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OXIMES AS INHIBITORS OF ACETYLHOLINESTERASE– A STRUCTURE-ACTIVITY RELATIONSHIP (SAR) STUDY

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Running title: Oximes inhibitory effects on acetylcholinesterase

ABSTRACT

Acetylcholinesterase (AChE) reactivators (oximes) are generally used as antidotes in case of nerve agent poisoning. Because of their affinity to AChE, they may also act as weak inhibitors of AChE. Their inhibition potency against AChE was determined by an *in vitro* method based on the interaction between AChE and oxime reactivator in the concentration range 10^{-1} to 10^{-8} M. We used eel AChE for these assays. We found that AChE inhibition strongly depends on the oxime structure. The aim of the present study is to describe the structure-activity relationship (SAR) between oxime structure and inhibition of AChE. AChE reactivators tested include both monoquaternary and bisquaternary structures with the oxime group in different positions on the pyridine ring and with changes in the connecting linker in the case of the bisquaternary compounds.

We found AChE inhibition to be highest in bisquaternary oximes that have a longer linker length and have the oxime group in the ortho position. Increased AChE inhibition in monoquaternary oximes was highest when the *meta* position was occupied by the oxime nucleophile. In addition, different substituents in the connecting chain (in case of bisquaternary oximes) modulated their inhibition potency.

Keywords: acetylcholinesterase, inhibitor, reactivator, oxime, structure-activity relationship (SAR), antidote

Introduction

Acetylcholinesterase (AChE; 3.1.1.7.) is an enzyme that belongs to the family of α/β hydrolases. AChE splits the neurotransmitter acetylcholine (ACh) in synaptic clefts with subsequent release of choline and acetic acid. This vital enzyme is found in the central and peripheral nervous systems, neuromuscular junctions and in the hematopoietic system. It plays a key role in the termination of cholinergic transmission in all cholinergic junctions [1]. Organophosphates (OPs) represented by nerve agents and pesticides are AChE inhibitors. However, OP inhibition of AChE is usually irreversible and thereby extremely toxic. Inhibited AChE results in the accumulation of ACh in cholinergic clefts. Increased ACh concentration produces overstimulation of cholinergic receptors in all parts of the human body leading to, if left untreated, cholinergic crisis and often death [2].

Current medicinal therapy for OP intoxication combines an anticholinergic drug (mainly atropine, benactyzine), an anticonvulsant (diazepam), and an AChE reactivator (pralidoxime, obidoxime, HI-6). Anticholinergic drugs decrease the interaction of ACh with cholinergic receptors, but they do not address the root cause of intoxication, AChE inhibition [3]. On the other hand, AChE reactivators (generally called oximes) work as nucleophilic agents that remove the organophosphate thus restoring activity of inhibited AChE and re-establishing its physiological function [4, 5].

Although oximes are mainly used for their reactivation potency, their inhibition of AChE must be also considered. It is known that oximes are able to bind to AChE as reversible inhibitors either at the AChE active site or at the allosteric site, and sometimes at both sites [6]. Binding as a reversible inhibitor to the allosteric site induces indirect protection of the active site by changes in AChE structure [6]. Moreover, the binding affinities of oximes to the OP-AChE complex are slightly higher than to the free AChE [7].

Previous studies have suggested that the effects of oxime reactivators could not be entirely explained by their AChE reactivation potency [8, 9]. Melchers et al. [10] posit that the oxime, HI-6, might affect the GABA-ergic neurotransmission in the central nervous system (CNS). Furthermore, oximes can have effects on various steps of the cholinergic transmission e.g. inhibition of ACh synthesis [8], alteration of ACh release from neurons [11], and interaction with pre- and postsynaptic receptors [12]. It is possible that these additional pharmacological effects could lead to new oxime targets and improve our knowledge of their effects in the whole organism [13].

The goal of the present study is to determine how oxime structure (Figure 1) influences inhibition of AChE. Therefore, 26 structurally different oximes were tested. Knowledge obtained in this study will be used for subsequent synthesis of oximes with controlled (weak or strong - depending on the need) inhibition of AChE. Moreover, the structure activity relationships that we obtain will be useful for designing AChE reactivators with mainly peripheral action, which may successfully be used in prophylaxis against organophosphorus compounds or for the treatment of Myasthenia gravis. Hereafter we will refer to oxime-based reactivators as ‘reactivators’ to distinguish the functional group from the whole compound.

Methods

Chemicals

All tested reactivators were previously synthesized at the Department of Toxicology, Faculty of Military Health Science, Hradec Kralove, Czech Republic [14]. Phosphate buffer, eel AChE, DTNB (*5,5´*- dithiobis (2-nitrobenzoic) acid) and acetylthiocholine iodide were purchased from Sigma – Aldrich (Prague, Czech Republic).

The measurement of IC_{50} of AChE

The activities of AChE were evaluated by the standard spectrophotometric Ellman's method. Acetylthiocholine iodide was used as a substrate and DTNB was used as the chromogen. Wavelength 412 nm was used for *in vitro* measurement [15, 16]. The spectrophotometer a Helios Alpha (Thermo Scientific, Great Britain) was used for absorbance determinations. The results were analyzed with the standard statistic software Prisma 4.0.

In vitro measurement was as follows: Solution of eel AChE (90 µl, activity was previously established) was pipetted into the cuvette. Subsequently, 10 µl of the selected reactivator in concentrations from 10^{-1} to 10^{-8} M were added. This mixture was incubated for 10 min under laboratory temperature (20 ± 2 °C). Then, 200 µl of DTNB and 600 µl of phosphate buffer (0.1M, pH 7.4) were added. The reaction was started by adding acetylthiocholine iodide (100 µl).

Oximes in higher concentrations may split DTNB, this process is known as oximolysis and produces false-positive results [17]. To eliminate this issue, a portion of the eel AChE was replaced by distillated water. Subsequently the same portions of other substances were added. Acquired measurements were deducted from AChE activity values.

Actual activity of the enzyme (the blind sample) was established for all concentration series. The reactivator was replaced by water in cuvette and obtained values were calculated as 100% of the enzymes activity.

Results and Discussion

Reactivators of AChE are used as common antidotes in the therapy of nerve agent poisonings [18]. A vast number of monopyridinium and bispyridinium oximes have been synthesized and tested for their efficacy within last sixty years [19, 20]. However, none of the tested oximes has been identified as effective against all OP inhibitors [21, 22].

Reactivators have been shown to restore cholinesterase´s (ChE) activity [23]. The oxime reactivation process is dependent on the nucleophilicity and orientation of the oxime as well as on the structure of the OP-AChE conjugate [24]. However, structure–activity relationships describing oxime reactivation efficacy are poorly understood [22].

There are several important structural factors that influence oxime reactivation potency [25]. At least one oxime group in the reactivator structure is necessary for the reactivation process [7]. Other functional groups, their number and position on the pyridinium ring directly influence oxime pharmacokinetics profile, toxicity and also reactivation potency [26]. It is also known that the bisquaternary reactivators have higher potency to reactivate inhibited AChE compared to monoquaternary ones [22]. It is evident, that reactivators may simultaneously act as reversible inhibitors of AChE. This fact is confirmed in the present study. The AChE inhibition results are summarized in Tables 1-5 as IC_{50} values. In Figure 2, we present an example of a sigmoidal inhibition curve that was obtained for every AChE reactivator assay. Here we show the qualitative relationship between the reactivator chemical structure and its ability to inhibit AChE is shown and discussed in order of importance.

Bisquaternary Linker Length

Based on our results, the connecting linker in the bisquaternary oxime structure is the most important moiety influencing its inhibition potency. In this regard, we show (as depicted in Table 1) that oxime inhibition increases with increased number of methylenes in the connecting linker. The reactivators with a longer linker (approximately 9-10 carbons) achieved the maximum inhibition potency with an IC_{50} between $1x10^{-4}$ to $5x10^{-4}$ M. These values should be considered as very similar. Although the connecting linker does not play any role in the dephosphorylation process, it plays a major role in distribution, elimination and AChE reactivation rates (e.g. in the binding mechanism) [27]. IC_{50} values are shown in Table 2 for reactivators with substitution of a methylene group by another atom (for example oxygen) or insertion of a double bond. Rotation around a double bond is not favorable and double bonds are shorter. Reactivators with a double bond in the connecting linker have less favorable orientation at the rim in the anionic site of enzyme. Despite this fact, a double bond contributes weakly to the AChE inhibition in comparison to the longer linker length. Juxtapose these inhibition data with commonly used nerve agent antidotes (trimedoxime, K027, HI-6, obidoxime) which have short linkers (C_3-C_4), and one sees the trade-offs for the sufficient reactivation of irreversibly inhibited AChE [22].

Oxime Moiety Position on Pyridine Ring, Monoquaternary and Bisquaternary Reactivators

Another parameter followed in this study was change of oxime group position by oximes with 4 or 3 carbons in the connecting linker. These results are summarized in Table 3 and 4. The highest inhibitory efficacy has the bisquarternary reactivator with substituent in the *ortho*

position, the least inhibiting reactivator with a substituent in the *para* position. The last group of oximes (Table 5) is created by monopyridine reactivators. The oxime in the *meta* position has the highest inhibitory efficacy. We can contrast these results with optimum AChE reactivation that requires bisquaternary reactivators to have the oxime moiety to be in the *para* position and monoquaternary reactivators to have the oxime moiety in the *ortho* position.

Conclusions

The qualitative structure-activity relationships observed in this study should be used for design of novel peripherally acting AChE reactivators or reversible AChE inhibitors [28]. Oxime reactivators have been primarily designed to reactivate inhibited AChE (post exposure treatment), but they may also act as prophylaxis before the nerve agent intoxication. Combination of ChE and oxime pre-treatment is used as a pseudo-catalytic bioscavenger. This combination of enzyme and reactivator increases prophylactic potential [29]. The results of this study allow us to dissect the chemical properties important to reversible inhibition from reactivation of AChE. In addition, novel structures of reversible AChE inhibitors can be used as treatment of myasthenia gravis (MG) or in anesthetic practice to reverse the skeletal muscle relaxation induced by non-depolarising neuromuscular blocking agents [30] could also be designed based on information gained from the current study.

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Figure 1. The typical structure of bisquaternary reactivator of AChE

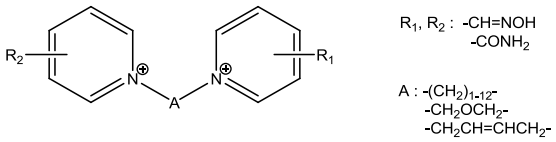


Figure 2. Inhibition of AChE by reactivator K 197 - relationship between K 197 concentration and percentage of AChE activity.

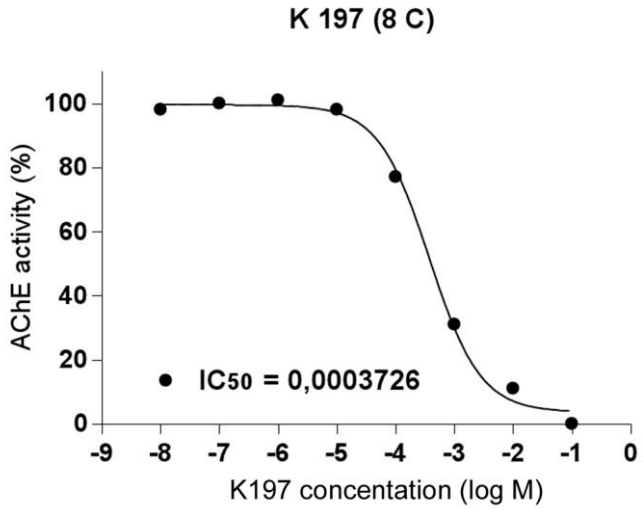
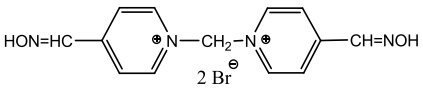
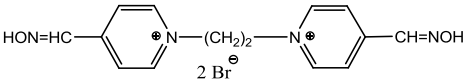
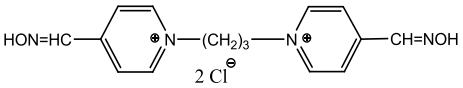
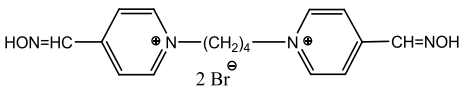
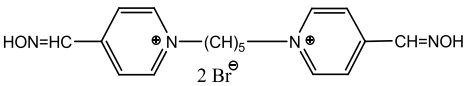
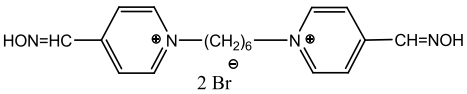


Table 1: IC_{50} values of AChE reactivators with various length of connecting chain between the pyridinium rings.

No.	Names of oximes	MW	Chemical structure	Log P	Result IC50 (M)
1	**K154**	418,08		-6,99	0,2274
2	**K191**	432,11		-7,1	0,1246
3	**K018**	446,14		-7,04	0,0519
4	**K074**	460,16		-6,53	0,0043
5	**K305**	474,19		-6,08	0,0009
6	**K194**	488,22		-5,64	0,0009

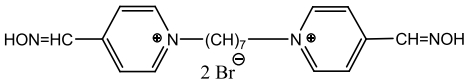
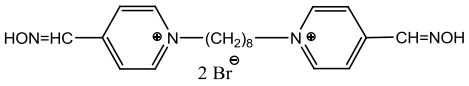
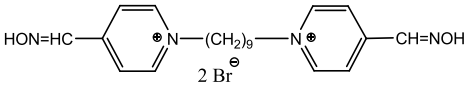
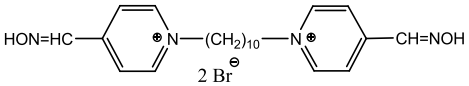
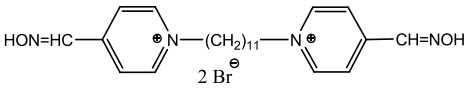
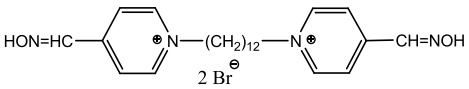
7	**K309**	502,24		-5,19	0,0007
8	**K197**	516,27		-4,75	0,0004
9	**K310**	530,3		-4,3	0,0003
10	**K338**	544,32		-3,86	0,0001
11	**K339**	558,35		-3,41	0,0004
12	**K340**	572,38		-2,97	0,0005

Table 5. IC_{50} values of monopyridine AChE reactivators with various position of oxime group on pyridinium ring.

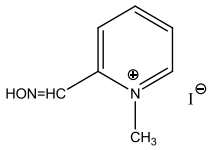
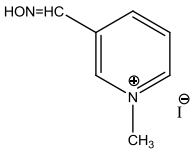
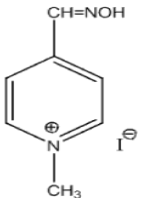
No.	Name of oximes	MW	Chemical structure	Log P	Result IC50 (M)
1	**2PAM**	264,06		-3,26	0,0549
2	**3PAM**	264,06		-3,47	0,0271
3	**4PAM**	264,06		-3,47	0,0643

Table 3. IC_{50} values of bispyridine AChE reactivators with 4C connecting chain with various position of oxime group on pyridinium ring.

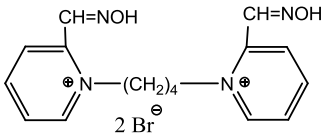
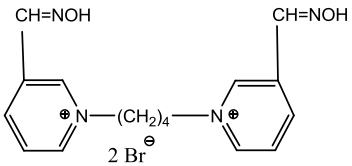
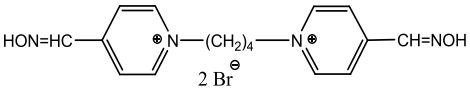
No.	Names of oximes	MW	Chemical structure	Log P	Result IC50 (M)
1	**K033**	460,16		-6,11	0,0011
2	**K101**	460,16		-6,53	0,0048
3	**K074**	460,16		-6,53	0,0043

Table 4. IC_{50} values of bispyridine AChE reactivators with 3C connecting chain with various position of oxime group on pyridinium ring.

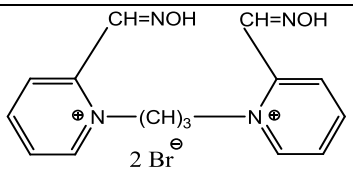
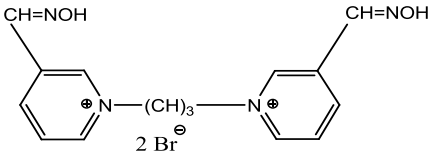
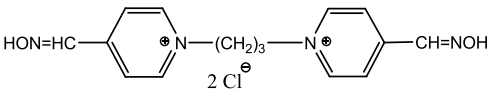
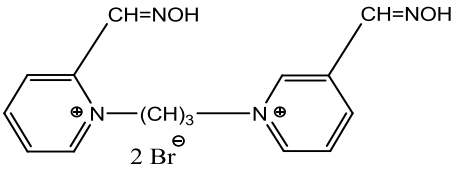
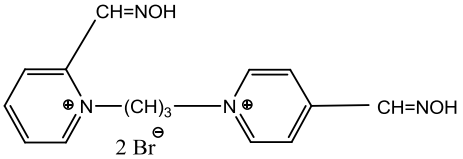
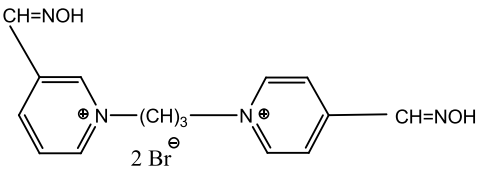
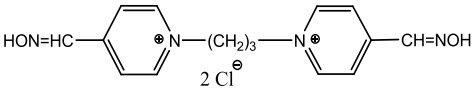
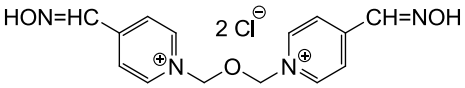
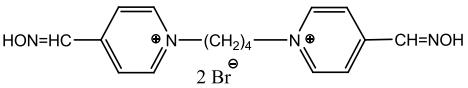
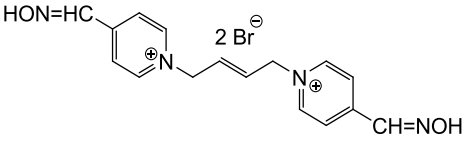
No.	Names of oximes	MW	Chemical structure	Log P	Result IC50 (M)
1	**K005**	446,14		-6,63	0,0004
2	**K099**	446,14		-7,04	0,0195
3	**K018**	446,14		-7,04	0,0519
4	**K207**	446,14		-6,83	0,0177
5	**K208**	446,14		-6,83	0,0031
6	**K209**	446,14		-7,04	0,0138

Table 2. IC_{50} values of various bispyridine AChE reactivators - substitution and double bond in connecting linker.

No.	Names of oximes	MW	Chemical structure	Log P	Result IC50 (M)
1	**K018**	446,16		-7,04	0,0519
2	**K318**	359,21		-6,93	0,0367
3	**K074**	460,16		-6,53	0,0043
4	**K075**	458,15		-6,58	0,0067