Structural Requirements of Acetylcholinesterase Reactivators

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Abstract: Nerve agents (sarin, soman, cyclosarin, tabun and VX agent) and pesticides (paraoxon, chlorpyrifos, TEPP) represent extremely toxic group of organophosphorus compounds (OPCs). These compounds inhibit enzyme acetylcholinesterase (AChE, EC 3.1.1.7) via its phosphorylation or phosphonylation at the serine hydroxy group in its active site. Afterwards, AChE is not able to serve its physiological function and intoxicated organism is died due to overstimulation of cholinergic nervous system.

The current standard treatment of poisoning with highly toxic OPCs usually consists of the combined administration of anticholinergic drugs (preferably atropine) and AChE reactivators (called "oximes"). Anticholinergic drugs block effects of accumulated neurotransmitter acetylcholine at nicotinic and muscarinic receptor sites, while oximes reactivate AChE inhibited by OPCs.

Unfortunately, none from the currently used oximes is sufficiently effective against all known nerve agents and pesticides. Therefore, to find new oximes able to sufficiently reactivate inhibited AChE (regardless of the type of OPCs) is still very important task for medicinal chemistry with the aim to improve the efficacy of antidotal treatment of the acute poisonings mentioned.

In this paper, the relationship between chemical structure of AChE reactivators and their ability to reactivate AChE inhibited by several nerve agents and pesticides is summarized. It is shown that there are several structural fragments possibly involving in the structure of proposed AChE reactivators. Finally, an attempt of a future course of new AChE reactivators development is discussed.

Keywords: Acetylcholinesterase, nerve agent, pesticide, antidote, reactivator, structural requirements.

I. INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is a crucial enzyme in the human body. It is affiliated with Alzheimer´s and Parkinson´s diseases and with many other degenerative disorders. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine and terminates impulse transmission at cholinergic synapses. AChE is the key enzyme that is targeted by many different compounds such as competitive inhibitors (Alzheimer´s disease drugs – Tacrine, Galantamine; Nerve agents prophylactics – SAD-128 (1,3-bis(4-*t*-butylpyridinium) 2-oxapropan dichloride), Pyridostigmine) and irreversible inhibitors (Chemical warfare agents – sarin, tabun; Pesticides – chlorpyrifos, paraoxon) [1].

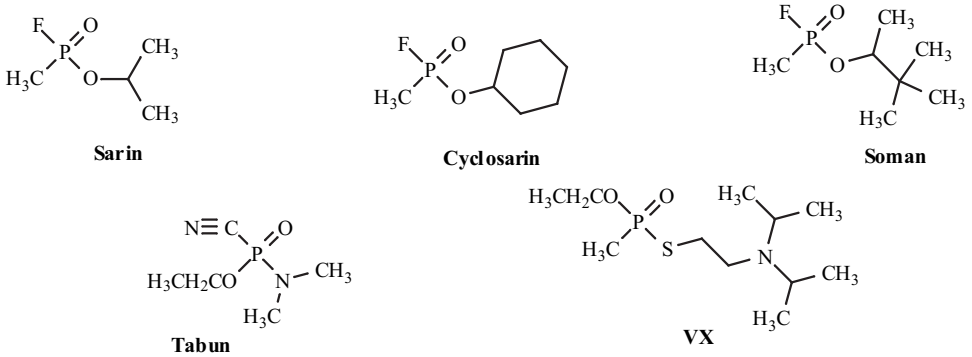

Fig. (1). Members of nerve agents family.

Nerve agents (NA) are special group of chemical warfare agents. NA are the most significant chemical warfare agents. They are extremely toxic. From the chemical point of view, they are reactive organophosphorus compounds –

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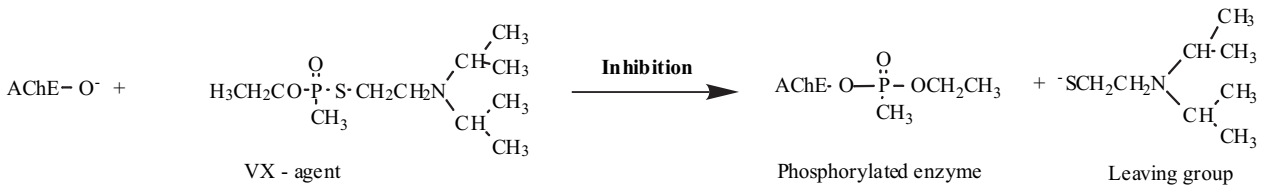


Fig. (2). Inhibition of AChE by nerve agent – VX agent.

phosphates, phosphonates or their thio-analogs. Sarin (GB; O-isopropylmethylfluorophosphonate), cyclosarin (GF; O-cyclohexylmethylfluorophosphonate), soman (GD; O-pinacolylmethylfluorophosphonate), tabun (GA; O-ethyl-N,N-dimethyl phosphoramidocyanidate) and VX agent (O-ethyl-S-(2-diisopropylaminoethyl)-methylthiophosphonate) belong to the main members of the NA group (Fig. **1**) [2].

The main toxic effect of these compounds is caused by irreversible inhibition of the AChE (Fig. **2**). NA inhibit AChE via its phosphorylation or phosphonylation at the serine hydroxy group in its active site [3]. Afterwards, AChE is not able to serve its physiological function (splitting of the neuromediator acetylcholine) and due to the acetylcholine excess, cholinergic overstimulation at the cholinergic synapses of both peripheral and central nervous system occurs. This precipitates a cholinergic crisis characterized by miosis, increased tracheobronchial and salivary secretion, broncho-constriction, fasciculation, behavioral incapacitation, muscular weakness and convulsions, culminating in death caused by respiratory failure [4].

The current standard treatment of poisoning with highly toxic OPCs usually consists of the combined administration of anticholinergic drugs (preferably atropine) and AChE reactivators (called "oximes" according to their functional group). Anticholinergic drugs block effects of accumulated acetylcholine at muscarinic receptor sites, while oximes reactivate AChE inhibited by OPCs [5].

Pralidoxime (2-PAM; 2-hydroxyiminomethyl-1-methylpyridinium chloride), obidoxime (Toxogonin®; 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride) and H-oxime HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)–2-oxa-propane dichloride) are considered to be the most important AChE reactivators (Fig. **3**) [6]. Other AChE reactivators are currently in development process in different countries of the word – methoxime (MMC-4; MMB-4; 1,1-bis(4-hydroxyiminomethylpyridinium)-methane dichloride), HLö-7 (1-(2,4-bis(hydroxyiminomethylpyridinium))-3-(4-carbamoylpyridinium)–2-oxa-propane dichloride), trimedoxime (TMB-4; 1,3-bis(4-hydroxyiminomethylpyridinium)-propane dibromide) and K048 (1-(4-hydroxyiminomethyl pyridinium)-3-(4-carbamoylpyridinium)–propane dibromide), respectively (Fig. **3**) [7-16].

These compounds are mono- or bisquaternary pyridinium salts having in its molecule functional oxime group able to

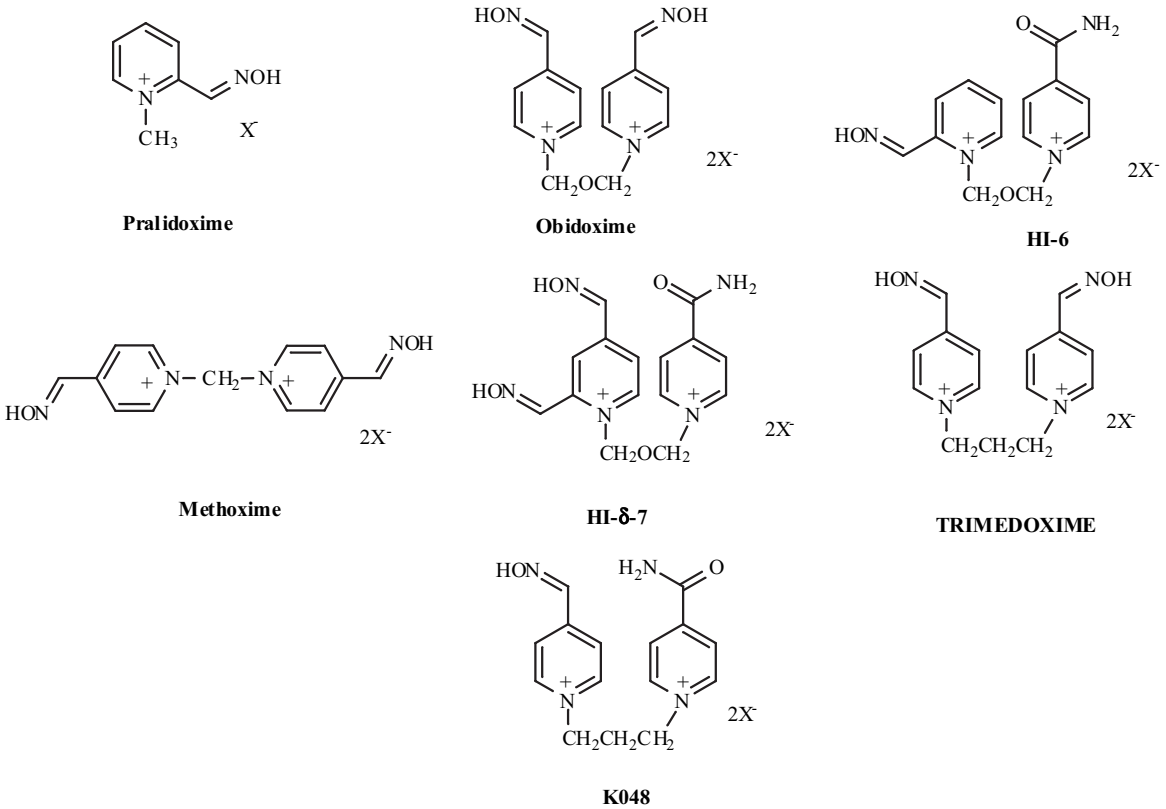


Fig. (3). Currently used and promising AChE reactivators.

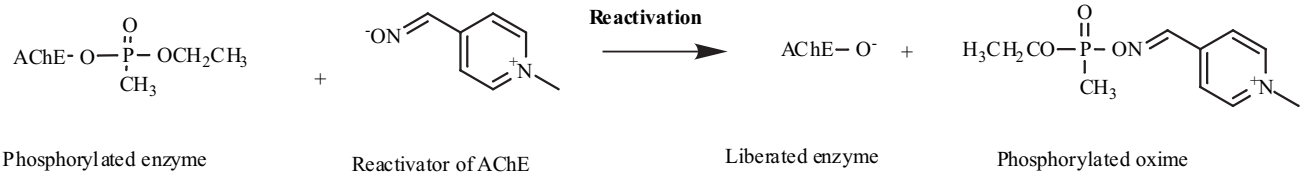


Fig. (4). Reactivatioin of VX agent-inhibited AChE.

split the bond between irreversibly bounded inhibitor (organophosphorus residue) and enzyme to release free functional enzyme normally acting in the organism [17] (Fig. **4**).

Unfortunately, none from the above mentioned AChE reactivators is sufficiently effective against all known NA and pesticides. Therefore, to find another oxime able to be sufficiently effective against inhibited AChE regardless of the type of OPCs is still very important task for medicinal chemistry with the aim to improve the efficacy of antidotal treatment of these acute poisonings. Therefore, many scientific institution over the world are interested in the synthesis of the new AChE reactivators [18-22].

In this work, the relationship between chemical structure of AChE reactivators and their ability to reactivate AChE inhibited by several nerve agents is summarized. The purpose is to show that there are several structural fragments, which should be involved in the structure of proposed AChE reactivators. Finally, we would like to predict a future course of new AChE reactivators development.

II. STRUCTURE OF ACETYLCHOLINESTERASE REACTIVATORS

There is a lack of informations about relationship between structure and reactivation potency of AChE reactivators. Some authors use computer-aided calculations for prediction of new more potent AChE reactivators [22-27]. Unfortunately, different biological data (biological activities) are commonly used as input data. These differences are caused by the use of miscellaneous design of biological measurement – e.g. using different method of measurement (Elmann method versus potentiometric method), using different species tested (laboratory animal versus human enzyme source), using *in vitro* versus *in vivo* methods (correlation *in vitro* and *in vivo*) etc. In the text bellow, results obtained using our unique *in vitro* method are presented [16].

Potentiometric method: Rat brain homogenate is used as the source of the enzyme. The brain homogenate is inhibited by nerve agent (30 min) and then AChE reactivator is added for 10 min. Afterwards, substrate (ACh – acetylcholine) is added into the solution and the measurement starts. ACh is splitted and released acetic acid is titrated using natrium hydroxide. Consumption of the natrium hydroxide is proportional to the enzyme activity allowing to calculate reactivation potency of tested AChE reactivators [16].

We are using the rat brain homogenate because in the brain there is the center of the breathing, which is believed to be the main cause of the death after OPCs intoxications. Moreover, in our experiment we are working with ACh, which is AChE native substrate, on the contrary to acetylthiocholine (ASCh) which is using in Ellman´s method. Disadvantage of our method consists in the presence of other different enzymes and substances which can interact with the tested oximes and due to this fact influence their potencies.

Due to the fact that we have had during last years many results in synthesis and *in vitro* evaluation in this area, we think that the range of this work will be adequate and that this work could help to the chemists, who will synthesize new structures of AChE reactivators.

There are five most important structural factors, which influence the affinity of the AChE reactivators toward inhibited AChE: presence of the quaternary nitrogen in the reactivator´s molecule, length and rigidity of the connection chain between two pyridinium rings, presence of the oxime group, position of the oxime group at the pyridinium ring and number of oxime group in the reactivator structure.

1). Presence of the Quaternary Nitrogen in the Reactivator´s Structure

It is generally known, that the anionic center of AChE binds the charged quaternary group of the choline moiety of acetylcholine, and also other quaternary ligands including quaternary AChE reactivators [28-30]. Owing to the presence of the quaternary nitrogen in the structure of AChE reactivator, also oximes have increased affinity to both intact and inhibited AChE. In fact, this structural feature allows entrance of the molecule of AChE reactivator into the enzyme cavity.

Table 1. Affinity of Pralidoxime (Monoquaternary Oxime) and HI-6 (Bisquaternary Oxime) Towards Intact and Nerve Agent-Inhibited AChE

AChE Reactivator	K_{DIS} [μM]	$K_{R\text{-}GB}$ [μM]	$K_{R\text{-}GF}$ [μM]	$K_{R\text{-}VX}$ [μM]	$K_{R\text{-}GA}$ [μM]
Pralidoxime	210	354	12000	127	575
HI-6	24	9	12	130	6

K_{DIS} - Affinity towards intact AChE; $K_{R\text{-}GB}$, $K_{R\text{-}GF}$, $K_{R\text{-}VX}$, $K_{R\text{-}GA}$ – affinity towards sarin, cyclosarin, VX respectively tabun-inhibited AChE.

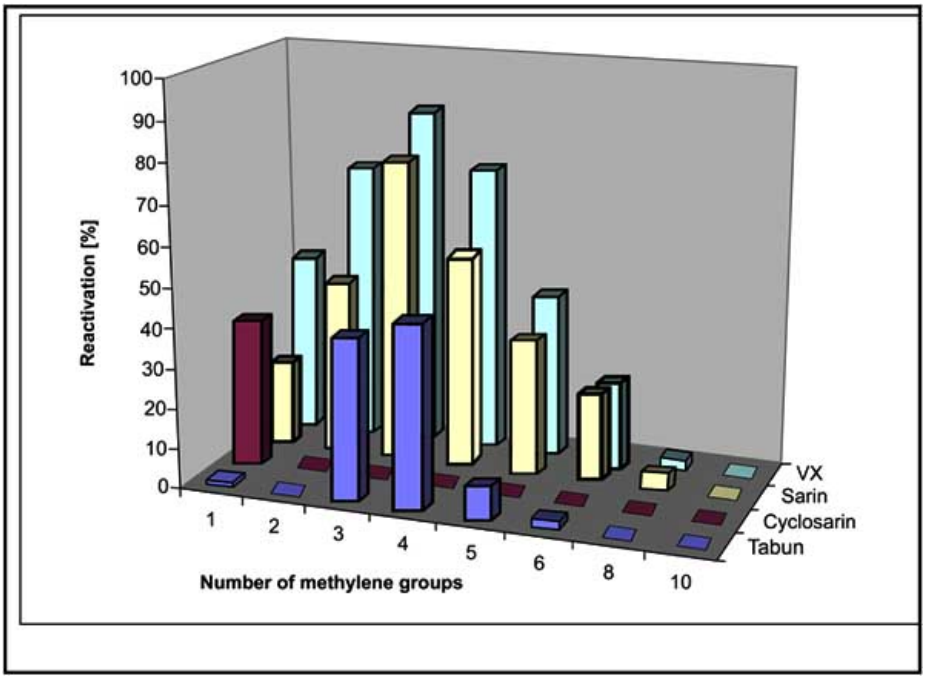


Fig. (5). Dependence of the reactivation potency on the number of methylene groups and on the type of the OPC inhibitor [38].

On the other hand, there is discussion about the penetration of the quaternary charged compounds (especially AChE reactivators) through the blood-brain barier (BBB). *In vivo* reactivation of nerve agent-inhibited brain AChE was determined by Kassa [31] and Bajgar [32]. The partial ability of oximes to penetrate the blood brain barrier (about 10%) was also recently confirmed by Sakurada *et al*. [33].

Commonly used AChE reactivators have one (pralidoxime) or two (HI-6) quaternary nitrogens in their molecules. According to our results, affinity of bisquaternary oximes towards intact and inhibited AChE is generally higher in comparison with monoquaternary ones [12,34-36] (Table **1**).

2). Length and the Rigidity of the Connection Chain Between Pyridinium Rings

The length of the connecting chain between both pyridinium rings (for bisquaternary pyridinium reactivators) plays an important role in its potency to reactivate nerve agent-inhibited AChE [22,37,38]. For *n*-methylene linkage chain, there exists dependence between the length of the connection chain and nerve agent used for inhibition. This dependence is shown in (Fig. **5**) [38].

As it is shown, the ideal length of connecting chain for reactivation of tabun, sarin or VX -inhibited AChE is 3 or 4 methylene groups. On the other hand, methoxime (contains one methylene group) seems to be the most potent reactivator of cyclosarin inhibited AChE [34,38].

Table 2. Reactivation Potency of Several Non-*n*-Methylene Bisquaternary Pyridinium AChE Reactivators (Concentration of Oxime 0.001 M, 30 Min Inhibition with Nerve Agent, 10 Min Reactivation, pH 7.6, 25 °C)

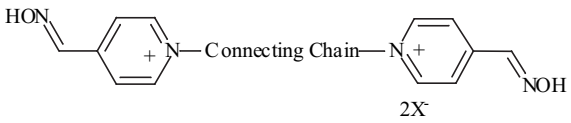
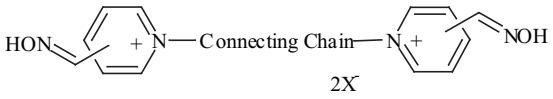
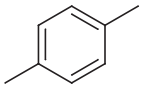
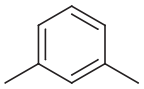
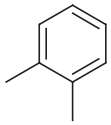
		**Reactivation potency [%]**			
AChE Reactivator	Connecting Chain	Sarin	Cyclosarin	VX	Tabun
Trimedoxime	$CH_2CH_2CH_2$	7	0	85	41
K074	$CH_2CH_2CH_2CH_2$	9	0	72	46
TO 057	$CH_2CH_2SO_2CH_2CH_2$	8	8	26	8
TO 058	$CH_2CH_2S^+(CH_3)CH_2CH_2$	5	0	46	9
Obidoxime	CH_2OCH_2	4	0	79	0
TO 052	CH_2COCH_2	2	3	71	8
K075	$CH_2CH{=}CHCH_2$	13	0	87	34

Table 3. Reactivation Potency of Several Rigid Bisquaternary Pyridinium AChE Reactivators (Concentration of Oxime 0.001 M, 30 Min Inhibition With Pesticide Chlorpyrifos, 10 min Reactivation, pH 7.6, 25 °C)

	**Reactivation potency [%]**		
Connecting Chain	**2***	**3***	**4***
$CH_2CH_2CH_2$	19	16	79
$CH_2CH_2CH_2CH_2$	24	6	50
$CH_2CH{=}CHCH_2$	4	13	25
$CH_2C{-}CCH_2$	0	0	0
	41	8	34
	26	5	37
	19	0	20

*Position of the oxime group at the pyridinium rings.

Unfortunately, this rule is satisfied just in the case of *n*-methylene linkage chains. Compounds with oxygen, sulphur or other structural fragments differing from the methylene incorporated into the connection chain do not fulfil the above mentioned rule [12,39-41] (Table **2**).

These differences could be caused by the presence of the free electrons in the connection chain and subsequent interactions of this part of the reactivator´s molecule with the cavity of the enzyme.

The important structural factor influencing the reactivation process is the "rigidity" of the linking chain. Owing to the rigidity of the connection chain, spatial orientation of the pyridinium rings in the enzyme cavity is limited. Compounds with certain level of rigidity in the connection chain were synthesized with the aim to elucidate the influence of rigidity on reactivation potency. Alkene, alkyne and xylene moieties were inserted into the linkage chain [42,43]. As it can be seen in (Table **3**), incorporation of the rigidity of the connection chain (but-2-ene or but-2-ine) rapidly decreases the reactivation potency of AChE reactivators.

In the case of xylene linkers, the above mentioned results were not obtained. However, other important factor can be considered. It is influence of the angles involved in the connection chain structure. The only bonds accessible for free rotation in these molecules are methylene junctions in contrast to free rotation in the molecules with propane or butane linking chain. These rigid xylene junctions cause the non-coplanary formation of each pyridinium ring to the phenylene ring. Therefore, there could be determined angles between bonds outgoing from phenylene ring (Fig. **6**). From this point of view, the most promising are compounds with bigger angles meaning further distance of pyridinium rings [42].

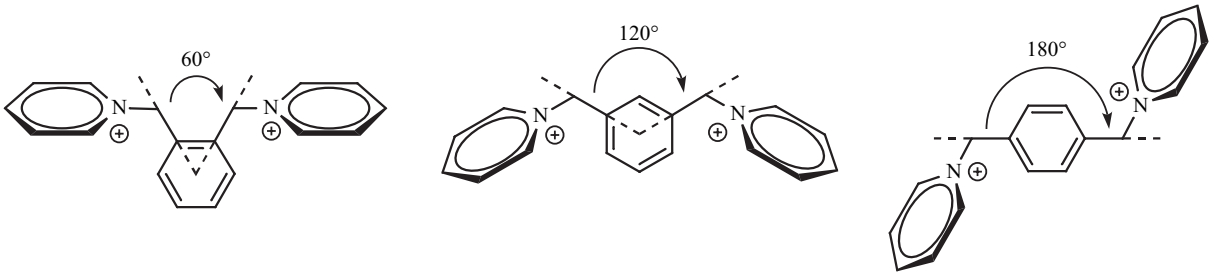
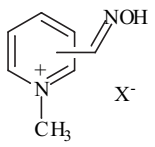


Fig. (6). Angles between bonds outgoing from phenylene ring.

Table 4. Reactivation Potency of Monoquaternary Pyridinium AChE Reactivators (Concentration of Oxime 0.001 M, 30 Min Inhibition with Nerve Agent or Pesticide, 10 Min Reactivation, pH 7.6, 25 °C)

	**Reactivation potency [%]**	
Oxime Group Position	Cyclosarin	Chlorpyrifos
2	0	80
3	6	0
4	3	7

3). Presence of the Oxime Group in the Oxime Structure

Presence of the oxime group in the structure of reactivator is other substantial structural factor. Former, hydroxyiminoacetone, hydroxamic acids, geminal dioles, ketoximes were used as potential nucleophilic agents for break of the bond between inhibitor and enzyme [20,44-46]. Aldoxime group seems to be currently entirely used, and therefore it is involved in all newly synthesized AChE reactivators.

Oxime group (-CH=NOH) in the environment of human body dissociates and the arisen oximate anion (-CH=NO$^-$) acts as nucleophile and cleaves the bond between enzyme and phosphorus atom of the inhibitor (Fig. **4**) [17,44].

4). Position of the Oxime Group

Other important structural factor is also connected with the oxime group. It is the position of the oxime group at the quaternary pyridinium ring. As it can be seen in the Tables **4** and **5**, there are significant differences in the reactivation potency of the oximes regarding the oxime group position.

There exists no general rule that all oximes in the given position are able to reactivate all nerve agent-caused inhibitions. However, general standard is that reactivators containing oxime group in position two and four are more effective compared to the oximes in the positioin three. This fact is due to the difference in pK_a between oximes in position two and four versus by those in position three [44].

Although rule of oxime position for all nerve agents is unrealistic, there are some relationships between position of oxime group and nerve agent. It means that the position of the oxime group is, as well as the length of connecting chain between quaternary pyridinium rings, nerve agent-dependent. For example, cyclosarin-inhibited AChE is the best reactivated by reactivators with oxime in position two [41]. On the other hand, reactivators with oxime group at the pyridinium ring in position four are currently considered to be the most potent for reactivation of tabun-inhibited AChE [12]. Also pesticides-induced poisonings are best-treatable with AChE reactivators with oxime group at the position four [47,48]. Thanks to the very good reactivability of sarin and VX-inhibited AChE, both reactivators (with oxime groups in the position two and four) are potent reactivators of inhibited AChE [35,49].

5). Number of Oximes Group in the Reactivator´s Molecule

Number of oxime groups in the reactivator´s molecule is the fifth structural factor discussed in this article. One molecule of AChE reactivator can contain one, two, three or four oxime groups at one and two or three pyridinium rings. Examples of different structures with their biological activities are shown in Table **6** [12, 50].

Table 5. Reactivation Potency of Monoquaternary Pyridinium AChE Reactivators (Concentration of Oxime 0.001 M, 30 Min Inhibition with Nerve Agent or Pesticide, 10 min Reactivation, pH 7.6, 25 °C)

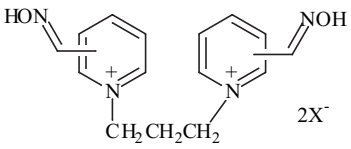
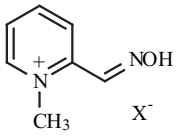
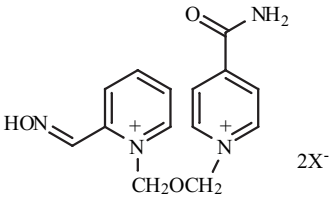
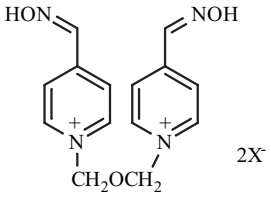
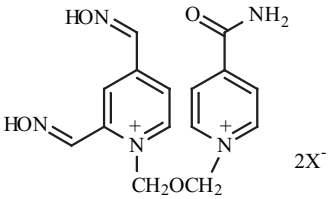
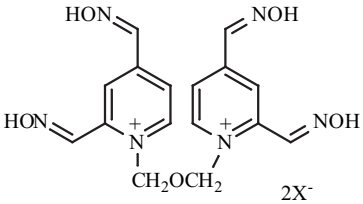
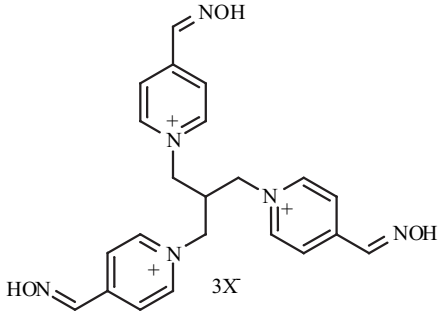
	**Reactivation potency [%]**	
Oxime Group Position	Cyclosarin	Chlorpyrifos
2	1	19
3	2	16
4	0	79

Table 6. Reactivation Potency Of Different Reactivators With Different Number Of Oxime Groups At Different Number of Pyridinium Rings. (Concentration of Oxime 0.001 M, 30 Min Inhibition with Tabun, 10 min Reactivation, pH 7.6, 25 °C)

Structure of AChE reactivator	Description	Reactivation potency [%]
 Pralidoxime	• One oxime group at one pyridinium ring • One pyridinium ring	4
 HI-6	• One oxime group at one pyridinium ring • Two pyridinium rings	6
 Obidoxime	• Two oxime groups at two pyridinium rings • Two pyridinium rings	37
 HLö-7	• Two oxime groups at one pyridinium ring • Two pyridinium rings	3
 Tetroxime	• Four oxime groups at two pyridinium rings • Two pyridinium rings	1
 TO055	• Three oxime groups at three pyridinium rings • Three pyridinium rings	2

As it can be seen, number of oxime groups does not increase reactivation potency of potential AChE reactivator. This fact could be connected with increased size of the AChE reactivator´s molecule. Owing to the fact, that main role in the reactivation process has just the first oxime group (with the lowest pKa) the presence of the second oxime group is not so strict.

FUTURE AChE REACTIVATORS DEVELOPMENT

There are many ways how to improve reactivation potency of currently used AChE reactivators. Bellow, we would like to outline some different approaches, which should be considered as eventual solution of this challenge:

- Modification or replacement of the functional oxime group by another more potent nucleophile group.
- Coupling of the oxime containing part of the AChE reactivator with quaternary compound with higher affinity towards AChE than quaternary pyridinium salt (e.g. quinolinium, isoquinolinium, acridinium etc.).
- Coupling of the oxime containing part of the AChE reactivator with non-quaternary compounds with high affinity towards AChE (e.g. tacrine, galantamine etc.).
- Coupling of the oxime containing part of the AChE reactivator with some for organism essential substances (sugars, aminoacids, steroids, etc.) which could serve as enhancers of penetration through the blood brain barrier
- Using click chemistry for in situ synthesis of AChE reactivators.

CONCLUSION

Our obtained data are in good agreement with data presented by other authors. For example, all currently promising AChE reactivators are bisquaternary pyridinium salts with three membered connection chains (obidoxime, trimedoxime, HI-6 and Hlö-7) [51-55]. All these AChE reactivators have oxime groups at the positions two or/and four. As it is clearly seen in all these articles, there is also fulfil the rule that position of oxime groups is nerve-agent dependent. For example, Puu *et al*. [55] confirmed that currently the most potent reactivator of tabun-inhibited AChE is obidoxime (oxime groups in position four).

Owing to the threat of organophosphate exposures, not only to pesticides but also to nerve agents, it is very important to develop universal – broad spectrum AChE reactivator, which could be used in the case of all organophosphorus agent intoxications. Although current antidotes against organophosphorus intoxications consist also of prophylactics (pyridostigmine or BuChE as nerve agents-scavenger), AChE reactivators are still needed especially in the case of intoxications with high doses of organophosphates, for which prophylactic treatment is not effective. For these above-mentioned reasons, new AChE reactivators are and will be still developed.

Our work summarizes relationship between AChE reactivators and their reactivation activity, and should be in future help for scientists interesting in the area of AChE reactivators development.

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