A COMPLEX PROCESS OF THE DEVELOPMENT OF NEW ACETYLCHOLINESTERASE REACTIVATORS - FROM PREDICTION TO IN VIVO EVALUATION

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ABSTRACT
The mechanism of intoxication with organophosphorus compounds, including highly toxic nerve agents, is based on the irreversible inhibition of acetylcholinesterase that is followed by an accumulation of acetylcholine at peripheral and central cholinergic synapses, which in turn leads to the clinical manifestation of various signs and symptoms summarized as acute cholinergic crisis. Nerve agent poisoning is commonly treated using a combination of a cholinolytic drug to counteract the accumulation of acetylcholine at muscarinic receptors and acetylcholinesterase reactivators (pralidoxime or obidoxime) to reactivate nerve agent-inhibited acetylcholinesterase.
There is a strong interest in developing new, more potent acetylcholinesterase reactivators. The development of acetylcholinesterase reactivators consists of several steps: description of nerve agent intoxication mechanism on molecular basis (molecular design), prediction of biologically active structure of acetylcholinesterase reactivators (artificial neural networks), their synthesis, *in vitro* and *in vivo* evaluation of their potency to counteract acute toxicity of nerve agents.

INTRODUCTION
Organophosphorous (OP) compounds are ubiquitous as pesticides, in veterinary medicine and in industry. The highly toxic OP compounds, called nerve agents, were developed, produced and weaponized as chemical warfare agents and, in addition, they have been misused by terrorists in two Japanese cities Matsumoto (1994) and Tokyo (1995) (Bajgar, 2004). OP compounds irreversibly inhibit the enzyme acetylcholinesterase (AChE, EC 3.1.1.7), which is responsible for terminating the neurotransmitter action of acetylcholine (ACh) at various cholinergic nerve endings. Irreversible AChE inhibition results in the accumulation of ACh at cholinergic receptor sites, producing continuous stimulation of cholinergic fibres throughout the central and peripheral nervous systems (Marrs, 1993).

Currently , a combination of an antimuscarinic agent (preferably atropine), and an AChE reactivator (called an oxime according to its chemical structure) is recommended for the treatment of OP poisoning (Bajgar, 2004). Atropine blocks the effects of accumulated ACh-induced overstimulation of peripheral muscarinic receptor sites, whereas reactivators repair the biochemical lesion by dephosphorylating the enzymatic molecule, AChE, and restoring its

activity (Kassa, 2002). Unfortunately, currently available oximes (pralidoxime, obidoxime and HI-6), used in clinical toxicology to counteract the acute effects of OP pesticides and introduced into some armies to counteract the acute effects of nerve agents, have been shown to be rather ineffective against certain nerve agents, especially soman, cyclosarin and tabun (Cabal et al., 2004; Kassa, 2002). As a result, the development of new, more potent AChE reactivators with sufficient efficacy to reactivate phosphonylated or phosphorylated AChE and thereby decrease the acute toxicity of OP compounds regardless of their chemical structure is still a very important task for toxicological research institutes throughout the world. Ideally, the process of the development of oximes should be as effective and quick as possible. Our Department of Toxicology has tried to optimize this process and the final structure of this process is described in this work.

THE PROCESS OF THE DEVELOPMENT OF NEW AChE REACTIVATORS

The entire developmental process consists of five phases

- prediction of new AChE reactivator structures using artificial neural networks (ANN)
- description of the reactivation process using molecular design
- synthesis of new AChE reactivators
- *in vitro* testing, and
- *in vivo* testing.

A short description of individual developmental steps is shown in Figure 1 and described below.

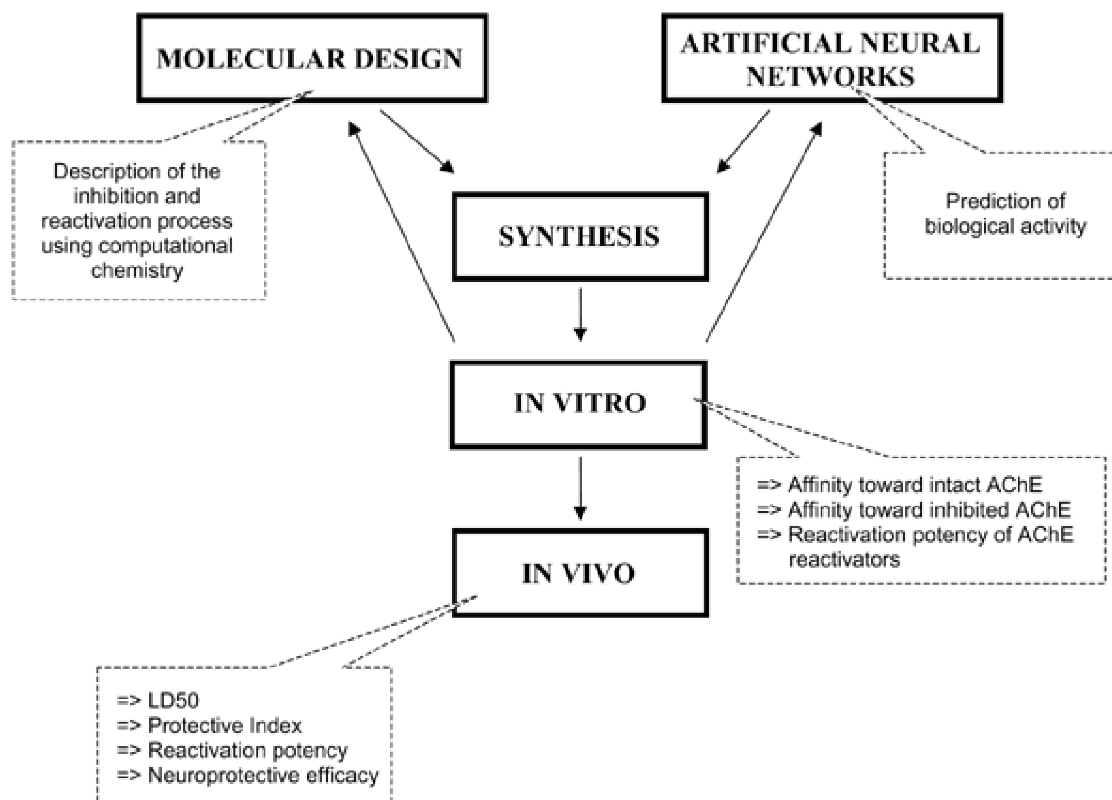

Figure 1. Description of the developmental process

Prediction using artificial neural networks
The first step of the developmental process is to use artificial neural networks (ANN) to predict AChE behavior. Using known biological activities of different substances, there is the possibility to “learn” how the ANN works without detailed knowledge of the exact interaction between a compound and an organism. Chemometric methods are able to estimate biological activity of chemical compounds. These are combined using calculations and are used to estimate the biological activities of potential antidotes without actually synthesizing them. In our development process, ANN are used for the prediction of the appropriate structure of new AChE reactivators. Biological activity and structure of currently used AChE reactivators are used as an input data set. Then, the model of relationships between chemical structure and biological activity is calculated. Afterwards, we are able to predict new, more potent reactivators of AChE inhibited by nerve agents based on these models (Dohnal and Kuca, 2004). *Editors note:* Another article describing the use of ANN and QSAR to AChE reactivators is "Structural bioinformatics and QSAR analysis applied to the acetylcholinesterase and bispyridinium aldoximes" by P.P. Mager and A. Weber in Drug Des Discov. 2003;18(4):127-50.

Molecular design
Molecular modeling is used for a study of AChE conformational changes caused by substances such as OP compounds (nerve agents, pesticides). This study is performed using molecular dynamics that calculate intramolecular energies of modified residues. Reconformational changes in AChE structure caused by reactivators are then examined. The influence of these substances towards the enzyme is evaluated on the basis of known structures and by docking method and subsequent molecular design simulations. The acquired description of interactions and their quantification obtained from interaction energies of model systems serve as proposition for new, more potent AChE reactivators (Wiesner et al., 2005).

Synthesis of new AChE reactivators
All the promising AChE reactivators predicted by ANN and molecular design methods are synthesized using special methods developed especially for the synthesis of currently used AChE reactivators. Over the last three years, we have synthesized more than twenty AChE reactivators (Kuca et al., 2003a; 2003b; 2004a; 2004b). All of these synthesized substances are mono or bis quaternary pyridinium rings, connected mostly with three or four membered linkage chains. In all their structures, the oxime group is functioning as a nucleophile able to split the bond between enzyme and inhibitor. The oxime group is usually located in the position two or four on the pyridinium ring.

In vitro testing
The reactivation potency of each synthesized AChE reactivator is first evaluated using *in vitro* experiments, with the help of potentiometric method of the measurements of AChE activity (Kuca and Kassa, 2003). The homogenates from rat, pig and human brains are used as a source of AChE, although commercially available pure enzymes can also be used. The protein in AChE homogenate (0.5 mL) is mixed with isopropanol solution of nerve agent to achieve 95% AChE inhibition and incubated at 25 °C for 30 min. After incubation, 2.5 mL of 3 M NaCl and distilled water are added to a final volume of 23 mL. Then, 2 mL of 0.02 M acetylcholine iodide is added and the enzyme activity is assayed. The activities of intact (a_o) and nerve agent-inhibited (a_i) AChE are estimated. After the nerve agent-inhibited AChE is incubated for 10 min with a solution of an oxime reactivator, the activity of the reactivated AChE (a_r) is obtained. The activity values a_0, a_i and a_r, are calculated from the slopes of initial

parts of titration curves. Each value is the arithmetic mean of two independent measurements. The kinetics of the reactivation process may be represented by the scheme:

where EI is the nerve agent-inhibited enzyme, R is the reactivator, E is the reactivated enzyme, EIR is the intermediate complex, and P is the product, usually a phosphonylated, unstable oxime. K_R and k_R are the dissociation constants and the rate constants for decomposition of the intermediate complex, respectively. For all the oximes whose reactivation abilities are screened, the percentage of reactivation (% R) is calculated from equation

$$\%\mathrm{R} = \left[1 - \frac{a_0 - a_r}{a_0 - a_i}\right].100$$

The percentage of reactivation of currently used reactivators is measured in the range of oxime concentrations from 10^{-7} to 10^{-2} M. Kinetic constants of reactivation are obtained from the equation

$$k_{app} = k_0 + k_R \frac{c_R}{K_R + c_R}.$$

where

$$k_{app} = -\frac{1}{t}\ln\frac{a_0 - a_r}{a_0 - a_i}$$

The dissociation constant of the oxime-reactivator complex with inactive, i.e. non-phosphonylated AChE (K_R), is obtained from the equation.

$$a_i = \frac{a_0 c_s}{c_s + K_M(1 + \frac{c_R}{K_R})}$$

using a non-linear regression analysis of a_i on c_R, where a_o is enzymatic activity of non-nerve agent-inhibited AChE, a_i is enzyme activity of nerve agent-inhibited AChE, c_s is concentration of substrate, c_R is concentration of oxime and K_M is Michaelis constant for acetylcholine as substrate, equal 0.19 mM. Second-order rate constants of reactivation (k_r), which represent overall reactivation ability, are calculated from the equation (Patocka and Bielavsky, 1975)

$$k_r = k_R / K_R$$

Results of *in vitro* experiments are used as input data for ANN prediction and the most potent AChE reactivators are selected for *in vivo* experiments.

In vivo testing

Before starting *in vivo* evaluation of the reactivating ability and therapeutical potency of the chosen (and newly synthesized) AChE reactivators, their toxicity is evaluated by determining the median LD_{50} in mice and rats. The LD_{50} values and their 95% confidence limits are assessed using probit-logarithmical analysis of death occuring within 24 h after i.m. administration of AChE reactivators at five different doses with six mice per dose (Tallarida and Murray, 1987). After the LD_{50} is established, the therapeutic efficacies of AChE reactivators against nerve agents are assessed by the evaluation of their ability to prevent lethality. The efficacy of tested AChE reactivators is expressed as protective ratio (LD_{50} value of nerve agent in protected animals/ LD_{50} value of nerve agent in unprotected animals).

The reactivating efficacy of oximes is measured by the calculation of percentage of reactivation of nerve agent-inhibited AChE, free AChE, in peripheral (mostly blood and diaphragm) and central (central nervous system) compartments in rats by a spectrophotometric method (Ellman et al., 1961). The reactivation rate is calculated using the AChE activity values: 1- [((oxime + atropine) – (saline))/((atropine control) – (saline))] x 100 (Clement et al., 1992). The AChE activity is expressed as a percentage of control (no nerve agent present) values.

Finally, the potency of the oximes to eliminate signs of neurotoxicity (neuroprotective efficacy) is evaluated using Functional observational battery (FOB) consisting of more than 40 measurements of sensory, motor and autonomic nervous functions. Some of these are relative scores, the others are measured in absolute units (Frantik and Hornychova, 1995; Hornychova et al., 1995; Moser et al., 1997; Kassa and Krejcova, 2003; Krejcova and Kassa, 2004). Scored parameters are shown in Table 1. The sequence of evaluations and observations in the FOB are as described. The first evaluation is obtained when the nerve agent-poisoned rats are in the home cage. The observer evaluates each animal`s posture, palpebral closure and involuntary motor movements. Then, each rat is removed from the home cage and briefly held in the hand. The exploratory activity, piloerection and other skin abnormalities are noted, too. Salivation and nose secretion are also registered and scored. Then, the rats are placed on a flat surface, which serves as an open field. A timer is started for three minutes during which the frequency of rearing responses is recorded. At the same time, gait characteristics are noted and ranked and arousal, stereotypy and bizarre behaviors and abnormal posture are evaluated. At the end of the third minute, the number of fecal boluses and urine pools on the absorbent pad is registered. A reflex testing consisting of recording each rat`s response to the frontal approach of the blunt end of a pen, a touch of the pen to the posterior flank and an auditory clic stimulus is also used. The responsiveness to a pinch on the tail and the ability of pupils to constrict in response to light are then assessed. These measures are followed by a test for the aerial righting reflex and by the measurements of forelimb and hindlimb grip strength, body weight, rectal temperature and finally hindlimb landing foot splay. The whole battery of tests requires approximately 6-8 minutes per rat. The observer of behavior does not know the experimental design.

After the FOB, the motor activity data are collected, using an apparatus for testing of a spontaneous motor activity of laboratory animals (constructed in the Faculty of Military Health Sciences, Hradec Kralove, Czech Republic). The animals are placed for a short period (10 minutes) in the measuring cage and their movements (total, horizontal and vertical activity) are recorded.

Data collected with the FOB and motor activity assessment include categorial, ordinal and continuous values. Statistical analyses of the results are performed on a PC with a special interactive programme NTX (Frantik and Hornychová, 1995). The categorial and ordinal values are formulated as contingency tables and judged consecutively by Chi-squared test of homogeneity, Concordance-Discordance test and Kruskal-Wallis test, respectively. The continual data are assessed by successive statistical tests: CI for Delta, Barlett test for Equality of Variance, Williams test and Test for Distribution Functions (Roth et al., 1962). The differences are considered significant when $P < 0.05$.

RESULTS

This developmental process is commonly used at our Department of Toxicology to evaluate the reactivating effectiveness of newly developed oximes as well as currently available oximes against nerve agents. There are several examples.

Kinetic parameters (dissociation constant: K_{dis} and K_R; rate constants: k_R and k_r) shown in Table 2 characterize the ability of new oximes to reactivate tabun-inhibited AChE *in vitro.* The values of the constant K_R characterizing the affinity of oximes to tabun-inhibited AChE indicate that the affinity of obidoxime to the enzyme-inhibitor complex is the highest among all oximes tested. The values of the constant k_R express the breakdown of the intermediate complex. The highest value of this constant for oxime K048 was obtained. The values of this constant decrease in the following order: K048 > Obidoxime > K027 > HI-6. Obidoxime has the highest bimolecular constant of reactivation (k_r, representing overall reactivation ability), followed by HI-6, K048 and K027 (5.6, 18 and 22.9 fold lower compared with obidoxime, respectively) (Kuca et al., 2005).

Table 2. Kinetic parameters of the reactivation of tabun-inhibited AChE in rat brain homogenate in vitro

Oxime	K_{dis} [μM	K_R [μM]	k_R [min.$^{-1}$]	k_r [M^{-1}.min.$^{-1}$]
Obidoxime	280	3.2	0.020	6250
HI-6	24	6.3	0.007	1111
K027	5888	54	0.0148	273
K048	228	93	0.0324	348

The ability of oximes to reactivate tabun-inhibited AChE in rat blood, diaphragm and brain *in vivo* is shown in Table 3. Both newly developed oximes seemed to be effective reactivators of tabun-inhibited AChE. While K048 was shown to be the best reactivator of tabun-inhibited AChE among studied oximes in the peripheral compartment (blood, diaphragm), K027 appeared to be the best reactivator of tabun-inhibited AChE in the central compartment (brain) among the oximes studied. Nevertheless, the difference in reactivating efficacy of tabun-inhibited AChE between newly developed oximes (K027, K048) and some commonly used oximes (obidoxime and trimedoxime) was not significant. On the other hand, HI-6 has significantly lower potency in reactivating tabun-inhibited AChE in peripheral as well as central compartments (Kassa et al., 2005).

Table 3. Rate of reactivation of tabun-inhibited AChE by oximes in rat blood, diaphragm and brain in vivo.

TREATMENT	AChE activity (μkat/L or μkat/kg)		
	Blood	Diaphragm	Brain
Atropine	2.23 ± 0.59[a]	11.93 ± 4.07[a]	82.8 ± 20.5[a]
Atropine + obidoxime (% reactivation[b])	5.36 ± 0.46 (18.1[*x])	27.36 ± 3.92 (25.6[*])	126.5 ± 17.1 (20.7[*])
Atropine + HI-6 (% reactivation)	3.28 ± 0.61 (6.1)	24.98 ± 5.54 (21.6[*])	104.8 ± 21.3 (10.5)
Atropine + trimedoxime (% reactivation)	5.18 ± 0.75 (17.1[*x])	29.59 ± 4.18 (29.3[*])	112.3 ± 12.7 (14.0)
Atropine + K027 (% reactivation)	5.14 ± 0.77 (16.9[*x])	29.88 ± 5.69 (29.8[*])	129.3 ± 23.8 (22.1[*])
Atropine + K048 (% reactivation)	5.94 ± 0.91 (21.5[*x])	31.78 ± 2.65 (33.0[*])	115.4 ± 20.6 (15.5)

[a] Means ± S.E.M., N = 8. The untreated control value for rat blood AChE activity was 19.5 (μkat/L), for diaphragm AChE activity 72.0 μkat/kg and for brain AChE activity 293.3 μkat/kg.
[b] Percent reactivation was determined using the AChE activity values: 1- [((oxime + atropine) – (saline))/((atropine control) – (saline))] x 100.
[*] Significantly different from the atropine group at a level of $P < 0.05$, [x] significantly different from the atropine + HI-6 group at a level of $P < 0.05$ as determined by the Student`s test.

The observation of neurotoxic signs indicated that many functional disorders of poisoned organisms outlasted at least 24 hours not only in non-treated tabun-poisoned rats (e.g. decreased exploratory activity, impairment of gait and motility, decrease in the distance between hindpaws after a jump, forelimb and hindlimb grip strength, body weight, body temperature and spontaneous horizontal as well as vertical motor activity) but also in tabun-poisoned rats treated with atropine alone or combined with pralidoxime or the oxime HI-6. While obidoxime in combination with atropine was able to eliminate some tabun-induced signs of neurotoxicity observed at 24 hours following tabun challenge with the exception of a decrease in hindlimb grip strength, exploratory activity, body temperature and spontaneous motor activity, neither atropine alone nor atropine in combination with pralidoxime or the oxime HI-6 was able to eliminate or at least to decrease the intensity of most of above mentioned tabun-induced signs of neurotoxicity. In addition, involuntary clonic movements are even observed to be more intensive in rats treated with atropine alone or atropine in combination with pralidoxime compared to non-treated tabun-poisoned rats (Table 4) (Kassa and Krejcova, 2003).

CONCLUSION

The whole developmental process as presented here seems to be very useful for the development of new antidotes against chemical warfare agents. It permits us to select the most promising antidotes from many chemical substances very effectively and quickly. That is why the Department of Toxicology of Faculty of Military Health Sciences is very active in the development of new AChE reactivators. We are able to evaluate with the help of this developmental process tens of chemical substances per year to find promising antidotes for further pre-clinical and clinical examination. Recently, the developmental process has been

used to find new, sufficiently effective asymmetric bispyridinium oximes against tabun (Kuca and Kassa, 2003; Kuca and Kassa, 2004).

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