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RESEARCH PAPER

In vitro Evaluation of Neutral Oximes as Reactivators of Parathion-inhibited Electric Eel Acetylcholinesterase

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ABSTRACT

Organophosphorus (OP) compounds are irreversible inhibitors of acetylcholinesterase (AChE) commonly used as pesticides and, unfortunately, as nerve agents in terrorist attacks. These compounds are highly soluble easily crossing the blood-brain barrier (BBB). Clinically, oximes such as pralidoxime and obidoxime are used for the reactivation of AChE. These oximes are not effective to reactivate AChE inhibited by different OPs besides the fact that they are permanently charged and do not readily cross the BBB. This work evaluated the ability of ten neutral oximes to reactivate parathion-inhibited eel AChE. Because oximes can bind to AChE as reversible inhibitors, this property was also evaluated, with pralidoxime (2-PAM) used as a reference compound. Unlike 2-PAM, which inhibited AChE in a concentration-dependent way, neutral oximes did not follow the linear order of AChE inhibition. Neutral ligands can present affinity for the periferic anionic site (PAS) site. Neutral oximes 1 and 2 (200 µM) reactivated parathion-inhibited eel AChE by 9 per cent and 11 per cent, respectively; but neither of them surpassed the reactivation efficacy of 2-PAM (25 per cent). Neutral oximes 1 and 2 reactivated AChE at a safe concentration for humans. Both neutral oximes 1 and 2 are good non-quaternary moieties for the synthesis of conjugates with enhanced reactivation potency and BBB penetration.

Keywords: Acetylcholinesterase; Eel AChE; Reactivator; Oxime; Pralidoxime; Parathion

NOMENCLATURE

OP	Organophosphorus
AChE	Acetylcholinesterase
ACh	Acetylcholine
CNS	Central nervous system
2-PAM	Pralidoxime
TMB-4	Trimedoxime
HI-6	Asoxime
BBB	Blood-brain barrier
PAS	Peripheral anionic site

1. INTRODUCTION

Parathion (O,O-diethyl-O-4-nitro-phenylthiophosphate), an organophosphorus (OP) insecticide with acaricide properties, has been widely applied in agriculture over the past decades[1]. Application of parathion is still legal in many developing countries, leading to elevated cases of human poisoning, despite having been listed as 'extremely hazardous' by the World Health Organisation and banned in many developed countries due to its high toxicity[2]. Among pesticides, OPs are the most toxic to vertebrates, accounting for 2/3 (over three million cases) of human poisoning death worldwide[3]. Toxic exposure to OP may occur through inhalation, ingestion or transdermal exposure[4].

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In addition to their use as insecticides, some OPs (Sarin, Soman, Tabun, VX) are 'nerve agents' and have been used as chemical weapons in terrorist attacks[5]. OPs have the capacity to irreversibly inhibit AChE and butyrylcholinesterase activities by phosphorylation of the serine residue in their active sites[6]. Inhibition of AChE and butyrylcholinesterase results in ACh accumulation in cholinergic synapses of the peripheral and central nervous systems. Increased ACh overstimulates muscarinic and nicotinic receptors, resulting in peripheral muscarinic (salivation, lacrymation, nausea, bradycardia, bronchosecretion), nicotinic (skeletal muscle fasciculations, diaphragm and intercostal paralysis) and central (muscle tremors, convulsions, coma and respiratory depression) manifestations that lead to death[7-9].

Current antidotal regimens approved for human treatment of OP poisoning consist of a combination of muscarinic receptor antagonists (atropine), anticonvulsants (benzodiazepines) and AChE reactivators (oximes), such as obidoxime, 2-PAM, TMB-4 and HI-6[10,11]. These oximes have a high affinity for AChE and have strong nucleophilic character. The first step to reactivation is associated to attack of oxime at phosphorus atom of the phosphorylated enzyme, removing the phosphoryl group from serine at the active site of AChE[12,13]. AChE catalytic properties can be modified by the reversible binding of oximes at different catalytic sites (active

or allosteric)[14]. Although approved as antidotes, these oximes are not sufficiently effective to reactivate AChE inhibited by the different OPs[15,16]. The mono-quaternary oxime 2-PAM is very efficient in reactivating AChE inhibited with sarin or VX[17] but is not effective against tabun or soman[18]. Obidoxime is the most potent and most efficacious oxime in reactivating AChE inhibited by various classes of OP insecticides and tabun, but was inferior to oxime HI-6 against soman, sarin, cyclosarin and VX[19]. A significant drawback to these oximes is they are permanently charged and do not readily cross the blood brain barrier (BBB)[20]. As a result, they show only limited activity in the CNS, which is a major target of OPs. Thus, effective reactivators as antidotes are increasingly needed against a broader spectrum of nerve agents[21].

Introducing non-quaternary organic compounds has been a novel method of efficiently penetrate the BBB for reactivation of brain AChE[22]. Non-charged oximes have previously been developed with improved BBB penetration[23-29] and sufficient reactivation of AChE inhibited by nerve agents and insecticides[24,30]. Even with improved BBB penetration and reactivation of AChE, the specific oxime structures with superior reactivating potency to those in use remain unknown.

The work proposed here evaluated the ability of ten known neutral oximes (Fig. 1) to reactivate parathion-inhibited electric eel AChE. To our knowledge, this work is the first to describe the interactions of these oximes with AChE. Understanding the activity of various neutral oximes will be useful for subsequent design and synthesis of conjugates containing non-quaternary oximes that are capable of binding to both sites of AChE, thereby leading to enhanced reactivation potency.

2. MATERIALS AND METHODS

2.1 Chemicals

Electric eel AChE (EC.3.1.1.7), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine-iodide (ATCI), 2-pyridine aldoxime (2-PAM), parathion (O, O-diethyl O-4-nitrophenyl tiophosphate), 2-bromo-, 3-bromo-, 4-bromobenzaldehyde, 2-chloro-, 4-chlorobenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, 3,4-(methylenedioxy) benzaldehyde, 4-nitrobenzaldehyde, 4-pyridinecarboxaldehyde and 4-cyanepyridine were acquired from Sigma-Aldrich (Brazil). Dichloromethane, ethyl acetate, hexane, ethanol and methanol were purchased from Tedia (Brazil).

2.2 Synthesis of Oxime Derivatives

All neutral oximes (Fig. 1) were prepared by reaction of the respective aldehydes with hydroxylamine hydrochloride. Aldehyde (6 mmol), 6 mL of distilled water, 20 mL of ethanol and hydroxylamine hydrochloride (18 mmol) were combined in a 50 mL round flask. All reactions were conducted with microwave irradiation (P = 80 W) for 15 min, except reactions employing 4-hydroxy-3-methoxybenzaldehyde and 4-cyanepyridine, which were conducted at 60 ºC for 24 h under constant stirring. All reactions were monitored by thin layer chromatography (TLC – hexane:ethyl acetate-1:1) until the aldehydes were totally consumed. Chromatographic plates were examined under ultraviolet light (254 nm). Products were extracted with dichloromethane (3 x 25 mL) and the organic phase was separated and dried with sodium sulfate. Finally, the solvent was removed in a rotatory evaporator and the product was purified by flash chromatography on a silica gel column using a gradient of polarity (hexane:ethyl acetate)[31]. All neutral oximes were characterised by mass spectrometry and ^{1}H NMR, and the signals were compared with those reported in the literature. All mass spectra presented molecular ion values compatible with the expected values. ^{1}H NMR showed signals around δ 8.00 – 7.20, which were associated with iminic hydrogen (-CH=N-O-). This value was compatible with the syn isomer, because the anti isomer has lower values of δ[32].

2.3 Enzyme Activity Determinations

AChE activity was monitored spectrophotometrically (Vmax Microplate reader; Molecular Device) at 405 nm with an Ellman assay[33] modified[34]. AChE stock solution (stock A) (25 units/mL) was prepared in phosphate buffer (100 mM, pH 7.4). An aliquot of stock A was then diluted 60 times with phosphate buffer to give stock B. ATCI (20 mM) was prepared in distilled water. DTNB (10 mM) was prepared in phosphate buffer (100 mM, pH 7.4). 2-PAM (dissolved in distilled water), parathion (dissolved in ethanol) and neutral oximes (dissolved in methanol) were prepared at a concentration of 10 mM and diluted appropriately in phosphate buffer (100 mM, pH 7.4) to the desired concentrations immediately before use. All solutions were kept on ice during the experiment. The final ethanol or methanol concentration in the assay medium was less than 1 per cent and did not inhibit the enzyme activity at that concentration. All experiments were performed at 25±2°C. The values depicted in the figures are the average of three independent assays performed in triplicate in a 96-wells plate.

2.4 *In-vitro* Inhibition of AChE

All experimental wells received AChE stock B, DTNB (0.25 mM), and phosphate buffer (control – enzyme activity) or neutral oximes solutions (10^{-3} mM, 10^{-2} mM, 5 x 10^{-2} mM, 10^{-1} mM, and 2x 10^{-1} mM). The mixture was incubated for 10 min at 25 °C. Then, ATCI (0.5 mM) was added to all wells and the plate was read immediately for 2 min. The spontaneous and oxime induced hydrolysis of the substrate (oximolysis) were evaluated by replacing enzyme for buffer and the activities were corrected for these two parameters. Inhibition is given relative to the control (non-inhibited enzyme; 100 per cent activity). All concentrations refer to final concentrations. The volume of the sample in each well was 0.2 mL.

2.5 *In-vitro* Reactivation of AChE

The incubation mixture was prepared by the addition of parathion (0.1 mM) to a mixture of AChE (stock B) and DTNB (0.25 mM). The mixture was allowed to stand for 60 min at 25°C to give 76 ± 1 per cent inhibition of enzyme activity. Then, the neutral oximes solutions (2x10^{-1} mM; 1 mM) were added to start reactivation. After 10 min of reactivation, ATCI (0.5 mM) was added and the plate reading was done immediately for 2 min. The control enzyme activity at 70 min (without inhibitor and oxime) and the inhibited enzyme activity (without oxime) were determined as described above. All concentrations given above are the final concentrations in the well. The volume of

the sample in each well was 0.2 mL.

Percentage reactivation was calculated using the following equation[34],

$$\% \text{ Reactivation} = (E_r - E_i / E_o - E_i) \times 100$$

where E_0 is the control enzyme activity at 70 min (without inhibitor and oxime), E_i is the inhibited enzyme activity (without oxime) determined as described above and E_r is the activity of reactivated enzyme after incubation with the oxime test compounds. Spontaneous reactivation of inhibited AChE was assayed using the same protocol, the reaction mixture contained enzyme and parathion without oxime. Under these conditions spontaneous reactivation was found to be insignificant. All the values were corrected for their oximolysis.

2.6 Statistical Analysis

All calculations were performed using graph pad prism 5 software (San Diego, CA, USA). The results were analysed by analysis of variance (ANOVA). p values less than 0.05 were considered statistically significant. The results were expressed as means ± SD of three independent assays, each one performed in triplicate.

3. RESULTS

3.1 *In-vitro* Inhibition of AChE by Neutral Oximes

Because oximes bind to AChE as reversible inhibitors and form complexes with AChE either in the active site, allosteric site or in both sites of the enzyme, the inhibition capacity of the neutral oximes 1 to 10 (Fig. 1) was evaluated.

The results of the inhibition experiments are depicted in Fig. 2(a) and 2(b). Inhibition is given relative to the control (non-inhibited enzyme presenting 100 per cent activity). 2-PAM was the reference compound.

As seen from Figs. 2(a) and 2(b), 2-PAM showed higher affinity for AChE than neutral oximes, inhibiting the enzyme in a concentration-dependent manner. In general, the neutral oximes were not good inhibitors. The greatest inhibitory potency (37 per cent) was observed for neutral oxime 8 at a concentration of 200 µM. At 200 µM, neutral oximes 1, 2 and 7 inhibited the enzyme by only 13 per cent, 10 per cent and 23 per cent, respectively. Neutral oxime 6 had no inhibitory effect. Neutral oximes 3-5, 9 and 10 significantly inhibited the enzyme at concentrations from 10 µM to 200 µM, but the

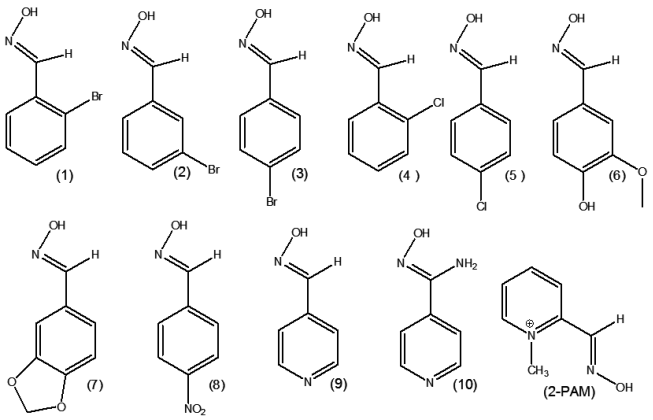

Figure 1. Chemical structure of neutral oximes.

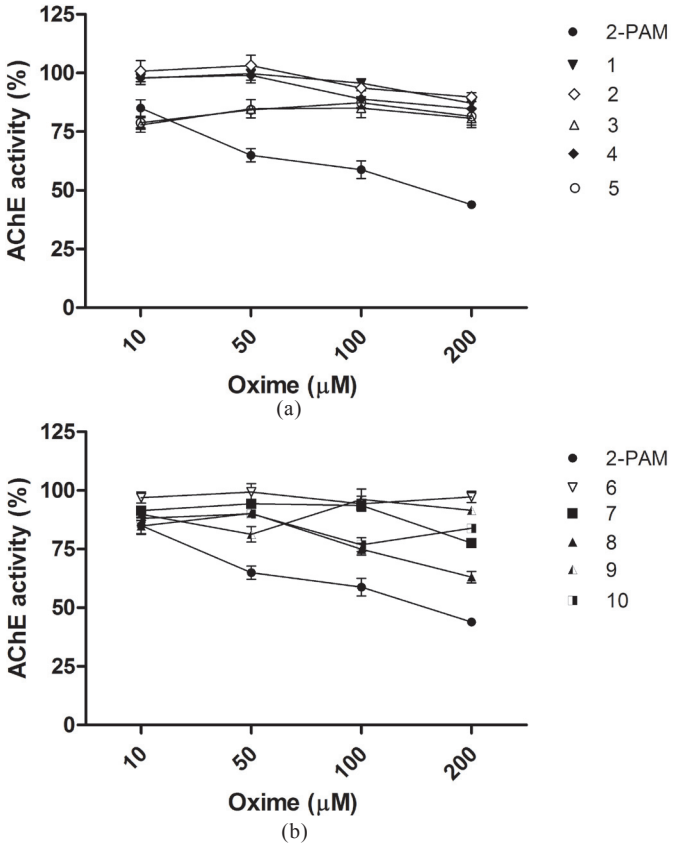

Figure 2. Reversible inhibition of eel AChE by (a) neutral oximes 1-5 and 2-PAM and (b) neutral oximes 6-10 and 2-PAM. The values are the average ± S.D of three independent assays, each one performed in triplicate. *P<0.05 ANOVA.

inhibition percentage remained at approximately 20 per cent even with increasing concentrations of inhibitor.

3.2 *In-vitro* Reactivation of AChE

The *in-vitro* reactivation of parathion-inhibited eel AChE by neutral oximes is depicted in Table 1. The results were compared with the standard oxime reactivator 2-PAM. From these data, it can be seen that neutral oximes 1 and 2 reactivated parathion-inhibited eel AChE by 9 per cent and 11 per cent, respectively, at a concentration of 200 µM. However, neither neutral oxime 1 or 2 surpassed the reactivation efficacy of 2-PAM (25 per cent). Regardless, it is worth noting that at a concentration of 1000 µM, neutral oximes 2 and 5 reactivated 24 per cent and 19 per cent of AChE activity, respectively, while 2-PAM was not able to reactivate the enzyme.

It is known that at elevated concentrations, 2-PAM has esterase-like activity against acetylthiocholine[35-37]. Figure 3 shows the intense oximolysis (esterase-like activity) of ATCI by 2-PAM at 1000 µM and the real activity (activity observed – oximolysis) in the presence of 2-PAM. The results show that better reactivation of 2-PAM occurred at 10 µM.

Indeed 2-PAM is much better reactivator than the neutral oximes since at a concentration of 10 µM reactivation of AChE-parathion inhibited was 42 per cent (Fig. 4). None of the neutral oximes exhibited oximolysis or were able to reactivate parathion-inhibited AChE at concentrations below 200 µM.

Table 1. Reactivation potency of neutral oximes for parathion-inhibited eel acetylcholinesterase

Reactivator	% Reactivation = $[(E_r-E_i)/(E_o-E_i)]$ x 100	
	eel AChE (% Reactivation ± S.D.)	
	200 µM[a]	1000 µM[a]
2-PAM	25.4 ± 3.6	0
1	9.2 ± 2.8	0
2	11.0 ± 3.7	24.1 ± 3.8
3	0	3.6 ± 2.0
4	0	0
5	0	19.5 ± 3.3
6	0	0
7	5.0 ± 0.4	0
8	0	0
9	0	0
10	0	0

[a]Reactivator concentration.
The values are the average ± S.D. of three independent assays, each one performed in triplicate. *P<0.05 ANOVA.

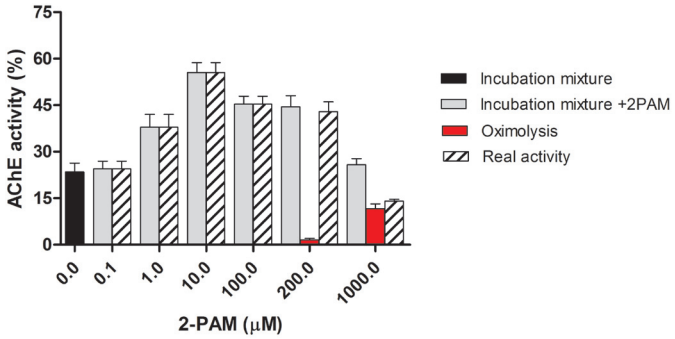

Figure 3. Activity of parathion-inhibited AChE with increasing concentrations of 2-PAM. The values are the average ± S.D. of three independent assays, each one performed in triplicate, compared to non-inhibited enzyme. Oximolysis refers to non-enzymatic hydrolysis of ATCI. *P<0.05 ANOVA.

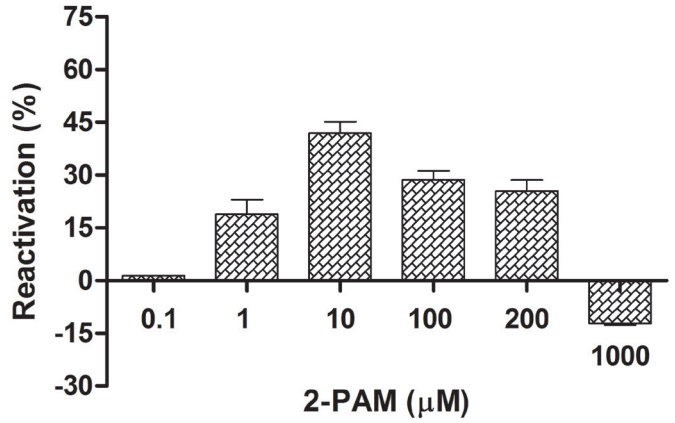

Figure 4. Calculated reactivation of parathion-inhibited AChE with increasing concentrations of 2-PAM. The values are the average ± S.D. of three independent assays, each one performed in triplicate. *P<0.05 ANOVA.

Neutral oximes pKa values (ACD/Labs Software; data not shown) were around 10.

4. DISCUSSION

Oximes can bind to AChE as reversible inhibitors by binding to the active site, allosteric site or both sites. A concentration-dependent inhibition is usually observed when the inhibitor binds to the active site. The neutral oximes tested in this study were not good inhibitors of AChE. The result was expected because it has been shown that the absence of charge affects the reactivity of the nucleophilic oxime moiety and also reduces its affinity for the active site in AChE[38]. Instead, 2-PAM inhibitory activity is attributed to the binding of this compound to an anionic site in the active site of AChE[39,40]. Because it has been shown that neutral ligand can exhibit affinity for the PAS site[24,25], the low inhibitory power observed for the neutral oximes could be attributed to their binding to the PAS, which may modulate the catalytic activity of the active site. The main component of the PAS is an aspartate residue (D74) that is part of an omega loop (Cys65-Cys92) that allosterically links PAS to the active site[41,42]. In general, compounds commonly shown to be AChE reactivators have lower inhibitory potency[43].

According to the results (table 1), neutral oximes 1, 2 and 5 exhibited significant reactivation of parathion-inhibited AChE. 2-bromine aldoxime (neutral oxime 1) reactivated the complex at a concentration of 200 µM, 3-bromine aldoxime (neutral oxime 2) was able to reactivates AChE at both 200 µM and 1000 µM concentrations and 4-chlorine aldoxime (neutral oxime 5) reactivated the enzyme at a concentration of 1000 µM. The human non-toxic concentration of one reactivator was found to be 10^{-4} M and lower[44]. So, although an increase in AChE activity of 5-10 per cent allows for survival in cases of organophosphorus intoxication[45,46], neutral oxime 5 could not be designated as a good reactivator. At a concentration of 200 µM, neutral oxime 1 presented reactivation potency similar to that of neutral oxime 2 and was unable to reactivate AChE at a concentration of 1000 µM. Both oximes differ only in the position of bromine. It seems that bromine at position 3 increases the affinity towards the enzyme. None of the neutral oximes surpassed the reactivation efficacy of 2-PAM. However, it was not the goal of this study to develop a better reactivator than 2-PAM.

Our goal was to find structures capable of reactivating AChE via PAS that could serve as PAS ligand moieties in the development of conjugates able to bind to both sites of AChE. Neutral oximes 1 and 2 are possible candidates to be a PAS ligand moiety because they reactivated the parathion-inhibited AChE at a concentration non-toxic in humans. Moreover, the non-ionic character of these oximes should increase the lipophilicity and BBB penetration of the conjugates. Although we know that the structural and functional differences between human, animal and electric eel AChE may result in a different affinity and reactivity of oximes, we have been working with electric eel AChE due to its ready availability, which facilitates screening assays.

Some reports have demonstrated that an allosteric enhancement of reactivation of carbamoylated or phosphorylated acetylcholinesterases occurred through PAS

occupation by peripheral site ligands[47-49]. Furthermore, it was also shown that oxime-mediated dephosphorylation was accelerated in the presence of a ligand with affinity for the PAS[50]. De Koning[24,25], *et al*. have presented a novel approach to the design of AChE non-ionic reactivators that can cross the BBB more efficiently. They assessed molecules whose characteristics were considered to be neutral and that exhibited a relatively weak affinity for the PAS. Molecules with these characteristics would be linked to a reactivating moiety via a spacer to enable these structures to interact with PAS and AS.

5. CONCLUSIONS

In this study, we assessed the *in-vitro* reactivation efficacy of ten neutral mono oximes against parathion-inhibited AChE. Based upon this study, oximes 1 and 2 showed promising reactivation activity. Knowledge obtained here will be useful for the subsequent design and synthesis of new non-ionic conjugate reactivators with potentially improved BBB penetration. These types of reactivators could be useful for the treatment of intoxication by OP. Although these molecules are not new, this is the first time that these oximes have been tested against parathion-inhibited AChE.

REFERENCES

1. Wu, C. & Linden, K.G. Degradation and byproduct formation of parathion in aqueous solutions by UV and UV/H_2O_2 treatment. *Water Res.*, 2008, **42**, 4780-4790. doi: 10.1016/j.watres.2008.08.023
2. US EPA [United States Environmental Protection Agency]. R.E.D Facts: Ethyl-parathion. EPA-738-F00-009. http://www.epa.gov/oppsrrd1/REDs/factsheets/0155fct.pdf. 2000. Acessed 8 Jan 2013.
3. Carey, L.; Dunn, C. & Gaspari, R.J. Central respiratory failure during acute organophosphate poisoning. *Respir. Physiol. Neurobiol.*, 2013, **189**, 403– 410. doi: 10.1016/j.resp.2013.07.022
4. Eddleston, M.; Buckley, N.A.; Eyer, P. & Dawson, A.H. Management of acute organophosphorus pesticide poisoning. *Lancet*, 2008, **371**, 597–607. doi: 10.1016/S0140-6736(07)61202-1
5. Vale, A.; Marrs, T.C. & Rice, P. Chemical terrorism and nerve agents. *Medicine*, 2011, **40**, 77-79. doi: 10.1016/j.mpmed.2011.11.017
6. Taylor, P.; Radic, Z.; Hosca, N.A.; Camp, S.; Marchot, P. & Berman, H.A. Structural basis for the specificity of cholinesterase catalysis and inhibition. *Toxicol. Lett.*, 1995, **82–83**, 453–458. doi: doi:10.1016/0378-4274(95)03575-3
7. Jokanovic, M. & Prostran, M. Pyridinium oximes as cholinesterase reactivators. Structure-activity relationship and efficacy in the treatment of poisoning with organophosphorus compounds. *Curr. Med. Chem.*, 2009, **16**, 2177-2188. doi: 10.2174/092986709788612729
8. Edwards, F.L. & Tchounwou, P.B. Environmental toxicology and health effects associated with methyl parathion exposure – a scientific review. *Int. J. Environ. Res. Public Health.*, 2005, **2**, 430-441. doi: 10.3390/ijerph2005030007
9. Tafuri, J. & Roberts, J. Organophosphate poisoning. *Ann. Emerg. Med.*, 1987, 16, 193–202. doi: 10.1016/S0196-0644(87)80015-X
10. Kumar, S.V.; Fareedullah, Md.; Sudhakari, Y.; Venkateswarlu, B. & Kumar, E.A. Current review on organophosphorus poisoning. *Arch. Appl. Sci. Res.*, 2010, **2**, 199-215.
11. Jokanovic, M. Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. *Toxicol. Lett*., 2009, **190**, 107-115. doi: 10.1016/j.toxlet.2009.07.025
12. Acharya, J.; Rana, H. & Kauskik, M.P. Synthesis and *in-vitro* evaluation of xylene linked carbamoyl bis-pyridinium monooximes as reactivators of organophosphorus (OP) inhibited electric eel acetylcholinesterase (AChE). *Eur. J. Med. Chem.*, 2011, **46**, 3926-3933. doi: 10.1016/j.ejmech.2011.05.064
13. Bharate, S.B.; Guo, L.; Reeves, T.E.; Cerasoli, D.M. & Thompson, C.M. Bisquaternary pyridinium oximes: Comparison of *in-vitro* reactivation potency of compounds bearing aliphatic linkers and heteroaromatic linkers for paraoxon-inhibited electric eel and recombinant human acetylcholinesterase. *Bioorg. Med. Chem.*, 2010, **18**, 787-794. doi: 10.1016/j.bmc.2009.11.052
14. Jokanovic, M. & Stojiljkovic, M.P. Current understanding of the application of pyridinium oximes as cholinesterase reactivators in treatment of organophosphate poisoning. *Eur. J. Pharmacol.*, 2006, **553**, 10-17. doi: 10.1016/j.ejphar.2006.09.054
15. Bajgar, J.; Fusek, J.; Kuca, K.; Bartosova, L. & Jun, D. Treatment of organophosphate intoxication using cholinesterase reactivators: Facts and fiction. *Mini Rev. Med. Chem.*, 2007, **7**, 461-466. doi: 10.2174/138955707780619581
16. Eyer, P.; Worek, F. Oximes. In: Marrs, T.C.; Maynard, R.L. & Sidell, F.R. (Eds.), Chemical warfare agents: Toxicology and treatment. John Wiley & Sons Ltd, Chichester, 2007. pp. 305–329.
17. Mesic, M.; Deljac, A.; Deljac, V.; Binenfeld, Z.; Kilibarda, V.; Maksimovic, M. & Kovacevic, V. Reactivations of acetylcholinesterase inhibited by organophosphorus compounds. Imidazole derivatives. II. *Acta Pharm. Jugosl.*, 1991, **41**, 203–210.
18. Koplovitz, I. & Stewart, J.R. A comparison of HI-6 and 2-PAM against soman, tabun, sarin and VX in the rabbit. *Toxicol. Lett.*, 1994, **70**, 269–279. doi: 10.1016/0378-4274(94)90121-X
19. Worek, F.; Bäcker, M.; Thiermann, H.; Szinicz, L.; Mast, U.; Klimmek, R. & Eyer, P. Reappraisal of indications and limitations of oxime therapy in organophosphate poisoning. *Hum. Exp. Toxicol.*, 1997, **16**, 466-472. doi: 10.1177/096032719701600808
20. Sakurada, K.; Matsubara, K.; Shimizu, K.; Shiono, H.; Seto, Y.; Tsuge, K.; Yoshino, M.; Sakai, I.; Mukoyama, H. & Takatori, T. Pralidoxime iodide (2-PAM) penetrate across the blood-brain barrier. *Neurochem. Res*., 2003,

28, 1401-1407.
10.1023/A:1024960819430

21. Gupta, B.; Sharma, R.; Singh, N.; Kuca, K.; Acharya, J.R. & Ghosh, K.K. *In-vitro* reactivation kinetics of paraoxon- and DFP inhibited electric eel AChE using mono- and bis-pyridinium oximes. *Arch. Toxicol.*, 2014, **88**, 381-390. doi: 10.1007/s00204-013-1136-z
22. Kalasz, H.; Nurulain, S.M.; Veress, G.; Antus, S.; Darvas, F.; Adeghate, E.; Adem, A.; Hashemi, F. & Tekes, K. Mini review on blood–brain barrier penetration of pyridinium aldoximes. *J. Appl. Toxicol.*, 2015, **35**, 116–123. doi: 10.1002/jat.3048
23. Garcia, G.E.; Campbell, A.J.; Olson, J.; Moorad-Doctor, D. & Morthole, V.I. Novel oximes as blood–brain barrier penetrating cholinesterase reactivators. *Chem. Biol. Interact.*, 2010, **187**, 199–206. doi: 10.1016/j.cbi.2010.02.033
24. De Koning, M.C.; van Grol, M. & Noort, D. Peripheral site ligand conjugation to a non-quaternary oxime enhances reactivation of nerve agent-inhibited human acetylcholinesterase. *Toxicol. Lett.*, 2011a, **206**, 54–59. doi: 10.1016/j.toxlet.2011.04.004
25. De Koning, M.C.; Joosen, M.J.A.; Noort, D.; van Zuylen, A. & Tromp, M.C. Peripheral site ligand-oxime conjugates: A novel concept towards reactivation of nerve agent-inhibited human acetylcholinesterase. *Bioorg. Med. Chem.*, 2011b, **19**, 588–594. doi: 10.1016/j.bmc.2010.10.059
26. Kalisiak, J.; Ralph, E.C.; Zhang, J. & Cashman, J.R. Amidine-oximes: Reactivators for organophosphate exposure. *J. Med. Chem.*, 2011, **54**, 3319–3330. doi: 10.1021/jm200054r
27. Kalisiak, J.; Ralph, E.C. & Cashman, J.R. Nonquaternary reactivators for organophosphate-inhibited cholinesterases. *J. Med. Chem.*, 2012, **55**, 465–474. doi: 10.1021/jm201364d
28. Mercey, G.; Verdelet, T.; Saint-Andre, G.; Gillon, E.; Wagner, A.; Baati, R.; Jean, L.; Nachon, F. & Renard, P.Y. First efficient uncharged reactivators for the dephosphylation of poisoned human acetylcholinesterase. *Chem. Commun.*, 2011, **47**, 5295–5297. doi: 10.1039/c1cc10787a
29. Radic, Z.; Sit, R. K.; Kovarik, Z.; Berend, S.; Garcia, E.; Zhang, L.; Amitai, G.; Green, C.; Radić, B.; Fokin, V.V.; Sharpless, K.B. & Taylor, P. Refinement of structural leads for centrally acting oxime reactivators of phosphylated cholinesterases. *J. Biol. Chem.*, 2012, **287**, 11798–11809. doi: 10.1074/jbc.M111.333732
30. Dube, S.N.; Ghosh, A.K.; Jeevarathinam, K.; Kumar, D.; Das Gupta, S.; Pant, B.P.; Batra, B.S. & Jaiswal, D.K. Studies on the efficacy of diethyxime as an antidote against organophosphorus intoxication in rats. *Jpn. J. Pharmacol.*, 1986, **41**, 267–271. doi: 10.1254/jjp.41.267
31. Rodrigues, R.C. & De Aguiar.; A.P. A simple and efficient method for the synthesis of nitrile oxide from aldoxime using trichloroisocyanuric acid. *Synth. Commun.*, 2001, **31**, 3075-3080. doi: 10.1081/SCC-100105879
32. Groenendijk, G.W.T.; De Grip, W.J. & Daemen, F.J.M. Identification and characterization of syn- and anti-isomers of retinaloximes. *Anal. Biochem.*, 1979, **99**, 304-310. doi:10.1016/S0003-2697(79)80011-1
33. Ellman, G.L.; Courtney, K.D.; Andres, J.R.V. & Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase. *Biochem. Pharmacol.*, 1961, **7**, 88–95. doi:10.1016/0006-2952(61)90145-9
34. Acharya, J.; Gupta, A.K.; Mazumder, A. & Dubey, D.K. In-vitro reactivation of sarin inhibited electric eel acetylcholinesterase by bis-pyridinium oximes bearing methoxy ether linkages. *Toxicol. In-vitro*, 2008, **22**, 525–530. doi: 10.1016/j.tiv.2007.10.006
35. Sakurada, K.; Ikegaya, H.; Ohta, H.; Akutsu, T. & Takatori, T. Hydrolysis of an acetylthiocholine by pralidoxime iodide (2-PAM). *Toxicol. Lett.*, 2006, **166**, 255-260. doi: 10.1016/j.toxlet.2006.07.339
36. Jafari, M. & Pourheidari, G. The reactivation effect of pralidoxime in human blood on parathion and paraoxon-induced cholinesterase inhibition. *DARU J. Pharm. Sci.*, 2006, **14**, 37-43.
37. Nadarajah, B. The effect of pralidoxime chloride in the assay of acetylcholinesterase using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). *J. Anal. Toxicol.*, 1992, **16**, 192-193. doi: 10.1093/jat/16.3.192
38. Kuca, K.; Jun, D. & Musilek, K. Structural requirements of acetylcholinesterase reactivators. *Mini Rev. Med. Chem.*, 2006, **6**, 269–277. doi: 10.2174/138955706776073510
39. Rosenberry T.L. Acetylcholinesterase. *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1975, **43**, 103-218. doi: 10.1002/9780470122884.ch3
40. Froede, H.C. & Wilson, I.B. Acetylcholinesterase. *In* The Enzymes. Edited by P.D. Boyer, Ed. 3rd. New York, Academic Press, 1971, pp. 87–114.
41. Johnson, G. & Moore, S.W. The peripheral anionic site of acetylcholinesterase: structure, functions and potential role in rational drug design. *Curr. Pharm. Des.*, 2006, **12**, 217-225. doi: 10.2174/138161206775193127
42. Hosea, N.A.; Radic, Z.; Tsigelny, I.; Berman, H.A.; Quinn, D.M. & Taylor, P. Aspartate 74 as a primary determinant in acetylcholinesterase governing specificity to cationic organophosphonates. *Biochem.*, 1996, **35**, 10995-11004. doi: 10.1021/bi9611220
43. Sepsova, V.; Karasova, J.Z.; Korabecny, J.; Dolezal, R.; Zemek, F.; Bennion, B.J. & Kuca, K. Oximes: Inhibitors of human recombinant acetylcholinesterase. A structure-activity relationship (SAR) study. *Int. J. Mol. Sci.*, 2013, **14**, 16882-16900. doi: 10.3390/ijms140816882
44. Kuca, K. & Kassa, J. A comparison of the ability of a new byspyridinium oxime-1-

(hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium) butane dibromide and currently used oximes to reactivate nerve agent-inhibited rat brain acetylcholinesterase by *in-vitro* methods. *J. Enzyme Inhib. Med. Chem.*, 2003, **18**, 529-535.
doi: 10.1080/14756360310001605552

45. Bajgar, J.; Fusek, J.; Kassa, J.; Jun, D.; Kuca, K. & Hajek, P. An attempt to assess functionally minimal acetylcholinesterase activity necessary for survival of rats intoxicated with nerve agents. *Chem. Biol. Interact.*, 2008, **175**, 281-285.
doi: 10.1016/j.cbi.2008.05.015
46. Oh, K.A.; Yang, Y.; Jun, D.; Kuca, K. & Jung, Y.S. Bispyridiumaldoxime reactivators connected with $CH_2O(CH_2)$ (n)OCH_2 linkers between pyridinium rings and their reactivity against VX. *Bioorg. Med. Chem. Lett.*, 2006, **16**, 4852-4855.
doi: 10.1016/j.bmcl.2006.06.063
47. Rosenberry, T.L.; Johnson, J.L.; Cusack, B.; Thomas, J.L.; Emani, S. & Venkatasubban, K.S. Interactions between the peripheral site and the acylation site in acetylcholinesterase. *Chem. Biol. Interact.*, 2005, **157–158**, 181-189.
doi: 10.1016/j.cbi.2005.10.027
48. Tomlinson, G.; Mutus, B. & McLennan, I. Modulation of acetylcholinesterase activity by peripheral site ligands. *Mol. Pharmacol.*, 1980, **18**, 33-39.
49. Rosenberry, T.L.; Sonoda, L.K.; Dekat, S.E.; Cusack, B. & Johnson, J.L. Monitoring the reaction of carbachol with acetylcholinesterase by thioflavin T fluorescence and acetylthiocholine hydrolysis. *Chem. Biol. Interact.*, 2008, **175**, 235-241.
doi: 10.1016/j.cbi.2008.06.002
50. Harris, L.W.; Heyl, W.C.; Stitcher, D.L. & Broomfield, C.A. Effects of 1,1′-oxydimethylene bis-(4-tert-butylpyridinium chloride) (SAD-128) and decamethonium on reactivation of soman- and sarin-inhibited cholinesterase by oximes. *Biochem. Pharmacol.*, 1978, **27**, 757-761.
doi:10.1016/0006-2952(78)90516-6

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