Pyridinium Oxime Reactivators of Cholinesterase Inhibited by Diisopropyl-Fluorophosphate (DFP): Predictive Value of *In-Vitro* Testing for *In-Vivo* Efficacy

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Abstract: Poisoning with organophosphorus cholinesterase inhibitors (OPCs) poses a serious global threat. Therapy comprises the use of atropine and pyridinium oximes to reactivate acetylcholinesterase (AChE). Clinical experience with established oximes (pralidoxime and obidoxime) is disappointing and several experimental potential alternatives (K oximes) have been developed.

This review summarizes data on these oximes, when used in exposure to the OPC diisopropylfluorophosphate (DFP). *In vitro* testing includes determination of IC_{50} (intrinsic oxime AChE inhibitory activity), of tan α (reactivation capacity) and in silico estimation of LogP (lipophilicity/hydrophilicity) of the individual oximes. *In vivo* approaches encompass determination of toxicity (LD_{50}) and of protective efficacy (reduction of relative risk of death after DFP exposure in rats).

Correlations between the different *in vitro* and *in vivo* data available reveal that an oxime with a low *in vitro* AChE inhibitory activity (high IC_{50}) is rather non-toxic and reduces DFP-induced mortality (low cumulative relative risk). Oximes with a high *in vitro* AChE reactivation potency (high tan α) also have a high *in vitro* AChE inhibitory activity (low IC_{50}) and have a low LD_{50} *in vivo*, implying high toxicity. Less hydrophilic oximes have strong *in vitro* AChE inhibitory activity, are better *in vitro* AChE reactivators, but are also more toxic *in vivo* and are associated with a high cumulative risk of death after DFP exposure in rats, implying low *in vivo* efficacy. *In vitro* reactivation capacity of human red blood cell (RBC)-AChE has no predictive value for *in vivo* (rat) efficacy, at least in the case of DFP exposure.

Key Words: organophosphate, aldoxime, DFP, cholinesterase, LogP, relative risk.

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POISONING WITH ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS (OPCS)

Organophosphorus esters (organophosphates and organophosphonates) are serine esterase and protease inhibitors widely used in agriculture as insecticides and acaricides, in industry and technology as softening agents and additives to lubricants, and some of them are declared as chemical warfare agents [1-4]. Sarin (GB) and VX have been involved in terrorist attacks in Japan, highlighting the major terrorist threat that these compounds constitute. The likelihood of the use of organophosphorus cholinesterase inhibitors (OPCs) by terrorist organizations is related to the relative ease of production of these substances, certainly within the means of even moderately sophisticated organizations [4].

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The inhibition of esterases (butyrylcholine: 3.1.1.8 and acetylcholine: 3.1.1.7) results from reacting covalently with the active centre serine, i.e. by phosphylation (phosphorylation or phosphonylation), and translates into an "endogenous acetylcholine poisoning" [2, 5].

The effects of poisoning with organophosphates and organophosphonates are well known and have been described extensively [2, 6-11]. The inhibition of cholinesterases causes accumulation of acetylcholine and results in an initial sympathomimetic response due to stimulation of nicotinic receptors in the adrenal medulla, followed by a longer-lasting parasympathomimetic response due to the stimulation of muscarinic synapses. Both responses can and must be controlled by appropriate medications [9]. In addition, organophosphates and organophosphonates cause an acetylcholine overflow at neuromuscular junctions with resultant depolarizing block, requiring artificial ventilation.

The number of yearly casualties due to accidental or intentional exposure to organophosphates and organophospho-

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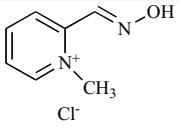
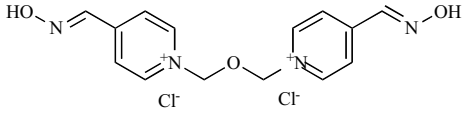
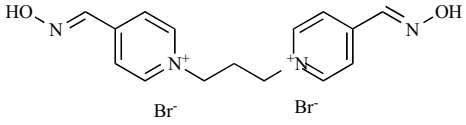
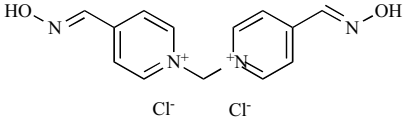
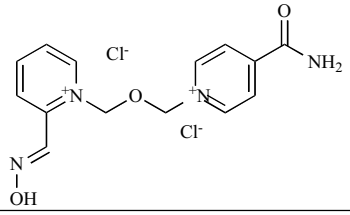
Oxime	Structure
Pralidoxime 2-PAM	
Obidoxime LueH-6	
Trimedoxime TMB-4	
Methoxime MMB-4, MMC-4	
HI-6	

Fig. (1). Chemical structure of ***established*** oxime reactivators of organophosphorus-inhibited cholinesterase. Pralidoxime (2-PAM) is a monopyridinium aldoxime with the functional group at position two. The other oximes are bisquaternary symmetric (obidoxime, trimedoxime and methoxime) or asymmetric (HI-6) pyridinium aldoximes with the functional aldoxime group at position two (HI-6) or four (obidoxime, trimedoxime and methoxime) of the pyridine ring(s).

nates is very high [12, 13]. Jeyaratnam estimated "that there may be 1 million serious unintentional poisonings each year and in addition 2 million people hospitalized for suicide attempts with pesticides". In his view "this necessarily reflects only a fraction of the real problem". On the basis of a survey of self-reported minor poisoning carried out in the Asian region, he estimated "that there could be as many as 25 million agricultural workers in the developing world suffering an episode of poisoning each year" [14].

The therapy of OPC poisoning is known by the catchy acronym **A FLOP** = **A**tropine, **FL**uids, **O**xygen, **P**ralidoxime [15]. The mnemonic is an oversimplification in as much as it does not include the GABA-agonists (benzodiazepines) used to control convulsions. A more comprehensive version would be **A FLOOD** = **A**tropine, **FL**uids, **O**xygen, **O**xime, **D**iazepam.

Oximes are the only enzyme reactivators clinically available [10, 16-18]. Pralidoxime (2-PAM) is the oxime used in the United States. 2-PAM is used as an adjunct to (but not as a substitute for) atropine in the treatment of poisoning by most cholinesterase inhibitors. Clinically while atropine relieves muscarinic signs and symptoms 2-PAM is supposed to shorten the duration of respiratory muscle paralysis by reactivation of cholinesterases [18, 19].

Clinical experience with 2-PAM (and other oximes) is, however, disappointing and its routine use has been questioned [12, 20-25]. In addition it is known that oximes are not or not equally effective against all available OPCs. There is a clear demand for "broad spectrum" cholinesterase reactivators with high efficacy.

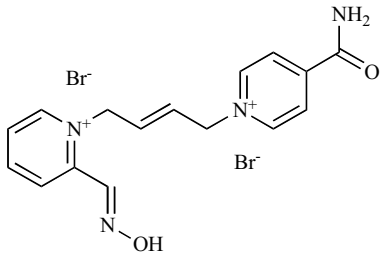
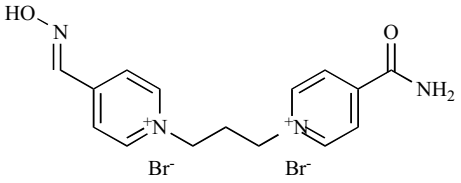
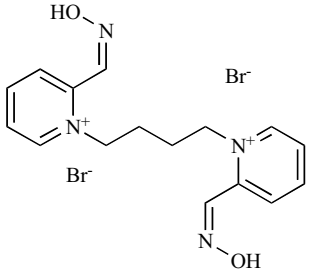
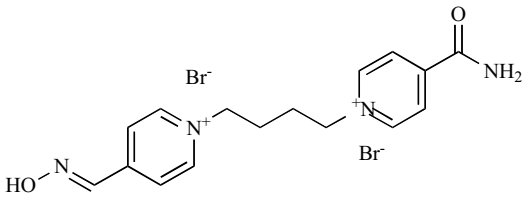
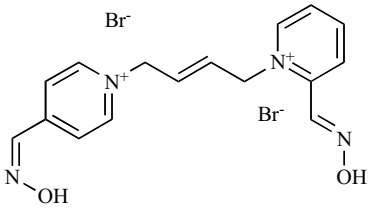
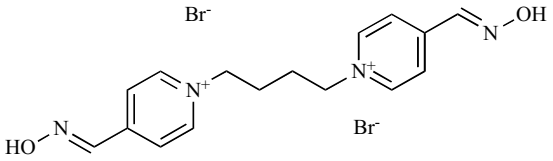
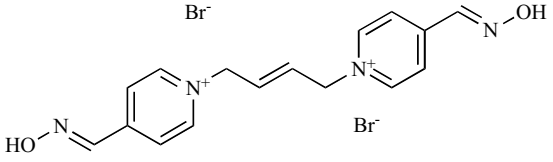
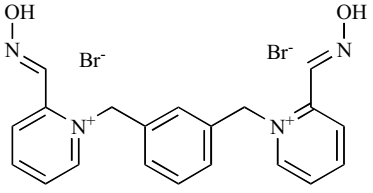
Over the years, different groups developed new potential reactivators of cholinesterase inhibited by OPCs.

At present, the most important oximes in antidotal treatment of OPC poisoning [26, 27] are 2-PAM [16, 19], obidoxime, trimedoxime, methoxime and HI-6 (Fig. **1**).

Trimedoxime (TMB-4) was first synthesized by Poziomek *et al*. in 1958. It was the first promising bisquaternary AChE reactivator. It has been introduced by several military forces, however, due to its toxicity it was mostly replaced by less toxic oximes such as obidoxime and methoxime [28, 29]. It is still used in auto-injectors available in Israel and elsewhere [30].

Methoxime (MMB-4; MMC-4) was synthesized and tested by Hobbiger and Sadler in the UK [31]. This reactivator is employed by the Czech Army to be used in case of nerve agent exposure.

Obidoxime, developed by Luettringhaus and Hagedorn in Germany was initially known by the acronym LueH-6. It is the oxime most commonly used in Continental Europe [32]. HI-6 is apparently the most efficacious of the H-series of oximes developed by Hagedorn (Ilse) but associated with stability issues making storage difficult [33, 34]. Apparently a product called Transant® containing HI-6 for transdermal application is available in Eastern Europe [35] (Fig. **1**).

Oxime	Structure
BI-6	
K-27	
K-33	
K-48	
K-53	
K-74	
K-75	
K-107	

(Fig. (2). Contd….)

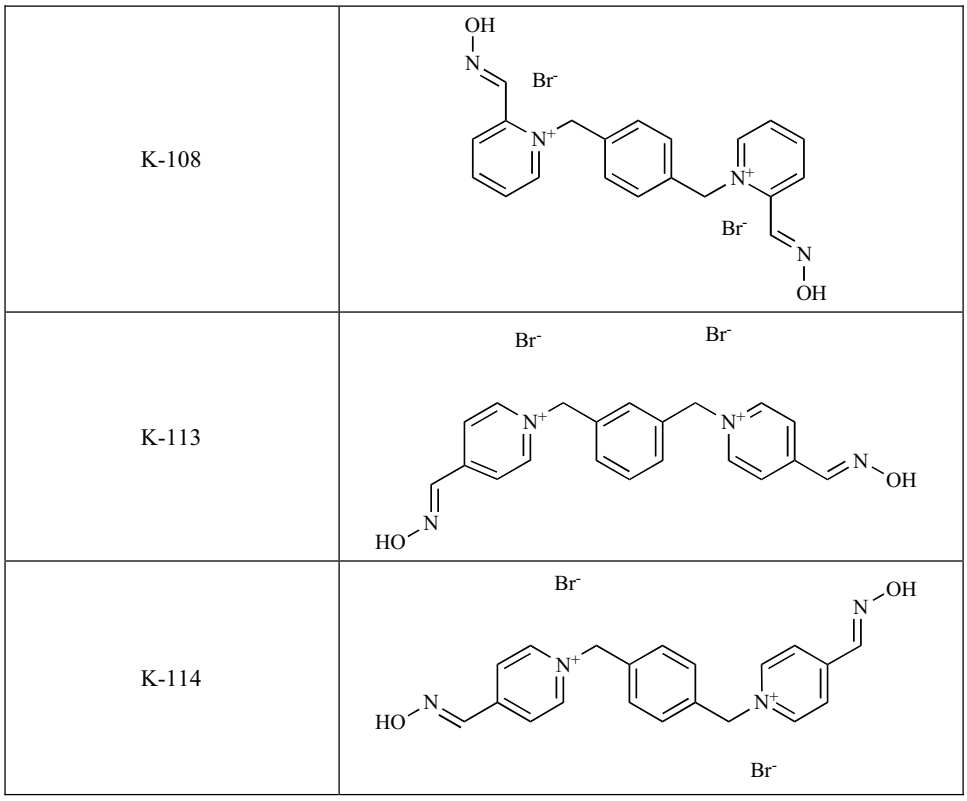


Fig. (2). Chemical structure of ***experimental*** oxime reactivators of organophosphorus-inhibited cholinesterase. From a chemical point of view, the newly developed oximes are bisquaternary symmetric (K-33, K-74, K-75, K-107, K-108, K-113, K-114) or asymmetric (BI-6, K-27, K-48, K-53) pyridinium aldoximes with the functional aldoxime group at position two (BI-6, K-33, K-107, K-108), four (K-27, K-48, K-74, K-75, K-113, K-114) or both (K-53) of the pyridine rings. K-107, K-108, K-113 and K-114 contain a xylene linker.

The K-series of reactivators were developed by Kuča and later by Musilek working in the Department of Toxicology at the Faculty of Military Health Sciences (University of Defense, Hradec Kralove, Czech Republic) at the same institution; K apparently stands for Kamil which is the first name of both Kuča and Musilek [2, 36-52] (Fig. **2**).

Their chemical structures were derived from the structures of existing esterase reactivators, especially 2-PAM, obidoxime (LuH-6) and HI-6. From a chemical point of view, the newly developed oximes are bisquaternary symmetric (K-33, K-74, K-75, K-107, K-108, K-113, K-114) or asymmetric (BI-6, K-27, K-48, K-53) pyridinium aldoximes with the functional aldoxime group at position two (BI-6, K-33, K-107, K-108), four (K-27, K-48, K-74, K-75, K-113, K-114) or both (K-53) of the pyridine rings.

DFP (Fig. **3**) was developed in the early 1940s by McCombie and Saunders in the United Kingdom, while performing research on warfare agents. McCombie and Saunders' work was based on earlier research by Willy Lange and Gerda von Krueger in Germany [53, 54]. This OPC, which is commonly used in research as a substitute for organophosphonates, is capable of inducing organophosphate-induced delayed neuropathy (OPIDN) in susceptible animals [55-59].

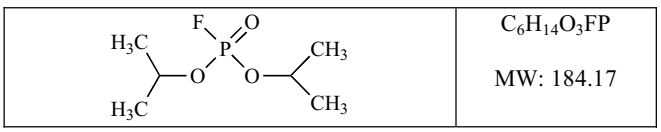


Fig. (3). Chemical structure of Diisopropyl-Fluorophosphate (DFP). The structural analog of sarin was developed in the 1940s by McCombie and Saunders in the United Kingdom [82].

Due to ethical reasons, testing of novel oxime compounds in humans is not possible, emphasizing the paramount importance of *in vitro* and animal *in vivo* testing. Because *in vivo* studies are time-consuming and associated with considerable stress for the animals, it would be desirable to have *in vitro* tests with good predictive value for *in vivo* efficacy in order to pick out the most promising oximes among the newly developed compounds. The present review summarizes *in vitro* data obtained using DFP and correlates them with the respective *in vivo* results.

IN VITRO PARAMETERS OF OXIME EFFICACY AND TOXICITY

In vitro testing (using either blood or brain tissue) centers around the ability of the oximes to increase esterase activity despite the presence of an OPC. The results are quantified using various approaches such as IC_{50} calculations, Schild plots and K determinations as well as tan α (IC_{50} shift) representations [47, 59]. In addition, physico-chemical properties of the compounds can be considered for predictive purposes [60] with lipophilicity/hydrophilicity having recently been emphasized [61].

RBC-AChE Activity

Human red blood cell (RBC) acetylcholine esterase (AChE) serves as an *in vitro* test system for cholinesterase activity. RBC AChE activity is measured in diluted whole blood samples in the presence of the selective butyrylcholinesterase inhibitor ethopropazine as previously described by Worek *et al*. [62]. The assay, which is based on Ellman's method, measures the reduction of Dithiobis-Nitrobenzoic Acid (DTNB) to nitrobenzoate (TNB^-) by thiocholine, the product of acetylthiocholine hydrolysis [63]. Values are normalized to the hemoglobin content [64].

IC_{50} value of DFP for RBC AChE**:** Using this *in vitro* assay, the DFP concentration required to inhibit RBC AChE activity by 50% (IC_{50}) is determined. After assessing enzyme activity in the absence of DFP, AChE activity is measured in the presence of different concentrations of the inhibitor DFP, which are added together with DTNB and ethopropazine before the incubation period, i.e. 20 min prior to addition of acetylthiocholine. Enzyme activities are corrected for oxime-induced thiocholine-ester cleaving activity [65], and the IC_{50} is determined using the SlideWrite™ (Advanced Graphics Software Inc, Encinitas, CA-USA) software, applying the user-defined equation $y=a_0/[1+(x/a_1)^{a2}]$, where a_1 corresponds to the IC_{50} value.

By this approach, an IC_{50} value of 118 ± 3 nM [95% confidence interval (CI): 111-127] has been obtained for DFP [59].

IC_{50} Value of Various Oximes for RBC AChE

Oximes given alone also have an inhibitory effect upon AChE activity (intrinsic AChE inhibitory activity). As for DFP, the IC_{50} value of each oxime can be determined measuring human RBC AChE activities in the absence of and then after addition of the respective oxime in different concentrations.

IC_{50} values of the various oximes are listed in Table **1**. The established oximes 2-PAM, obidoxime, trimedoxime and methoxime exhibit relatively high IC_{50} values (500 μM and above), implying that they hardly inhibit RBC AChE. Some K oximes (K-27, K-48) also show relatively high IC_{50} values (around 500 μM), whereas the IC_{50} of others (K-107, K-108, K-113, K-114) is relatively low (< 20 μM), indicating that the latter K oximes may be more toxic than the established ones.

IC_{50} Shift *In Vitro* in Human Blood

Oximes can reactivate AChE inhibited by an OPC; in a similar way they also protect AChE from inhibition by the OPC. This protective effect can be quantified in the RBC *in vitro* system: When oximes are administered together with DFP, higher DFP concentrations are necessary to obtain the same degree of enzyme inhibition: with increasing oxime concentration, the DFP IC_{50} value apparently rises. IC_{50} (DFP for RBC-AChE) is determined as described above in the absence of, and then in the presence of increasing oxime concentrations (range: 0-5 μmol/L, final concentration at the onset of incubation). If the IC_{50} does not change at these concentrations, higher dosages are added [59]. Concentration ranges tested for the different oximes are described in Table **1**. An example, showing the relationship between oxime (K-53) concentration and IC_{50} is depicted in Fig. **4**: the IC_{50} of DFP "shifts" in the presence of K-53 to higher values. The calculated IC_{50} values are plotted against the oxime concentrations to obtain an IC_{50} shift curve (equation: $y=a_0+ a_1 x$), where a_1 represents the slope (tangent: tan α) of the IC_{50} shift graph: $\mathbf{IC_{50} = IC_{50/DFP} + \tan \alpha * (Oxime\ concentration).}$ The tan α can be used to quantify the magnitude of the protective effect (nM IC_{50} increase per μM reactivator). The IC_{50} shift [tan α (nM/μM)], a parameter for *in vitro* reactivation potency which can be determined relatively easily, has no units.

Tan α values are low (< 2 nM/μM) for some of the established oximes (2-PAM, obidoxime, HI-6) and some K-type oximes (K-27), suggesting low reactivation capacity (Table **1**). Some of the new oximes (K-107, K-108, K-113, K-114) show values that are one order of magnitude higher, indicating distinctly better *in vitro* AChE reactivation.

Binding Constant K of Oximes for RBC-AChE

Another *in vitro* parameter employed to quantify the reactivation potency of oximes is the dissociation equilibrium constant K. It is derived from the Schild equation, which has originally been developed to analyse the interaction between agonists and antagonists [66, 67]. It can also be applied to describe the relation between oxime concentrations and OPC concentrations necessary to inhibit 50% of the enzyme (IC_{50}). However, in contrast to the tan α graph, the logarithm is used for graphical representation of the data in the Schild plot. Furthermore, instead of the IC_{50}, the dose ratio (DR) is depicted as a function of oxime concentration. The DR is defined as the IC_{50} of the inhibitor determined in the presence of a protective agent (in this case the oxime) divided by the IC_{50} of the inhibitor (DFP) for the enzyme studied in the absence of the oxime ($\mathbf{IC_{50/DFP}}$) [66, 67].

The Schild equation (**DR - 1 = oxime dose / K)** can be derived mathematically from the above-mentioned equation as follows:

$IC_{50} = IC_{50/DFP} + \tan \alpha * (Oxime\ concentration)$

$IC_{50} - IC_{50/DFP} = IC_{50/DFP} (IC_{50} / IC_{50/DFP} - 1) = \tan \alpha * (Oxime\ concentration)$

$(IC_{50} / IC_{50/DFP})$ is defined as the dose ratio (DR), hence:

$IC_{50/DFP} * (DR - 1) = \tan \alpha * (Oxime\ concentration)$

$(DR - 1) = \tan \alpha / IC_{50/DFP} * (Oxime\ concentration)$

If: $IC_{50/DFP} / \tan \alpha = K$ we obtain the Schild equation:

$(DR - 1) = (Oxime\ concentration)/K$

Similarly, it can be shown that K is the amount of the antagonist (oxime) that needs to be added so that double the quantity of the agonist (DFP) is necessary to attain the same effect ($\mathbf{IC_{50}}$), i.e. inhibition of 50% of the AChE activity.

If the performed measurements (IC_{50}) are depicted as log (DR -1) for the dependent variable and -log (oxime concentration) for the independent variable, the Schild plot allows the graphical estimation of the K value (Fig. **5**). Due to the linear relationship assumed between $\mathbf{IC_{50}}$ and oxime concentration, the slope of the graph is close to one, indicative of a competitive mechanism of interaction (between DFP and the oxime). The intercept of the graph with the x-axis (-log K) is ≈ 4.9 in the case of K-53 (Fig. **5**).

Table 1. Synopsis of the Data Used for Analysis. The second column gives the dose range of the oxime in which a linear relationship between oxime dose and IC_{50} of DFP was observed *in vitro*. The third column lists the intrinsic acetylcholinesterase (AChE) inhibitory activity of the oxime (IC_{50}), when administered (*in vitro)* alone, i.e. without organophosphate, to human red blood cell (RBC) acetylcholinesterase (AChE). Values represent means ± standard deviation, brackets: 95% confidence interval. Column four shows the values of the tangent of the angle α, formed by the graph of the IC_{50} shift with a horizontal line, which is an *in vitro* indicator for the reactivation capacity of an oxime. Column five lists the binding constant K, as determined by the Schild plot using the equation: *dose ratio - 1 = x/ K*. For mathematical reasons described earlier, the product of tan α and the binding constant K (column six) should be equal to the IC_{50} of the organophosphate used for inhibition (IC_{50} DFP ≈ 120 nM). Column seven gives the Log*P* of various oximes, column eight the intrinsic toxicity as quantified by LD_{50} while the final column (nine) gives the Risk Ratio of death (RR) over time when animals are treated for DFP exposure with the oxime indicated. Oximes were administered in an equitoxic dosage, i.e. half the LD_{01} [72]. * data from [59], ** data from [60], *** data from [72, 73]. n.a.: not determined

Oxime	Dosage Range Used *In Vitro* [μM] *	IC_{50} [μM] of Oxime for Human RBC AChE *	tan α [nM/μM] *	Binding Constant K [μM] *	tan α * K ≈ $IC_{50\ DFP}$*	Log*P* of Oxime **	LD_{50} of Oxime [μmol/kg Body Weight] ***	RR of Death Over Time (DFP Alone = 1) ***
Pralidoxime 2-PAM	0-100	592 ± 26 (535 - 650)	0.8 ± 0.05 (0.7 - 0.9)	158	126	-2.31	700	0.61 0.49 – 0.78
Obidoxime Lue H-6	0-100	702 ± 39 (606 - 798)	1.5 ± 0.07 (1.3 -1.7)	79	119	-3.12	493	0.23 0.17 -0.30
Trimedoxime TMB-4	0-10	652 ± 93 (413 - 891)	5.2 ± 0.4 (3.5 - 6.9)	36	187	-2.07	344	0.45 0.22 -0.68
Methoxime MMC-4	0-5	1514 ± 157 (1111-1917)	5.9 ± 0.3 (5.1 - 6.8)	25	147	-2.60	n.a.	0.26 0.08 -0.43
HI-6	0-80	310 ± 28 (239-381)	0.8 ± 0.06 (0.6 -1.0)	158	126	-3.39	n.a.	0.35 0.17 -0.54
BI-6	0-80	83 ± 5 (72 – 95)	0.7 ± 0.1 (0.5 - 0.8)	141	99	-2.81	480	n.a.
K-27	0-100	414 ± 28 (346-483)	0.9 ± 0.1 (0.8 - 1.0)	112	99	-2.66	1373	0.19 0.10 -0.28
K-33	0-20	21 ± 0.7 (20-23)	6.8 ± 0.2 (6.1 - 7.4)	20	136	-1.78	136	n.a.
K-48	0-100	461 ± 18 (417-505)	1.3 ± 0.1 (1 - 1.6)	100	130	-2.61	534	0.28 0.22 -0.37
K-53	0-10	115 ± 10 (88 – 141)	8 ± 0.5 (6.6 - 9.3)	13	112	-2.05	81	0.22 0.16 -0.27
K-74	0-10	103 ± 3 (94 – 111)	1.4 ± 0.3 (0.7 - 2.0)	47	66	-1.96	109	0.38 0.27 -0.49
K-75	0-10	63 ± 6 (46 -80)	7.3 ± 1 (4.5 -10)	21	159	-2.02	199	0.29 0.14 -0.44
K-107	0-5	6 ± 0.7 (4.4 - 7.9)	17 ± 1 (14 -19)	7	119	-0.66	6	0.97 0.67 -1.26
K-108	0-5	8 ± 0.3 (7.5 - 9)	20 ± 1 (17 – 23)	5	100	-0.66	3	0.91 0.62 -1.19
K-113	0-5	9 ± 0.6 (6-11)	16 ± 2 (11-21)	7	102	-0.87	15	0.73 0.55 -0.90
K-114	0-5	13 ± 0.9 (11-15)	14 ± 1 (12-15)	9	126	-0.67	n.a.	n.a.

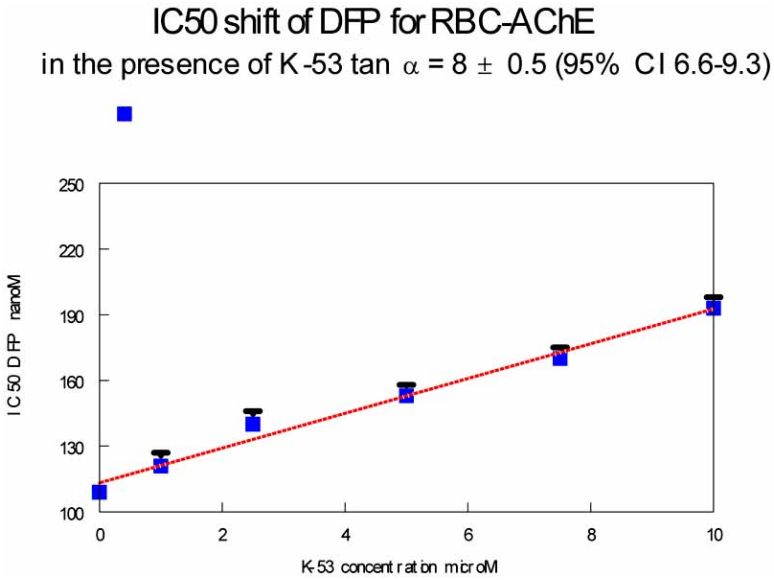


Fig. (4). IC_{50} shift of DFP for human red blood cell (RBC) acetylcholinesterase (AChE) in the presence of increasing concentrations of K-53: increasing the K-53 concentration from 0 to 10 μM increases the IC_{50} of DFP for RBC-AChE from 118 to 192 nM (Δ = 74 nM). The slope of the graph is tan $\alpha = 8 \pm 0.5$ (95% CI = 6.6 - 9.3).

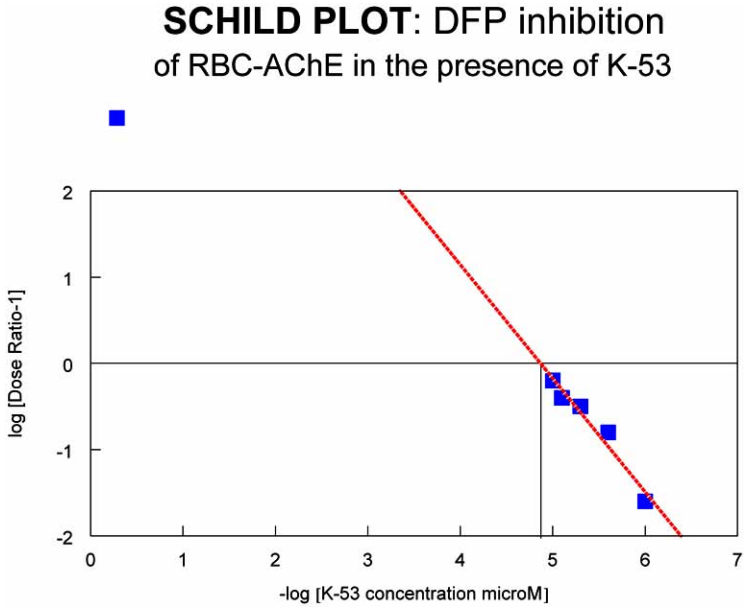


Fig. (5). Estimation of the dissociation equilibrium constant (binding constant) K of K-53 for human red blood cell (RBC) acetylcholinesterase (AChE) using the Schild equation and IC_{50} DFP shift data. According to the Schild equation ***K*** equals ***(Oxime concentration)/ (DR-1).*** DR, the dose ratio, is the IC_{50} of the inhibitor determined in the presence of a protective agent (in this case: K-53 oxime) divided by the IC_{50} of the inhibitor (DFP) for the enzyme studied. When using the Schild plot for graphical estimation of the K value, the y data are depicted as log (DR -1) and the x data are shown as -log (Oxime concentration).

The curve fit ($r^2 = 0.96$) is a line $y = a_0 + a_1 x$ where a_1 represents the slope of the line [a_1 = -1.3; 95% CI = -(0.8 to 1.8)]. The slope of the Schild plot (a_1) is close to one, indicative of a competitive mechanism of interaction (between DFP and the oxime). The intercept of the graph with the x-axis (-log K) is ≈ 4.9. Therefore the calculated dissociation equilibrium constant (binding constant) K of K-53 for red blood cell (RBC)-AChE in the presence of DFP is ≈ 13 μM. In case of a competitive mechanism (slope of the Schild plot $a_1 \approx 1$) the dissociation equilibrium constant (binding constant) K is equal to the inhibitory constant.

Considerable differences between various oximes have been detected (Table **1**), with K values [μM] ranging from 5-10 for K-107, K-108, K-113, K-114 to about 160 for 2-PAM and HI-6.

Due to the above mentioned equation [$\mathbf{IC_{50/DFP}}$ / **tan α = K**], the product of K and tan α is expected to be equal to $\mathbf{IC_{50/DFP}}$. For the great majority of oximes studied, the values obtained for K * tan α (around 120, Table **1**), come indeed very close to the $\mathbf{IC_{50/DFP}}$ value of 118 measured directly for DFP.

Because of this relationship between K and tan α, calculation of the K values of the respective oximes yields results comparable to those obtained for tan α, allowing essentially

the same conclusions, i.e. low reactivation capacity (K > 100) for some of the established oximes (2-PAM, HI-6) and K-27, and better AChE reactivation (K < 10) for K-107, K-108, K-113 and K-114 (Table **1**).

Log*P*

The concept of partition of substances between oil and water has apparently been introduced over a century ago by Berthelot [68]. The octanol-water partition coefficient [log*P*] has first been shown to yield correlation with biological activities by Hansch *et al*. [69] and Leo *et al* [70]. Log*P* values have been calculated using the PrologP module of the Pallas 3413 software (CompuDrug Inc., Sedona, AZ, USA) [60]. Details of the algorithm used for calculations are given by Molnar *et al*. [71]. 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 authors emphasize that their neural network-based method (pseudo-linear algorithms) combines the precision of non-linear approaches with the transparency of the early linear methods. 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.

All tested oximes are hydrophilic, as indicated by a negative log*P* value (Table **1**). The most hydrophilic substances are HI-6 and obidoxime (log*P* < -3), but also BI-6, K-27 and K-48 (log*P* <-2.5), whereas K-107, K-108, K-113 and K-114 are much less hydrophilic (log*P* > -1).

IN VIVO PARAMETERS OF OXIME EFFICACY AND TOXICITY

In vivo testing of oximes includes evaluation of their acute toxicity (LD_{50}) [72] and assessment of their *in vivo* efficacy to protect from mortality induced by DFP [72, 73].

LD_{50} Values of Oximes

Acute toxicity of the individual oximes has been evaluated in the step-wise fashion according to the Acute Toxic Class Method [72, 74, 75]. LD values and their 95% confidence limits were assessed in rats using logarithmical analysis of death occurring within 48 hours after i.p. injection of each oxime at different doses.

By far the highest LD_{50} value (LD_{50} > 1000 μMol/kg body weight) is observed for K-27. 2-PAM, obidoxime and K-48 are also relatively non-toxic (LD_{50} = 500- 1000 μMol/kg body weight). In contrast, LD_{50} values for K-107, K-108 are below 10 μMol/kg body weight, indicating much higher toxicity.

Relative Risks of Death (RR)

In vivo efficacy of oximes was quantitated by statistical analysis of the mortality data after DFP exposure in rats, as described [72, 73]. Oximes were administered in an equitoxic dosage, i.e. half the LD_{01} [72]. For each of the time points, the respective hazards ratios (relative risks of death) were estimated using Cox proportional hazards model [76]. Both DFP dose 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. No Bonferroni correction for multiple comparisons was applied and an $\alpha \leq 0.05$ was considered significant.

The most efficacious oxime was K-27, reducing the RR to below 20%. An RR reduction below 30% was observed for obidoxime, methoxime, K-48, K-53 and K-75, whereas K-107 and K-108 did not have a significant influence upon DFP-induced mortality (Table **1**).

CORRELATION ANALYSIS

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 described above have been correlated in a pair-wise manner. The nonparametric 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 [77]. No Bonferroni correction for multiple comparisons has been applied, and a statistical significance level α of 0.05 has been used. Only correlations with a rank correlation coefficient $|R| \geq 0.6$ have been considered.

IC_{50} of Oximes (*In Vitro*) Versus LD_{50} of Oximes (*In Vivo*)

The IC_{50} of the various oximes, which measures their intrinsic AChE inhibitory activity, is positively correlated with the respective LD_{50} values ($R = 0.78$, $p \leq 0.005$; Fig. **6**). This implies that an oxime with a low *in vitro* AChE inhibitory activity (high IC_{50}) is rather non-toxic *in vivo* (high LD_{50}). The IC_{50} is therefore a relatively good *in vitro* predictor for the *in vivo* toxicity of an oxime.

IC_{50} Versus Cumulative Relative Risk (RR)

The IC_{50} of the various oximes, which measures their intrinsic AChE inhibitory activity, is negatively correlated ($R = -0.66$, $p \leq 0.05$; Fig. **7**) with their ability to reduce mortality in the presence of DFP (cumulative RR). 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. This is most likely due to the fact that a low IC_{50} is associated with low toxicity, allowing to administer the oxime in a higher dosage, thereby counteracting DFP-induced AChE inhibition more efficiently [72].

Tan α (*In Vitro*) Versus IC_{50} of Oximes (*In Vitro*)

Tan α values of the respective oximes, representing an assumed indicator of *in vitro* AChE reactivation potency, are negatively correlated with the IC_{50} of the various oximes, which measures their intrinsic AChE inhibitory activity ($R = -0.65$, $p \leq 0.01$; Fig. **8**). This implies that an oxime with a high *in vitro* AChE reactivation potency (high tan α) has a high *in vitro* AChE inhibitory activity (low IC_{50}). Good *in vitro* reactivation is therefore associated with high intrinsic AChE inhibition, implying high toxicity.

Tan α (*In Vitro*) Versus LD_{50} of Oximes (*In Vivo*)

Tan α values of the respective oximes, representing an assumed indicator of *in vitro* AChE reactivation potency, are negatively correlated with the LD_{50} of the oxime ($R = -0.87$, $p \leq 0.001$; Fig. **9**), implying that oximes with good *in vitro* reactivation capacity (high tan α), have a low LD_{50} *in vivo*,

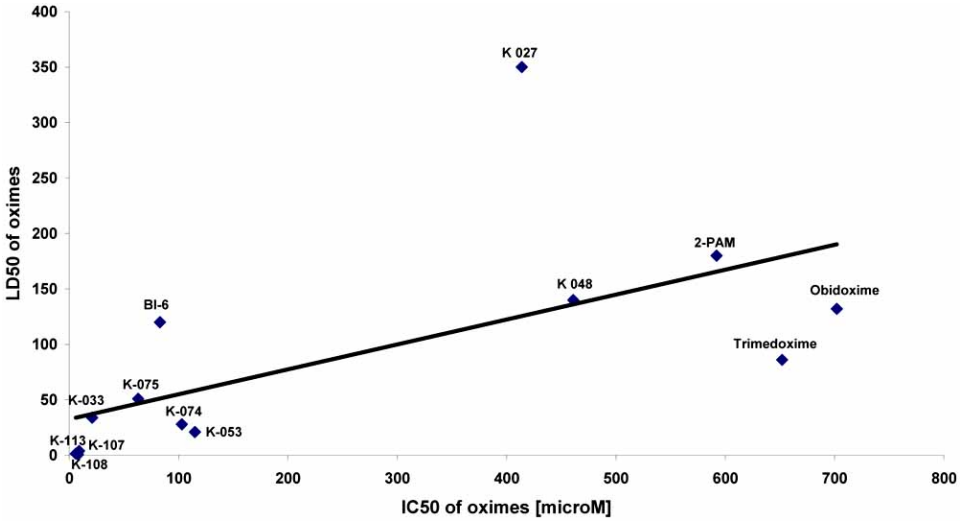


Fig. (6). Scatter plot of LD_{50} versus IC_{50} of oximes with least squares linear regression line.

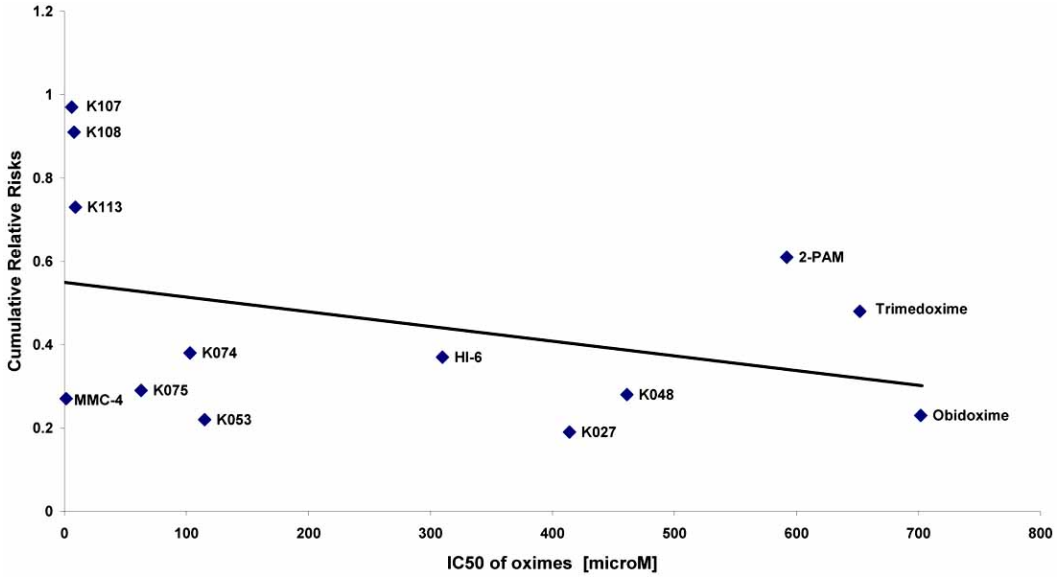


Fig. (7). Scatter plot of cumulative relative risk after DFP exposure versus IC_{50} of oximes with least squares linear regression line.

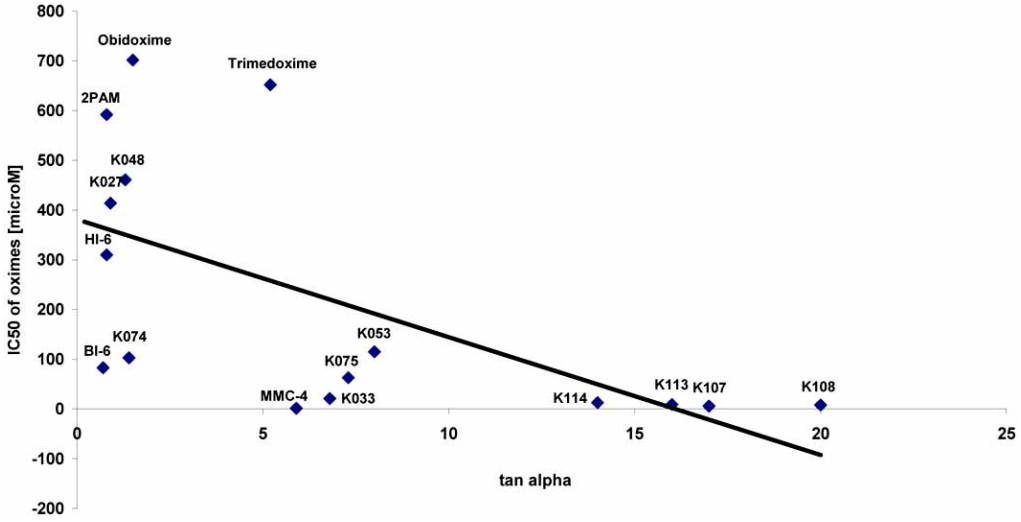


Fig. (8). Scatter plot of IC_{50} versus tan α of oximes with least squares linear regression line.

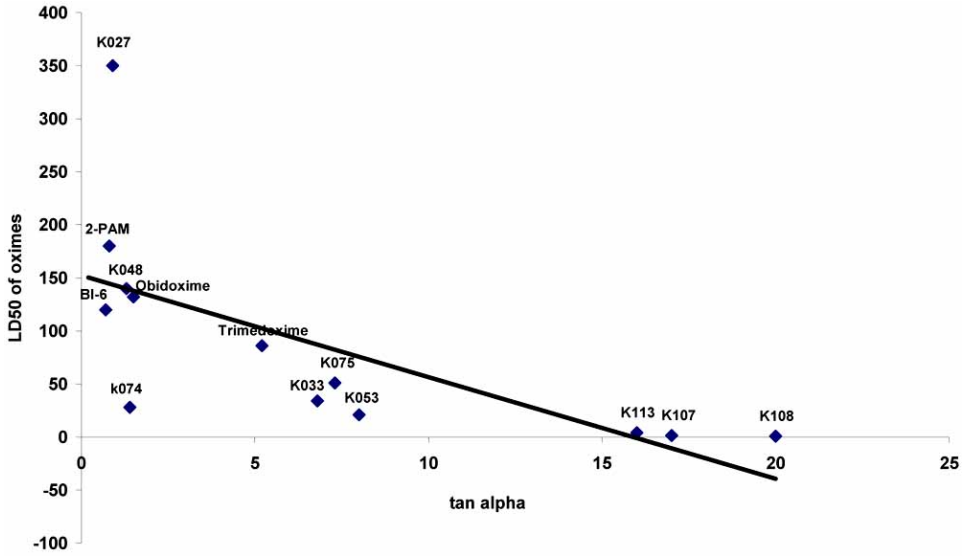


Fig. (9). Scatter plot of LD_{50} versus tan α of oximes with least squares linear regression line.

implying high toxicity. This observation is consistent with the correlation between tan α values and IC_{50} as well as with the correlation between IC_{50} and LD_{50} values.

LogP (*In Vitro*) Versus IC_{50} of Oximes (*In Vitro*)

LogP values of the respective oximes, representing an indicator of their lipophilicity, are negatively correlated with their IC_{50} ($R = -0.77$, $p \leq 0.001$; Fig. **10**), implying that oximes with high LogP values (less hydrophilic) have strong *in vitro* AChE inhibitory activity (low IC_{50}). Less hydrophilic oximes thus more strongly inhibit RBC AChE.

LogP (*in Vitro*) Versus Tan α (*In Vitro*)

LogP values of the respective oximes, representing an indicator of their lipophilicity, are positively correlated with their tan α values ($R = 0.85$, $p \leq 0.001$; Fig. **11**), implying that oximes with high LogP values (less hydrophilic) have good *in vitro* reactivation capacity (high tan α). Less hydrophilic oximes are thus better *in vitro* RBC AChE reactivators.

LogP (*In Vitro*) Versus LD_{50} of Oximes (*In Vivo*)

LogP values of the respective oximes, representing an indicator of their lipophilicity, are negatively correlated with their LD_{50} values ($R = -0.85$, $p \leq 0.001$; Fig. **12**), implying that oximes with high LogP values (less hydrophilic) have high *in vivo* toxicity (low LD_{50}). Less hydrophilic substances are thus more toxic *in vivo*. This observation is consistent with the correlation between LogP and IC_{50} as well as with the correlation between IC_{50} and LD_{50} values.

LogP (*In Vitro*) Versus Cumulative Relative Risk of Death (*In Vivo*)

LogP values of the respective oximes, representing an indicator of their lipophilicity, are positively correlated with the cumulative RR ($R = 0.69$, $p \leq 0.01$; Fig. **13**). Oximes with high LogP values (less hydrophilic) are therefore asso-

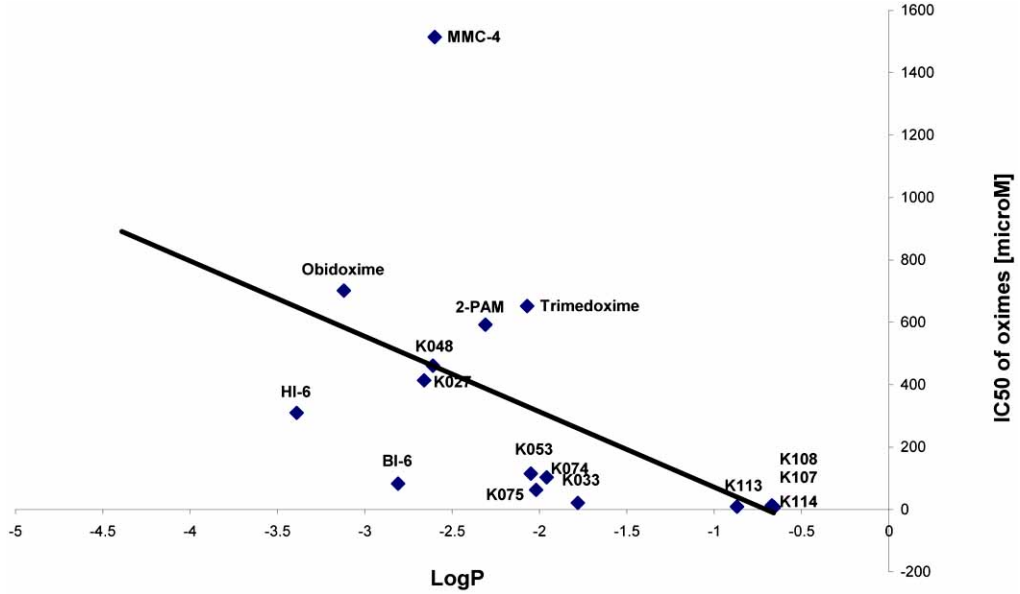


Fig. (10). Scatter plot of IC_{50} versus LogP of oximes with least squares linear regression line.

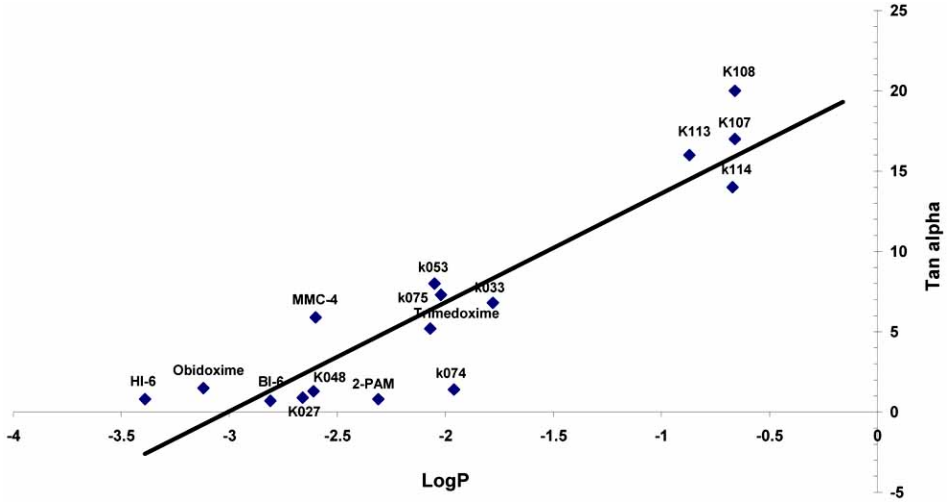


Fig. (11). Scatter plot of tan α versus LogP of oximes with least squares linear regression line.

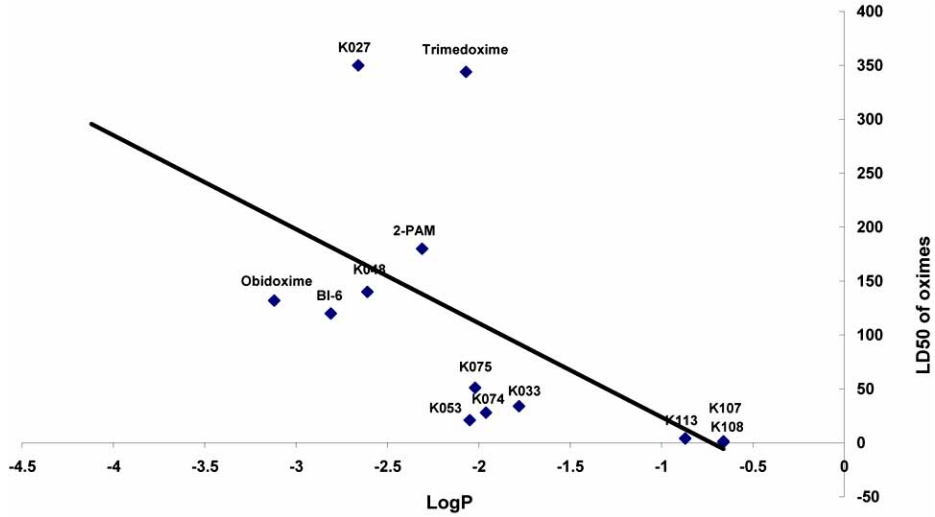


Fig. (12). Scatter plot of LD_{50} of oximes versus their LogP value with least squares linear regression line.

ciated with a high cumulative relative risk of death after DFP exposure, implying low *in vivo* efficacy.

Tan α (*In Vitro*) and Cumulative Relative Risk of Death (*In Vivo*)

No correlation was observed between the tan α value (*in vitro*: human RBCs) and the cumulative relative risk of death (*in vivo: rats*) (R = 0.39, p = 0.18; Fig. **14**). This implies that, at least in the case of DFP exposure, *in vitro* reactivation capacity of human RBC-AChE has no predictive value for *in vivo* efficacy in rats.

Considering the results presented above (Table **2**), the pivotal predictive ability of LogP becomes evident. All the reactivators (various oximes) structurally share a common bispyridinium backbone. Variations in LogP values thus truly reflect the influence of the variables (number and position of the aldoxime group(s) and length and composition of the linking bridge between the pyridinium rings) on the physical, chemical and biological properties of the respective compounds. The correlations derived can be summarized as shown in Fig. **15**.

A low hydrophilicity/high lipophilicity translates *in vitro* (outer yellow triangle) into a superior ability to reactivate red blood cell AChE as evidenced by the high tan α value (which is linearly inversely related to the dissociation constant *K*) and in a higher intrinsic AChE inhibitory effect of oximes (low IC_{50} value). At the same time, a low hydrophilicity/high lipophilicity translates *in vivo* (inner blue triangle) into a high intrinsic toxicity (low LD_{50} value) and most importantly in a low survival (high cumulative relative risk). While some of the correlations are post hoc intuitively obvious others are less so.

One implicit assumption is that *in vitro* activity translates into *in vivo* enzyme protection and therefore increase in survival. As such, tan α (or the dissociation constant *K*) was expected to inversely correlate with the risk ratio; we have been unable to demonstrate such a relationship, when using DFP as an OPC. In other words: in the RBC AChE model used, *in vitro* reactivation ability (human blood) does not translate into *in vivo* efficacy (rat survival). Several explanations for this unexpected observation are conceivable.

The lack of correlation may be related to the fact that *in vitro* experiments were performed using human material,

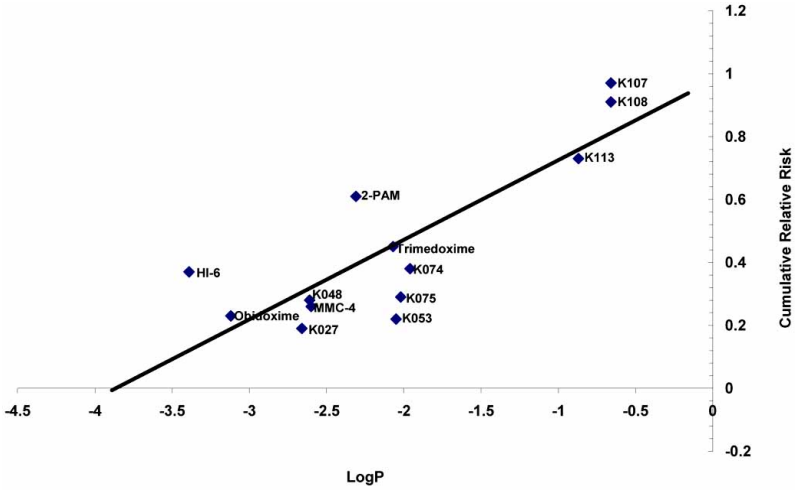


Fig. (13). Scatter plot of Cumulative Relative Risk after DFP exposure of death versus LogP of oximes with least squares linear regression line.

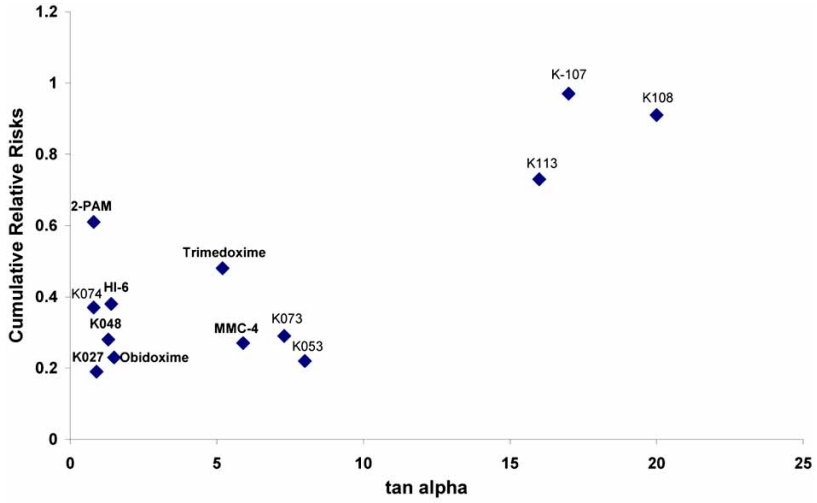


Fig. (14). Scatter plot of Cumulative relative risk of death after DFP exposure versus tan α of oximes.

Table 2. **Synopsis of the Correlation Results.** The nonparametric 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 [77]. No Bonferroni correction for multiple comparisons has been applied, and a statistical significance level α of 0.05 has been used. Only correlations with a rank correlation coefficient $|R| \geq 0.5$ were considered. 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.

Variable Pairs	Spearman Correlation	Statistical Significance (Two Tailed)
IC_{50} of oximes (*in vitro*) versus LD_{50} of oximes (*in vivo*)	0.78	0.002
IC_{50} of oximes (*in vitro*) versus Cumulative relative risk of death (*in vivo*)	-0.66	0.029
Tan alpha (*in vitro*) versus IC_{50} of oximes (*in vitro*)	-0.65	0.006
Tan alpha (*in vitro*) versus LD_{50} of oximes (*in vivo*)	-0.87	≤0.001
Tan alpha (*in vitro*) versus Cumulative relative risk of death (*in vivo*)	0.39	0.184
LogP (*in vitro*) versus IC_{50} of oximes (*in vitro*)	-0.77	0.000
LogP (*in vitro*) versus Tan alpha (*in vitro*)	0.85	≤ 0.001
LogP (*in vitro*) versus LD_{50} of oximes (*in vivo*)	-0.85	≤ 0.001
LogP (*in vitro*) versus Cumulative Relative Risk of death (*in vivo*)	0.69	0.009

while the *in vivo* experiments were done on rodents. Species differences in AChE expression, inhibition by OPC and reactivation by oximes may explain the observed lack of correlation [12]. Another explanation may be the formation of phosphylated oximes. It is well known that oximes, while reactivating the enzyme, become phosphylated (phosphorylated or phosphonylated) themselves [78-80]. These phosphylated oximes are potent AChE inhibitors, sometimes being even more potent than the initial phosphylating agent [78]. A plasma enzyme destroys these phosphylated oximes. It has been shown that this enzyme reacts very little with phosphoryl oxime from DFP [80] and it may therefore well be that phosphylated oximes are relatively stable after DFP exposure and able to re-inhibit AChE. Given the observation that this phosphylated oxime-catalyzing enzyme loses its catalytic activity in the presence of EDTA [80], which is added to the *in vitro* RBC AChE preparation [59], it appears likely that the stability of phosphylated oximes is different *in vitro* from *in vivo*, thus contributing to the discrepancy between *in vivo* and *in vitro* results.

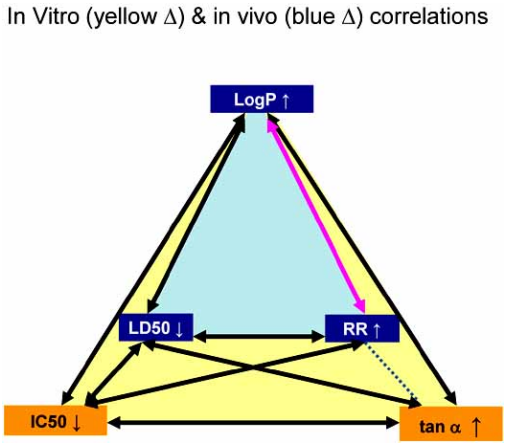


Fig. (15). Graphical representation of the different correlations observed: outer (yellow) triangle represents correlations between *in vitro* parameters, inner (blue) triangle represents correlations with *in vivo*-derived data. Arrows depict established correlations; dotted line depicts lack of significant correlation. The clinically most important correlation (LogP <–> RR) is highlighted in magenta.

The most likely reason for the lack of correlation observed between *in vitro* reactivation capacity and *in vivo* efficacy is the higher toxicity of oximes with good *in vitro* reactivation capacity. Toxicity of efficacious oximes is reflected by the correlation between *in vitro* reactivation (tan α) and both *in vitro* AChE inhibitory activity (IC_{50}) and LD_{50}. Due to their high 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 [72].

The second implicit assumption is related to lipophilicity and capacity to increase survival after OPC intoxication: it is generally believed that substances crossing the blood brain barrier are more efficient in preventing death in acute severe OPC poisoning [81]. Because less hydrophilic oximes are more likely to gain access to the central nervous system [61], thereby achieving higher brain tissue concentrations, less hydrophilic oximes should be in a better position to reactivate brain esterase and should thereby increase survival. The reviewed data indicate that in fact the opposite is true: fat solubility correlates with both high intrinsic toxicity (low LD_{50}) and reduced survival (high risk ratio).

Based on the available data for DFP, the most hydrophilic compounds have the lowest intrinsic toxicity both *in vitro* (high IC_{50}) and *in vivo* (high LD_{50}) and are the most efficacious in increasing survival (low risk ratio). Again one can only speculate that hydrophilicity favors less the creation of more stable toxic phosphylated oximes.

These conclusions are, however, based only on data derived from experiments with DFP and need therefore to be validated in studies using other OPCs.

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