ORIGINAL ARTICLE

Efficacy of structural homologues and isomers of pralidoxime in reactivation of immobilised acetylcholinesterase inhibited with sarin, cyclosarin and soman

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Abstract

OBJECTIVES: Quantification of efficacy of monopyridinium isomers and homologs derived from clinically used Pralidoxime within reactivation of acetylcholinesterase inhibited with organophosphorus nerve agents.
METHODS: This work uses the colorimetric biosensor called Detehit – cotton cloth with immobilized enzyme acetylcholinesterase. Biosensor is based on the modificated Ellman's method.
RESULTS: The highest reactivation was observed with sarin-inhibited acetylcholinesterase. Substantially lower reactivation was found with the cyclosarin-inhibited enzyme whereas AChE, inhibited by soman could not be effectively reactivated under the given conditions (enzyme inhibition for 2 minutes and subsequent treatment with the reactivator for 15 minutes).
CONCLUSION: Our work gives comparison of efficacy of reactivators in dependence on the lenght of alkylene chain and position of aldoxime functional group. Evaluation of effectivity of aldoxime reactivators is provided by simple means. The method allows rapid *in vitro* evaluation of the reactivators without being disturbed by excess of the organophosphate or reactivator.

INTRODUCTION

The group of nerve paralytic compounds occupies a significant position among chemical warfare agents. The first member of this group was synthesized as early as in the thirties of the last century by Gerhard Schrader at the I.G. Farben company in attempts to develop new pesticides.

These extremely toxic compounds irreversibly inhibit acetylcholinesterase, an enzyme that hydrolytically cleaves the neurotransmitter acetylcholine in the synaptic cleft of the nervous system. Deactivation of the enzyme in the organism results in cumulation of acetylcholine in nerve endings which manifests itself by nicotinic, muscarinic and central toxic effects. To eliminate them, causal and functional antidotes are employed. Acetylcholinesterase is the target point for carbamates and many other drugs as well. This fact was used for example in study of Alzheimer's disease (Dhawan *et al.* 2008).

Functional antidotes are compounds that hinder overstimulation of cholinergic receptors. Atropin is the most employed compound damping prevalently muscarinic effects. On the other hand, causal antidotes recover the acetylcholinesterase function. These compounds, the so-called reactivators, have

Abbreviations & Units:

AChE – acetylcholinesterase
2-PA – *syn*-Pyridine-2-aldoxime
2-PAM – 2-hydroxyiminomethyl-1-methylpyridinium iodide, pralidoxime
2-PAE – 2-hydroxyiminomethyl-1-ethylpyridinium iodide
2-PAP – 2-hydroxyiminomethyl-1-propylpyridinium iodide
3-PA – pyridine-3-aldoxime
3-PAM – 3-hydroxyiminomethyl-1-methylpyridinium iodide
3-PAE – 3-hydroxyiminomethyl-1-ethylpyridinium iodide
3-PAP – 3-hydroxyiminomethyl-1-propylpyridinium iodide
4-PA – pyridine-4-aldoxime
4-PAM – 4-hydroxyiminomethyl-1-methylpyridinium iodide
4-PAE – 4-hydroxyiminomethyl-1-ethylpyridinium iodide
4-PAP – 4-hydroxyiminomethyl-1-propylpyridinium iodide
GB – isopropyl-methylphosphonofluoridate, sarin
GD – 1,2,2-trimethylpropyl-methylphosphonofluoridate, soman
GF – *O*-cyclohexyl-methylphosphonofluoridate, cyclosarin

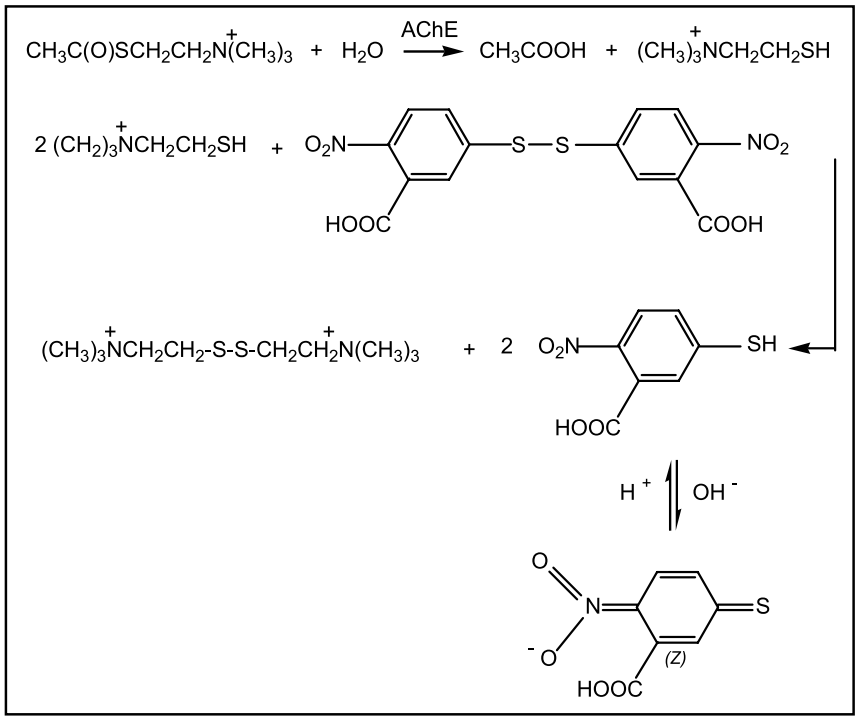
been studied since the beginning of the fifties of the last century. Since that time, a great number of compounds of various structure have been studied. Among them are hydroxylamines, ketoximes, hydroxamic compounds and many others. The most effective reactivators proved to be compounds with an aldoxime functional group. The first such compound was pralidoxime, chemically 2-hydroxyiminomethyl-1-methylpyridinium iodide (2-PAM) which has also found use in the clinical practice. Unfortunately, it is not very effective in reactivation of soman-inhibited AChE (Loomis & Safalsky, 1963; Fleisher *et al.* 1967; Harris *et al.* 1990). For soman inhibited acetylcholinesterase should be effective new group of bispyridinium reactivators e.g. so called HI-6 (Soukup *et al.* 2008; Kassa, 2002).

The high efficacy of this reactivator prompted the preparation and investigation of its isomers and homologues, e.g., isomers with the oxime group in position 3 and 4, a sulfone analogue or pyridine-2-aldoxime N-oxide and its two isomers (Augustinson *et al.* 1960). Analogues with two nitrogen atoms in the benzene nucleus, the so-called N-methylidazine aldoximes and ketoximes, have been studied at the Institute of Organic Chemistry, Prague Institute of Chemical Technology; compared with compound 2-PAM, these derivatives proved to be comparably or less effective (Cabal *et al.* 1997). Also another group of reactivators was synthesized in which the aldoxime group is replaced by a ketoxime group (Picha *et al.* 2005), and also methylthiol analogues of pralidoxime. Among reactivators tested are also compounds with quinuclidinium heterocyclic system, further aldoxime compounds with parent pyridinium-quinuclidinium and quinuclidinium-imidazolium structure (Bevandic *et al.* 1985; Primozic *et al.* 2004).

The intoxication by nerve paralytic compounds can be assessed by several analytical methods the eldest of which consists in determination of degree of inhibition of cholinesterases. The most popular is the modified Ellman's method of thiol determination. This method (see ***Scheme 1***.) is most often used in clinical biochemistry (Ellman *et al.* 1961).

The cleavage of substrate to thiocholine manifests itself by yellow coloration of the reaction zone due to formation of the reduced form of Ellman's reagent. In the presence of an inhibitor the indicator fabric remains white or has a yellowish color, depending on the degree of inhibition.

Various modifications of this reaction are utilized in means of detection such as the Detehit biosensor (Tusarova & Halamek, 2001). This detector is used by the Czech Army, not only as an individual detector but also in a number of means of chemical reconnaissance and checking. It contains a strip consisting of a cotton fabric zone with immobilized and stabilized acetylcholinesterase and a detector paper with acetylthiocholine iodide and 5,5´-dithiobis(2-nitrobenzoic acid). Acetylcholinesterase is immobilized in the form of stable enzyme chimera on polysacharide (cellulose). The enzyme remains in the solid phase and its use is polyvalent. As an advantage, an excess of organophosphate as well as reactivator may be removed from the fabric by simple washing out with water.

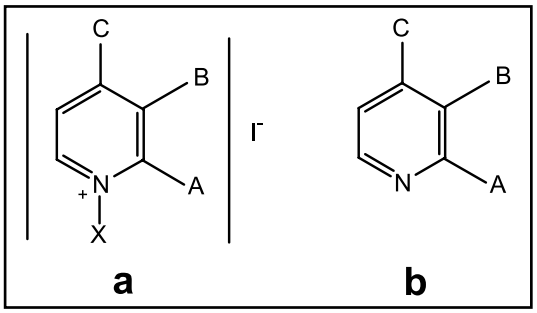


Scheme1. Ellman's method for determination of acetylthiocholine hydrolysis products.

Table 1. Overviw of acetylcholinesterase´s reactivators structure.

Reactivator	A	B	C	X
2-PA*	-CH=N-OH	-	-	-
2-PAM	-CH=N-OH	-	-	$-CH_3$
2-PAE	-CH=N-OH	-	-	$-C_2H_5$
2-PAP	-CH=N-OH	-	-	$-C_3H_7$
3-PA*	-	-CH=N-OH	-	-
3-PAM	-	-CH=N-OH	-	$-CH_3$
3-PAE	-	-CH=N-OH	-	$-C_2H_5$
3-PAP	-	-CH=N-OH	-	$-C_3H_7$
4-PA*	-	-	-CH=N-OH	-
4-PAM	-	-	-CH=N-OH	$-CH_3$
4-PAE	-	-	-CH=N-OH	$-C_2H_5$
4-PAP	-	-	-CH=N-OH	$-C_3H_7$

A,B,C ... location of aldoxime functional group;
X ... the lenght of alkyl chain on pyridine nitrogen;
* ...compounds are not pyridinium jodides.



Scheme 2. Structure of acetylcholinesterase reactivators
a) Homologs and isomers of pralidoxime iodide;
b) Isomers of pyridine aldoxime type; A,B,C ... location of aldoxime functional group; X ... alkyl chain

MATERIAL AND METHODS

Cellulose fabric with immobilized and stabilized porcine brain AChE (Oritest company, Czech Republic), was inhibited by nerve agents GB, GF and GD (VOZ Laboratories, 072, Zemianské Kostolany, Slovak Republic) by immersion into an aqueous solution of the inhibitor for 2 minutes to reduce the enzyme activity to 10–20% of the original value. The concentrations of organophosphates were in the order of 10^{-5}–10^{-4} mg.cm^{-3}. Then, the fabric was washed with distilled water and immersed into an aqueous solution of the reactivator (see ***Table 1***. and ***Scheme 2***.) of concentration PA 8,2.10^{-4} M; PAM 3,8.10^{-4} M; PAE 3,6.10^{-4} M and PAB 3,4.10^{-4} M for 15 minutes. The reactivation by pyridinium oximes without an alkyl chain was performed also in a phosphate buffer, pH 5. After this time, the fabric with reactivated AChE was washed with distilled water and for 1 minute superimposed with the indicator paper with substrate and chromogenic reagent (Oritest company, Czech Republic). The time course of the fabric surface coloration change was then measured at 410 nm at 10 second time intervals. The measurement proper was commenced 20 seconds after removal of the indicator paper. Each measurement was carried out 7–10 times at temperatures ranging from 20 to 25 ^{o}C. Objective colorimetric measurements were performed on a ULTRA SCAN XE spectrometer (Hunter Lab, USA).

Reactivators of acetylcholinesterase were prepared by Kroupa-Balex company, Pardubice, Czech Republic and Sigma-Aldrich Chemie GMbH, Steinheim, Germany.

RESULTS AND DISCUSSION

The effect of length of the alkyl chain at the heterocyclic nitrogen in monopyridinium aldoximes was investigated. Pyridinium aldoximes without an alkyl do not significantly reactivate AChE that has been inhibited by selected nerve compounds: the reactivation values do not exceed 10%, even in the case of acetylcholinesterase-sarin complex which otherwise is relatively easily cleavable by nucleophilic reagents and higher concentration than other aldoximes with alkyl chain (8,2.10^{-4} M) (***Figure 1***). The efficacy does not change significantly even after change of medium, i.e., in phosphate buffer or on acidification with 1M HCl to pH 5, at which pH an active form of pyridinium aldoxime may arise. A more significant change is observed when an alkyl group is attached to the heterocyclic nitrogen atom. With increasing length of the alkyl groups the efficacy of the individual reactivators moderately decreases. In the case of acetylcholinesterase-sarin complex, for some isomers the second member of the homologous series is more effective. Evidently, the alkyl chain plays here an important role. Although the substituent at the pyridinium nitrogen is not capable of nucleophilic attack at the bond between the serine hydroxyl and the organophosphate, it affects the position of the reactivator at the enzyme active site so that its functional group approaches this bond. Reactivation of soman-inhibited enzyme is difficult, apparently due to the rapid ageing of the enzyme-inhibitor complex which leads to a form not capable of reactivation.

Also the position of the aldoxime group affects the reactivation efficacy, as shown by comparison of results obtained with the position isomers (***Figure 2***). These isomers involves nucleophilic functional group in ortho, metha or para position (2, 3 or 4) in pyridine heterocyclic system. Aldoxime group play a pivotal function in cleavage of the binding of the organophosphate to the esteratic site of the enzyme. Optimal location of this group affects reactivation efficacy. The comparison confirms the highest reactivation ability for compounds with the aldoxime functional group in the ortho-position. This is most marked in the case of sarin-inhibited acetylcholinesterase whereas with the AChE-cyclosarin complex no such dependence has been found and the ability to reactivate this complex

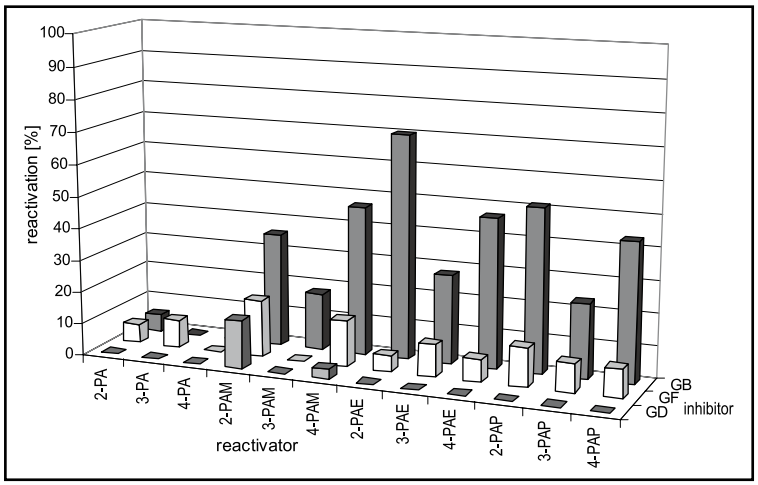


Figure 1. Efficacy [%] of homologues of 2-PAM in reactivation of immobilized acetylcholinesterase inhibited by GB, GFor GD.
Inhibition time 2 minutes, reactivation 15 minutes, reactivator concentration: PA 8,2.10^{-4} M; PAM 3,8.10^{-4} M; PAE 3,6.10^{-4} M and PAB 3,4.10^{-4} M (0,1 mg.cm^{-3})

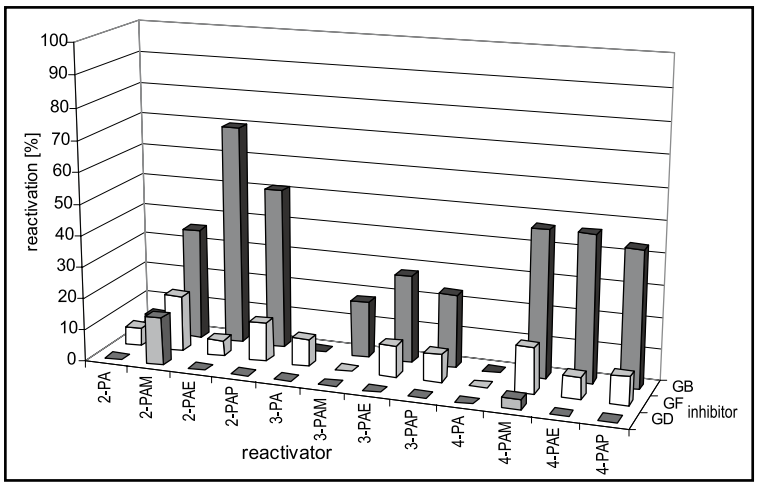


Figure 2. Efficacy [%] of position isomers derived from 2-PAM in reactivation of immobilized acetylcholinesterase inhibited by GB, GF or GD.
Inhibition time 2 minutes, reactivation 15 minutes, reactivator concentration: PA 8,2.10^{-4} M; PAM 3,8.10^{-4} M; PAE 3,6.10^{-4} M and PAB 3,4.10^{-4} M (0,1 mg.cm^{-3})

by the inhibitors employed is low. 2-PAE achieved 70% in reactivation of acetylcholinesterase-sarin complex. Isomers with functional group in position para had almost identical efficacy, 45% (not for compounds without an alkyl chain). The lowest ability to reactivate sarin-inhibited acetylcholinesterase was observed for meta isomers. This location is not appropriate in reactivation of inhibited enzyme.

The results, obtained with immobilized enzyme, are in accord with those based on use of the free enzyme; however, as concerns arrangement and experimental execution, the immobilized enzyme method is incomparably simpler. Using this modified Ellman's method with immobilized acetylcholinesterase on cotton cloth, it is possible to determine in a short time the coloration change of the fabric caused by products of reaction between the substrate and the chromogenic reagent. The reactivation measurement is not influenced by an excess of the organophosphate or reactivator and, as one of a few, this method enables to determine enzyme reactivation very rapidly. It is very important in view of possibility of misuse the organophosphorus compounds (nerve agents or pesticides).

REFERENCES

1 Augustinson KL, Hasslquist H, Larsson L (1960). Pyridine-N-oxide –aldoximes. Their synthesis and chemical and biochemical properties. Acta Chem. Scand. **14:** 1253–1260.
2 Bevandic Z, Deljac A, Maksimovic M, Boskovic B, Binenfeld Z (1985). Methylthio analogues of PAM-2, TMB-4 and Obidoxim as antidotes in organophosphate poisonings. Acta Pharmacol. Jug. **35:** 213–218.
3 Cabal J, Hampl F, Liska F, Patocka J, Petrlikova S, Sevcikova K (1997). Synteza a reaktivacni vlastnosti N-methydiaziniovych aldoximu – reaktivace acetylcholinestrazy fosfonylovane sarinem. [(Synthesis and reactivation properties of N-methydiazin aldoximes – reactivation of acetylcholinesterase phosphonylated with sarin.) (In Czech language)]. Chem. Listy. **91:** 1031.
4 Dhawan N, Puangco J, Jandial R (2008). Review in search of a treatment for Alzheimer's disease and potential immunosuppresive therapeutic interventions. Neuroendocrinol. Lett. **29:** 410-420.
5 Ellman GL, Courtney KD, Andres V jr., Featherstone RM (1961). A new and rapid colorimetric determination of acetylcholiesterase activity. Biochem. Pharmacol. **7:** 88–95.
6 Fleisher JH, Harris LW, Murtha EF (1967). Reactivation by pyridinium aldoxime methochloride (PAM) of inhibited cholinesterase activity in dogs after poisoning with pinacolyl methylphosphonofluoridate (soman). J. Pharmacol. Exp. Ther. **156:** 345–351.
7 Harris LW, Anderson DR, Lennox WJ, Woodard CL, Pastelak A, Vanderpool BA (1990). Evaluation of several oximes as reactivators of unaged soman-inhibited whole blood acetylcholiesterase in rabbits. Biochem. Pharm. **40:** 2677–2682.
8 Kassa J (2002). Review Of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. J. Toxicol. **40:** 803-816
9 Loomis TA, Safalsky B (1963). Antidotal action of pyridinium oximes in anticholinesterase poisoning; comparative effects of soman, sarin and neostigmine on neuromuscular function. Toxicol. Appl. Pharmacol. **5:** 685–701.
10 Picha J, Kuca K, Klvala M, Kohout M, Cabal J, Liska F (2005). A new group of monoquaternary reactivators of acetylcholinesterase inhibited by nerve agents. J. Enzyme Inhib. Med. Chem. **20:** 233–237.
11 Primozic I, Odzak R, Tomic S, Simeon-Rudolf V, Reiner E (2004). Pyridinium, imidazolium, and quinucludinium oximes: synthesis, interaction with native and phosphylated cholinesterases, and antidotes against organophosphorus compounds. J. Med. Chem. Def. **2:** 1–30.
12 Soukup O, Pohanka M, Tobin G, Jun D, Fusek J, Musilek K, Marek J, Kassa J, Kuča J (2008). The effect of HI-6 on cholinesterases and on the cholinergic system of the rat bladder. Neuroendocrinol. Lett. **29:** 759-762.
13 Tusarova I, Halamek E (2001). Biosenzor pro detekci a rozliseni inhibitoru cholinesteraz, zpusob pripravy zony biosenzoru s imobilizovanou cholinesterazou, zpusob detekce inhibitoru cholinesteraz a zpusob rozlisení inhibitoru cholinesteraz. [(Biosensor for detection and differentiating of cholinesterase's inhibitors, preparation of zone of biosensor with immobilized acetylcholinesterase.) (In Czech language)] Patent 288 576, Czech Republic.