Defence Life Science Journal, Vol. 01, No. 2, September 2016, pp. 162-166, DOI : 10.14429/dlsj.1.10736

In-vitro Evaluation of Bis-pyridinium Oximes Connected by Xylene Linkers as Reactivators of DFP-inhibited Electric EEL Acetylcholinesterase

Jyotiranjan Acharya*, Hitendra N. Karade, G. Raviraju, Sanatan Ghosh, and Arvind Kumar Gupta

Process Technology Development Division, Defence Research & Development Establishment, Gwalior - 474 002 India
**E-mail: jracharya01@gmail.com*

ABSTRACT

Bis-pyridinium oximes connected by xylene linkers were synthesised and their *in-vitro* reactivation efficacy was evaluated for DFP inhibited AChE. The reactivation efficacy data were compared with those of 2-PAM and obidoxime. However, it was observed that none of these oximes were able to surpass the reactivation efficacy of 2-PAM and obidoxime in reactivating DFP inhibited AChE. 2-PAM and obidoxime respectively exhibited 52 per cent and 43 per cent reactivation of DFP inhibited AChE, where as the synthesised oximes **3a, 3d,** and **3f** showed 37 per cent, 30 per cent, and 31 per cent reactivation, respectively within 10 min at 10^{-3} M.

Keywords: Bis-pyridinium oximes, Reactivators, 2-PAM, DFP, organophosphorus pesticides, nerve agents, acetylcholinesterase, obidoxime

NOMENCLATURE

AChE	Acetylcholinesterase
DFP	Diisopropyl phosphorofluoridate
GC	Gas chromatography
IR	Infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
TLC	Thin layer chromatography

1. INTRODUCTION

Organophosphorus (OP) pesticides such as *O*,*O*-diethyl-*O*-(4-nitrophenyl) phosphate (paraxon), diisopropyl chlorophosphate (DFP) have been used as pesticides extensively causing approximately three million pesticide related poisoning (both intentional and occupational) world wide resulting in the death of three hundred thousands of human lives annually[1,2]. Further, a large volume of organophosphorus (OP) based chemical warfare (CW) agents viz. tabun, soman, sarin and VX are still available (Fig.1). In spite of the continued efforts by the world community to prevent production, storage and use of these deadly CW agents, they have frequently been used during war[3,4] and terrorism[5,6] displays that they constitute a major threat for the civilisation.

The acute toxicity of OP compounds is attributed to their irreversible inhibition of cholinesterase (ChE) family of enzymes including acetylcholinesterase (AChE)[7], a critically important central nervous system (CNS) and peripheral nervous system (PNS) enzyme that hydrolyses the neurotransmitter acetylcholine (ACh), into choline and acetate anion[8,9]. The resultant increase in the level of ACh at cholinergic synapses produces an acute cholinergic crisis characterised by miosis, increased tracheobronchial and salivary secretions, bradycardia and convulsions resulting in death by respiratory failures[10].

Currently pyridostigmine bromide, a spontaneously reactivating inhibitor of enzyme AChE to prevent irreversible inhibition of AChE by OP compounds has been used as a pretreatment measure of OP poisoning. However, therapeutic treatment of OP poisoning consists of a combination of anticholiergic drugs such as atropine and a reactivator such as oxime (Fig. 2) to reactivate OP inhibited AChE[11,12]. Currently, pyridiniumaldoximes are the only clinically used reactivators available organophosphorus poisoning. OP inhibited enzyme is generally reactivated by the attack of a strong nucleophile (oximino anion) at the electron defficient P-atom of the OP-AChE complex. Oximes such as 2-(Hydroxyiminomethyl)-1-methylpyridinium chloride (2-PAM) and several other oximes are developed in the past as reactivators for OP poisoning[13,14]. One of the most widely used reactivator is 2-PAM, developed

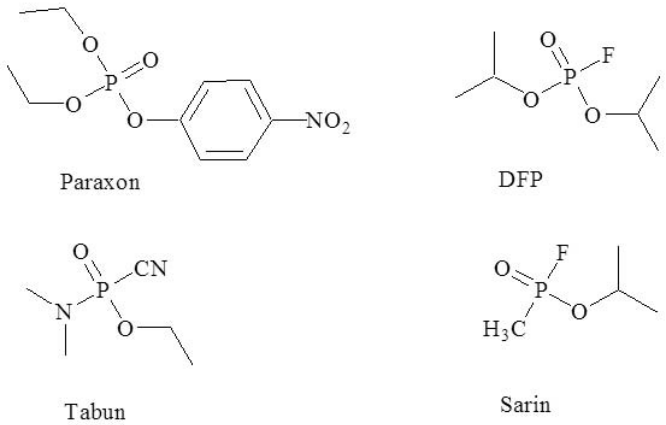


Figure 1. Organophosphorus pesticides and nerve agents.

Received : 20 March 2016, Revised : 15 June 2016
Accepted : 16 June 2016, Online published : 07 October 2016

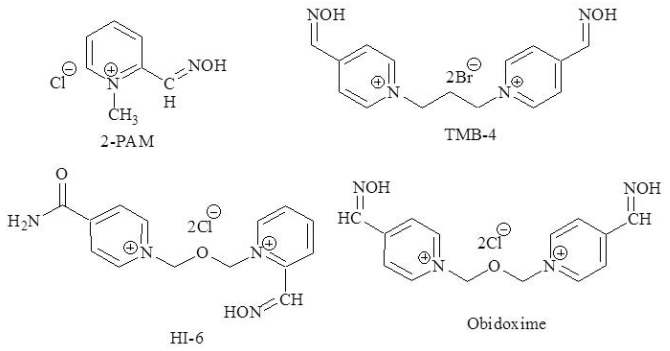


Figure 2. Oximes used as reactivators of OP inhibited AChE.

in the late 1950s by Ginsberg and Wilson[15]. While 2-PAM is effective against sarin, diisopropylphosphoro-fluoridate (DFP) and ethyldimethyl phosphoramidocyanidate (tabun); its efficacy against 3, 3'-dimethyl-2-butyl methyl phosphonofluoridate (soman) is marginal[11]. Further, 1-[(4-carbamoylpyridino)-methoxy methyl]-2-[(hydroxyimino)methyl] pyridinium dichloride (HI-6) though effective against soman, is unstable. Therefore, research in this area is focused on to find a least toxic and stable oxime which would be effective against all the nerve agents[11,16-17]. Mono- and bis-pyridinium oximes have been used as AChE reactivators in OP intoxication[18,19]. The standard oximes available today as antidotes are obidoxime (toxogonin), HI-6 and TMB-4. The oxime HI-6 has been reported as an effective reactivator of non-aged soman inhibited human AChE[18-21]. Further an effective therapy by a single oxime for a broad spectrum of OP pesticides and nerve agents is still lacking.

Recently several bis-pyridinim-aldoximes connected by a variable length alkylene and bis-methoxy alkane chain were reported as AChE reactivators[21-23]. Some of these oximes have shown enhanced reactivation potential against OP inhibited AChE and led us to further explore the synthesis of bis-pyridinium oximes using a variety of linkers. In continuation of our work on the reactivators for OP-inhibited AChE[25], here in the evaluation of a series of bis-pyridinium oximes connected by xylene bridge as reactivators of DFP-inhibited AChE is reported.

Hence, in the present work we have carried out a systematic study on the synthesis and *in-vitro* reactivation efficacy of bis-pyridinium oximes connected by xylene linker between two pyridinium rings against DFP inhibited electric eel AChE.

2. MATERIALS AND METHODS

2.1 Chemicals

Electric eel AChE (EC.3.1.1.7), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholineiodide, and 2-, 3- & 4-pyridinealdoxime, α,α'-dibromoxylene were purchased from Sigma-Aldrich, USA and used without further purification. Potassium dihydrogen-phosphate and dipotassium-hydrogen phosphate were purchased from E. Merck (India) and used without further purification. Solvents (acetonitrile, acetone, methanol) were purchased from s.d. Fine Chemicals (India) and dried before use. DFP was prepared in this laboratory with > 98 per cent purity (GC and ^{31}P NMR). 2-PAM was prepared according to the reported method[15]. The bis-pyridinium oximes and obidoxime were synthesised, characterised by their ^{1}H NMR and ^{13}C NMR chemical shift values and their purity was checked by TLC (cellulose, DSO, Fluka) with 1-butanol: acetic acid: water (3:1:1) as solvent system[24].

2.2 Synthesis of Bis-pyridinium Oximes

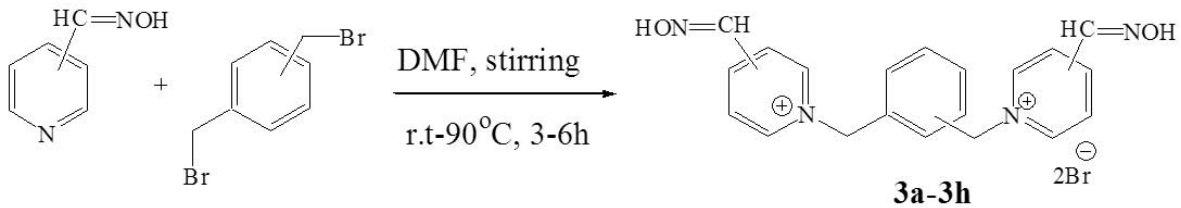
The synthesis of bis-pyridinium oximes **(3a-3h)** were reported earlier[26]. The reaction involved alkylations of pyridine-aldoxime (**1**) with isomeric α,α'-dibromoxylene (**2**) (Scheme 1) to form the desired oximes (**3**) (as shown in Table 1) and characterised by their IR ^{1}H and ^{13}C NMR chemical shifts[26].

2.3 In-*vitro* Reactivation Study

The in-vitro reactivation efficacy of the synthesised oximes (**3a-3h**) were evaluated against DFP inhibited AChE in phosphate buffer (0.1 M, pH 8.0 at 37 °C) using enzyme assay protocol of Ellman[27], *et al.* Values depicted in figures are average of three runs with maximum relative standard deviation of ±2 per cent. AChE stock solution (stock A) was prepared in phosphate buffer (pH 7.6, 0.1 M) (352 units/0.5 mL). An aliquot of stock A was then diluted 50 times with phosphate buffer to give stock B. Stock solution of DFP (1.08 x 10^{-2} M) was freshly prepared in isopropanol and stored under refrigeration. It was then diluted appropriately with triple distilled water just before use. All oxime stock solutions were prepared in triple distilled water. DTNB stock (10 mM) and acetylthiocholineiodide (75 mM) were prepared in phosphate

Table1. Structure of bis-pyridinum oximes evaluated against DFP-inhibited AChE

Oxime	-CH=NOH	Xylene
3a	4	*para-*
3b	3	*para-*
3c	2	*para-*
3d	4	*meta-*
3e	3	*meta-*
3f	4	*ortho-*
3g	3	*ortho-*
3h	2	*ortho-*



Scheme 1. Synthesis of bis-pyridinium oximes

buffer (pH 7.6, 0.1 M) and distilled water, respectively. Fifty microlitre of DFP (1.08 x 10^{-4} M) was added to a mixture of 50 µL enzyme (stock B) in 350 µL phosphate buffer pH 8.0 (0.1 M) to prepare the DFP inhibited enzyme. The mixture was allowed to stand for 15 min at ambient temperature to give 95-97 per cent inhibition of enzyme activity. Further increase in the inhibition of enzyme activity was not observed even after 1 h of the inhibition with DFP at this concentration. It was then followed by addition of 50 µL of oxime test solution (1 x 10^{-2} M, 1 x 10^{-3} M) to start reactivation. The final volume of the reactivation cocktail was 500 µL. The final concentration of DFP was 1.08x10^{-5} M and oxime was diluted 10 fold in the reactivation cocktail. After 10 minutes of reactivation the enzyme activity was assayed by Ellman's method (Fig. 3). Twnwty microlitre of reactivation cocktail was added to a cuvette containing 50 µL DTNB in phosphate buffer (pH 8.0, 0.1M). The enzyme activity was then assayed by addition of 50 µL of substrate to the cuvette against a blank containing reactivation cocktail without substrate. The final volume of the assay mixture was adjusted to 3 mL and final concentration of DTNB and substrate was 0.16 mM and 1.25 mM, respectively. The reactivation of inhibited enzyme was then studied at an interval of 10 minutes and followed up to 1h (as shown in Fig. 4). Percentage reactivation was calculated using the following Eqn[24,26].

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

where E_o is the initial enzyme activity at 0 min (without inhibitor and oxime), E_i is the activity of inhibited enzyme determined in the similar manner 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 DFP but no oxime. Under these conditions spontaneous reactivation was found to be insignificant. Values are corrected for their oxime induced hydrolysis.

3. RESULTS

The reactivation of DFP inhibited AChE by these oximes was studied at two different concentrations i.e., 10^{-3} M and 10^{-4} M. However, it was observed that none of these oximes

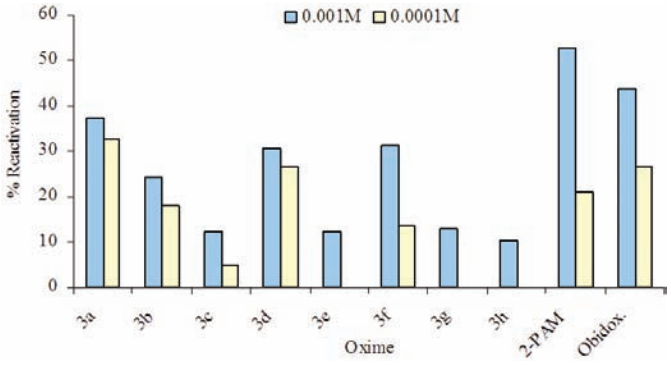


Figure 3. Reactivation efficay of the oximes against DFP-inhibited AChE. Source of enzyme: electric eel, inhibitor agent: DFP, inhibitor concentration: 1.08x10^{-5} M, time of inhibition: 15 min, time of reactivation: 10 min, pH: 8.0 and Temperature: 37 °C. The values are average of three runs with maximum S.D. of ±2 per cent.

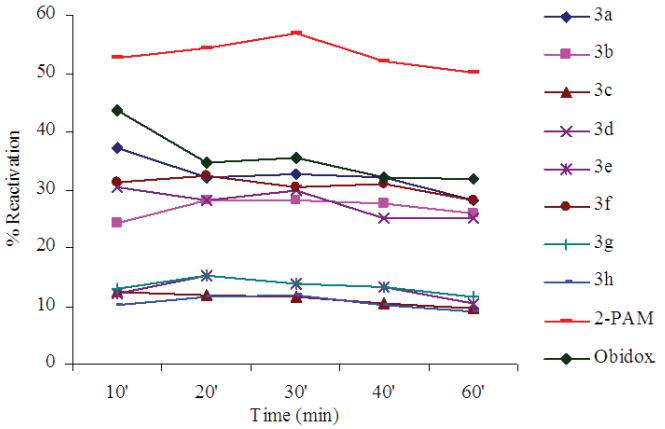


Figure 4. Reactivation profile of DFP inhibited AChE with tested oximes at 1x10^{-4} M.

surpassed the reactivation efficacy of 2-PAM and obidoxime in reactivating DFP inhibited AChE. 2-PAM and obidoxime respectively exhibited 52 per cent and 43 per cent reactivation of DFP inhibited AChE where as the synthesised oximes **3a, 3d** and **3f** showed 37 per cent, 30 per cent and 31 per cent, respectively within 10 minutes at 10^{-3} M (as shown in Fig. 3). Earlier it was demonstrated that **3b** reactivated the sarin-inhibited AChE more effectively in comparison to the 2-PAM even at a lower concentration.[26] It can also be noted that all the 4-isomers (oximino moiety in the 4-position of pyridinium ring) of bis-pyridinium oximes were found to be superior in terms of reactivation potential than their 3- and 2-isomers for DFP inhibited AChE where as 3-isomers of the same were found most potent in reactivating sarin inhibited AChE. It further corroborates earlier study (bis-methoxy ethane linkages) that reactivation of inhibited enzyme also depends on the structure of the inhibitor as well as that of reactivator.[17]

Further, from the study of the effect of different isomers of xylene bridge (as shown in Figs. 3 and 4), it was observed that the *para*-isomers in the xylene linkage showed the highest reactivation followed by *meta*- and *ortho*-isomers (**3b > 3e > 3g**).

The time dependent reactivation profile by following the reactivation up to 60 min of oxime addition was also investigated and has been presented in Fig. 4. However, in case of DFP inhibition no time dependent reactivation was observed in Fig. 4. The maximum reactivation was observed within 10 minutes of addition of oxime, after which it began to fall sharply. This is in fact, contrast to the earlier study involving other class of bis-pyridinium oximes, where reactivation of OP inhibited AChE increases with time[26,24].

4. DISCUSSION

The oxime induced reactivation of OP-inhibited AChE depend on various factors such as number of pyridinium rings (mono or bis-pyridinium), position of the oxime group chemical structure and length of theconnecting chain in bis-pyridinium oximes[22,23] as well as the structure of the OP inhibitor[28]. Better reactivation with the studied bis-pyridinium oximes bearing oxime function at 4th position could be attributed to electronic and steric effects[29]. The higher reactivation efficacy of **3a** and

3d for DFP inhibited AChE at the lower concentration 10^{-4} M in comparison to 2-PAM and obidoxime showed that they can be promising candidates for *in-vivo* reactivation as well. The higher reactivation potency of the oximes bearing *p*-xylene linkers as compared to their *ortho*- and *meta*- analogues may be attributed to the fact that the pyridinium rings connected to the p-xylene linkers are far away from each other giving rise to suitable orientation to be fit in the 20Å active-site gorge of the enzyme AChE, where one pyridinium ring might reside at the ring of the active site allowing the other ring to penetrate through the gorge to bind to the anionic site. The oximino moiety on the second ring suitably orients itself to attack on the phosphorus atom of the phosphorylated enzyme. Further, the xylene group in the linkers might provide suitable non-bonding hydrophobic interactions with the aryl residues present in the active-site gorge of the enzyme.

In conclusion, we have synthesised series of new bis-pyridinium oximes and evaluated their *in-vitro* reactivation efficacy against DFP inhibited AChE. Based upon this study, **3a** and **3d** may prove a useful therapeutic candidates for the reactivation of AChE inhibited by DFP. The detailed study of antidotal efficacy including *in-vivo* reactivation against DFP and other nerve agents remains to be explored.

REFERENCES

1. Eyer, P. The role of oximes in the management of organophosphorus pesticide poisoning. *Toxicol. Rev.*, 2003, **22**, 165-90. doi: 10.2165/00139709-200322030-00004
2. Roberts, D. M.; Karunarathna, A.; Buckley, N. A.; Manuweera, G.; Sheriff, M. H. R. & Eddleston, M. Influence of pesticide regulation on acute poisoning deaths in Sri Lanka. *Bull. World Health Org.,* 2003, **81** (11), 789-798.
3. MacIlwain, C. Study proves Iraq used nerve gas. *Nature*, 1993, **363**, 3. doi: 10.1038/363003b0
4. The Nobel Peace Prize **2013**, <http://www.opcw.org/news/article/opcwreceives-2013-nobel-prize-for-peace/
5. Nagao, M.; Takatori, T.; Matsuda, Y.; Nakajima, M.; Iwase, H. & Iwadate, K. Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. *Toxicol. Appl. Pharmacol.*, 1997, **144**, 198-203. doi: 10.1006/taap.1997.8110
6. Okumura, T. ; Hisaoka, T.; Yamada, A.; Naito, T.; Isonuma, H.; Okumura, S.; Miura, K.; Sakurada, M.; Maekawa, H.; Ishimatsu, S.; Takasu, N. & Suzuki, K. The Tokyo subway sarin attack: Lessons learned**.** *Toxicol. Appl. Pharmacol.*, 2005, **207**, 471-476. doi: 10.1016/j.taap.2005.02.032
7. Bajgar, J. Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment**.** *Adv. Clinical Chem.*, 2004, **38**, 151-216. doi: 10.1016/S0065-2423(04)38006-6
8. Kuca, K.; Bielavsky, J.; Cabal, J. & Kassa, J. Synthesis of a new reactivator of tabun-inhibited acetylcholinesterase. *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3545-3547. doi: 10.1016/S0960-894X(03)00751-0
9. Patocka, J.; Kuca, K. & Jun, D. Acetylcholinesterase and butyrylcholinesterase important enzymes of human body. *Acta Medica (Hardec Kralove)*, 2004, **47**, 215-228.
10. Koelle, G.B. Pharmacological basis of therapeutics. Edited by L.S. Goodman & A. Gilman, Macmillan Co., New York, 1970. pp 442-465.
11. Gray, A.P. Design and structure activity relationship of antidotes to organophosphorus antichlonesterase agents. *Drug Metabolism Rev.*, 1984, **15**, 557-589.
12. Leadbeater, L.; Inns, R.H. & Rylands, J.M. Treatment of poisoning by soman. *Fundamental Appl. Toxicol.*, 1985, **5**, S225-31.
13. Krejcova, G. & Kassa, J. Neuroprotective efficacy of pharmacological pretreatment and antidotal treatment in tabun-poisoned rats. *Toxicology*, 2003, **185**, 129-139. doi: 10.1016/S0300-483X(02)00599-1
14. Musilek, K.; Kuca, K.; Jun, D. & Dolezal, M. Progress in synthesis of new acetylcholine reactivators during the period 1990-2004. *Curr. Org. Chem.*, 2007, **11**, 229-238. doi: 10.2174/138527207779316417
15. Ginsburg, S. & Wilson, I.B. Oximes of the pyridine series. *J. Am. Chem. Soc.*, 1957, **79**, 481-5. doi: 10.1021/ja01559a067
16. Thiermann, H.; Seidl, S. & Eyer, P. HI 6 dimethanosulfonate has better dissolution properties than HI 6 dichloride for application in dry/wet autoinjectors. *Int. J. Pharma.* 1996, **137**, 167-176. doi: 10.1016/0378-5173(96)04511-5
17. Lallement, G.; Clarencon, D.; Brochier, G.; Baubichon, D.; Galonnier, M.; Blanchet, G. & Mestries, J.C. Efficacy of atropine/pralidoxime/diazepam or atropine/HI-6/ prodiazepam in primates intoxicated by soman. *Pharmacol. Biochem. Behavior*, 1997, **56**, 325-332. doi: 10.1016/S0091-3057(96)00292-4
18. Sterri, S.H.; Lyngaas, S. & Fonnum, F. Cholinesterase and carboxylesterase activities in soman poisoned rats treated with bispyridinium mono-oximes HI-6 and HS-6. *Biochem. Pharmacol.*, 1983, **32**, 1646-649. doi: 10.1016/0006-2952(83)90062-X
19. Schoene, K.; Steinhanses, J. & Oldiges, H. Reactivation of soman inhibited acetylcholinesterase in vitro and protection against soman in vivo by bispyridinium-2-aldoximes. *Biochem. Pharmacol.*, 1983, **32**, 1649-651. doi: 10.1016/0006-2952(83)90343-X
20. Kim, T.H.; Kuca, K.; Jun, D. & Jung, Y.S. Design and synthesis of new bis-pyridinium oxime reactivators for acetylcholinesterase inhibited by organophosphorus nerve agents. *Bioorg. Med. Chem. Lett.*, **2005**, 16, 2914-917. doi: 10.1016/j.bmcl.2005.03.060
21. Musilek, K.; Kula, K.; Jun, D.; Dehnal, V. & Dolezal, M. Synthesis of the novel series of bispyridinium compounds bearing (E)-but-2-ene linker and evaluation of their reactivation activity against chloropyrifos-inhibited acetylcholinesterase**.** *Bioorg. Med. Chem. Lett.* 2006, **16**, 662-27. doi: 10.1016/j.bmcl.2005.10.059
22. Chennamaneni, S.R.; Venkateshwarulu, V.; Achaiah, G.; Quaternary salts of 4, 3' and 4, 4' bis-pyridinium monooximes: Synthesis and biological activity. *Bioorg. Med. Chem. Lett.*, 2005, **15**, 3076-3080. doi: 10.1016/j.bmcl.2006.01.065
23. Rao, C.S.; Venkateswahlu, V. & Achaiah, G. Quaternary

salts of 4,3' and 4,4' bis-pyridinium monooximes. Part 2: Synthesis and biological activity. *Bioorg. Med. Chem. Lett.*, 2006. **16**, 2134-2138. doi: 10.1016/j.bmcl.2006.01.065

24. Acharya, J.; Gupta, A.K.; Mazumder, A. & Dubey, D. K. *In-vitro* reactivation of sarin inhibited electric eel acetylcholinesterase by bispyridinium oximes bearing methoxy ether linkages. *Toxicol. Invitro* 2008, **22**, 525-530. doi: 10.1016/j.tiv.2007.10.006
25. Sharma, R.; Gupta, B.; Singh, N.; Acharya, J.; Musilek, K.; Kuca, K. & Ghosh K. K. Development and Structural Modifications of Cholinesterase Reactivators against Chemical Warfare Agents in Last Decade: A Review. *Mini Rev. Med. Chem.* 2015, **15**(1), 58-72. doi: 10.2174/1389557514666141128102837
26. Acharya, J.; Gupta, A.K.; Mazumder, A. & Dubey, D. K., In-vitro regeneration of sarin inhibited electric eel acetylcholinesterase by bis-pyridinium oximes bearing xylene linker. *Eur. J. Med. Chem*., 2009. **44**, 1326-30. doi: 10.1016/j.ejmech.2008.02.020
27. Ellman, G.L.; Courtny, K. D.; Andres, V. Jr. & Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, 1961, **16**, 88-95. doi: 10.1016/0006-2952(61)90145-9
28. Kuca K. & Patočka J. Reactivation of Cyclosarin-inhibited Rat Brain Acetylcholinesterase by Pyridinium–Oximes. *J. Enz. Inhib. Med. Chem.*, 2004, **19**, 39-43.
29. Valiveti, A.K.; Bhalerao, U.M.; Acharya, J.; Karade, H.N.; Acharya, B.N.; Raviraju, G.; Halve, A.K.; Kaushik M.P. Synthesis and in vitro kinetic evaluation of *N*-thiazolylacetamide monoquaternary pyridinium oximes as reactivators of sarin, *O*-ethylsarin and VX inhibited human acetylcholinesterase (*h*AChE). *Bioorg. Med. Chem.* 2015, **23**, 4899-4910. doi: 10.1016/j.bmc.2015.05.027

Conflict of Interest: None

ACKNOWLEDGEMENT

Authors thank Dr Lokendra Singh, Director, Defence Research and Development Establishment, Gwalior for his keen interest in this work. The authors are also thankful to Dr D.K. Dubey, Associate Director and Head PTD Division for his valuable suggestions.

CONTRIBUTORS

Dr Jyotiranjan Acharya received his MSc (Organic Chemistry) and PhD (Medicinal Chemistry) from Jiwaji University, Gwalior, India, in 1999 and 2008, respectively. Currently, he is a Scientist in Defence Research & Development Establishment, Gwalior, India. His area of interest includes : Development of antidotes against anticholinesterase agents.

Dr Hitendra N. Karade did his MSc (Organic Chemistry) from Amaravati University, Amaravati, India in 2002 and PhD in Chemistry from Jiwaji University, Gwalior, India in 2008. Currently, he is a Scientist in Defence Research & Development Establishment, Gwalior, India. His area of interest includes : Development of antidotes against anticholinesterase agents as well as synthesis of small bioactive molecules.

Mr G. Raviraju received his MSc in physical chemistry from Andhra University, Vishakapatnam, India in 2009. Currently he is Scientist in Defence Research & Development Establishment, Gwalior, India. His area of interest includes : Development of antidotes against anticholinesterase agents.

Mr Sanatan Ghosh received his MSc degree in chemistry from The University of Burdwan, Burdwan, West Bengal in 2013. Currently he is Senior Technical Assistant in Defence Research & Development Establishment (DRDE) Gwalior, India. His area of interest includes : Design synthesis and evaluation of antidotes against nerve agent poisoning.

Dr Aravind Kumar Gupta received his MSc in chemistry and PhD from Jiwaji University, Gwalior, India in 1987 and 2003, respectively. Currently he is Scientist in Defence Research & Development Establishment, Gwalior, India. His area of interest includes : Synthesis, characterisation and analysis of chemical warfare agents and their antidotes.