7155±1438 4867-9443	6503±3466 987-12018
K-48	10,375±2844 5850-14,900	3998±1320 1897-4364	4022±1323 1921-6133
K-53	9045±1177 7172-10,918	4350±1358 2189-6511	5955±3482 414-11,496
K-74	4238±3498 0-9804	4943±1601 2094-7190	2175±1609 0-4735
K-75	8100±2049 4839-11,361	6120±2084 2804-9436	2648±2727 0-6987
K-107	1523±2825 0-6018	165±93 17-313	0±0 ---
K-108	840±1461 0-3165	2183±2776 0-6599	0±0 ---
K-113	2700±2375 0-6479	2543±3397 0-7949	105±172 0-379

Table 12: Cumulative survival time in minutes with seven time points and three progressive doses i.e. 6, 10 and 14 µmol/rat of DFP. The first row in each box shows the cumulative survival in minutes and second row shows the 95% confidence interval of the data.

4.3.1.2 Kaplan Meir survival function

Figure 2 and figure 3 shows the Kaplan Meir survival plot for the survival data of Paraoxon and DFP challenged rats with subsequent oximes treatment respectively. Table 13 and 14 depicts the Kaplan Meir survival analysis, showing means of survival times for the entire treatment regimen along with standard error and 95% confidence interval.

Group	**Estimate**	**Std. Error**	**95% Confidence Interval**
G1:POX	309	99	114-504
G2: POX+2-PAM	752	146	465-1038
G3:POX+OBIDOXIME	966	153	666-1266
G4:POX+K027	2061	145	1777-2346
G5:POX+K048	1652	155	1348-1957
G6:POX+K053	1620	160	1305-1935
G7:POX + K074	1449	152	1152-1746
G8:POX + K075	1639	161	1324-1954
G9:POX + K107	815	147	527-1103
G10:POX + K108	747	145	462-1031
G11:POX +K113	549	129	296-802

Table 13: Kaplan-Meir survival test showing means for survival time (minutes) after challenging the rats with three different doses of POX separately and then treatment with oximes. Group with the highest survival time is highlighted with green colour (K-027) and the least with yellow colour.

Group	**Estimate**	**Std. Error**	**95% Confidence Interval**
G1:DFP	152	67	20-283
G2: DFP+2-PAM	429	102	229-629
G3:DFP+OBIDOXIME	1703	161	1389-2019
G4:DFP+K027	1823	128	1572-2073
G5:DFP+K048	1298	149	1005-1591
G6:DFP+K053	1579	138	1309
G7:DFP + K074	979	134	717-1241
G8:DFP + K075	1328	143	1049-1608
G9:DFP + K107	140	58	2-254
G10:DFP + K108	198	77	48-349
G11:DFP +K113	383	107	173-591

Table14: Kaplan-Meir survival test showing means for survival time (minutes) after challenging the rats with three different doses of DFP separately and then treatment with oximes. Group with the highest survival time is highlighted with green colour (K-027) and the least with yellow colour.

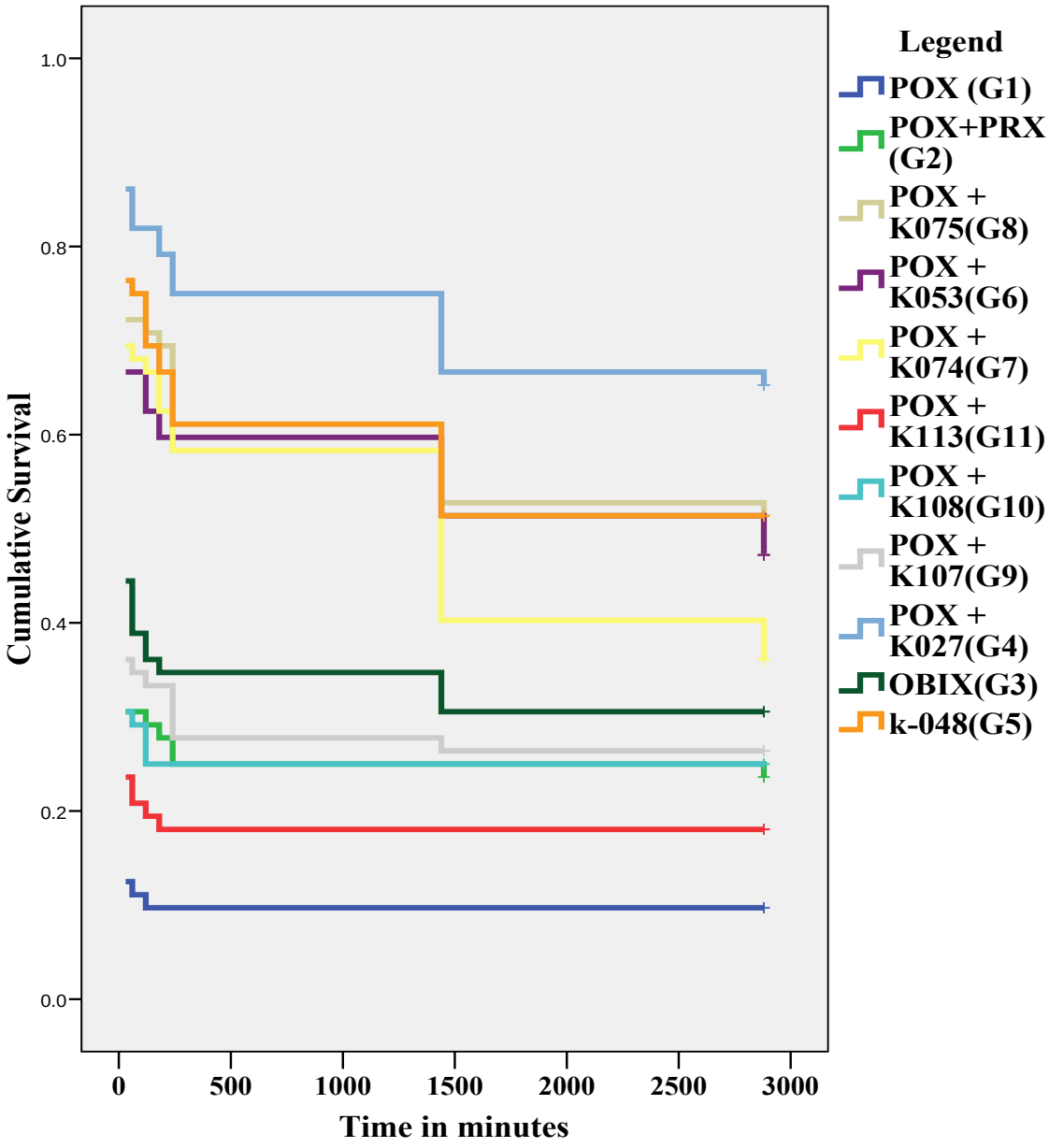


Figure 2: Kaplan-Meier survival function plot for paraoxon induced mortality.

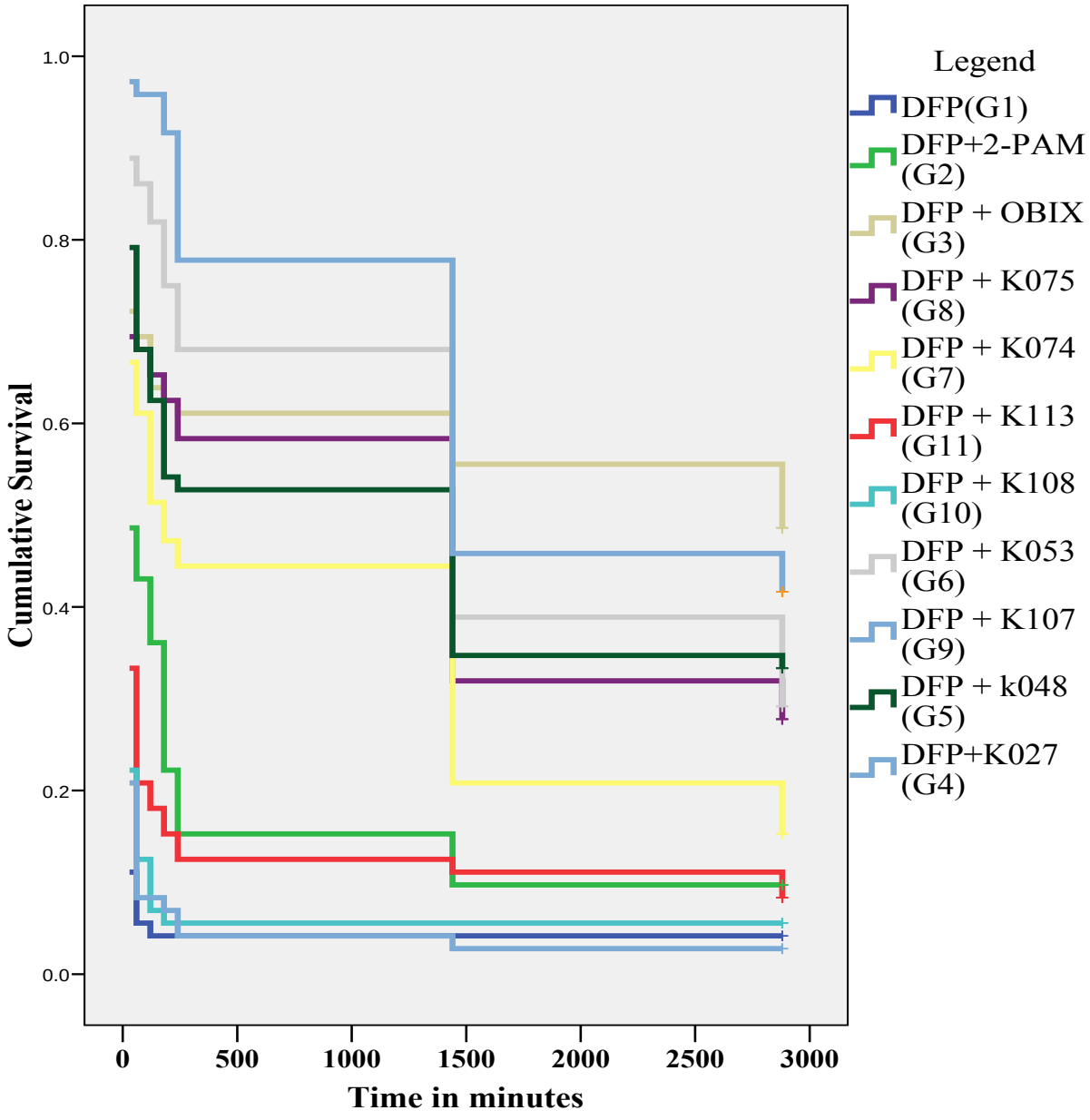


Figure 3: Kaplan-Meier survival function plot for paraoxon induced mortality

4.3.1.3 Relative risk of death (Cox regression)

4.3.1.3(a) 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. 4. It was compared with untreated animals (group 1, RR = 1) and adjusted for paraoxon dose (high/low). Statistical comparison (table 15 & 17) between the different oxime treatments was performed on the cumulative relative risk, i.e. the area under the RR time curve (Fig. 4).

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). Pair-wise comparison between the different oxime treatments, performed on the cumulative RR, i.e. the area under the RR time curve, showed significant differences between the various substances tested (table 15&17). 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 (30-45%) was also achieved by K-48 and the three new bispyridinium oximes, which contain two aldoxime groups, but no xylene linker: 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

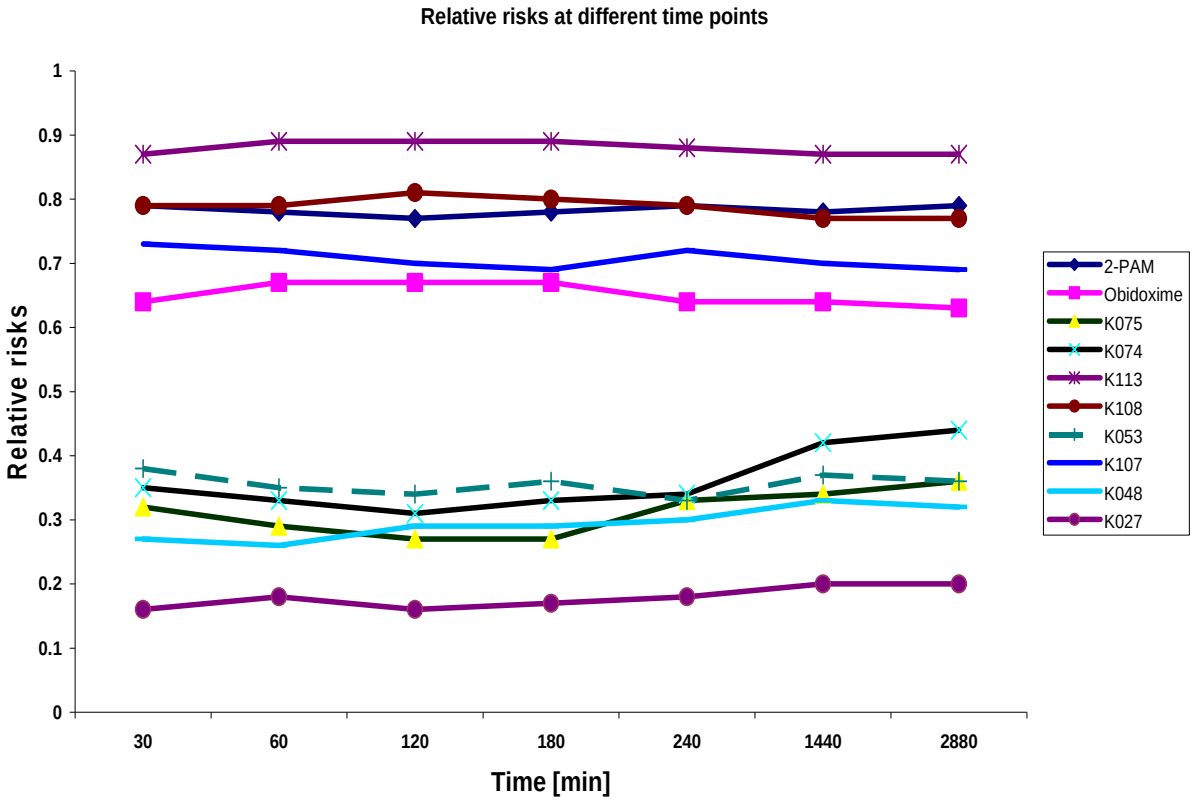


Figure 4: Relative risk (RR) of death estimated by Cox (1972) analysis in animals exposed to intraperitoneal (i.p.) injection of **paraoxon** and adjusted for paraoxon dose (high/low) for each of the time points examined (30 min, 1, 2, 3, 4, 24, 48 h). Two conventional oximes, pralidoxime (2-PAM) and obidoxime, are compared with eight experimental K-oximes (K-027, K-048, K-053, K-074, K-075, K-107, K-108 and K-113) and with no treatment (group 1, RR = 1). The injected oxime dose was approximately half the LD_{01}. Best protection is conferred by K-027. K-048, K-053, K-074 and K-075 reduce mortality to about 30-50%. Obidoxime, K-107, K-108, 2-PAM and K-113 are poorly effective (RR ≥ 70%).

Group	treatment	RR (±SD)	95% CI	p-value
1	Paraoxon	1	reference	reference
2	Paraoxon + 2-PAM	0.78±0.06	0.69-0.87	P≤0.01
3	Paraoxon + Obidoxime	0.64±0.14	0.42-0.85	P≤0.01 [a]
4	Paraoxon + K-027	0.20±0.06	0.11-0.29	P≤0.01 [b, a, c]
5	Paraoxon + K-048	0.32±0.08	0.19-0.45	P≤0.01 [b, a,]
6	Paraoxon + K-053	0.36±0.07	0.26-0.47	P≤0.01 [b, a,]
7	Paraoxon + K-074	0.42±0.07	0.31-0.53	P≤0.01 [b, a]
8	Paraoxon + K-075	0.35±0.14	0.12-0.57	P≤0.01 [b, a]
9	Paraoxon + K-107	0.70±0.08	0.58-0.81	P≤0.01
10	Paraoxon + K-108	0.77±0.09	0.63-0.91	P≤0.01
11	Paraoxon + K-113	0.87±0.11	0.69-1.04	P≤0.01

a. $p \leq 0.05$ compared to K-113

b. $p \leq 0.05$ compared to 2-PAM, Obidoxime, K-107 & K-108.

c $p \leq 0.05$ compared to K-048, K-053, & K-074

Table 15: Cox analysis of the cumulative relative risk (RR) of death, including 95% confidence interval (CI), of animal exposed to paraoxon and adjusted for paraoxon dose (high/low). The cumulative RR was assessed by determining the area under the RR-time curve (figure 5) for two established oximes and new experimental K-oximes.

4.3.1.3(b) DFP 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 figure 5. It was compared with untreated animals (group 1, RR = 1) and adjusted for paraoxon dose (high/low). Statistical comparison (table 16, 18) between the different oxime treatments was performed on the cumulative relative risk, i.e. the area under the RR time curve.

Pair-wise comparison between the different oxime treatments, performed on the cumulative relative risk, i.e. the area under the RR time curve, showed significant differences between the various compounds tested (Table 16 &18). Based on these data, oximes could be divided into the following groups;

Best protection was obtained by K027 (R=0.19), which was significantly more effective than all the other tested oximes, except obidoxime, K053, and K-075 (Table 16 &18).

Good protection was achieved with obidoxime (RR=0.19), K-053 (RR0.22) and K-075 (RR=0.29). The differences between these three oximes were not significant and they were significantly superior to all other oximes except K-048 and K-027.

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. However, the difference from Obidoxime, K-53 and K-74 was not significant but less efficacious than K-027.

The two new K-oximes K-107 and K108 did not significantly reduce DFP induced mortality (Table 16).

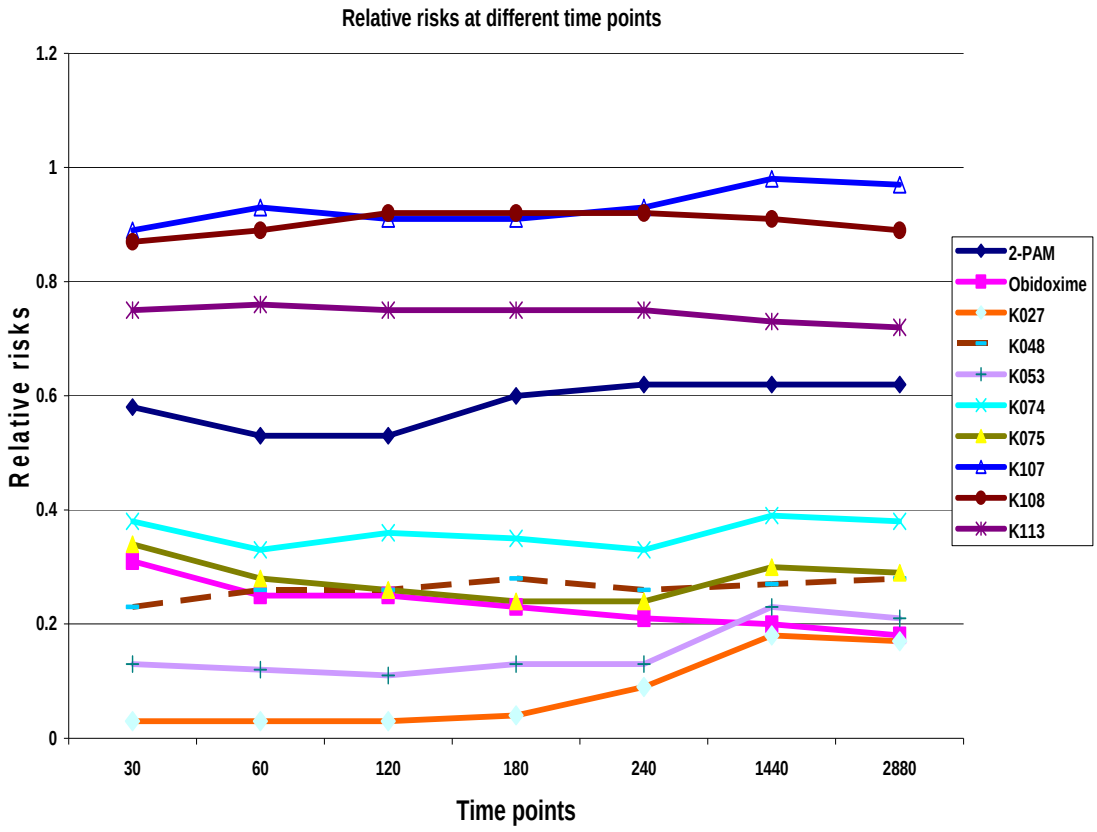


Figure 5 Relative risk (RR) of death estimated by Cox (1972) analysis in animals exposed to intraperitoneal (i.p.) injection of the organophosphate diisopropylfluorophosphate **(DFP)** for each of the time points examined (30 min, 1, 2, 3, 4, 24, 48 h). Two established oximes, pralidoxime (2-PAM) and Obidoxime, are compared with eight experimental K-oximes (K-027, K-048, K-053, K-074, K-075, K-107, K-108 and K-113) and with no treatment (group 1, RR = 1). The injected oxime dose was approximately half the LD_{01}. Best protection is conferred by **K-027**.Obidoxime, K-048, K-053, K-074 and K-075 confer moderate to good protection. 2-PAM and K-113 are poorly effective, and no significant effect has been observed for K107 and K108.

Groups	Relative Risk (RR ±SD)	95% CI	p-value (Mann Whitney test)
DFP	1	1 --- 1	---
DFP + 2-PAM	0.62±0.09	0.48-0.76	P≤0.05
DFP + Obidoxime	0.19±0.07	0.08-0.30	P≤0.05
DFP + K027	0.16±0.06	0.07-0.25	P≤0.05
DFP + K048	0.28±0.06	0.18-0.37	P≤0.05
DFP + K053	0.22±0.03	0.16-0.27	P≤0.05
DFP + K074	0.38±0.07	0.27-0.49	P≤0.05
DFP + K075	0.29±0.10	0.14-0.44	P≤0.05
DFP + K107	0.97±0.19	0.67-1.26	**N.S**
DFP + K108	0.91±0.18	0.62-1.19	**N.S.**
DFP + K113	0.73±0.11	0.55-0.90	P≤0.05

a. $p \leq 0.05$ compared to 2-PAM and K113

b. $p \leq 0.05$ compared to K027

c. $p \leq 0.05$ compared to Obidoxime

d $p \leq 0.05$ compared to K053

Table 16: Cox analysis of the cumulative relative risk (RR) of death, including 95% confidence interval (CI), of animal exposed to DFP and adjusted for DFP dose (high/low). The cumulative RR was assessed by determining the area under the RR-time curve (figure 5) for two established oximes and new experimental K-oximes.

	2-PAM	OBIDOXIME	K027	K048	K053	K074	K075	K107	K108	K113
2-PAM	-	0.146	0.020	0.020	0.020	0.020	0.020	0.081	0.886	0.146
OBIDOXIME	0.146	-	0.021	0.021	0.021	0.043	0.021	0.386	0.083	0.043
K027	0020	0.021	-	0.043	0.029	0.021	0.110	0.021	0.021	0.021
K048	0.020	0.021	0.043	-	0.564	0.149	0.885	0.021	0.021	0.021
K053	0.020	0.021	0.029	0.564	-	0.468	1.000	0.021	0.021	0.021
K074	0.020	0.043	0.021	0.149	0.468	-	0.564	0.021	0.021	0.021
K075	0.020	0.021	0.110	0.885	1.000	0.564	-	0.021	0.021	0.021
K107	0.081	0.386	0.021	0.021	0.021	0.021	0.021	-	0.248	0.059
K108	0.084	0.083	0.021	0.021	0.021	0.021	0.021	0.248	-	0.191
K113	0.146	0.043	0.021	0.021	0.021	0.021	0.021	0.059	0.191	-

Table 17: P-values for the comparison among all tested oximes after challenging the rats with ***paraoxon***. Mann Whitney test was used for these p-values and $p \leq .0.05$ was set for statistical significance.

	2-PAM	OBIDOXIME	K027	K048	K053	K074	K075	K107	K108	K113
2-PAM	-	0.021	0.021	0.021	0.021	0.021	0.021	0.043	0.021	0.248
OBIDOXIME	0.021	-	0.554	0.146	0.773	0.020	0.149	0.021	0.021	0.021
K027	0.021	0.554	-	0.041	0.146	0.020	0.058	0.021	0.020	0.020
K048	0.021	0.146	0.041	-	0.146	0.081	0.772	0.020	0.020	0.020
K053	0.021	0.773	0.146	0.146	-	0.021	0.248	0.021	0.021	0.021
K074	0.021	0.020	0.020	0.081	0.021	-	0.083	0.021	0.021	0.021
K075	0.021	0.149	0.05	0.772	0.248	0.083	-	0.021	0.021	0.021
K107	0.043	0.021	0.021	0.020	0.021	0.021	0.021	-	0.773	0.110
K108	0.021	0.021	0.020	0.020	0.021	0.021	0.021	0.773	-	0.083
K113	0.248	0.021	0.020	0.020	0.021	0.021	0.021	0.110	0.083	-

Table 18: P-values for the comparison among all tested oximes after challenging the rats with ***DFP.*** Mann Whitney test was used for these p-values and $p \leq .0.05$ was set for statistical significance.

4.4 Comparative efficacy

Figure 6 reveals the differences in efficacy for structurally two different OPC exposures for oximes treatment.

Statistically, there is no significant difference for Paraoxon and DFP. However, if look at figure 6, it is evident that except K107 and K108 all the oximes are more efficacious for DFP exposure than paraoxon and K107 and K108 although poorly effective or not effective but better for paraoxon than DFP.

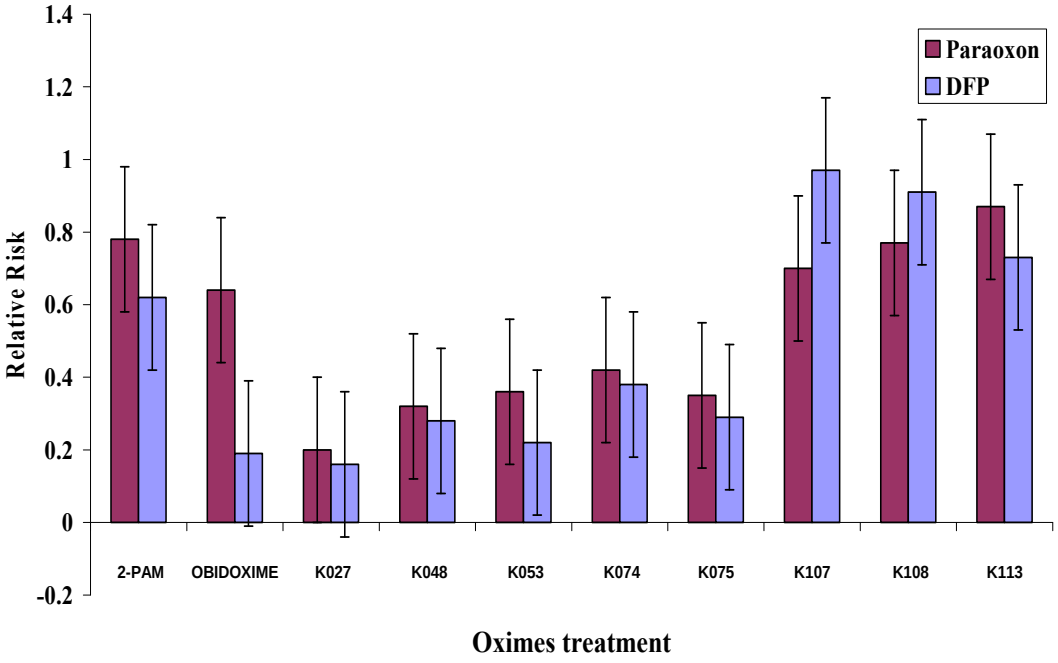
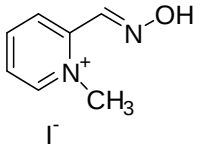
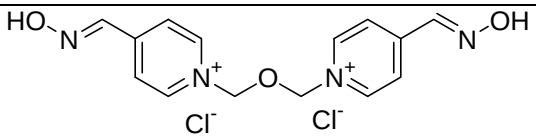
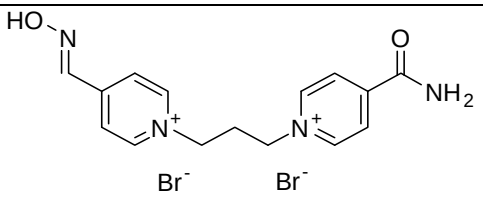
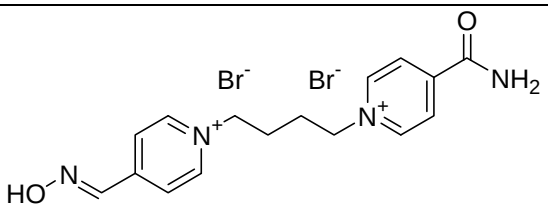
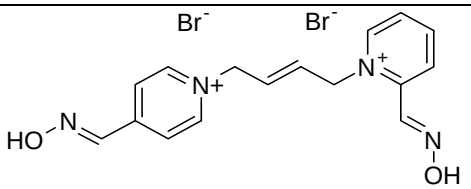


Figure 6: Comparative efficacies (RR±SD) of different experimental K-oximes and established oxime against Wister rats. It is evident from the figure that 2-PAM is not promisingly effective oxime; Obidoxime is good for DFP and statistically different for paraoxon. Among the eight experimental K-oximes, five are better and moreover; their efficacy is not significantly different for two different OPC.

4.5 Calculated Log*P*

All tested oximes are hydrophilic, as indicated by negative Log*P* value (Table 19). 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)

Oxime	Structure	Log*P* (oxime)
2-PAM		-2.31
Obidoxime		-3.12
K-27		-2.66
K-48		-2.61
K-53		-2.05

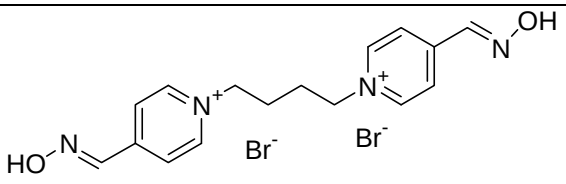
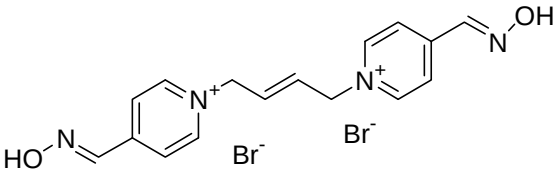
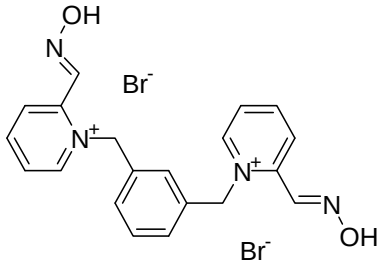
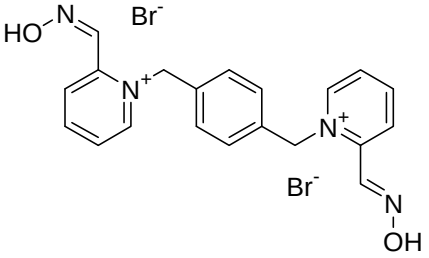
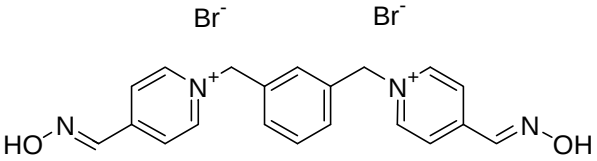
K-74		-1.96
K-75		-2.02
K-107		-0.66
K-108		-0.66
K-113		-0.87

Table 19; Oximes with chemical structure and calculated Log*P* value. It may be noted that all the oximes have negative Log*P* values indicating that they are more hydrophilic than lipophilic.

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

Table 20 corroborates the data for correlation study. These *in vitro* results were obtained from Petroianu and Kalasz 2007; Petroianu and Lorke 2008, and Lorke et al. 2008b.Oximes given alone also have an inhibitory effect upon AChE activity. As for paraoxon and DFP, the IC_{50} value of each oxime can be determined by measuring human RBC-AChE activities in the absence of and in the presence of respective oxime in different concentrations. According to table 20, the established oximes pralidoxime and obidoxime have relatively high IC_{50} value (< 500 μM) implying that they hardly inhibit RBC-AChE and hence their intrinsic toxicity is low. K-027 and K- 048 also show relatively high IC_{50} (around 500 μM). Other K-oximes like K-107, K108 and K113 are relatively low indicate that they are more toxic than the other tested oximes.

Tan α (IC_{50} shift) is a parameter for in vitro reactivation potency. Tan α can be used to quantify the magnitude of the protective effect (nM IC_{50} increase per μM reactivator). The table 20 reveals that Tan α for 2-PAM, obidoxime and K-027 are low, suggesting low reactivation capacity. The experimental K-oximes viz. K-107, K108 and K113 showing distinctly better in vitro AChE reactivation capacity. Moreover, it can be clearly noticed that the reactivation potency of oximes for two different organophosphorus compounds is different. The oximes which is very good for paraoxon like 2-PAM is poor for DFP and the oximes like K-107, K108 and K113 suggests promising reactivator is very poor in *vivo* result.

In order to determine the predictive value of *in vitro* testing (human blood) for *in vivo* efficacy (rat), the various *in vivo* and *in vitro* parameters have been correlated in a pair-wise manner (table 21a, b, and c)**,** using nonparametric spearman correlation coefficient that is rho (σ) and described as below.

In short, *in vitro* reactivation capacity of human red blood cell RBC-AChE has no predictive value for *in vivo* (rat) efficacy.

4.6.1 IC_{50} of oximes (in vitro) versus LD_{50} of oximes (in vivo)

The IC_{50} of the tested oximes, which measures their intrinsic AChE inhibitory activity is positively and strongly correlated with the respective LD_{50} values (σ =0.83, $p \leq 0.003$; figure 7). This implies that an oxime with a low in vitro AChE inhibitory activity (high IC_{50}) value is rather non toxic in vivo (high LD_{50}).The IC_{50} is therefore a relatively good predictor for the in vivo toxicity of the oxime.

4.6.2 Tan α (in vitro) versus LD_{50} of oximes (in vivo)

The correlation between this pair of variable yields a statistically significant and strong negative correlation (σ = -0.95, $p \leq 0.000$; figure 8). The correlation states that oximes with good in vitro reactivation capability (high tan α), have a low LD_{50} in vivo, means high toxicity.

4.6.3 Tan α (in vitro) versus IC_{50} of oximes (in vitro)

The correlation between this pair of variable yields a statistically significant and strong negative correlation (σ = -0.81, $p \leq 0.005$; figure 9).Conclusion is same as in 3.4.2.

4.6.4 Log*P* (in vitro) versus LD_{50} of oximes (in vivo)

Log*P* values of the respective oximes represent an indicator of their lipophilicity or hydrophilicity. The correlation between this pair of variable is strong negative with statistical significance (σ = -0.95, $p \leq 0.001$; figure 10). It implies that oxime with high Log*P* values (less hydrophilic) have high in vivo toxicity that is low LD_{50} values. Less hydrophilic substances are thus more toxic in vivo.

4.6.5 Log*P* (in vitro) versus IC_{50} of oximes (in vitro)

The correlation between this pair of variable is strong negative with statistical significance(σ = -0.94, $p \leq 0.000$; figure 11). It implies that oxime with high Log*P* values (less hydrophilic) have high in vitro toxicity that is low IC_{50} values.

4.6.6 Log*P* (in vitro) versus Tan α (in vitro)

Log*P* values are positively, moderately but statistically significant correlated with their tan α values ($\sigma = 0.77$, $p \leq 0.010$; figure 12). It may be concluded that oximes with high Log*P* values (less hydrophilic) have good in vitro reactivation capacity (high tan **α).** Less hydrophilic oximes are thus better in vitro RBC AChE reactivators.

4.6.7 IC_{50} of oximes (in vitro) versus cumulative relative risk of death (in vivo)-POX

The variable has negative weak correlation and also not statistically significant ($\sigma = -0.30$, $p \leq 0.405$; figure 13).

4.6.8 Tan α (in vitro) versus cumulative relative risk of death (in vivo) - POX

The variable has positive weak correlation and also not statistically significant ($\sigma = 0.30$ $p \leq 0.293$; figure 14).

4.6.9 Log*P* (in vitro) versus cumulative relative risk of death (in vivo) - POX

The variable has positive weak correlation and also not statistically significant ($\sigma = 0.30$ $p \leq 0.293$; figure 15)

4.6.10 IC_{50} of oximes (in vitro) versus cumulative relative risk of death (in vivo)-DFP

IC_{50} values of the respective oximes are negatively and moderately correlated with the cumulative RR ($\sigma = -0.73$, $p \leq 0.017$; figure 16).Correlation is also statistically significant. This implies that an oxime with a low in vitro AChE inhibitory activity (high IC_{50}) reduces DFP induced mortality (low cumulative RR).The IC_{50} is therefore also an in vitro predictor for the in vivo efficacy of an oxime.

4.6.11 Tan α (in vitro) versus cumulative relative risk of death (in vivo) - DFP

No correlation or weak correlation was noted here (σ = 0.58, $p \leq 0.077$; figure 17).This implies that at least in case of DFP exposure, in vitro reactivation capacity of human RBC-AChE has no predictive value for in vivo efficacy in rats.

4.6.12 Log*P* (in vitro) versus cumulative relative risk of death (in vivo) - DFP

LogP values are positively and strongly correlated with the cumulative RR (σ = 0.89 $p \leq 0.001$; figure 18). Correlation is statistically significant. It indicates that oximes with high LogP values (less hydrophilic) are associated with a high cumulative relative risk of death after DFP exposure implying low in vivo efficacy.

Oxime	Dosage Range used In vitro [µM]	IC_{50}[µM] of oxime for human RBC-AChE	tan α [nM/µM] **(Paraoxon)**	tan α [nM/µM] **(DFP)**
Pralidoxime	0-100	592±26 (535-650)	0.31± 0.01 (0.29-0.34)	0.8±0.05 (0.7-0.9)
Obidoxime	0-100	702±39 (606-798)	7.3 ± 1 (5.8-8.8)	1.5±0.07 (1.3-1.7)
K-027	0-100	414±28 (346-483)	3.9 ± 0.2 (3.4-4.4)	0.9±0.1 (0.8-1.0)
K-048	0-100	461±18 (417-505)	1.5 ± 0.2 (0.8-2.0)	1.3±0.1 (1.0-1.6)
K-053	0-10	115±10 (88-141)	2.5 ± 0.4 (1.5 - 3.6)	8±0.5 (6.6-9.3
K-074	0-10	103±3 (94-111)	5.3 ± 0.5 (4.1 – 6.4)	1.4±0.3 (0.7-2.0)
K-075	0-10	63±6 (46-80)	4 ± 0.3 (3.2 - 4.9)	7.3±1.0 (4.5-10)
K-107	0-5	6±0.7 (4.4-7.9)	5.6 ± 0.6 (3.8-7.4)	17±1 (14-19)
K-108	0-5	8±0.3 (7.5-9)	4.9 ± 0.5 (3.5-6.2)	20±1 (17-23)
K113	0-5	9±0.6 (6-11)	7.1 ± 0.4 (5.9-8.3)	16±2 (11-21)

Table 20 Synopsis of the data used for correlation analysis. The second column gives the dose range of the oxime in which a linear relationship between oxime dose and IC50 of DFP was observed in vitro. The third column lists the intrinsic acetylcholinesterase (AChE) inhibitory activity of the oxime (IC50) when administered in vitro alone, that is without organophosphate, to human red blood cell acetylcholinesterase activity. Values presents means ±standard deviation, bracket: 95% confidence interval. Column four shows the values of the tangent of the angleα, formed by the graph of the IC_{50} shift with a horizontal line (for DFP), which is an *in vitro* indicator for the reactivation capacity of an oxime. Column five shows the values of the tangent of the angle α, formed by the graph of the IC_{50} shift with a horizontal line (for paraoxon).

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 21-b (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 21-c (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 21a, b, c: Synopsis of the correlation results. Spearman rank correlation coefficient (σ) has been employed for data analysis. 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.

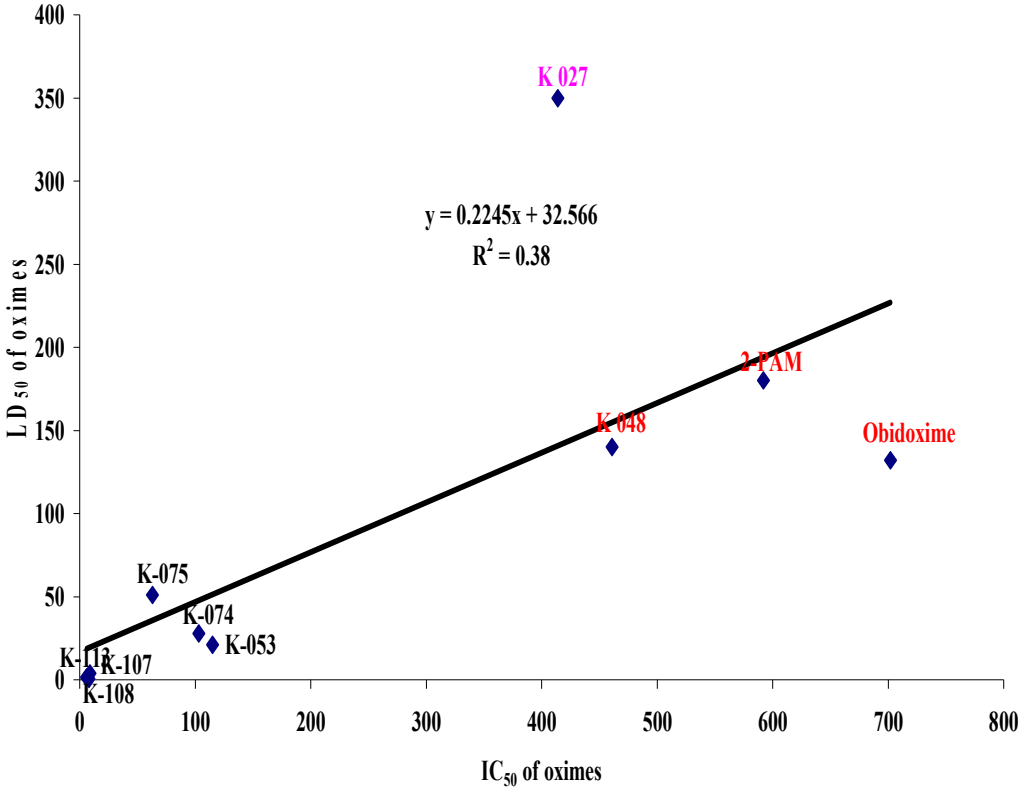


Figure 7; Scatter plot of LD_{50} versus IC_{50} of oximes with least square linear regression line.

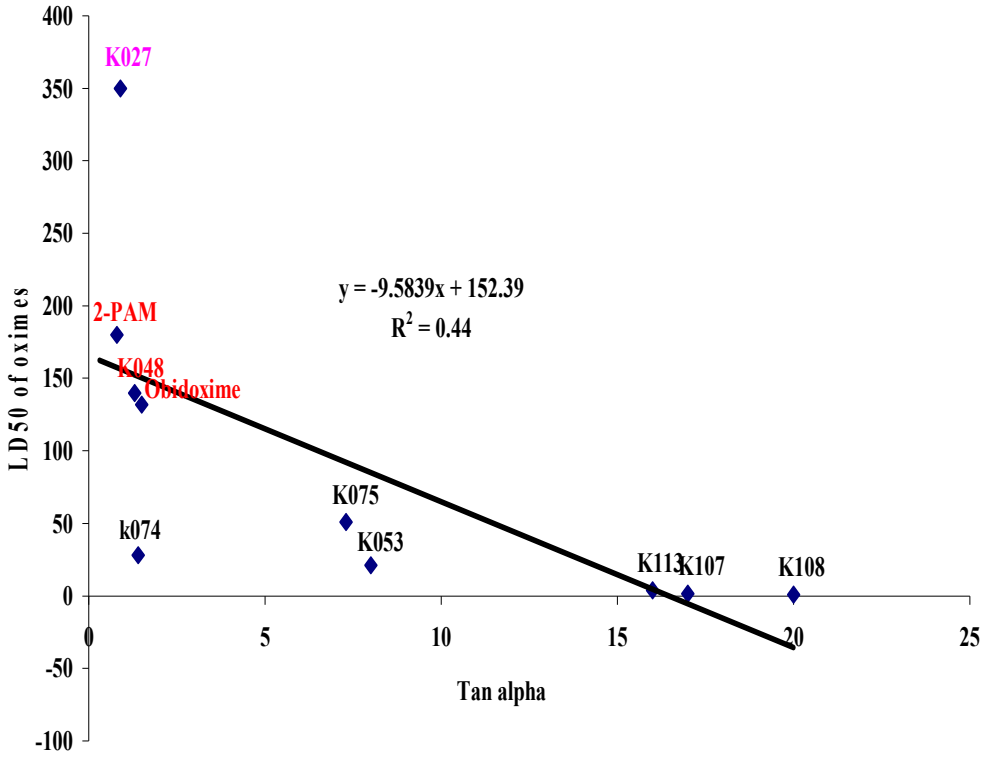


Figure 8; Scatter plot of LD_{50} versus tan α of oximes with least square linear regression line.

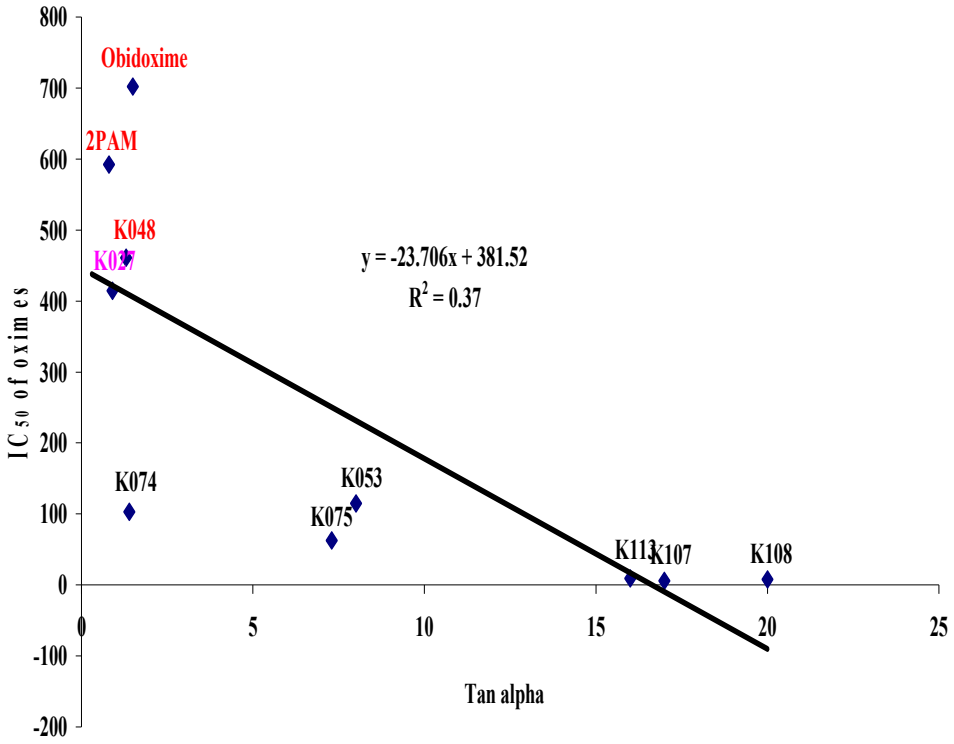


Figure 9; Scatter plot of IC_{50} of oximes versus tan α of oximes with least square linear regression line

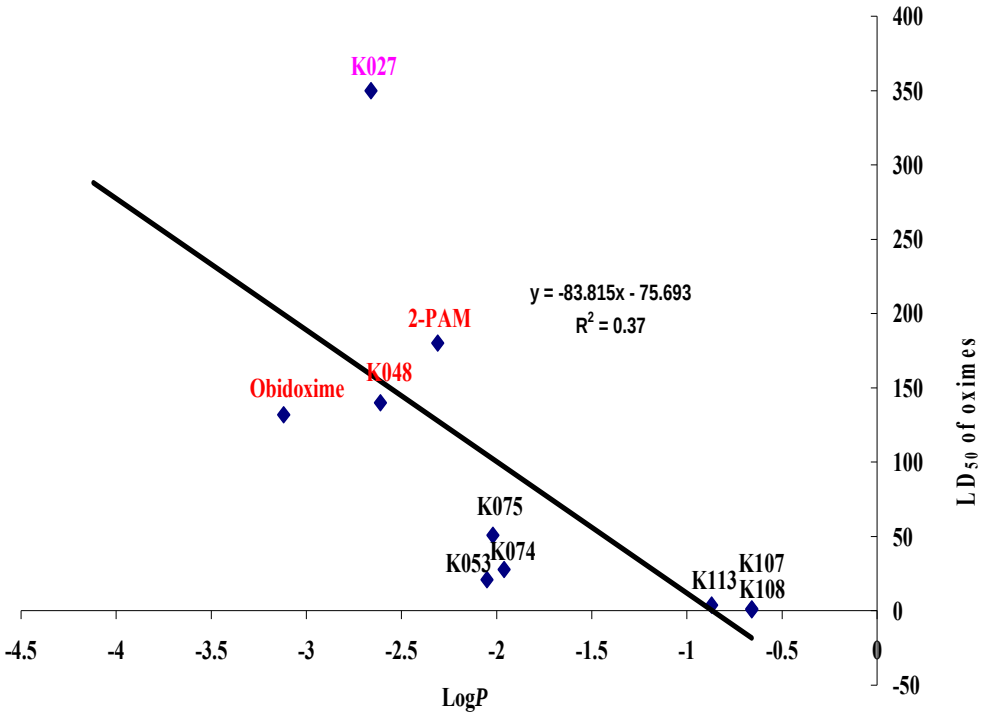


Figure 10; Scatter plot of LD_{50} of oximes versus Log*P* of oximes with least square linear regression line.

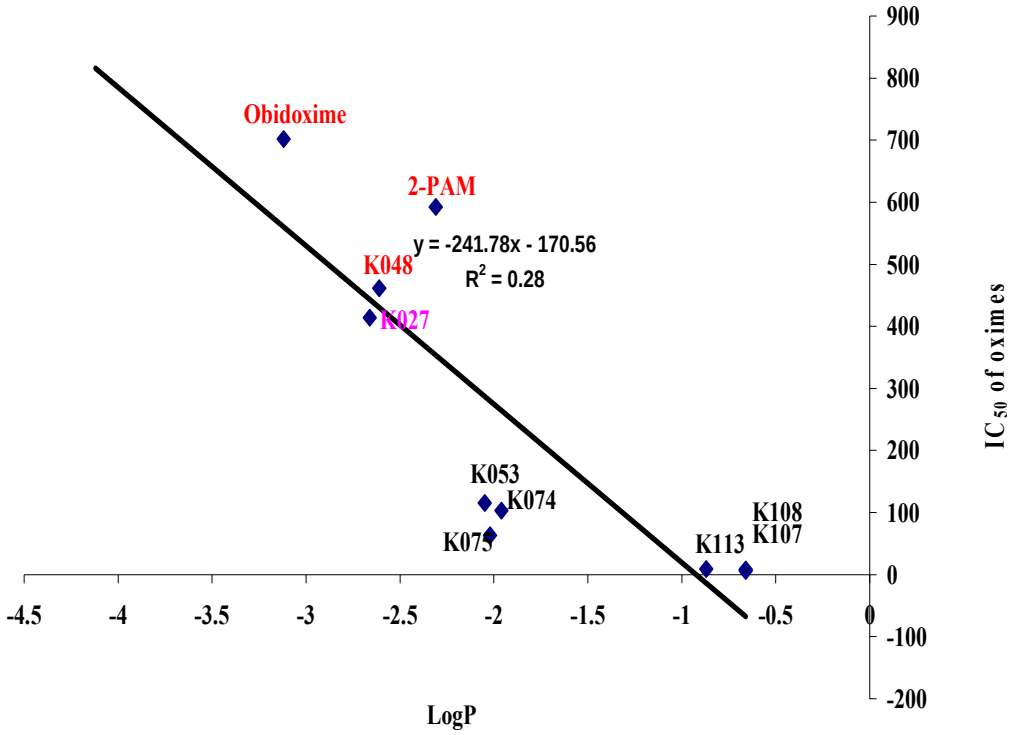


Figure 11; Scatter plot of IC_{50} of oximes versus Log*P* of oximes with least square linear regression line.

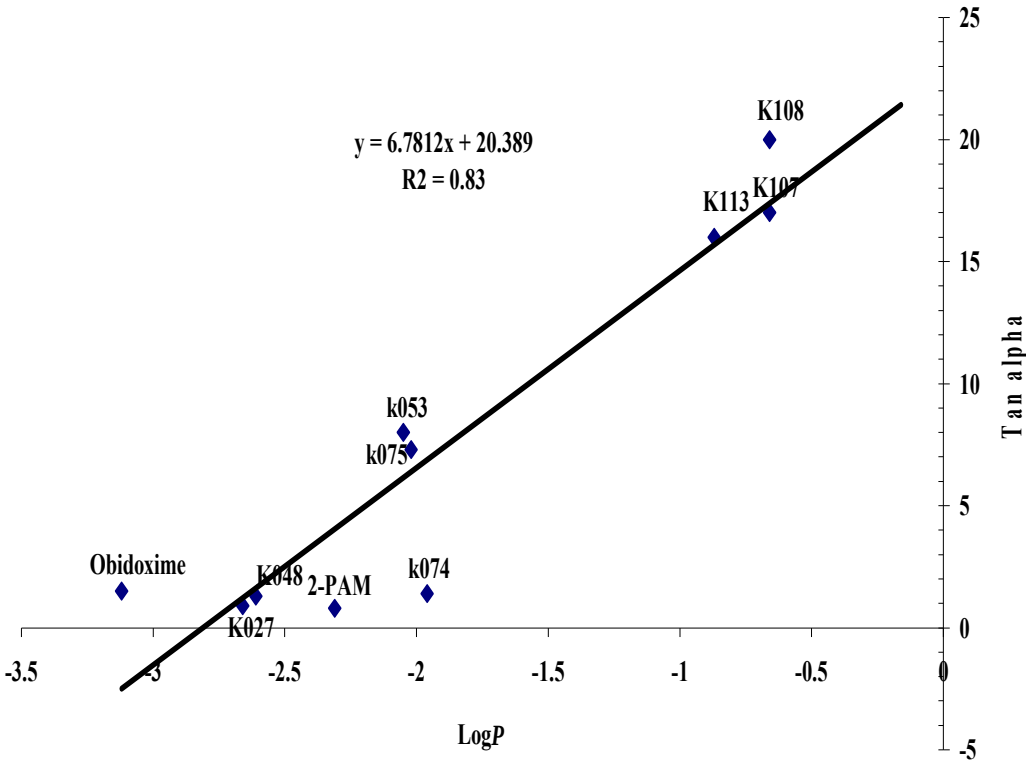


Figure 12; Scatter plot of tan α of oximes versus Log***P*** of oximes with least square linear regression line.

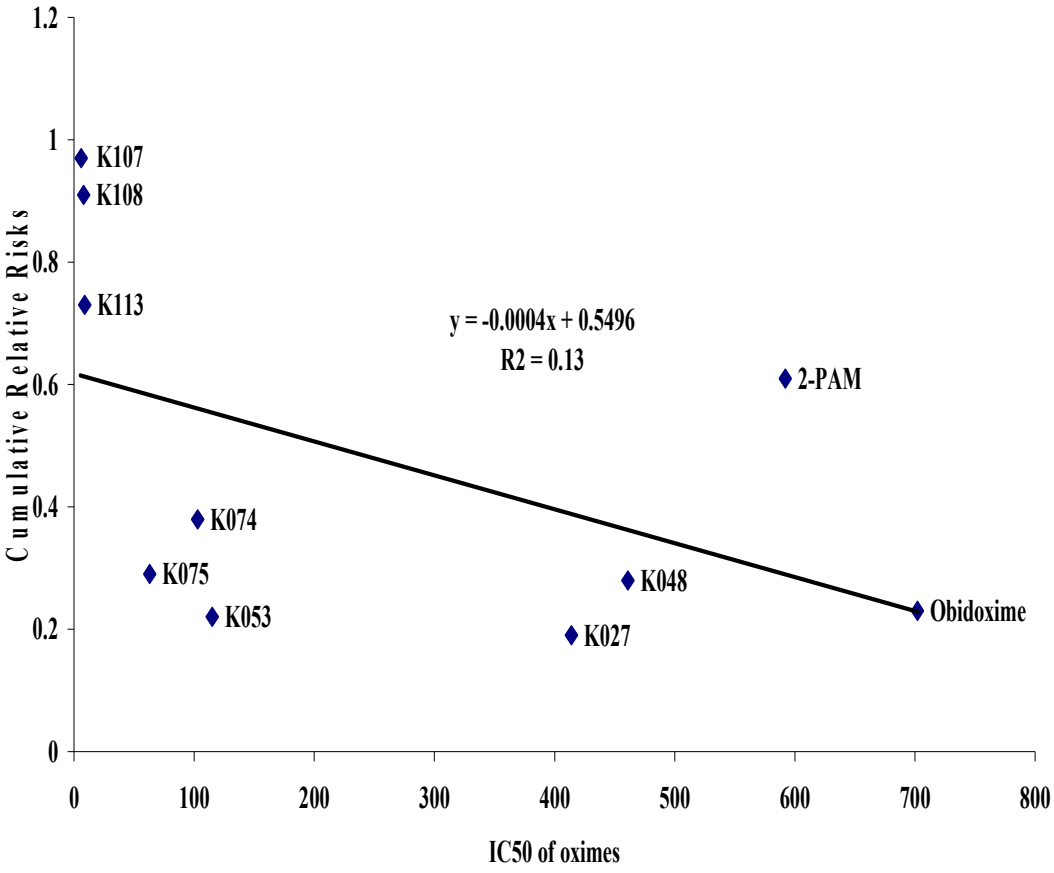


Figure 13; Scatter plot of cumulative relative risk after paraoxon exposure versus IC_{50} of oximes with least square linear regression line.

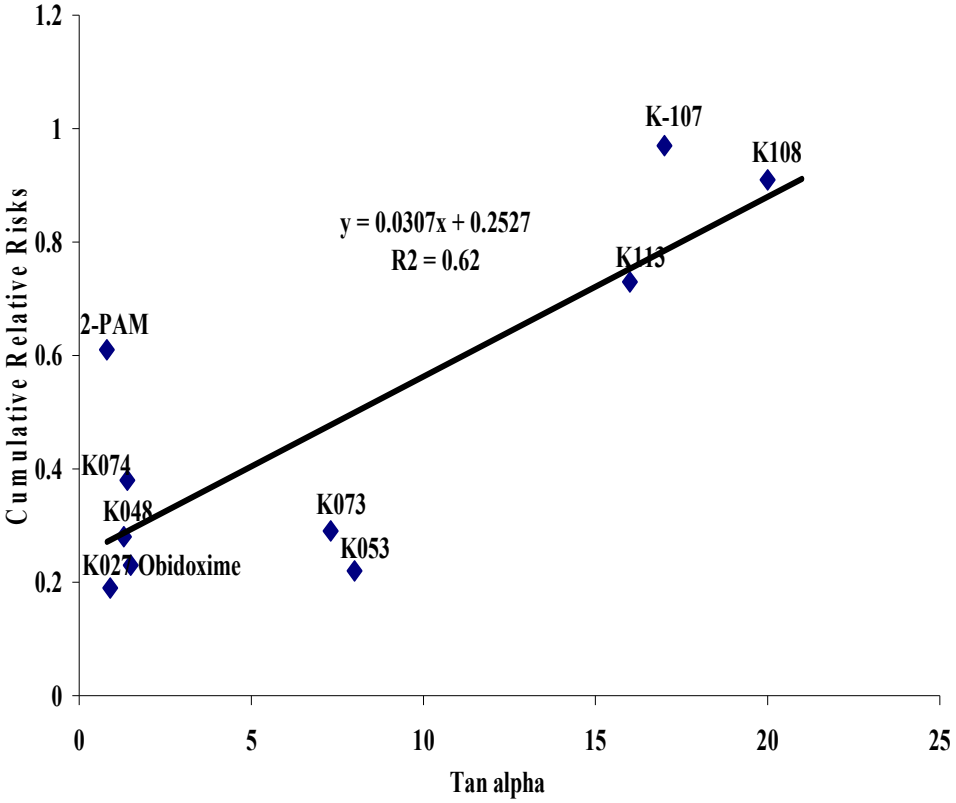


Figure 14; Scatter plot of cumulative relative risk after paraoxon exposure versus tan α of oximes with least square linear regression line

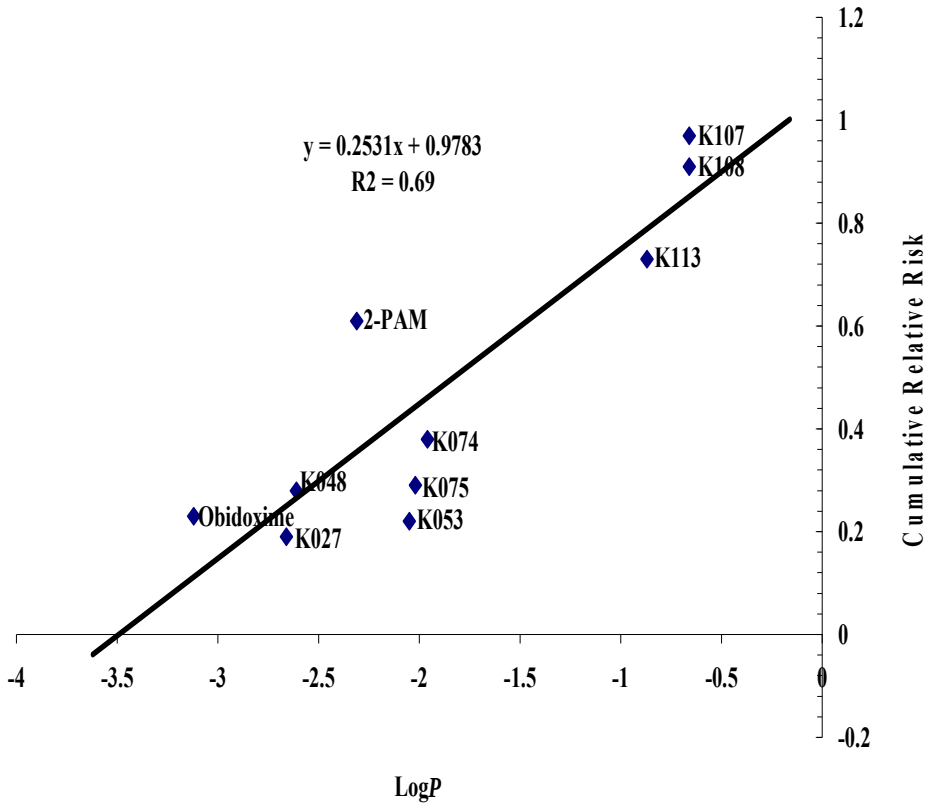


Figure 15; Scatter plot of cumulative relative risk after paraoxon exposure versus Log*P* of oximes with least square linear regression line

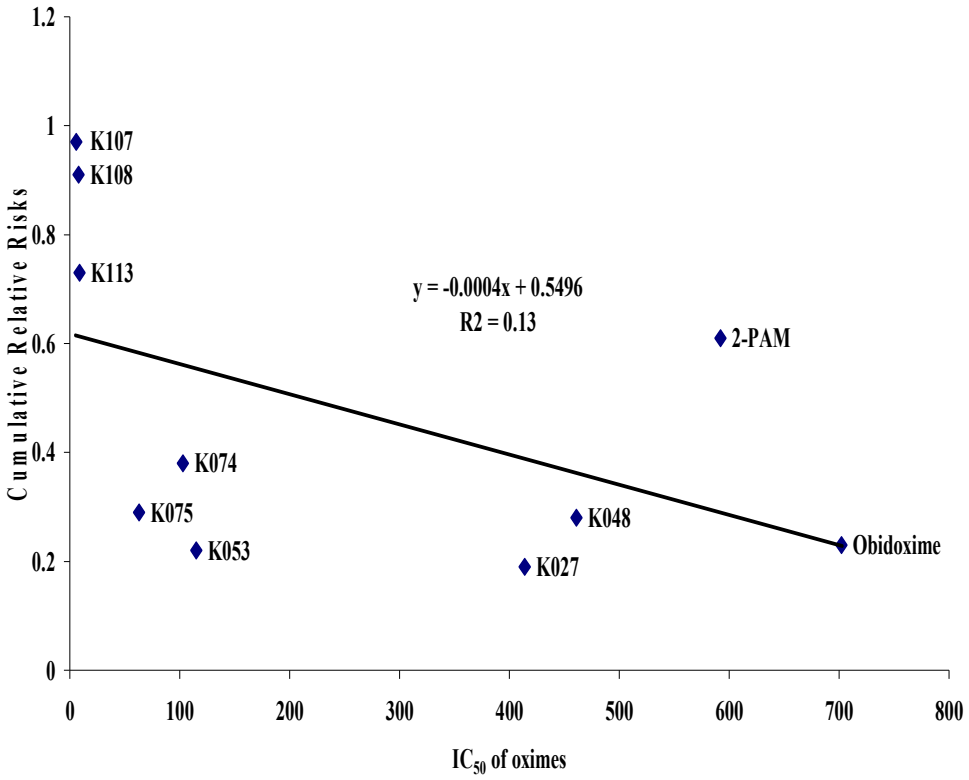


Figure 16; Scatter plot of cumulative relative risk after DFP exposure versus IC_{50} of oximes with least square linear regression line.

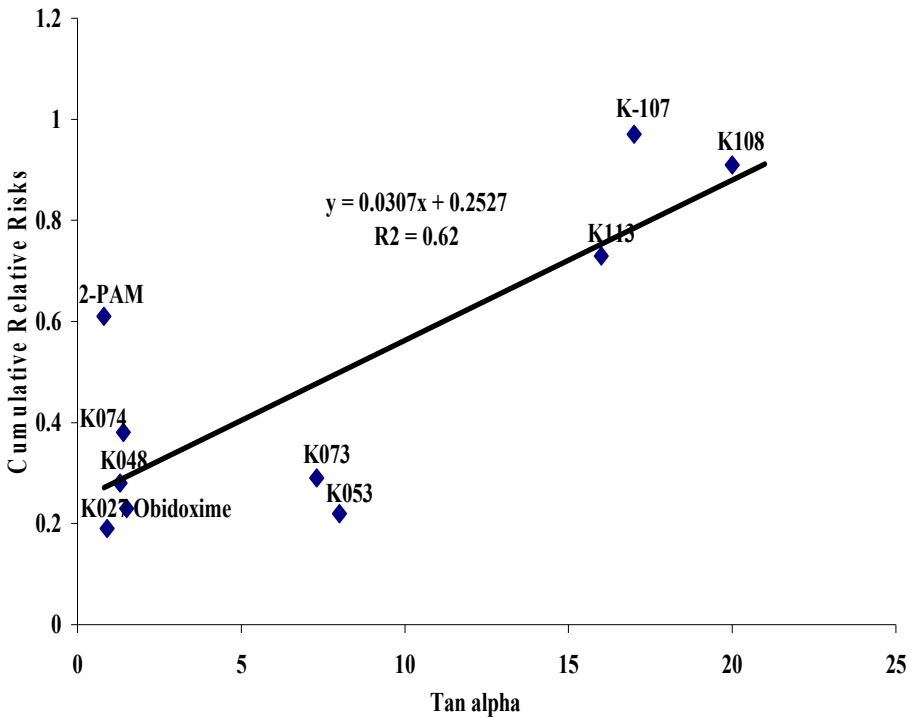


Figure 17; Scatter plot of cumulative relative risk after DFP exposure versus tan α of oximes with least square linear regression line

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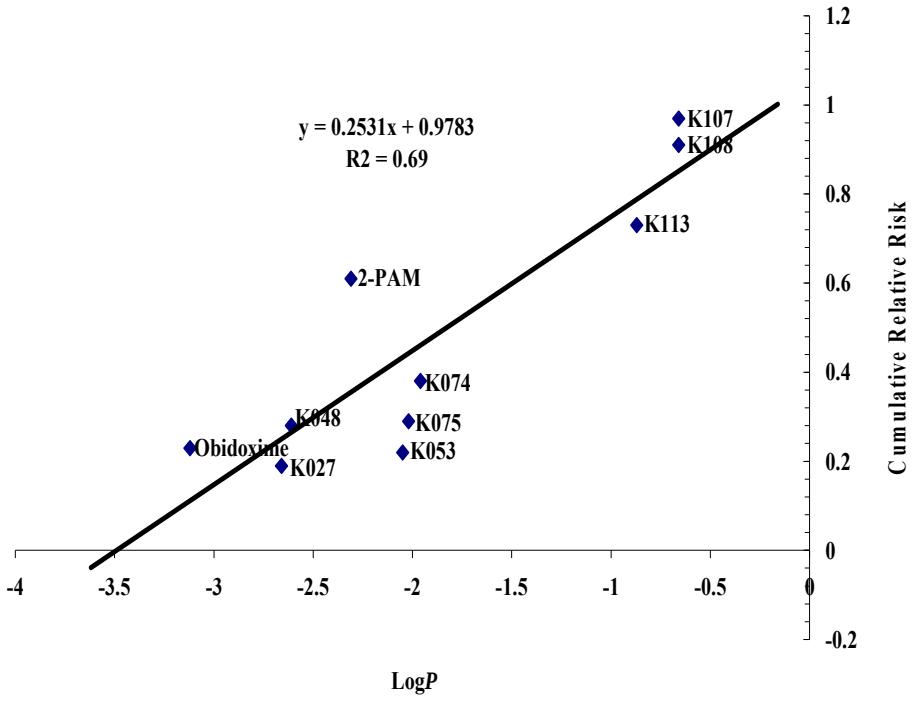

Figure 18; Scatter plot of cumulative relative risk after DFP exposure versus Log*P* of oximes with least square linear regression line

Chapter 5: Discussion

The present study has been undertaken to quantify the *in vivo* efficacy of eight experimental K-oximes in comparison to gold standard oxime Pralidoxime and Obidoxime by using two structurally different organophosphorus acetylcholine esterase inhibitor paraoxon and diisopropylfluorophosphate (DFP). Intrinsic toxicity of oximes in terms of LD_{50} (i.p) was determined to evaluate the intrinsic toxicity factor in OPC-inhibited AChE reactivation by oximes. The *in vitro* efficacy of tested oximes to protect human red blood cell AChE from inhibition by paraoxon (Petroianu and Kalasz 2007) and DFP (Petroianu and Lorke 2008) was correlated with *in vivo* efficacy against rats by same compounds. The lipophilicity/hydrophobicity of the tested oximes was also predicted by calculating Log*P* values. The experimental K-oxime K027 was found to be the best in *in vivo* efficacy (cumulative relative risk for paraoxon 0.19 and DFP 0.27) .Same pattern has been reported as well in *in vitro* efficacy against human RBC-AChE reactivation. It has the least intrinsic toxicity (LD_{50} 612 mg/kg average body weight) among the tested oximes. All the oximes were hydrophilic suggesting a mechanism of action other than on brain or atleast not by simple passive diffusion to brain. The standard oximes were less effective and also not equally effective against structurally two kinds of OPC.

5.1 Intrinsic toxicity (LD_{50}) of tested oximes.

Oximes are reversible competitive inhibitor of AChE (Patocka et al. 2005; Jokanovic and Stojiljkvic 2006) and their binding to cholinesterase cause inhibition of the enzyme activity. However, binding of oximes to the enzymes also protects cholinesterases from phosphylation by OP compounds (Primozic et al. 2004).

One of the basic criteria for oximes selection is their intrinsic toxicity or toxicity *per se*. Among the therapeutically available oximes, HI-6 is the least toxic (LD_{50} values ~860 µmol/kg body weight against rats with ip injection, obidoxime ~280-625 µmol/kg body weight and pralidoxime ~1230 µmol/kg body weight (Dawson

1994). Kassa et al. 2008 b reported LD_{50} of Obidoxime against rat by i.m as 176.4 -252.6 mg/kg body weight and for HI-6, it was in between 738-826.6 mg/kg body weight. Among all the available oximes, HI-6 is considered a more potent reactivator and Pralidoxime as less potent AChE reactivator although every oxime has some limitations. But it can be deduce that oxime with less intrinsic toxicity is more potent AChE reactivator. Berend et al. 2008 reported the LD_{50} of K027; 599 -755.3 mg/kg, K048 154.2-328 mg/kg, K074 19-24 mg/kg, against male mice. In the present work LD_{50} for 2-PAM was 121 mg/kg, Obidoxime 177mg/kg, K027 612 mg/kg, K48 246 mg/kg. Indeed, these lethal values are according to the earlier reported values and little differences in figure may be due to strain, age, species and other factors. Structurally, the most toxic substances turned out to be the K-oximes with a xylene linker that is K107, K108, and K113. Their LD_{50} values were below 4μmol which was 100 times more toxic than K027. In the present work, K027 was found to be the least toxic and most promising reactivator. The *in vitro* result by Lorke et al. 2008d also indicates the same toxicity pattern as obtained in the present *in vivo* work.

The efficacy of the reactivators is related to the nucleophilicity of the oximate and the decay rate of the intermediate phosphyloxime and is dependent upon the structure of the oxime and of the op moiety as well as on the architecture of the enzyme. **(**Worek and Thiermann 2007)**.**The phosphylated oximes particularly stable phosphoryl oxime formed during the reactivation process might be potent inhibitors of cholinesterase, which could cause re-inhibition of the previously reactivated enzyme **(**Luo et al. 1999; Kinderlen et al. 2005). Re-inhibition of AChE can be faster than reactivation in the case when a phosphylated oxime inhibits the enzyme at rate higher than that of its elimination or decay to non toxic products.

5.2 Comparison between LD_{50} and *in vitro* intrinsic inhibitory activity.

An in vitro indicator of oxime toxicity is its intrinsic inhibitory potency upon AChE and is measured by IC_{50}. *In vitro* studies on RBC-AChE by Petroianu and

Lorke 2008; Lorke et al. 2008b demonstrates that 2-PAM, obidoxime, K-027 and K-048 have low AChE inhibitory activity (IC_{50} = 400-700uM) and are least toxic in comparison to all other tested oximes. The *in vivo* result also translated the low toxicity by these oximes. K-53, K74, K75 are in mid range with regard to both intrinsic AChE inhibition (IC_{50} = 50-120 uM) and *in vivo* toxicity. The three most toxic oximes (K107, K108, K113) have the lowest IC_{50} values (5-10 uM).This indicates that that *in vitro* AChE inhibition is good predictor for *in vivo* toxicity.

5.3 Mortality reduction / survival analysis

Oximes have been used as an adjunct treatment in the organophosporus induced AChE inhibition/ intoxication. But the literature confirms that none of the therapeutically available oximes is universal broad spectrum cholinesterase reactivators (Kassa 2002; Jokanovic 2009). HI-6 is considered good for reactivating phosphonates (nerve agents) induced AChE inhibition (de Jong et al. 1989; Eyer et al. 1992; Eyer 2003; Lundy et al. 1992; Kassa and Cabal 1999; Kuca et al. 2005a) but not promising for organophosphorus insecticies (Worek et al. 1996). Obidoxime are preferred for the treatment of acute poisoning with organophosphorus insecticides because they are effective reactivators for organophosphorus insecticides inhibited AChE (Jokanovic and Maksimovic 1995; Petroianu et al. 2007b; Worek et al. 1996; Kassa et al.2005). 2-PAM is the least effective oximes (Cherian et al. 2005) .Experimental K-oximes have been derived with the basic structure of 2-PAM, Obidoxime and HI series of oximes (Petroianu 2007) and mostly research emphasis is being given on nerve agents, rather all the oximes were developed and being investigated with the target of nerve poisoning although the impact of intentional or unintentional poisoning by Op pesticides is not ignorable. In H-oximes, one oxime group is present at 2 or 4 positions in one of the pyridinium rings (Kokshareva et al. 2005). In K-027 also one oxime group is present at position 4 of pyridinium rings and all the other K-oximes have same one or two functional oxime group at position 2 or 4 like H-oximes. De Jong et al. 1989 reported that oximes with functional group at position 4 seemed to be superior to the oximes with functional group at 2.On the other hand obidoxime has an oxime functional group at position 4 of both pyridinium rings and is also found

to be potent reactivators for insecticides. In the present study the best protective enzyme K027 has the same position 4 functional group. The newly developed K-oximes are bisquaternary symmetric (K-074, K-075, K107, K108, K113) or asymmetric (K-027, K-048, K-053) pyridinium aldoximes with the functional aldoxime group at position 2 (K107, K108), 4 (K-027, K-048, K074, K075 and K-113) or both (K053) of the pyridine rings. K-027 and K-048 have only one functional aldoxime group, the other k-oximes have two. In addition, K107, K108 and K113 contain a xylene linker. Among the tested K-oximes, K107, K108 and K113 which are bisquaternary symmetric and contain a xylene linker were not effective against paraoxon and DFP induced mortality in the present study but found to be very effective against nerve agents like cyclosarin (Kuca et al. 2005b; Hrabinova et al. 2006).The efficacy of K-048, K074, K075 and K053 were comparable with standard obidoxime and better than 2-PAM. K-027 was the superior to all experimental K-oximes and established oximes .The order of potency against paraoxon was K-027 > K-048> K-075 > K-053 > K-074 > Obidoxime > K-107 > K108 > 2-PAM > K-113.and against DFP was K-027 > Obidoxime > K-053 > K-048 > K-075 > K-074 > 2-PAM > K-113. The best efficacy of K-027 and K-048 have been reported earlier by Petroianu et al. 2006b, 2007 b& c and Petroianu and Kalasz 2007; Lorke et al. 2008a & d and Lorke et al. 2009 against paraoxon and DFP. It was also found to be potent RBC-AChE reactivator in in vitro studies with ethyl paraoxon (Musilova et al. 2009), methyl paraoxon and DFP (Petroianu et al. 2006a; Petroianu and Kalasz 2007; Lorke et al. 2008b). The oxime was found promising and better than established oximes against OP- nerve agents like tabun, Sarin and cyclosarin (Calic et al. 2006; Kuca and Kassa 2003; Kassa et al. 2006; Kuca et al. 2006).K027 is good for reactivation of RBC-AChE, inhibited by OPCs poisoning except two nerve agents, cyclosarin and tabun (Kuca et al. 2010). Inadequate efficiency of 2-PAM has been reported by Cherian et al. 2005. Oh et al. (2008) demonstrated that the bis- pyridinium oximes showed stronger activity than mono pyridinium oxime like 2-PAM against DFP and paraoxon inhibited acetylcholinesterases in *in vitro* study. Jun et al. 2008 reported that AChE reactivation potency of obidoxime is far better than pralidoxime and HI-6 against paraoxon inhibited acetyl cholinesterase. Now the

question arises, what are the factors which make the K oxime specially K-027 a better promising oxime. Although, like other oximes which are extremely hydrophilic (Csermely et al. 2008), it is non lipophilic and does not sufficiently (only about 5%) cross the blood brain barrier (Petroianu et al. 2007c; Lorke et al. 2007; Lorke et al. 2008c) hence, it is mainly effected on peripheral nervous system. But the presence of carrier-mediated transport system to brain cannot be ruled out (Lorke et al. 2008c, Kalasz et al. 2009a, Shrot et al. 2009). According to Jokanovic 2009, oxime penetration through blood brain barrier is underestimated. Two processes may contribute to better penetration of oximes through the blood-brain barrier: the induction of local inflammatory processes and increase of brain blood flow (Short et al. 2009). Moreover, penetration of K oximes has also been reported as dose dependant (Kalasz et al. 2009b). It might be suggested that in addition to the structural peculiarity which makes the K027 proficient, some unknown physiological/ intoxication process also triggers by this oxime which gives good protection in case of OP intoxication even at supra lethal doses. The differential efficacy of oximes also related with the chemical structural of oximes and OPC (Fukuto 1971, Su et al.1983; Worek et al. 1998; Kuca and Kassa 2003; Cabal et al. 2004; Musilek et al. 2006a**;** Kuca et al. 2006; Kovarik et al. 2007; Maxwell et al. 2008)**.** Jokanovic and Maksimovic (1995) have studied the acute toxicity of 26 different OP insecticides in the rat and the efficacy of four different oximes was evaluated. They found that the success of therapy was dependent on the chemical structure of OPC. Oxime might exert a direct action other than ChE reactivation (Herman 1991; Busker et al.1991; Jokanovic 2009).The potency of oximes to reactivate AChE may be influenced by the number of quaternary pyridinium rings (Hammond et al. 2003), the position and number of oxime groups at the pyridinium ring (Lamb et al. 1964; Kuca and Kassa 2003). K-027 or other potent K series oximes might have additional protective effect by synergistic interaction with Carboxylesterase (CaE), an endogenous scavenger enzyme for OPC. Such probability was examined with HI6 against soman toxicity by Donald et al. 1990. HI-6 which is considered the best protective enzyme particularly OP nerve agents and as mentioned earlier somewhere else that K-oximes are based on the chemical structure of HI oximes, it has been suggested that the much higher

therapeutic potency of HI oximes in comparison with conventional oximes may be caused not only by the higher reactivating efficacy (de Jong et al. 1989; Kassa 1995) but also by other antidotal mechanism (Kassa 1998). Several other attractive mechanisms such as antimuscarinic (Kuhnen-Clausen 1972; Kloog et al. 1986; Chen et al. 1996; et al. 1996), antinicotinic and neuromuscular blocking properties (Kuba et al. 1974; Caratsch and Waser 1984; Alkondon et al. 1988; Tattersall 1993), ganglionic blocking actions (Lundy and Tremblay 1979) as well as on restoration of neuromuscular blockade and beneficial effects on cardiovascular and respiratory systems have been suggested to be involved with the additional protective actions of oximes. The higher doses of OPC induced neuromuscular block. The recovery of neuromuscular transmission is an important mechanism of antidotal action of oximes and this action is not connected to cholinesterase reactivation (Smith and Muir 1977). In case of acute OPC intoxication, a functional disorder of the cardiovascular system develops in parallel with respiratory depression. These effects are induced by OPC influence on central and ganglionic synapses as well as ACh stabilization in the peripheral cholinoreactive systems. Some other studies have also indicated that the reactivation mechanism alone is not sufficient to explain the antidotal effect of oximes against OP poisoning in experimental animals (Oldiges 1976; Schoene 1976, 1980; Su et al. 1986). The physico-chemical properties and pharmacokinetics parameters are also important in determining the differential oxime efficacy (Dishovsky 2005; Jokanovic 2009). Inhibition of the ACh ageing reaction, and direct chemical interaction with ACh in blood are also considered antidotal effect of some oximes (Kokshareva et al. 2005).

5.4 Comparison of the *in vivo* efficacy of different oximes to protect from mortality induced by various OPCs

In addition to the factors mentioned in 5.3 for differential oxime efficacy , it is a well-known fact that the mortality-reducing effect of oximes strongly depends upon the type of OPC administered (Johnson et al. 2000; Bajgar, 2004; Calic et al. 2006; Worek et al. 2007; Eddleston et al. 2008; Lorke et al. 2008b). For example, comparing the effect of K-027, K-048, 2-PAM, obidoxime, trimedoxime, HI-6 and

methoxime after exposure to different OPCs, it has been demonstrated that the ability of oxime reactivators to protect from mortality is considerably altered when the ethyl group of paraoxon is replaced by a methyl group (Petroianu and Kalasz 2007).DFP is structurally very different from paraoxon. Whereas paraoxon contains a nitro phenyl group and two ethyl groups bound to the three oxygen atoms of the phosphate molecule, DFP is a monoflourophosphate which is substituted by two isopropyl groups bound to two oxygen atoms by ester bonds and has no aromatic group. However, for most of the oximes tested, our data on the survival after paraoxon exposure [reduction of RR: ≈ 20% (K-027), 32% (K-048), 42% (K-074) and 35% (K-075)] are amazingly similar to those obtained after DFP exposure [RR: ≈ 16% (K-027), 28% (K-048), 38% (K-074) and 29% (K-075)] (Lorke et al. 2008d). Also, both after paraoxon and DFP exposure, K-107, K-108 and K113 were the least efficient oximes. However, some mortality-reducing effect of these oximes was observed after paraoxon intoxication, but not after DFP poisoning. One factor for the differential oxime efficacy against structurally different organophosphorus AChE inhibitor is that different OPC renders the AChE ageing with different rates (Kassa 1998) and different OPCs have different aging kinetics. Once the AChE becomes aged, oximes fail to reactivate it**.** A good oxime like K-027 should reveal protective effect by other mechanism as well including the inhibition of ageing process. The oxime efficacy is also dependent upon the strength of binding of oximes to ACh. Generally it depends upon the structure and nature of OPC also. Generally, nerve agents reduce the strength of binding of oximes to ACh and make their nucleophilic attack less effective. On the other hand, currently used oximes, specially pralidoxime and obidoxime, have low affinity for OPC-inhibited AChE and thus their reactivating potency, found in vitro is commonly lower in comparison with H-oximes (Kassa et al. 2005).

The only oxime affecting paraoxon- and DFP-induced mortality differentially in the present study was obidoxime, the oxime currently stored as an antidote in OPC poisoning in Continental Europe. Obidoxime shows excellent mortality-reducing potency after DFP exposure, reducing the RR to ≈ 20% (Lorke et al. 2008a and d),

whereas its efficacy is relatively poor after paraoxon poisoning (RR=64), which is in line with previous results (Petroianu et al. 2007a). This suggests that obidoxime does not qualify as a broad spectrum oxime-type AChE reactivator. The efficacy of 2-PAM, the other established oxime, was only moderate, both after paraoxon [RR=78] and DFP [RR=62] exposure, which is far inferior to the protective capacity of many new K-oximes (K-027, K-048, K-053, K-074 and K-075). The latter oximes should therefore be further tested as candidates to potentially replace obidoxime and 2-PAM.There are many factors that influence oxime efficacy. Some among them are the inhibitory potential of OPCs and its toxicokinetics, aging kinetics of the inhibited AChE, reactivating property of oximes and its pharmacokinetics (Antonijevic and Stojiljkovic 2007).

So, it might be suggested that superior efficacy of K 027 or other K-oximes may not be only due to their higher AChE reactivating potency rather at supra lethal doses of OPC, other aforementioned mechanisms might be playing role along with AChE reactivation. A detailed work is needed to establish the mechanism of superior efficacy of some K-oximes in comparison to established oximes.

5.5: Comparison between relative risk (RR) and *in vitro* protective activity:

A. Paraoxon induced AChE inhibition

The results show that by far the best *in vivo* protection is conferred by K-027, which reduces the RR of paraoxon-induced mortality to 20%, a value, which is in good agreement with previous studies (Petroianu et al. 2006b; 2007b &c, Lorke and Petroianu 2009). This effect is significantly superior to that of all other tested oximes. The three new bispyridinium oximes, which contain two aldoxime groups, but no xylene linker (K-053, K-074, K-075) and K-048 also markedly reduce mortality, though to a lesser degree (RR= 32-42%). In contrast, the three new oximes containing a xylene linker (K-107, K-108 and K-113) as well as the established oxime 2-PAM were only poorly effective *in vivo* (RR=70-87%). Whereas the very good *in vivo* performance of K-027 and the poor performance of 2-PAM are in good agreement with their *in vitro* AChE reactivation capacity (Petroianu et al. 2006a; Petroianu and Kalasz, 2007), the inferior *in vivo*

protection conferred by K-107, K-108 and K-113 is not reflected by the *in vitro* data. When the shift of the paraoxon IC_{50} values after increasing oxime doses, measured as the slope of the curve (tan α), is used as a measure to quantify *in vitro* the protective capacity of these oximes, the three tested oximes with xylene linker are about twice as effective as K-027, K-048 and K-075 (Petroianu and Kalasz 2007). Poor protective capacity of K-107, K-108 and K-113 is most probably due to their high toxicity (Lorke et al. 2008d). The three oximes bearing a xylene linker are about 100 times more toxic than K-27, which is the least toxic oxime tested. Their LD_{01} is 5-10 times lower than that of K-53, K-74 and K-75 and about 50 times lower than that of 2-PAM, obidoxime and K-48, which are also relatively non-toxic. Because of their considerably higher toxicity, which is most probably related to their strong intrinsic AChE inhibitory activity (Lorke et al. 2008b and d), K-107, K-108 and K-113 can only be administered in a dosage which is two orders of magnitude lower than that of K-027 and K-048. The excellent *in vivo* protective capacity of K-027 becomes even more evident considering that this oxime was administered in a relatively lower concentration, i.e. 10% of the LD_{01}, as compared to 50% of the LD_{01} for all the other oximes, because K-027 was administered in the same dosage as in several previous studies (Petroianu et al. 2006b; 2007 b & c; Lorke et al. 2008a & d).

B. DFP induced AChE inhibition

The present results show that the oximes tested reduce DFP induced mortality differentially. On the basis of statistical differences in the relative risk (RR) of death, these oximes can be divided into five groups: best protection is conferred by K-027, reducing the RR from 1 to 0.16. Obidoxime, K-053 and K075 reduce the RR to ~0.20-0.30; K074 and K048 are in the middle range RR= 0.28-0.30), but the two later groups overlap. 2-PAM and K113 are poorly effective (RR= 0.60-0.75) and K107 and K108 do not significantly reduce DFP induced mortality (RR ≈1).

The same oximes were previously tested *in vitro* for their capacity to protect human RBC AChE from DFP induced inhibition (Lorke et al. 2008b). The shift of the DFP IC_{50} values after increasing oxime doses, measured as the slope of

the curve (tan alpha) and the binding constant K, determined on the basis of Schild curve (Arunlakshana and Schild 1959; Cheng 2001), were used to evaluate the in vitro protective capacity of these oximes. Considering that low K and high tan alpha values indicate the good reactivation capacity, it was found that K107, K108 and K113 were the most promising substances. However, turned out to be virtually ineffective *in vivo*. In contrast, K-27, the most efficient oxime in vivo and 2-PAM which is only poorly effective *in vivo*, show similar in vitro reactivation characteristics, which are far less good that those of K107, K108 and K113.The most likely explanation for this unexpected finding is relatively high intrinsic toxicity of these oximes (i.e. K107, K108 and K113) or these oximes may be forming a very stable phosphorylyl oximes *in vivo* and causing inhibition of reactivated AChE. Such condition limits its use in the treatment of OPC poisoning (Luo et al. 1999; Herkenhoff et al. 2004; Kiderlen et al. 2005).

5.6 Relationship between *in vitro* and *in vivo* toxicity data.

In vitro testing system comprises the human blood (RBC) and it centers on the ability of the oximes to increase the esterase activity despite the presence of an OPC. The results are quantified using various approaches such as IC_{50} calculation, IC_{50} shift (tan α), Schilds plot and K (binding constant) determinations. The simplified interpretation of theses parameters are as follows; a high IC_{50} value means low toxicity (means AChE is being inhibited at higher concentrations),Tan α or IC_{50} shift can be used to quantify the magnitude of the protective effect i.e. reactivation potency (low tan α means low reactivation capacity and vice versa). Binding constant K can be used to describe the relationship between oxime concentrations and OPCs concentrations to inhibit 50% of the enzyme (IC_{50}).

In vivo model of toxicity includes the determination of LD_{50} for oximes themselves, relative risk of death after challenge with different doses of OPC and subsequent application of oximes. The main reason of death after OPC poisoning with OPC is inhibition of AChE, a neurotransmitter hydrolyzing enzyme. Other factors of death are respiratory depression and cardiac arrest.

Generally, the *in vitro* results shows some relationships with the corresponding *in vivo* parameter but sometimes, *in vitro* results do not predict or correlate with *in vivo* studies. Some of the reasons for this deviation may be as follows;

1. Chemical is not absorbed at all or is poorly absorbed in *in vivo* condition.
2. Chemical is rapidly metabolized to an active or inactive metabolite that has a different profile of activity or different duration of action than the parent compound.
3. Chemical is rapidly eliminated (e.g. through secretory mechanism).
4. Species of the two test systems are different.
5. Experimental conditions of the *in vitro* and *in vivo* experiments differed and might have led to the different effects then expected.
6. Effects elicited in *in vitro* and *in vivo* differ in characteristics.
7. *In vitro* data cannot predict the volume of distribution in central and peripheral compartment.
8. The types of data might not be comparable.
9. *In vitro* data cannot predict the rate constants for chemical movement between compartments.
10. *In vivo* effects of chemicals are due to an alteration in the higher order integration of an intact animal system which cannot be reflected in a less complex or simple *in vitro* system.

In the present study, the *in vivo* toxicity data was correlated with the *in vitro* toxicity data. There were nine pairs of correlation (table 21 and figure 7-18) with paraoxon and DFP induced mortality. 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. The generalized assumption is that *in vitro* activity translates into *in vivo* enzyme protection and therefore increases in survival. In case of paraoxon and DFP exposure, *in vitro* reactivation capacity of human RBC-AChE has no predictive value for *in vivo* efficacy in rats. The lack of correlation may be related to one of

the factor mentioned above. Moreover, another explanation may be the formation of phosphylated oximes in *in vivo* condition which is discussed in earlier mortality section. It appears that the stability of phosphylated oxime is different *in vivo* and *in vitro*. Another possibility is that due to high intrinsic toxicity, some oximes with good *in vitro* reactivation capacity can only be safely administered *in vivo* at a very low dosage, which may be insufficient to protect from DFP induced mortality. It cannot be ruled out that the mortality/survival is not under total control of AChE reactivation/inhibition rather it has been discussed in the earlier sections that oxime do protective effect other than AChE reactivation. So, there may be possibility that protective actions of oximes are due to other mechanism as well which is reflected by no correlation between *in vitro* AChE reactivation capacity and *in vivo* relative risk of death in case of paraoxon and DFP exposure and other parameters in case of paraoxon exposure (Petroianu and Lorke 2008)

5.7 Calculated Log*P* and relationship with *in vivo* and *in vitro* toxicity results.

In silico study, Log*P* values were calculated to predict the physico-chemical properties particularly the lipophilicity/hydrophilicity of the oximes and then establish the relationship with *in vivo* and *in vitro* experimental data. The result shows that all the tested oximes were hydrophilic, as indicated by negative Log*P* value (table 19). The Log*P* values are in line with Csermely et al. 2008 and Laufer et al. 2010 work. Correlation with all the *in vitro* and *in vivo* data except with paraoxon induced relative risk of death were found to be strongly and statistically significantly correlated (table 21).

It is generally believed that substances crossing the blood brain barrier are more efficient in preventing death in acute severe OPC poisoning (Bird et al. 2003). Secondly, less hydrophilic or more lipophilic compounds able to cross blood brain barrier. The tested oximes are not lipophilic means less chances to cross the blood brain barrier. Many studies have revealed that

only 5-10% or very minimal amount of the bioavailable oximes reached to the brain which may be therapeutically not relevant (Petroianu et al. 2007c; Csermely et al. 2008; Kalasz et al 2008; Lorke et al. 2008c) and the antidotal action of oximes is on peripheral (Jokanovic 2009) and in addition to AChE reactivation, other physiological mechanism also involves which prefers hydrophilicity of oximes. However, Kalasz et al. 2009b reported that the drug levels reached the central nervous system as a proportion of the amount injected. One can speculate that hydrophilicity favors the less creation of more stable toxic phosphylated oximes. Then again, no correlation with paraoxon induced toxicity data with calculated Log*P* shows that surviving capability of the oximes is due to other than AChE reactivation by oximes and secondly structurally different OPCs behaves differently with different oximes as we noticed here with DFP and paraoxon. However, these conclusions are made only on the basis of structurally two different OPCs and need therefore to be validated in studies using other OPCs.

Chapter 6: Conclusion

The following conclusions may be drawn from the present undertaken 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 and may prove a broad spectrum oxime. But much more work is needed to establish a concrete conclusion.

- 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 studies on different aspects including mechanism, organ toxicity and other harmful effects if any, should be done before declaring the K-oximes as superior to all. Other structurally different OPC should be used to verify its equal efficacy against diversified organophosphorus AChE inhibitors.

- 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.

Chapter 7: Summary

There are diversified groups of organophosphorus compounds ranging from moderate toxic insecticides to deadly poison nerve agents but all have same mechanism of action that is inhibition of AChE. Oximes are the compounds used to reactivate the inhibited AChE. The present study was undertaken to evaluate the *in vivo* death preventing efficacy of eight new oximes (K-oximes). Paraoxon and diisopropylfluorophosphate were used as OP AChE inhibitor. The efficacy was compared with two therapeutically available oximes, pralidoxime and obidoxime whose efficacy has been controversial in clinical use. Intrinsic lethal effect of oximes in terms of LD_{50} and LD_{01} was also determined. Moreover, the *in vivo* results were compared with the *in vitro* findings of the same compounds to predict whether *in vitro* system is sufficient to translate the result for therapeutic use. Log*P* values of the oximes were also calculated by software.

The *in vivo* efficacy data shows that established oximes 2-PAM is poor in efficacy against both paraoxon and DFP. Obidoxime is good for DFP but not for paraoxon. Among the eight new K-oximes, K-027 was found to be the superior to all the tested oximes (established and experimental) against both paraoxon and DFP. The order of efficacy of the oximes against paraoxon was K-027 > K-048> K-075 > K-053 > K-074 > Obidoxime > K-107 > K108 > 2-PAM > K-113 and against DFP was K-027 > Obidoxime > K-053 > K-048 > K-075 > K-074 > 2-PAM > K-113. Paraoxon induced toxicity data did not reveal correlation with comparable *in vitro* parameters but DFP data revealed showed moderate to strong correlation, suggesting that the mechanism of action of oximes include some other physiological processes, in addition to AChE reactivation.

The calculated Log*P* result shows that all the tested oximes were hydrophilic, suggesting that oximes do not cross the blood brain barrier by simple passive diffusion. Conclusively, it is suggested that K-027 appears to be a promising oxime and may be a candidate for future clinical oxime. However, more study is needed to translate the work for human use.

Chapter 8 Publications

8.1 Publications related to thesis

1. **Nurulain SM**, Lorke DE, Hasan MY, Shafiullah M, Kuca K , Musilek K.(2009): Efficacy of eight experimental bispyridinium oximes against paraoxon induced mortality: Comparison with the conventional oximes pralidoxime and obidoxime. *Neurotox Res, **16(1):** 60-67.*

2. Lorke DE, Hasan MY, **Nurulain SM**, Kuca K, Schmitt A, Petroianu G.(2009): Efficacy of two new assymetrical bispyridinium oximes(K-27 and K-48) in rats exposed to diisopropylphosphate comparison with pralidoxime, obidoxime, trimedoxime, methoxime, and HI-6. *Toxicol Mech Methods,* **19(4):** 327-333.

3. Lorke DE, **Nurulain SM**, Hasan MY, Kuča K, Musilek K, Petroianu GA (2008): Eight new bispyridinium oximes in comparison with the conventional oximes pralidoxime and obidoxime: in vivo efficacy to protect from diisopropylfluorophosphate toxicity. *J Appl Toxicol,* **28(7):** 920-928.

4. Lorke DE, Hasan MY, **Nurulain SM**, Shafiullah M, Nagelkerke N, Petroianu GA. (2008): Effect of intrathecal pralidoxime administration upon survival of rats exposed to the organophosphate paraoxon. *Neurotoxicology,* **29(4):** 663-70.

5. Lorke DE, Hasan MY, **Nurulain SM**, Sheen R, Kuca K, Petroianu GA. (2007): Entry of two new asymmetric bispyridinium oximes (K-27 and K-48) into the rat brain: comparison with obidoxime. *J Appl Toxicol,* **27(5**): 482-90.

6. Petroianu GA, **Nurulain SM,** Nagelkerke N, Shafiullah M, Kassa J, Kuca K. (2007): Five oximes (K-27, K-48, obidoxime, HI-6 and trimedoxime) in comparison with pralidoxime: survival in rats exposed to methyl-paraoxon. *J Appl Toxicol,* **27(5**): 453-7.

7. Petroianu GA, Arafat K, **Nurulain SM**, Kuca K, Kassa J. (2007): In vitro oxime reactivation of red blood cell acetylcholinesterase inhibited by methyl-paraoxon. *J Appl Toxicol*, **27(2):** 168-75.

8. Petroianu GA, Lorke DE, Hasan MY, Adem A, Sheen R, **Nurulain SM**, Kalasz H. (2007): Paraoxon has only a minimal effect on pralidoxime brain concentration in rats. *J Appl Toxicol,* **27(4)**: 350-7.

9. Petroianu GA, **Nurulain SM,** Arafat K, Rajan S, Hasan MY. (2006): Effect of pyridostigmine, pralidoxime and their combination on survival and cholinesterase activity in rats exposed to the organophosphate paraoxon. *Arch Toxicol,* **80(11):** 777-84.

10. Petroianu GA, **Nurulain SM,** Nagelkerke N, Al-Sultan MA, Kuca K, Kassa J. (2006): Five oximes (K-27, K-33, K-48, BI-6 and methoxime) in comparison with pralidoxime: survival in rats exposed to the organophosphate paraoxon. *J Appl Toxicol,* **26(3):** 262-8.

8.2 Other Publications

1. Padmanabhan R, Abdulrazzaq YM Bastaki SMA, **Nurulain SM**, Shafiullah M. (2010): Vigabatrin(VGB) administered during late gestation lowers maternal folate concentration and causes pregnancy loss, fetal growth restriction and skeletal hypoplasia in the mouse. Reproductive Toxicol. [Epub ahead of print]

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