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Synthesis and in vitro kinetic study of novel mono-pyridinium oximes as reactivators of organophosphorus (OP) inhibited human acetylcholinesterase (*h*AChE)

Aditya Kapil Valiveti [a], Uma M. Bhalerao [a], Jyotiranjan Acharya [a,*], Hitendra N. Karade [a], Raviraju Gundapu [a], Anand K. Halve [b], Mahabir Parshad Kaushik [a]

[a] Process Technology Development Division, Defence Research & Development Establishment, Jhansi road, Gwalior 474 002, India
[b] School of Studies in Chemistry, Jiwaji University, Gwalior, India

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ABSTRACT

A series of mono pyridinium oximes linked with arenylacetamides as side chains were synthesized and their in vitro reactivation potential was evaluated against human acetylcholinesterase (*h*AChE) inhibited by organophosphorus inhibitors (OP) such as sarin, VX and tabun. The reactivation data of the synthesized compounds were compared with those obtained with standard reactivators such as 2-PAM and obidoxime. The dissociation constant (K_D) and specific reactivity (k_r) of the oximes were also determined by performing reactivation kinetics against OP inhibited *h*AChE. Among the synthesized compounds, oximes 1-(2-(4-cyanophenylamino)-2-oxoethyl)-4-((hydroxyimino)methyl)pyridinium chloride (**12a**) and 4-((hydroxyimino)methyl)-1-(2-(4-methoxyphenylamino)-2-oxoethyl)pyridinium chloride (**2a**) were found most potent reactivators for *h*AChE inhibited by sarin. In case of VX inhibited *h*AChE majority of the oximes have shown good reactivation efficacies. Among these oximes 1-(2-(benzylamino)-2-oxoethyl)-4-((hydroxyimino)methyl)pyridinium chloride (**18a**), 4-((hydroxyimino)methyl)-1-(2-(4-(methoxycarbonyl)phenylamino)-2-oxoethyl)pyridinium-chloride (**14a**) and **12a** were found to surpass the reactivation potential of 2-PAM and obidoxime. However, the synthesized oximes showed marginal reactivation efficacies in case of tabun inhibited *h*AChE. The pKa value of the oximes were determined and correlated with their observed reactivation potential.

1. Introduction

Poisoning by organophosphorus (OP) nerve agents viz. VX, sarin and tabun (Fig. 1A) and pesticides pose serious life-threatening situations to the mankind [1]. The ready availability of the raw materials, ease in their preparation and extreme toxicities of these toxicants made them as chemical weapons of mass destruction [2]. The use of nerve agents on the civilian population was witnessed by several instances in the history viz. Iran–Iraq war (1980–1988), sarin attack in Tokyo subway (1995) and recent sarin gas attack at Damascus in Syrian civil war (August 2013) [3]. All these incidents produced severe casualties all over the world. In addition, approximately 300,000 deaths have been recorded annually because of intentional (suicidal) and unintentional (occupational) means of poisoning by OP pesticides and insecticides in the developing countries around the globe [4]. Despite serious and continued efforts to prevent synthesis, storage and use of these compounds by the Chemical Weapons Convention (CWC) [5], repeated use of chemical warfare agents during military conflicts and terrorist attacks indicate that they constitute a persistent threat for the civilization [6].

The OP compounds (Fig. 1A) exert their toxicity by inhibiting the activity of the enzyme acetylcholinesterase (AChE), an enzyme responsible for hydrolysis of neurotransmitter acetylcholine (ACh). This lead to the accumulation of endogenous ACh thereby triggering a variety of clinical manifestations in the autonomic nervous system (both central and peripheral nervous system) and finally leading to death due to respiratory failure [7].

Current medical protection against the toxicity of OP poisoning consists of a regimen of anti-cholinergic drugs, such as atropine to counteract the accumulation of acetylcholine, an anti convulsant e.g. diazepam to reduce the CNS related symptoms and an oxime reactivator to reactivate OP-inhibited AChE [8]. Quaternary pyridinium oximes such as 2-pyridinealdoxime methochloride

* Corresponding author. Tel.: +91 751 2390205, +91 751 2340245; fax: +91 751 2341148.
E-mail addresses: jracharya@rediffmail.com, j.acharya@drde.drdo.in (J. Acharya).

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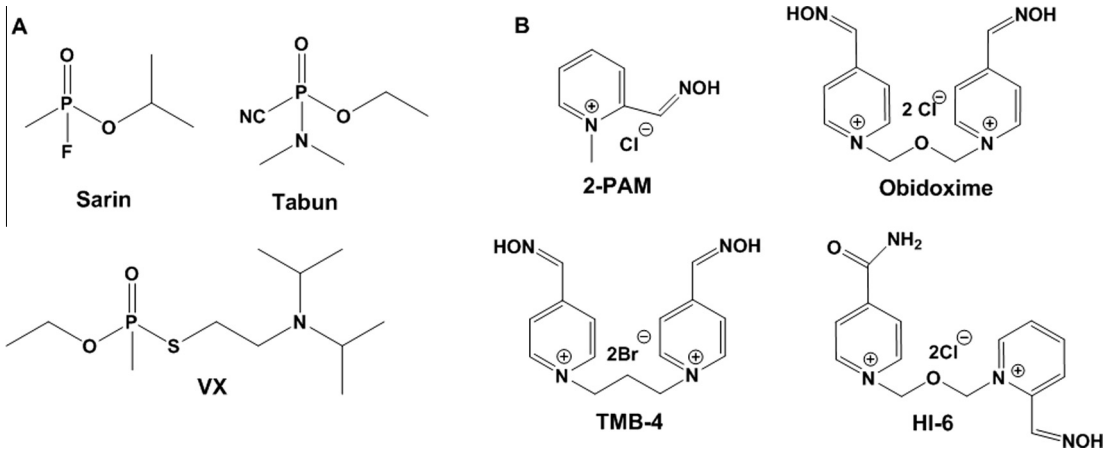


Fig. 1. (A) Structures of organophosphorus nerve agents; **(B)** structures of oxime reactivators.

(2-PAM), trimedoxime (TMB-4), obidoxime and HI-6 (Fig. 1B) are currently used as reactivators in the treatment of OP poisoning [9]. Though, these oximes have proven their efficacies against OP nerve agent poisoning, however there are certain limitations which constraint their scope in shaping them as universal antidote. 2-PAM is being used as an effective drug in many countries against OP poisoning, however its efficacy is limited to sarin and VX inhibited AChE and has marginal efficacy in case of other nerve agents. In view of the above, research is being continued for a far more effective reactivator that can be used as an antidote against a broad spectrum of nerve agents [10,11].

Recently several studies on bis- and mono-quaternary oximes connected with various bridging chains (prop-1,3-diyl, xylene linkers, aliphatic and heteroaromatic linkers) and side chain (benzyl, heterocyclic and functionalized aliphatic moieties) were reported for their efficacies against OP inhibited AChE [12–17]. Few of these reactivators have shown promising reactivation efficacies against specific OP inhibited AChE. Therefore further modifications in the structural features of AChE reactivator are highly essential in order to establish broad spectrum antidote in OP poisoning.

Bis-pyridinium oximes and their analogs have been widely studied against OP poisoning, however 2-PAM has been used worldwide [18]. Moreover, various studies have proved that the diffusion rate of monoquaternary oximes into the brain is higher in comparison to their bis-analogs [19]. Therefore, in continuation to our work on antidotes against nerve agents, herein we report the synthesis and in vitro evaluation of a series of mono-pyridinium oximes (connected to aromatic and aliphatic acetamide side chains) as reactivators of three different OP nerve agents (sarin, VX and tabun) inhibited *h*AChE.

2. Materials and methods

2.1. Materials

Substituted aromatic amines, benzylamine, cyclohexylamine, γ-aminobutyric acid, 6-aminocaproic acid, propargylamine, 2-, 3- and 4-pyridinealdoxime, acetylthiocholineiodide (ATChI), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), isopropanol (spectroscopic grade), potassium dihydrogenphosphate, dipotassium hydrogen phosphate, trizma-base and trizma-HCl were purchased from Sigma–Aldrich, USA and used without further purification. Glycine was obtained from E. Merck (India) and used without further purification. Chloroacetylchloride, anhydrous sodium sulfate, anhydrous sodium bicarbonate and anhydrous potassium carbonate were purchased from Qualigens, India. Solvents (dichloromethane, acetonitrile, acetone, and methanol) were purchased from S.D. Fine Chemicals (India) and dried and distilled before use. Sarin, tabun and VX were prepared in house with >98% purity (GC and ^{31}P NMR). 2-PAM was prepared according to the method of Wilson and Ginsburg [20]. Obidoxime was synthesized using reported methods [21]. The synthesized compounds as well as standards were characterized by their IR, ^{1}H NMR, ^{13}C NMR spectral and elemental analysis data (Supplementary information). The progress of reaction and purity of the compounds were checked by thin layer chromatography (TLC) using commercially available pre-coated silica on aluminum sheets purchased from E. Merck, India.

2.2. Synthesis of oximes

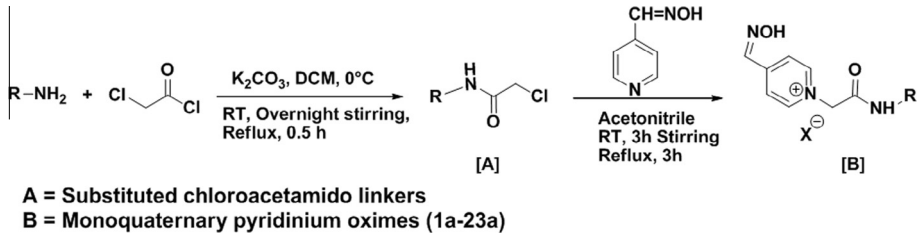
A two step simple (Scheme 1) protocol was used to synthesize the mono-pyridinium oximes (**1a**–**22a**).

2.2.1. Step 1: synthesis of 2-chloro-N-(4-nitrophenyl)acetamide

A suspension of 4-nitroaniline (7 g, 0.051 mol) and anhydrous potassium carbonate (7 g, 0.051 mol) in dichloromethane (30 mL) was stirred for 30 min at room temperature. The reaction mixture was cooled on an ice bath. To this was added an ice-cooled solution of chloroacetylchloride (5.7 g, 0.051 mol) in dichloromethane (20 mL) drop wise over a period of 30 min. The reaction mixture was stirred overnight at room temperature followed by reflux for additional 30 min. Excess solvent was removed and the residue was neutralized with aqueous sodium bicarbonate solution (5% w/v). The product obtained was filtered off and washed thoroughly with cold water. The crude product obtained was dried under vacuum (10 g, yield: 92%). TLC (chloroform, R_f = 0.5). The product was sufficiently pure and used in the next step directly.

2.2.2. Step 2: synthesis of 4-((hydroxyimino)methyl)-1-(2-(4-nitrophenylamino)-2-oxoethyl)pyridinium chloride

4-Pyridinealdoxime (1.0 g, 0.0082 mol) dissolved in dry acetonitrile (30 mL) taken in a two neck round bottom flask equipped with a magnetic stirrer, condenser and calcium chloride guard tube was stirred at room temperature. 2-chloro-*N*-(4-nitrophenyl) acetamide (1.76 g, 0.0082 mol) dissolved in dry acetonitrile (20 mL) was added to the reaction mixture slowly over a period of 10 min. The reaction mixture was then stirred for 3 h at room temperature followed by reflux for another 3 h. The product obtained was filtered off, washed with dry hot acetonitrile (2 × 15 mL) followed by dry hot acetone. Finally, the crude product was dried (0.52 g, yield 19%) and recrystallized from



Scheme 1. Synthetic route for the preparation of mono-pyridinium oximes (**1a–23a**).

Table 1
Physicochemical data and chemical structure of the synthesized oximes.

Oxime	Oxime position	X	–R	Yield (%)	MP (°C)	pKa
1a	4	Cl	Ph-4-CH_3	40	231–233	8.33
2a	4	Cl	Ph-4-OCH_3	43	216–218	8.31
3a	4	Cl	Ph-4-NO_2	19	242–244	8.22
4a	4	Cl	Ph-3-NO_2	57	258–260	8.49
5a	4	Cl	Ph-2-NO_2	50	260–262	8.21
6a	4	Cl	Ph-4-Cl	58	228–230	8.32
7a	4	Cl	Ph-3-Cl	32	207–209	8.13
8a	4	Cl	Ph-4-H	38	192–194	8.29
8b	2	I	Ph-4-H	15	202–204	8.08
9a	4	Cl	Ph-4-COOH	5	244–246	8.34
10a	4	Cl	Ph-3-COOH	19	219–221	8.23
11a	4	Cl	Ph-4-F	51	235–237	8.28
12a	4	Cl	Ph-4-CN	16	239–241	8.26
13a	4	Cl	Ph-3-CN	36	250–252	8.19
14a	4	Cl	Ph-4-$COOCH_3$	14	222–224	8.15
15a	4	Cl	Ph-4-$COOC_2H_5$	28	224–226	7.87
16a	4	Cl	Ph-4-$COCH_3$	41	219–221	8.25
17a	4	Cl	Ph-4-CF_3	35	241–243	8.37
18a	4	Cl	CH_2-Ph-4-H	50	145–147	8.27
19a	4	Cl	C_6H_{11}	76	188–190	8.43
20a	4	Cl	CH_2-$CONH_2$	14	–	–
21a	4	Cl	$(CH_2)_4$-COOH	35	194–196	8.42
22a	4	Cl	$(CH_2)_5$-COOH	22	180–182	8.39
23a	4	Cl	$CH_2C{\equiv}H$	11	–	8.11

methanol-acetone mixture. TLC (acetone/methanol (2:1), R_f = 0.10), m.p: 235–237 °C.

The physicochemical parameters of the synthesized oximes (**1a–23a**) were determined and depicted in Table 1. Elemental analyses were conducted on an ELEMENTAR, vario MICRO cube, Universal micro analyzer and the values were within ±0.4% of the calculated values. Infra-red (IR) spectra were obtained from KBr discs on a Bruker TENSOR-27 FTIR spectrophotometer. ^{1}H NMR (DMSO-d_6) spectra for the synthesized oximes were recorded on Bruker Avance 400 spectrometer at 400 MHz using tetramethylsilane (TMS) as internal standard and expressed in the δ (ppm) values. ^{13}C NMR (DMSO-d_6) was recorded using same instrument at 100 MHz (Supplementary information).

2.3. Enzyme assay, inhibition and reactivation

Human blood samples were collected from the volunteers as per norms. The hemoglobin free erythrocyte ghost AChE was prepared using the reported method [22] and stored at −80 °C until use. AChE activities were measured at 412 nm by using UV–Visible spectrophotometer (Cary 100, Agilent Technologies, USA) assisted with PCB 1500 water peltier system with a modified Ellman protocol [23,24]. Prior to start the experiments, aliquots of the erythrocyte ghosts were homogenized on an ice-bath with the help of an ultrasonic homogenizer (Model 3000, Biologics Inc., Manassas, USA), three times for 5 s with 30 s intervals to achieve homogenous matrix for the kinetic experiments [25,26]. Quartz cuvettes of 3 mL were used to measure the activity of the assay mixture. ATChI (0.48 mM, in distilled water) as substrate and DTNB as the colorimetric indicator (0.3 mM in 0.1 M phosphate buffer, pH = 7.4) were used to assay the activity. Oxime stock solutions and their dilutions were prepared freshly in triple distilled water. All the working solutions were kept on an ice bath until completion of the experiment. All experiments were performed at 37 °C in phosphate buffer at pH 7.4. All concentrations in the above assay mixture refer to the final concentrations.

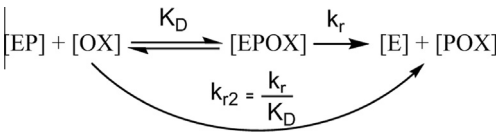
The stock solutions of OP inhibitors (sarin, VX and tabun) were prepared in isopropanol once in a week and were stored at −20 °C. The subsequent dilutions of OP inhibitors were freshly prepared every day in deionized water prior to start of the experiment. The enzyme *h*AChE taken in phosphate buffer (pH 7.4, 0.1 M) was inhibited by appropriate OP concentrations at 37 °C around 10–15 min to achieve 95–98% inhibition of the control activity. The residual inhibitor was removed by the concomitant extraction with six times excess volume of n-hexane [27–29].

The OP-inhibited *h*AChE was reactivated by the addition of oxime reactivator at a final concentration ranging from 10 to 1000 μM. An aliquot of 50 μL from the reactivation cocktail (total volume of cocktail: 600 μL, containing inhibition cocktail and oxime reactivator) at different time intervals (1, 3, 5, 7, 10, 15, 20, 25, 30, 40, 50 and 60 min) was transferred to the cuvette containing 3.0 mL of phosphate buffer and 100 μL of DTNB. The AChE activity was assayed by adding 20 μL of ATChI at 37 °C (final volume 3.17 mL). The oxime induced reactivation of the OP-inhibited AChE was monitored at different time intervals over a period of 60 min. Spontaneous reactivation of inhibited AChE was assayed using the same protocol (the reaction mixture containing enzyme and OP but no oxime). Under these conditions spontaneous reactivation was found to be insignificant. All the values were corrected for their oxime induced hydrolysis of ATChI.

2.4. Reactivation kinetics

The oxime assisted reactivation of the OP-inhibited AChE proceeds according to the Scheme 2.

Where [EP] is the phosphylated enzyme, [OX] is the reactivator, [EPOX] is the Michaelis-type complex between phosphylated-AChE and oxime reactivator, [E] is the reactivated enzyme, [POX] is the phosphylated oxime, K_D is the dissociation constant which is inversely proportional to the affinity of reactivator towards the phosphylated enzyme [EP], k_r is the rate constant for the displacement of phosphyl residue from [EPOX] by oxime and it expresses the reactivation efficiency of the reactivator.



Scheme 2. Oxime assisted reactivation of OP-inhibited AChE.

In case of complete reactivation and with [OX] ≫ $[EP]_o$ a pseudo first-order rate equation can be derived for the reactivation process as represented in Eq. (1)

$$k_{obs} = \frac{k_r[OX]}{K_D + [OX]} \quad (1)$$

where k_{obs} is the observed first-order rate constant of the reactivation at any given oxime concentration and it was calculated by non-linear regression analysis [24,25] using Eq. (2)

$$v_t = v_0 \times (1 - e^{-k_{obs} \times t}) \quad (2)$$

k_r and K_D were obtained by the non-linear fit of the relationship between k_{obs} versus [OX]. Both the rate constants k_r and K_D follow Michaelis–Menten type kinetics and the second order reactivation rate constant k_{r2} was obtained from the ratio of k_r and K_D.

The determined kinetic parameters and rate constants of the oximes were presented in the Table 2.

2.5. Data analysis

The kinetic rate constants were determined by processing the experimental data with non-linear regression analysis using curve fitting programs provided by Prism™ Vers. 6.0 (Graph Pad software, San Diego, USA).

2.6. Determination of acid dissociation constant (pKa)

The acid dissociation constants (pKa) of the oximes were determined using the method of Albert and Sergeant (1971) [30]. The method is based on direct determination of the ratio of molecular species (protonated) to dissociated (deprotonated) species in a series of non-absorbing buffer solutions. For this purpose, the spectra of molecular species were obtained first in buffer solution of particular pH in which compounds of interest would be present entirely in either form. Oxime stock solutions (15–35 μL, 5×10^{-3} M) were diluted to 3 mL in a cuvette containing either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide solution and the absorption spectra of oxime in acid or alkali were recorded over the wavelength range of 200–600 nm with a reference blank solution at 25 ± 1.0 °C. The spectra thus obtained in acid or alkali were of protonated (D_m) and deprotonated (D_i) molecules (Fig. 2). Ten different pH values, ranging from 5.94 to 10.03 were selected to determine the pKa of oximes. For this, appropriate buffers consisting of phosphate (pH: 5.94–7.60), tris (pH: 8.00–8.60) and glycine–NaOH (pH: 9.06–10.03) were used. Aqueous solutions

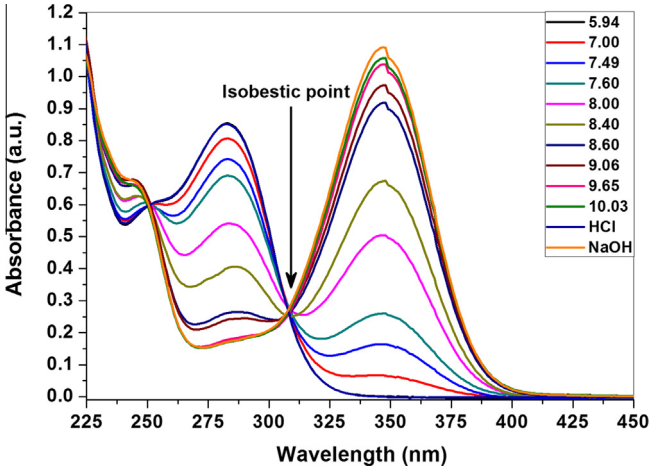


Fig. 2. Spectrophotometric determination of pKa of the oxime **12a.**

(15–35 μL) of oximes were diluted to 3 mL in each buffer and optical densities were determined at analytical wave lengths using buffer blank at 25 ± 1.0 °C. A set of ten values of pKa were obtained using the following Eq. (3):

$$pKa = pH + \log[(D_i - D)/(D - D_m)] \quad (3)$$

where, D_m and D_i corresponds to the optical density of protonated and deprotonated forms of the oxime, and D is the optical density in the buffer. The average value of the ten measurements was considered the pKa of the compound with respect to oximino functionality. UV–Visible spectrophotometer assisted with PCB 1500 water peltier system was used for spectrophotometric analysis. The temperature of the peltier system was adjusted at 25 ± 1 °C. The pH of the buffer solutions were determined by using Eutech 1500 Cyberscan pH meter. The pH meter was calibrated at 25 °C with standard buffer solutions pH 7.00 and 9.21.

2.6.1. Reagents

Oxime stock solutions (5×10^{-3} M) were freshly prepared in triple distilled water. Buffer solutions were prepared as per reported protocol [31]. The analytical wavelengths for the protonated and deprotonated species were obtained by using 0.1 M hydrochloric acid and 0.1 M sodium hydroxide solutions respectively.

3. Results and discussion

All the newly synthesized oximes were subjected to evaluation of their reactivation efficacies against three structurally different nerve agents (sarin, VX and tabun) inhibited *h*AChE. Depending on the structural features of nerve agent and the oxime reactivators used in this study, significant differences were observed in the reactivation efficacies and kinetic rate constants (k_r, K_D and k_{r2}) of the oximes. The nerve agents used in this study have sufficient aging time and hence the oxime induced reactivation was carried out for the reactivated enzyme but not for the aged one.

The affinity of an oxime toward the OP-AChE adduct has been interpreted by their respective K_D (Section 2.4) values. Large variations in the K_D values were observed among the tested oximes. Majority of the oximes showed higher affinity (lower K_D) toward sarin and VX inhibited AChE. On the other hand, lower affinities (higher K_D) were observed in case of the reactivation of tabun inhibited *h*AChE which were further characterized by their lower k_{r2} values. The reactivation efficacy of an oxime reactivator was assessed by calculating the reactivity rate constant k_r. The respective k_r values for the oximes were presented in Table 2. It was observed that the efficacy of the oximes to reactivate sarin and VX inhibited *h*AChE was significantly higher than that of tabun inhibited *h*AChE. Both the rate constants k_r (reactivity) and K_D (affinity) follow Michaelis–Menten type kinetics. The quantification of specific reactivity of an oxime was carried out by calculating its second order reactivation rate constant (k_{r2}) which depend on its k_r and K_D values (Table 2).

3.1. Reactivation of sarin inhibited hAChE

Moderate results were obtained in case of sarin inhibited *h*AChE. Few of the tested oximes have shown better reactivation efficacies than those of the standard oximes as evidenced by their higher second order rate constants. Among the studied oximes, **12a**, **2a**, **22a**, **9a** and **8b** have shown higher affinities (K_D) 4.51 μM; 6.62 μM; 8.83 μM; 9.24 μM; 13.27 μM respectively for the phosphylated complex of sarin inhibited AChE. This was further reflected by their higher second order rate constants (k_{r2}) 10.41 mM^{-1} min^{-1}; 9.92 mM^{-1} min^{-1}; 6.90 mM^{-1} min^{-1};

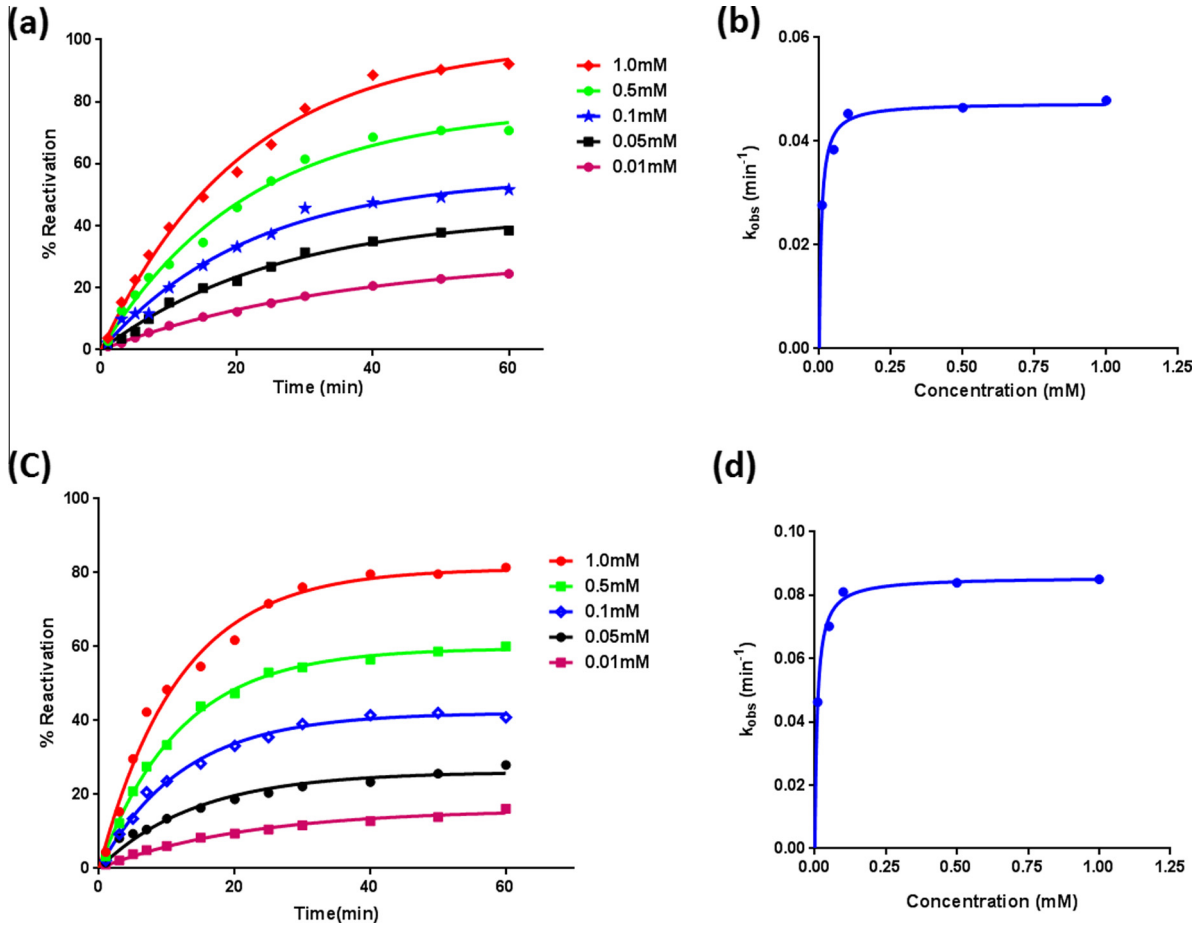


Fig. 3. Reactivation profile of oxime **18a** against sarin (a), VX inhibited *h*AChE (c); plot of k_{obs} *vs.* [**18a**] (mM) for sarin (b), VX inhibited *h*AChE (d).

6.67 mM^{-1} min^{-1}; 6.73 mM^{-1} min^{-1} respectively. The time dependent reactivation profile and k_{obs} for the oxime **18a** was illustrated in Fig. 3a and b. The lower K_D values of the oximes represent their greater affinity and better reactivity toward sarin-*h*AChE complex in the active site of the gorge. Oximes **18a** (6.14 mM^{-1} min^{-1}), **17a** (4.70 mM^{-1} min^{-1}), **23a** (4.59 mM^{-1} min^{-1}), **13a** (4.39 mM^{-1} min^{-1}) and **11a** (4.28 mM^{-1} min^{-1}) have also shown higher reactivity than that of the standard 2-PAM (4.07 mM^{-1} min^{-1}) and comparable reactivity with that of obidoxime (6.39 mM^{-1} min^{-1}). Further, oximes **1a**, **5a**, **6a**, **14a**, **15a** and **19a** showed comparable reactivity to those of 2-PAM and obidoxime (Table 2). Oximes **4a** (72.57 μM) and **3a** (41.41 μM) have displayed larger K_D values demonstrating their least reactivity toward the phosphylated complex of sarin-inhibited *h*AChE and thereby resulted in lower second order rate constants k_{r2}; 1.06 mM^{-1} min^{-1} and 1.57 mM^{-1} min^{-1} respectively.

3.2. Reactivation of VX inhibited hAChE

The reactivation efficacy of the synthesized oximes (**1a**–**23a**) to reactivate VX inhibited *h*AChE was presented in Table 2. It was observed that the K_D values for the oximes against VX inhibition were largely varied between 8 and 111 μM. Majority of the oximes have shown greater reactivation efficacy than that of the standard oxime 2-PAM. Among the oximes, **18a** (Fig. 3c and d) has shown greater affinity (K_D: 8.83 μM) toward phosphylated *h*AChE. This was further reflected by its higher second order rate constant (k_{r2}: 9.70 mM^{-1} min^{-1}) which indicate its higher reactivation efficacy among all the tested oximes. Again, oxime **14a** (K_D: 9.64 μM & k_{r2}: 8.61 mM^{-1} min^{-1}) and **12a** (K_D: 10.18 μM & k_{r2}: 7.41 mM^{-1} min^{-1}) have shown significantly higher reactivation efficacies than that of the standard 2-PAM (K_D: 26.59 μM & k_{r2}: 2.73 mM^{-1} min^{-1}) and comparable reactivity with obidoxime (K_D: 14.4 μM & k_{r2}: 9.03 mM^{-1} min^{-1}). Oximes **2a**, **6a**, **8a**, **8b**, **15a**, **16a**, **19a** and **23a** have shown higher reactivation efficacies than that of the standard 2-PAM as represented by their higher second order rate constant values (Table 2). However oxime **1a**, **9a**, **11a**, **17a** and **22a** have shown nominal reactivation efficacies against VX inhibited *h*AChE. These oximes also recorded significantly lower reactivation efficacies than that of obidoxime. Among all the synthesized compounds, oximes **21a** and **5a** displayed larger K_D (111.6 and 87.5 μM) and lower second order rate constant (k_{r2}) (0.52 mM^{-1} min^{-1} and 0.58 mM^{-1} min^{-1}) inferring their least affinity and reactivity towards the VX-*h*AChE complex.

3.3. Reactivation of tabun inhibited hAChE

Tabun inhibited AChE was least reactivated by any of the newly synthesized oximes. In the present study majority of the tested oximes failed to reactivate tabun inhibited AChE even at higher concentrations of the reactivator. The observed higher dissociation constants (K_D) of these oximes demonstrated their least affinity toward the highly resistant tabun inhibited *h*AChE. Among all the tested oximes, **8a** has recorded lower K_D (62.76 μM) and higher k_{r2} (0.91 mM^{-1} min^{-1}) thereby explaining its better affinity and reactivity than other oximes. Oximes **12a**, **14a** and **1a** have recorded nominal reactivity as revealed by their respective lower k_{r2} (0.75 mM^{-1} min^{-1}; 0.69 mM^{-1} min^{-1}; 0.72 mM^{-1} min^{-1})

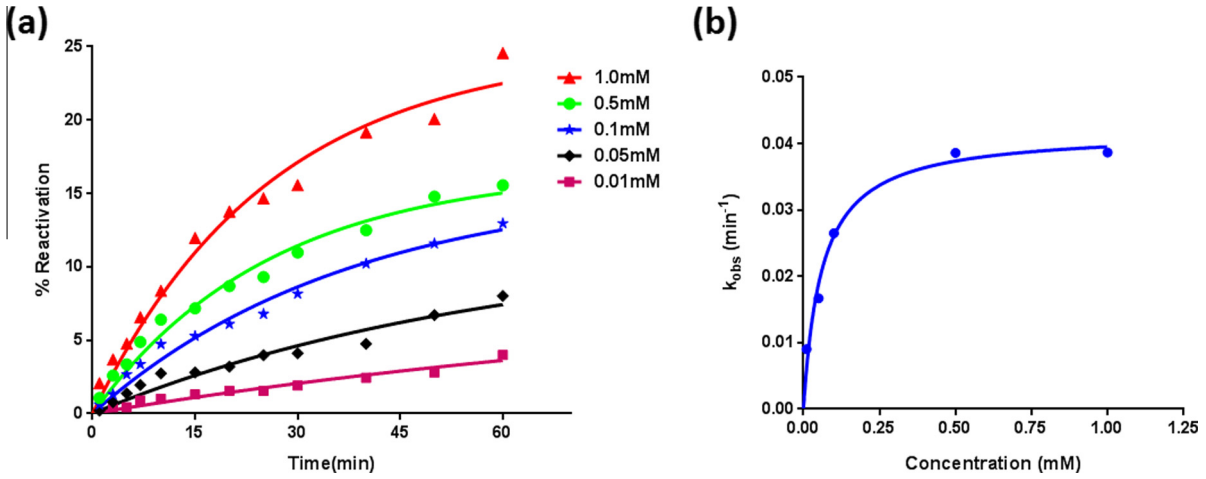


Fig. 4. Reactivation profile of oxime **14a** against Tabun-inhibited *h*AChE (a); plot of k_{obs} *vs.* [**14a**] (mM) for Tabun-inhibited *h*AChE (b).

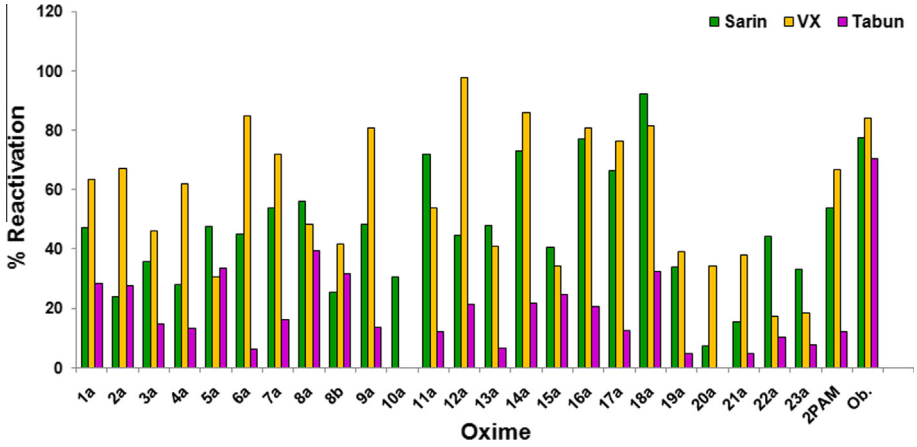


Fig. 5. Percentage reactivation efficacies of the oximes (**1a**–**23a**) in comparison to 2-PAM and obidoxime against sarin, VX and Tabun-inhibited *h*AChE. Oxime concentration: 1 mM, pH: 7.4, temperature: 37 °C, time of reactivation: 60 min.

values. Majority of these oximes (including 2-PAM) have failed to reactivate tabun inhibited AChE thereby explaining the resistivity of the tabun-AChE complex toward oxime assisted reactivation. However, only obidoxime has shown moderate reactivity (K_D: 50.73 μM and k_{r2}: 0.99 mM^{-1} min^{-1}) as compared to all other oximes. The time dependent reactivation profile and plot of k_{obs} *vs.* reactivator concentration [**14a**] was depicted in Fig. 4.

It is worth noticing (Table 2) that the tested oximes have shown several fold greater efficacies toward VX and sarin inhibited *h*AChE than that of tabun inhibited *h*AChE. The overall order of the reactivation efficacies of the oximes toward nerve agents were found to be VX > sarin > tabun.

The percentage reactivation efficacy of the oximes (**1a**–**23a**) at 1 mM oxime concentration in comparison to 2-PAM and obidoxime at 60 min against all the three nerve agents (sarin, VX and tabun) was illustrated in Fig. 5. Shortlisting a better reactivator solely on the basis of their percentage reactivation is difficult. Assessment of efficacy of an oxime has generally been made by evaluating their kinetic data (Table 2). Majority of the tested oximes have exhibited higher reactivation efficacies at concentration 1 mM against VX inhibited *h*AChE in comparison to sarin and Tabun (Fig. 5). Among the tested oximes **12a** (97%), **14a** (85%), **18a** (81%) and **16a** (80%) have shown higher reactivation efficacies indicating their appreciable reactivity of the VX inhibited *h*AChE complex. The second order rate constant k_{r2} signify the overall persistent reactivation efficacy of the oximes while the percentage reactivation efficacies (Fig. 5) reflect only the percentage of AChE reactivation at a particular concentration after certain time. In Fig. 5, 2-PAM and obidoxime (at 1 mM concentration) have shown higher percentage reactivation than **12a** (1 mM) at 60 min. But the kinetic data (Table 2) of **12a** has been found to be better than those of 2-PAM and obidoxime. Therefore the oxime **12a** has been identified as a superior reactivator than 2-PAM and obidoxime. Further, at higher oxime concentration (1 mM), oxime **18a** (92%), **11a** (71%) and **17a** (66%) have shown higher reactivation efficacy of sarin inhibited *h*AChE. However their affinities toward the phosphylated complex have shown to be lower as indicated by their lower second order rate constants. In case of tabun inhibited *h*AChE, all the oximes have recorded lower reactivation efficacies even at higher oxime concentration. However, oximes **5a**, **8a**, **8b** and **18b** have recorded nominal reactivation efficacies against tabun as compared to other nerve agents (VX and sarin) at 1 mM oxime concentration.

Several studies were made to highlight the importance of the interactions present in the gorge of OP inhibited AChE and concluded that the reactivators capable of π–π and cation-π interactions were highly significant in order to achieve a proper orientation of the reactivator toward the OP-*h*AChE complex [13–16]. In order to enhance the hydrophobicity of the reactivator, various aryl and aliphatic side chains with a variety of substituents

Table 2
Reactivation constants for oxime-induced reactivation of sarin, VX and tabun- inhibited *h*AChE.

Oxime	K_D μM (±SE)			k_r min^{-1} (±SE)			k_{r2} (mM^{-1} min^{-1})		
	Sarin	VX	Tabun	Sarin	VX	Tabun	Sarin	VX	Tabun
1a	21.02 (0.0043)	22.13 (0.0042)	92.46 (0.0253)	0.061 (0.0023)	0.072 (0.0026)	0.662 (0.005)	2.89	3.25	0.72
2a	6.62 (0.0009)	20.57 (0.0043)	139.2 (0.0351)	0.066 (0.0012)	0.085 (0.0033)	0.081 (0.006)	9.92	4.12	0.59
3a	41.41 (0.0126)	24.27 (0.0027)	191.1 (0.1279)	0.065 (0.0045)	0.051 (0.0011)	0.077 (0.0164)	1.57	2.07	0.41
4a	72.57 (0.0081)	38.46 (0.0039)	98.88 (0.0193)	0.077 (0.0022)	0.063 (0.0015)	0.033 (0.0017)	1.06	1.63	0.34
5a	24.92 (0.0032)	87.46 (0.0156)	94.79 (0.0115)	0.058 (0.0015)	0.051 (0.0025)	0.040 (0.0013)	2.33	0.58	0.42
6a	17.00 (0.0031)	11.31 (0.0022)	*	0.049 (0.0017)	0.053 (0.0017)	*	2.91	4.65	*
7a	28.86 (0.0029)	29.60 (0.0046)	127.3 (0.0336)	0.062 (0.0013)	0.034 (0.0011)	0.058 (0.0044)	2.13	1.15	0.45
8a	32.16 (0.0088)	12.06 (0.0015)	62.76 (0.0245)	0.064 (0.0037)	0.072 (0.0015)	0.057 (0.0056)	2.00	6.00	0.91
8b	13.27 (0.0013)	19.95 (0.0046)	77.80 (0.023)	0.089 (0.0015)	0.097 (0.0042)	0.056 (0.0045)	6.73	4.87	0.72
9a	9.24 (0.0013)	17.53 (0.0044)	*	0.062 (0.0014)	0.067 (0.0030)	*	6.67	3.81	*
10a	20.36 (0.0025)	*	*	0.026 (0.0006)	*	*	1.26	ns	*
11a	14.95 (0.0036)	16.13 (0.0039)	*	0.064 (0.0027)	0.061 (0.0026)	*	4.28	3.79	*
12a	4.51 (0.0008)	10.18 (0.0017)	74.28 (0.0167)	0.047 (0.0009)	0.075 (0.0019)	0.055 (0.0032)	10.41	7.41	0.75
13a	15.43 (0.0019)	45.14 (0.0075)	*	0.068 (0.0015)	0.091 (0.0035)	*	4.39	2.01	*
14a	36.45 (0.0027)	9.639 (0.0017)	60.85 (0.0132)	0.094 (0.0015)	0.083 (0.0023)	0.042 (0.0023)	2.58	8.61	0.69
15a	11.80 (0.0016)	14.48 (0.0032)	39.40 (0.0143)	0.034 (0.0007)	0.087 (0.0033)	0.023 (0.0018)	2.84	5.99	0.59
16a	27.44 (0.0051)	10.63 (0.0022)	103.3 (0.0192)	0.037 (0.0014)	0.066 (0.0021)	0.045 (0.0023)	1.33	6.23	0.43
17a	14.79 (0.0014)	16.21 (0.0032)	*	0.069 (0.0011)	0.059 (0.0021)	*	4.70	3.68	*
18a	7.721 (0.0013)	8.83 (0.0009)	71.96 (0.0143)	0.047 (0.0012)	0.086 (0.0013)	0.02 (0.001)	6.14	9.70	0.28
19a	24.94 (0.0061)	15.87 (0.0016)	*	0.079 (0.0038)	0.081 (0.0014)	*	3.15	5.08	*
20a	*	41.14 (0.0133)	*	*	0.049 (0.0036)	*	*	1.20	*
21a	31.53 (0.0021)	111.6 (0.030)	*	0.045 (0.0006)	0.058 (0.0044)	*	1.41	0.52	*
22a	8.831 (0.0013)	15.97 (0.0034)	*	0.061 (0.0014)	0.057 (0.0022)	*	6.90	3.56	*
23a	17.63 (0.0035)	15.24 (0.0037)	*	0.081 (0.0029)	0.088 (0.0036)	*	4.59	5.77	*
2PAM	20.46 (0.0036)	26.59 (0.0087)	*	0.083 (0.0028)	0.072 (0.0048)	*	4.07	2.73	*
Obid.	19.5 (0.0046)	14.4 (0.003)	50.73 (0.0235)	0.125 (0.0055)	0.129 (0.0047)	0.051 (0.0056)	6.39	9.03	0.99

* Insgnificant reactivation; data are means of ±SE ($n = 2$).

connected with the amide linkages to the quaternary *N*- atom of the pyridinium ring were chosen. In addition, the electron donor and acceptor capability of the acetamido moieties present in the reactivator might facilitate the conventional mode of interactions (H-bonding, salt bridge and hydrophobic interactions) between the gorge residues and the reactivator. This might promote the suitable orientation of the reactivator toward the active site of the AChE in the OP-*h*AChE complex.

The greater reactivation efficacies of majority of the oximes in case of VX inhibited *h*AChE might be due to the greater molecular access of the reactivator toward the VX-inhibited AChE than the other two nerve agents (sarin and tabun). After inhibition of the enzyme active site, the bulky residue of VX i.e. $-S(CH_2)_2N(C_3H_7)_2$ is departed from the active site thereby leaving more free space for the approach of the incoming nucleophile (oxime) [32]. This might be the plausible reason that majority of the tested oximes have exhibited significant efficacies in the reactivation of VX inhibited *h*AChE as indicated by their higher second order reactivation constants (Table 2).

Previously mono-pyridinium oximes having oxime group at the position 4-have been synthesized and evaluated for their reactivation efficacy against tabun inhibited *h*AChE [33]. Some of these reactivators were found to be most efficacious against tabun inhibited *h*AChE. However, in the present study majority of the 4-substituted pyridinium oximes were unresponsive toward reactivation of tabun inhibited *h*AChE.

In the present investigation few of the synthesized oximes containing aliphatic moieties attached to the pyridinium ring (**21a**, **22a**, **19a**, **20a**, and **23a)** have displayed an appreciable reactivity toward sarin and VX inhibited *h*AChE complex as compared to 2-PAM and obidoxime. However, none of these oximes have shown sufficient reactivation against tabun inhibited *h*AChE, thus representing their poor reactivity and affinity toward tabun-*h*AChE complex.

During the reactivation of OP inhibited *h*AChE, ionization of the oxime into oximate anion is essential to initiate cleavage of the P–O bond of the phosphylated complex. In order to assess the reactivation efficacies of new reactivators as antidotes against OP poisoning, determination of physico-chemical parameters such as acid dissociation constants (pKa) are important to understand the nucleophilicity of the oxime reactivator. Therefore, pKa of the oximes (**1a**–**23a**) were determined spectrophotometrically (Table 1 and Fig. 2) and were found in the range of 7.81–8.41. The lower range of these values indicated ease in their ionization. This was further reflected in their reactivation efficacies against sarin and VX Inhibited AChE. Oxime **4a** has recorded the highest pKa (8.49) among all the tested oximes and therefore characterized by its lower second order rate constant against all the three nerve agents inhibited *h*AChE. Overall the lower pKa values exhibited by the synthesized oximes might be the one of the reasons for their significantly higher reactivation efficacies observed in this study.

4. Conclusions

In the present investigation, a series of monoquaternary oximes were synthesized and evaluated for their reactivation efficacies against three different nerve agents (sarin, VX and tabun) inhibited *h*AChE. Significant reactivation efficacies against VX and sarin inhibited *h*AChE were observed by all the tested oximes. Oxime **12a** and **2a** have shown higher reactivation efficacies against sarin inhibited *h*AChE. Further, oximes **18a**, **14a** and **12a** have shown superior reactivation efficacies among all the tested oximes against VX inhibited *h*AChE. Based upon this study, the oximes **2a**, **8b**, **12a**, **14a**, **18a** and **22a** may provide a useful therapeutic potential for the reactivation of AChE inhibited by sarin and VX. The detailed study of antidotal efficacy including in vivo reactivation against sarin and other nerve agents is under progress and will be reported in due course.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2015.06.007.

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