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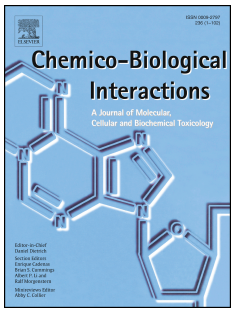

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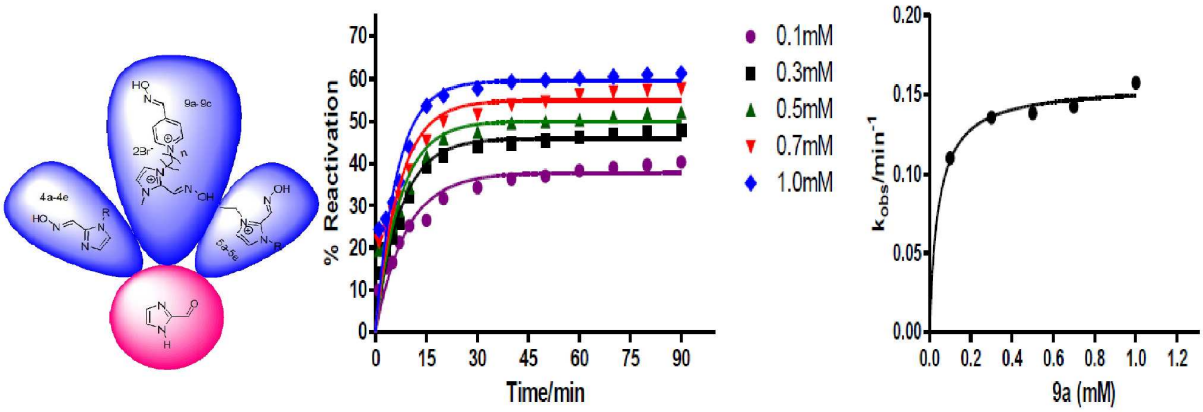

Synthesis and *in-vitro* reactivation screening of imidazolium aldoximes as reactivators of sarin and VX-inhibited human acetylcholinesterase (*h*AChE)

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Abstract

Post-treatment of organophosphate (OP) poisoning involves the application of oxime reactivator as an antidote. Structurally different oximes are widely studied to examine their kinetic and mechanistic behavior against OP-inhibited cholinesterase enzyme. A series of structurally related 1,3-disubstituted-2-[(hydroxyiminomethyl)alkyl]imidazolium halides (**5a-5e, 9a-9c**) were synthesized and further evaluated for their *in-vitro* reactivation ability to reactivate sarin- and VX- inhibited human acetylcholinesterase (*h*AChE). The observed results were compared with the reactivation efficacy of standard reactivators; 2-PAM and obidoxime. Amongst the synthesized oximes, **5a**, **9a** and **9b** were found to be most potent reactivators against sarin-inhibited *h*AChE while in case of VX only **9a** exhibited comparable reactivity with 2-PAM. Incorporation of pyridinium ring to the imidazole ring resulted in substantial increase in the reactivation strength of prepared reactivator. Physicochemical properties of synthesized reactivators have also been evaluated.

Keywords:- Organophosphates, Acetylcholinesterase, Oximes, Antidote, Reactivators, Reactivation kinetics.

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1. Introduction

The widespread, frequent use and misuse of highly perilous organophosphate (OP) compounds (Fig. 1) as pesticides (paraoxon, parathion, diazinon etc.) and nerve agents (sarin, soman, VX etc.) underlines the requirement of effective clinical therapy [1] in military conflicts (e.g. Gulf war, Syrian attack) and terrorist attacks (e.g. Subway attack in Tokyo) [2]. Nerve agents are rather toxic compounds and could be worn as weapons of mass destruction and thus represent a potential threat to humans worldwide. The toxic symptomatology of OP is reflected by the phosphylation of active serine residue of acetylcholinesterase (AChE). Consequently, its physiological function is discontinued and reflected in permanent stimulation of cholinergic receptors leading to failure of respiratory system and subsequent death from suffocation [3]. This indeed, requires the development of efficient, easily affordable and simply administered countermeasures for instant defense of common population from toxic OP exposures. Currently approved antidotal therapies against OP-intoxication entails co-administration of AChE "reactivator" such as 2-PAM, HI-6, obidoxime, trimedoxime, or similar agents (Fig. 2) with anticholinergic (atropine) and anticonvulsant (benzodiazepine) drugs. Reactivator restores the activity of enzyme while atropine antagonizes the effect of accumulated acetylcholine and benzodiazepine acts as an anticonvulsant [4].

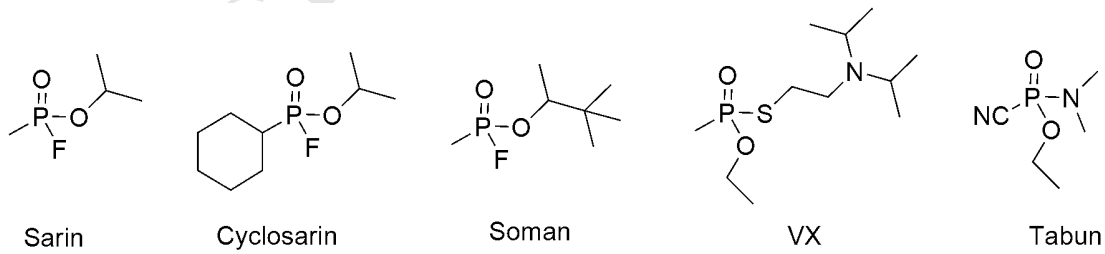


Fig.1. Structures of Chemical Warfare Agents

The interaction of AChE with inhibitors and reactivators is fully dependent on their structural features thus results into different inhibitory and reactivation potencies [5]. The available conventional oximes are not broad spectrum for all classes of OP-poisoning and this ineffectiveness encourages the synthesis of novel and efficient oximes with several structural modifications. In search of a universal antidote, several research groups have been engaged in synthesizing a number of oximes reactivators against all kind of OPs [5-9].

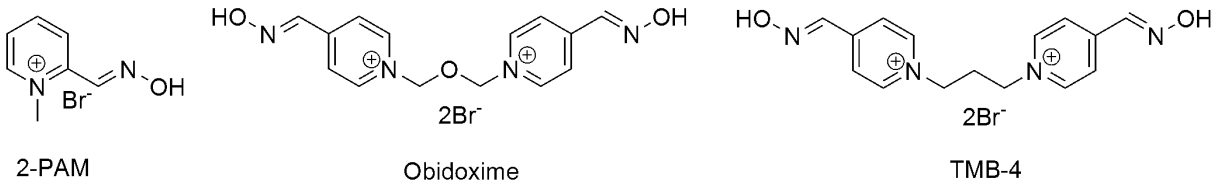


Fig. 2. Structures of Standard oximes

In order to characterize efficient reactivators, synthesized oximes are firstly evaluated for their physicochemical properties. Generally acid dissociation constant (pK_a), lipophilicity (log$P$), polar surface area (PSA), hydrogen bond donor and acceptor counts (HBD and HBA) are included as essential physicochemical properties or molecular descriptors [10].

Determination of pK_a of an oxime is essential to diagnose the effective pH at which the oximate moiety can attack the OP-AChE adduct to reactivate the enzyme [11]. Lipophilicity is first of the primary descriptors known for BBB penetration [12]. It describes the involvement of oxime molecule in lipid phase with respect to hydrophilic phase and their ability to penetrate the lipid bilayer membrane to reach the target site [13]. PSA defines the sum of polar atoms (nitrogen and oxygen) to which hydrogen is present in the oxime reactivator. It is a significant parameter to predict drug transport properties [14]. Hydrogen bond donor (articulated as the sum of OHs and NHs) and acceptor (represents the sum of Ns and Os) counts [15] also play crucial

role in prediction of minimal distribution of a molecule through BBB. Voicu et al. [14] well documented the function of several molecular descriptors as an assortment tool for a series of pyridinium aldoximes derivatives. They further [14] described their pharmacokinetic and pharmacodynamic importance and associated therapeutic consequences for oximes. However, the real potency of a reactivator is quantified by applying reactivation kinetics to inhibited-AChE along with the primary information facilitated by their physicochemical properties.

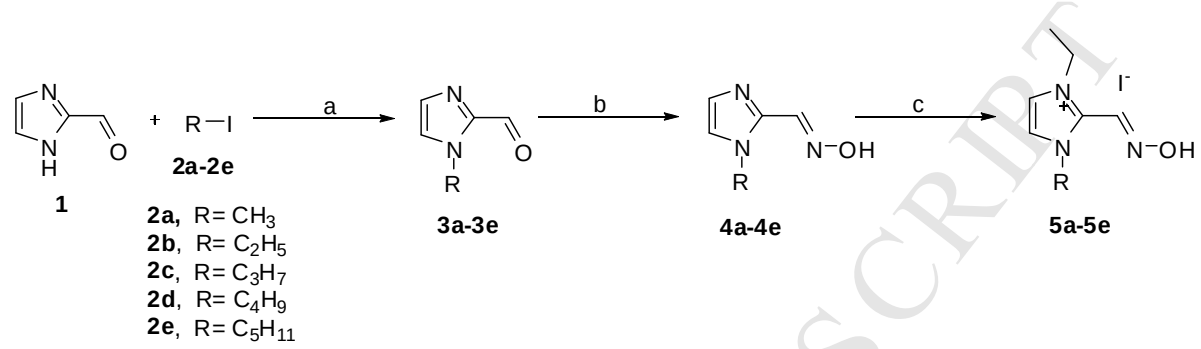
Tertiary and quaternary imidazolium aldoximes were broadly studied as mono-oxime reactivators of inhibited-AChE [16-17] and also as bis-oximes in combination with pyridinium and quinuclidinium oximes [18-19]. Some of these oxime reactivators were evaluated for both *in-vitro* and *in-vivo* investigations and observed as promising candidates for reactivation of tabun- and soman-inhibited AChE. Recently, Sit et al. [20] have synthesized a series of tertiary and quaternary imidazole-based aldoximes and studied their reactivation strength against different OP-inhibited BChE.

Therefore, in the sequence of development of antidotes against OP intoxication [21-24], herein, we report the synthesis of non-quaternary imidazole aldoxime, ethylimidazolium oximes and also bis-oximes in combination of *N*-alkylated 4-pyridinium aldoxime. Since efficacy of imidazolium oximes are generally reported with tabun and soman [18] hence we examined their physicochemical properties/molecular descriptors along with reactivation kinetics against sarin and VX-inhibited human AChE (*h*AChE).

2. Chemistry

The synthesis of non-quaternary imidazole-2-aldoxime derivatives (**4a-4e**) was achieved by N-alkylation of imidazole-2-carboxaldehyde (**1**) with the requisite alkyl iodide (**2a-2e**) by varying alkylated side chain at N1 position. This was then treated with hydroxylamine hydrochloride as

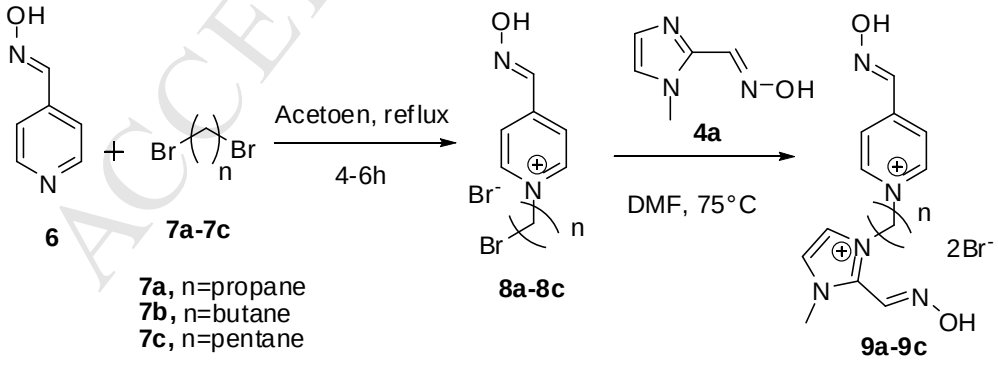
shown in Scheme 1. To increase the nucleophilicity and solubility of resulted compounds (**4a-4e**), process of quaternization has been undertaken. Further their quaternary salts (**5a-5e**) were prepared by reaction with iodoethane.



Reagents: a) K_2CO_3, DMF, 80℃; b) $NH_2OH.HCl$, Na_2CO_3, EtOH, 70℃; c) C_2H_5I, ACN, 75°C

Scheme 1. Synthesis of non-quaternary and quaternary imidazole aldoxime

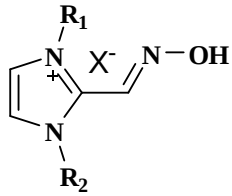
In addition to 10 non-quaternary and quaternary mono-imidazolium compounds, three bis-quaternary imidazolium compounds were also synthesized. Compounds (**9a-9c**) were synthesized by treatment of 4-PAM derivative of N-alkylated monopyridinium oxime (**8a-8c**) with 2-[(hydroxyimino) methyl]-l-methylimidazol (**4a**) (Scheme 2). Compounds (**9a-9c**) incorporate both the 4-[(hydroxyimino)- methylpyridinium moiety and the 2-[(hydroxyimino)-methyl] imidazolium moiety, thus combining the important antidotal properties of both types of reactivators. Elemental analysis of prepared compounds is presented in Table 1.



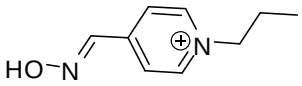
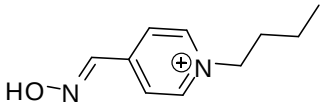
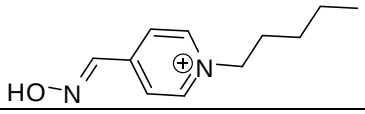
Scheme 2. Synthesis of bis-quaternary imidazole aldoxime

The prototype structure of a mono-imidazolium compound (**5a**) was established on its spectroscopic data. In the ^{1}H NMR spectrum, compound **5a,** exhibited the OH proton of oxime as singlet at δ 12.99, olefinic proton as singlet at δ 8.54, aromatic proton in range of δ 7.89-7.83, *N-CH_2 proton as quartet at δ 4.37 with *J* value 7.2 Hz. The N-CH_3 proton was observed as singlet at δ 3.91 and CH_3 as triplet at δ 1.38 in the aliphatic region with *J* value 7.2 Hz. Similarly the structure of bis-quaternary imidzolium oxime (**9a**) was also established using NMR techniques. The ^{1}H NMR spectrum of compound **9a** exhibited different peaks for OH group of oxime functionality at δ 12.96 and at δ 12.83. The aromatic protons of the pyridinium ring was observed at δ 9.11 and δ 8.26 with *J* value 6.4 Hz, olefinic proton as singlet at δ 8.58. Further the aromatic imidazolium proton was obtained at singlet; δ 8.26 and δ 7.94. *N-CH_2 proton of pyridinium ring was found as triplet at δ 4.71 and *N-CH_2 proton of imidazolium ring as triplet at δ 4.48. CH_3 proton was observed as singlet at δ 3.94 and CH_2 proton of the linker in aliphatic region of NMR spectrum at δ 2.50. All the synthesized compounds have shown their appropriate shifting value in ^{13}C NMR spectrum also.

Table 1. Elemental analysis and acid dissociation constants (pK_a) of synthesized compounds **5a–5e** and **9a-9c**.



Oximes	R_1	R_2	X^-	Yield (%)	pK_a
5a	C_2H_5	Methyl	I-	84	7.65±0.11
5b	C_2H_5	Ethyl	I-	74	7.79±0.09
5c	C_2H_5	Propyl	I-	79	7.92±0.13
5d	C_2H_5	Butyl	I-	78	7.96±0.12
5e	C_2H_5	Pentyl	I-	78	8.10±0.07

9a		Methyl	Br-	80	8.52±0.10
9b		Methyl	Br-	75	8.69±0.13
9c		Methyl	Br-	73	8.92±0.12

3.0 Materials and Methods

3.1 Materials

Acetylthiocholineiodide (ATChI), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 4-pyridinealdoxime, hydroxylamine hydrochloride, imidazole-2-carboxaldehyde, alkyl iodide, sodium carbonate and aliphatic linkers were purchased from Sigma-Aldrich, USA and used without further purification. Potassium dihydrogenphosphate and dipotassium hydrogenphosphate were obtained from E. Merck (India) and used without further purification. Solvents (DMF, acetonitrile, acetone, methanol) were purchased from SD Fine Chemicals (India) and dried before use. Nerve agents sarin and VX were prepared in Process Technology Development Division, Defence Research & Development Establishment, Jhansi Road, Gwalior (M.P.) 474002, India with >98% purity (GC and ^{31}P NMR). 2-PAM was prepared according to the method of Ginsburg and Wilson [25]. Obidoxime was synthesized using reported methods [26]. The oximes were synthesized and characterized by their ^{1}H NMR & ^{13}C NMR spectral data. Purity of the synthesized oximes was checked by TLC (cellulose, DSO, Fluka) with 1-butanol:acetic acid:water (3:1:1) as solvent system.

3.2 Determination of acid dissociation constant (pK_a)

The pK_a of all the oximes were determined by using the method of Albert and Sergeant [27]. 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 a particular pH in which compound of interest would be present wholly in either form. An aliquot (1-3ml) of oxime stock solutions (5 X 10^{-4} M) were diluted to 25ml buffer solution (combination of 70 mM di-sodium hydrogen phosphate and 70 mM potassium dihydrogen phosphate), Different pH values ranging from 6.35 to 10.32 were maintained using different composition of phosphate buffer, further pH was adjusted to the desired value by the addition of dilute sodium hydroxide solution (100 mM). After each pH adjustment, the solution was transferred into the cuvette and the absorption spectra were recorded over the wave-length range of 200–500 nm with a reference to blank solution at 25ºC ± 1˚C.

$$pK_a = pH_{exp} - \log \frac{Abs_{\Psi} - Abs_{HOx}}{Abs_{Ox} - Abs_{\Psi}} \qquad (1)$$

Where, Abs_{Ψ} represents the absorbance of partially ionized oxime species at particular pH; Abs_{HOx} is the absorbance of unionized form of oxime; and Abs_{Ox} is the absorbance of the completely ionized form of oxime. The average value of the 11 measurements was considered as the pK_a of the compound with respect to oximino functionality. Thermofisher scientific evolution 3000 UV-Visible double beam spectrophotometer with quartz cells of 10 mm was used for spectrometric analysis. The quartz cells were attached to a thermostatic peltier for maintaining the constant temperature (25 ±1.0ºC). pH values of buffers were determined using a Eutech (pH 700), pH meter equipped with Inlab$^{@}$ Expert Pro glass electrode with an accuracy of ±0.01 units. The pH meter was calibrated at 25ºC using the two point calibration method with commercially available standard buffer solutions pH 7.00 and 9.21.

3.3 Preparation of human erythrocyte ghost

The source of AChE was native intact human erythrocytes, prepared according to the method of Worek et al. [28]. Volunteers (n = 2) were male, aged 27-28 years, healthy, non-smoking, non-alcoholic and weighed ≈70 kg. Blood (10 mL each) was withdrawn into tubes containing EDTA, centrifuged (3000g, 10 min) and plasma was removed. Erythrocytes were washed three times with two volumes of phosphate buffer (0.1 M, pH 7.4). Packed erythrocytes were diluted in 20 volumes of hypotonic phosphate buffer (6.6 mM, pH 7.4) to facilitate hemolysis followed by centrifugation at 40,000g for 30 min at 4ºC. The supernatant was removed and pellets re-suspended in hypotonic phosphate buffer. After two additional washing cycles the pellets were combined and re-suspended in 3 mL phosphate buffer (0.1 M, pH 7.4). The erythrocyte ghosts were concentrated by centrifugation at 100,000g for 30 min at 4ºC. Aliquots of erythrocyte ghosts were stored at -80ºC until use. Prior to use, ghosts were homogenized on ice on homogenizer (Sonics vibra cell, USA); three times for 5s with 9s interval to achieve a homogenous matrix for the enzyme assay.

3.4 AChE Assay: Inhibition and Reactivation

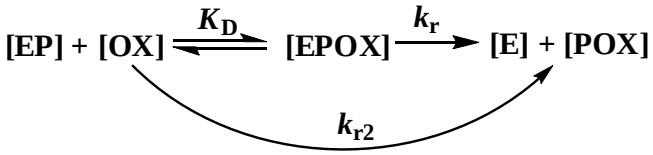
The activities of AChE were monitored spectrophotometrically by Perkin Elmer Lambda 35 UV-Visible double beam spectrophotometer at wavelength 412 nm with a modified Ellman assay [29] using quartz cuvettes and 0.48 mM ATChI as substrate and 0.32 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4). All experiments were performed at 37ºC temperature and pH 7.4. All mentioned concentrations refer to final concentrations. Oxime stock solutions and their dilutions were done freshly in triple distilled water. The stock solutions of OP inhibitors (sarin and VX) were prepared in isopropanol once in a week and were stored at -20ºC. The subsequent dilutions of these inhibitors were freshly prepared every day in deionized water just before to start the experiment. The hAChE was incubated with appropriate OP concentrations; sarin (0.5 x

10^{-7} M) and VX (0.5x 10^{-7} M) at 37ºC for 10–15 mins 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.

The reactivation of OP-inhibited hAChE was achieved by the addition of oxime reactivator at a final concentration ranging from 0.1 mM-1.0 mM. An aliquot of 50 µl of reactivation cocktail (total volume of cocktail; 750 µl, containing inhibition cocktail and oxime reactivator) were withdrawn at specified time intervals and diluted in to cuvettes containing 3000 µl phosphate buffer and 100 µl of DTNB to monitor change in the enzyme activity after addition of 20 µl ATChI. The oxime induced reactivation of the OP-inhibited AChE was monitored at different time intervals over a period of 60-90 mins. Spontaneous reactivation of inhibited AChE was assayed using the same protocol (the reaction mixture containing enzyme and OP but no oxime). In these conditions spontaneous reactivation was found to be insignificant. All the values were corrected for their oxime induced hydrolysis of ATChI.

3.5 Reactivation Kinetic Studies

The activity of enzyme in the control remained constant during the experiment. The reactivation percentage of inhibited enzyme was calculated as the ratio of the recovered enzyme activity and activity in the control. The apparent reactivation rate constant k_{obs} for each oxime concentration, the dissociation constant K_D of inhibited enzyme-oxime complex [EPOX] and the maximal reactivation rate constant *k*r, were calculated by non-linear fit using the standard oxime concentration-dependent reactivation Eq. 2 derived from the following Scheme 3 [30]. Calculation of kinetic constants was performed by non-linear regression analysis using curve fitting programs using Prism™ Vers. 6.0 (Graph Pad Software, San Diego, USA).



Scheme 3. Schematic representation of reactivation of OP-inhibited AChE by oxime

$$k_{obs} = \frac{k_r \text{ x } [OX]}{K_D + [OX]} \quad (2)$$

4.0 Results

The synthesized oximes may act as efficient reactivator if they have considerable nucleophilcity. The nucleophilicity of reactivators can be assessed by determining their pK_a values. Therefore acid dissociation constants (pK_a) of all the oximes (**5a-5e,** and **9a-9c**) were determined (Table 1) using the method of Albert and Sergeant [27]; Eq 1. pK_a is based on direct estimation of the ratio of molecular species (protonated) to ionised (deprotonated) species in a series of non-absorbing phosphate buffer solutions. A prototype spectrum of oxime **9a** is shown in Fig.3.

On the basis of pharmacokinetic principles, it is concluded that the blood brain barrier (BBB) is relatively impermeable to hydrophilic molecules like quaternary oximes due to their charged structures and hydrophilic oxime groups. Thus, make them inefficient to penetrate BBB and cure CNS-poisoning. The molecular descriptors serve as a screening tool for prediction of permeability of oximes (or lead compound) through biological membranes and are key parameters for efficient drug design [10]. Hence a variety of descriptors have been evaluated (Table 2) for synthesized oximes such as molecular weight (MW), polar surface area (PSA), hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), and lipophilicity (log*P*) using MarvinSketch version 6.2.2, 2014 (http://www.chemaxon.com). For favourable penetration through BBB, all oximes should have [14]; MW <500 Da; PSA≈ 60-70Å^2, HBD: <5, HBA: <10, log *P*: >2 to <5. Most of the tested reactivators fulfilled the mentioned requirements. Only

charged oximes (**5a-9c**) showed high negative log*P* values and may have insignificant ability for BBB permeability. It was observed that imidazole oximes in combination with pyridinium ring exceed in term of permissible PSA value and have a range of 77.87-80.70 Å^2.

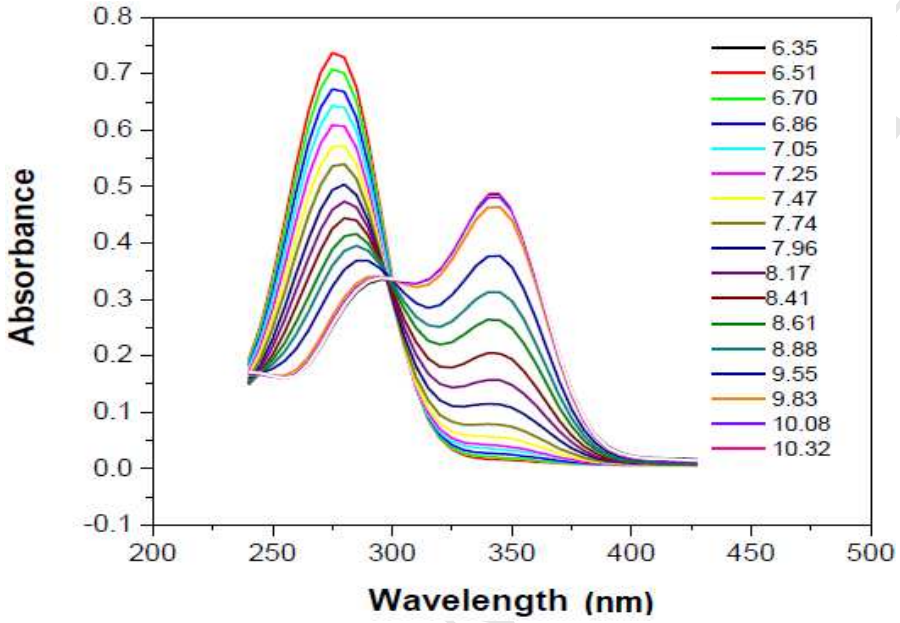


Fig. 3. Spectrophotometric determination of pK_a of oxime **9a**.

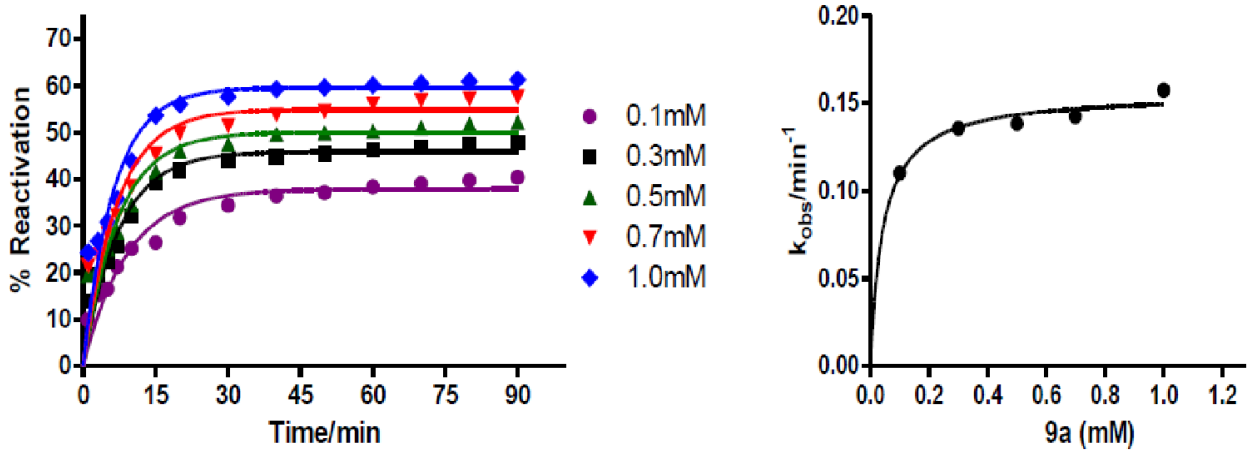
Table 2. Calculated values of other molecular descriptors of tested oximes (at pH 7.4)

Oximes	MW (Da)	PSA (Å^2)	HBD	HBA	*Log* P
5a	281.09	41.40	1	2	-3.21
5b	295.02	41.40	1	2	-2.85
5c	309.15	41.40	1	2	-2.33
5d	323.17	41.40	1	2	-1.88
5e	337.20	41.40	1	2	-1.44
9a	449.14	80.70	2	4	-7.14
9b	463.17	80.70	2	4	-6.63
9c	477.19	77.87	2	4	-6.18

All the synthesized oximes were subjected to kinetic analysis to evaluate their reactivation potential. Experimentally observed kinetic parameters for studied oximes (Table 3) against hAChE inhibited by sarin and VX revealed that the different affinity and reactivity are dependent

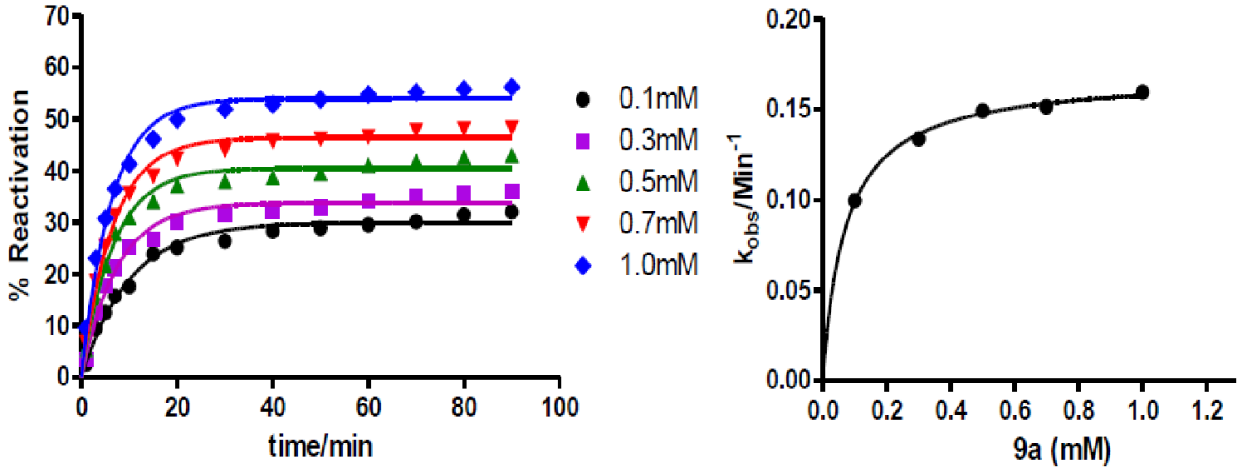
on the structures of both OP and oximes used. Synthesized oximes (**5a-5e** and **9a-9c**) were assayed for their *in-vitro* reactivation efficiency (Figs 4-5) at five different concentrations ranging from 0.1mM to 1.0mM using erythrocyte ghost *h*AChE inhibited by tested nerve agents over a period of 60-90 minutes (Table 3 and Fig. 6). The affinity of oximes toward phosphylated AChE was reflected by their K_D values. For both the nerve agents, all the studied molecules exhibited K_D values within the range of (21.86 μM- 144.40 μM). In case of sarin **5c** displayed comparatively higher K_D value i.e. 106.00 μM and only oxime **9a** showed comparable value with 2-PAM. For VX–AChE conjugate, synthesized oximes possessed slightly higher K_D values (71.68 μM to 144.40 μM) and displayed lower affinity toward OP-AChE complex as compared to standard 2-PAM and obidoxime.

The ability of the oxime for the de-phosphorylation of the phosphoryl residue from the OP-AChE adduct is articulated in terms of reactivity rate constant (k_r, min^{-1}). The rate constant k_r for the oximes **5a-5e** and **9a-9c** against the reactivation of sarin- and VX-inhibited AChE were found to be in the range of 0.040–0.156 min^{-1} and 0.076–0.169 min^{-1} respectively (Table 3). Bis-oxime **9a** (0.156 min^{-1}) showed comparable reactivity rate constant with obidoxime (0.170 min^{-1}) and was having much higher k_r value than 2-PAM (0.06 min^{-1}). In case of VX-inhibited AChE, again oxime **9a** (0.169 min^{-1}) showed higher reactivity rate constant than standard 2-PAM (0.110 min^{-1}) and displayed comparable values with obidoxime (0.18 min^{-1}).



(a) (b)

Fig. 4. Time and concentration-dependent reactivation of sarin-inhibited *h*AChE by **9a**. (a) Data were analyzed by non-linear regression analysis to determine k_{obs} (b) Plot of k_{obs} *vs* [**9a**] enabled the calculation of K_D and k_r.



(a) (b)

Fig. 5. Time and concentration-dependent reactivation of VX-inhibited *h*AChE by **9a**. (a) Data were analyzed by non-linear regression analysis to determine k_{obs} (b) Plot of k_{obs} *vs* [**9a**] enabled the calculation of K_D and k_r.

The second order rate constant k_{r2} gave the specific reactivity of the reactivator and is dependent on the value of K_D and k_r. In case of sarin- and VX-inhibited AChE the observed k_{r2} values of synthesized oximes **5a-5e** and **9a-9c** ranged between 0.56 to 3.59 $mM^{-1}min^{-1}$ and 0.73 to 2.36 $mM^{-1}min^{-1}$. Amongst the synthesized oximes, compound **9a** ($k_{r2;}$ 3.59 $mM^{-1}min^{-1}$) surpassed the second order rate constant value of 2-PAM (2.23 $mM^{-1}min^{-1}$), while **9b** (2.05 $mM^{-1}min^{-1}$) showed quite good reactivity against sarin inhibited erythrocyte ghost AChE. For VX-inhibited AChE, no tested oxime could cross the ability of 2-PAM and obidoxime but **9a** (2.36 $mM^{-1}min^{-1}$) displayed comparable k_{r2} value with standard 2-PAM (Table 3). Non-quaternary imidazole aldoximes (**4a-4e**) didn't show any remarkable reactivation efficiency against any of the OP inhibited *h*AChE.

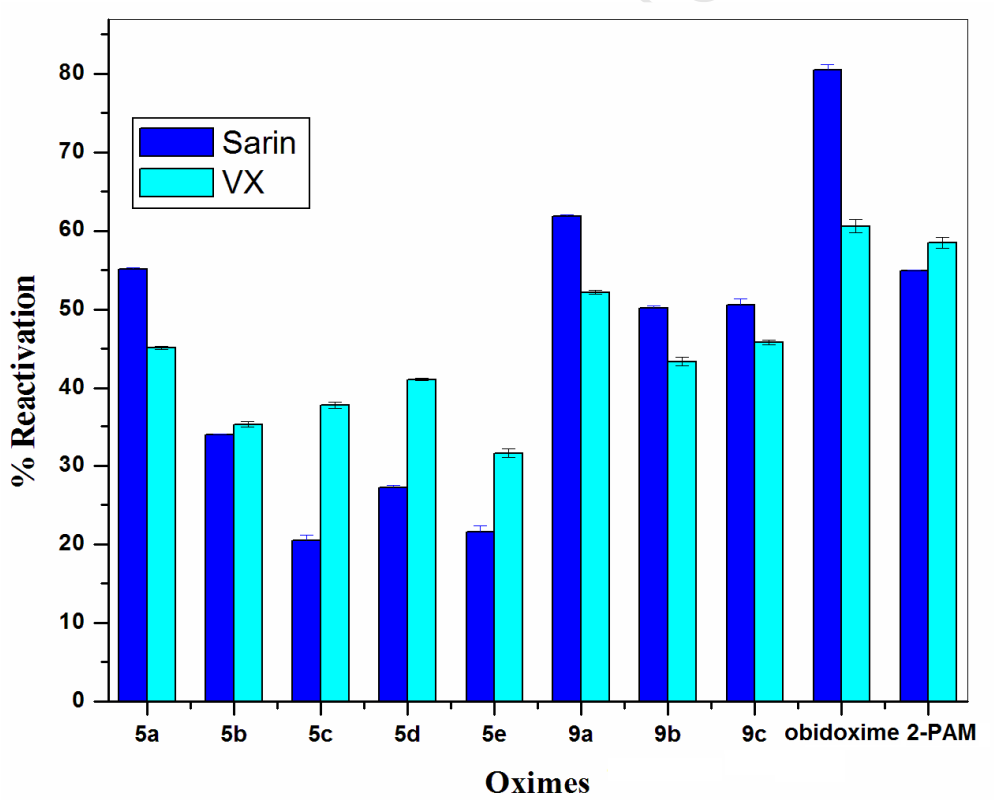


Fig. 6. Comparative percentage reactivation profile of synthesized oximes **5a–5e** and **9a-9c** and standard oximes at 90 min against sarin and VX inhibited *h*AChE (1.0 mM concentration of oximes, 0.1 M phosphate buffer, pH 7.4 and temperature 37 ˚C).

Table 3 Reactivation constants for oxime-induced reactivation of OP-inhibited AChE

Oximes	K_D (μM)		k_r (min^{-1})		k_{r2} ($mM^{-1}min^{-1}$)		%R		IC_{50} (mM)
	Sarin	VX	Sarin	VX	Sarin	VX	Sarin	VX	
5a	**58.14±4.4**	**74.14±4.4**	**0.077±0.0009**	**0.098±0.0011**	**1.72±0.51**	**1.32±0.42**	**55.15±0.21**	**45.15±0.21**	**1.39**
5b	70.21±3.6	83.22±18.6	0.040±0.0004	0.098±0.0037	0.56±0.10	1.18±0.20	33.90±0.14	35.25±0.35	1.97
5c	106.00±20.4	73.61±10.2	0.075±0.0041	0.076±0.0032	0.70±0.17	1.03±0.16	20.50±0.71	37.70±0.42	1.90
5d	88.16±6.9	78.45±11.0	0.054±0.0010	0.085±0.0025	0.61±0.14	1.08±0.12	27.20±0.28	41.10±0.14	2.08
5e	55.68±11.4	144.40±12.1	0.060±0.0020	0.105±0.0019	1.07±0.16	0.73±0.14	21.55±0.78	31.60±0.57	1.87
9a	**43.42±4.9**	**71.68±8.5**	**0.156±0.0022**	**0.169±0.0033**	**3.59±0.48**	**2.36±0.60**	**61.85**±0.21	**52.20**±0.28	**1.38**
9b	67.11±4.6	89.21±13.8	0.138±0.0013	0.110±0.0045	2.05±0.49	1.23±0.53	50.20±0.28	43.40±0.57	1.34
9c	75.27±2.4	84.81±7.7	0.081±0.0007	0.099±0.0030	1.07±0.45	1.18±0.74	50.55±0.78	45.80±0.28	1.17
2-PAM[a]	25.72±2.1	31.39±3.9	0.06±0.0004	0.110±0.0014	2.23±0.41	3.59±1.34	54.95±0.07	58.50±0.71	0.87
Obidoxime[a]	21.48±3.0	21.86±1.6	0.17±0.0019	0.180±0.0011	8.03±1.20	8.31±1.54	80.50±0.71	60.60±0.85	1.19

The pseudo first order rate constant k_{obs} was determined by non-linear regression analysis and these data were used for the calculation of the reactivity rate constant k_r, the dissociation constant K_D and the hybrid reactivation rate constant k_{r2} (from the ratio of k_r/K_D). Data are given as means ±SD (n = 2). Maximum reactivation gives the highest AChE activity after 90 mins incubation of inhibited AChE by the respective oxime. a= Ref.31.

In addition to the kinetic parameters, the inhibition potencies (IC_{50} values) of synthesized oximes (**5a-5e** and **9a-9c**) have also been determined and found in the range of 1.17 to 2.08 mM (Table 3). The observed IC_{50} values indicated that prepared oximes are less toxic in comparison to 2-PAM and obidoxime, except oxime **9c** which has least IC_{50} value (1.17mM).

5.0 Discussion

The reactivation of OP-inhibited AChE is affected by several factors such as the strength of nucleophile (oxime), the orientation of nucleophile with respect to phosphate group conjugated to the active centre serine and dealkylation of the OP–AChE conjugate (aging) [23]. Due to structural diversity of OP-based pesticides and nerve agents; none of the pyridinium based oxime reactivators (Fig. 2) is efficient to regenerate inhibited AChE [23]. Therefore, an effective and quick remedy for all kind of OP-poisoning is still lacking. Hence we tried to develop alternatives of pyridyl oximes with lower molecular weight and of smaller size. From the last few years, imidazolium aldoximes which have structural similarity to amidine oximes have drawn attention of researchers across the globe. In early 1990's, imidazolium aldoximes were studied extensively as cholinesterase reactivators [32-33] and recently a series of non-quaternary imidazolium aldoxime were reported against OP poisonings [20, 34]. In continuation of search of cholinesterase reactivators, we selected imidazole ring system. The selection of imidazole ring as compared to pyridinium ring was based on the synthetic limberness, sound isosteric similarity with pyridine, ease of quaternization [35] (for Coulombic attraction to anionic sites of AChE), and the standard requirement for oximes with pK_a near 8.0 [16]. Nevertheless, non-quaternary oxime resulted in decreased reactivation efficacy because they don't bind properly to the aromatic group of the anionic site, which is located at the bottom of the deep gorge of the AChE

[6]. This is the reason we have quternized the imidazole aldoxime and evaluated further. Apart from this we have also studied the effect combination of pyridinium and imidazolium ring on the reactivation of AChE inhibited by tested OP agents. Previously published uncharged oximes reactivators by Sit et al. [36] revealed that simple *N*-alkyl substituted imidazole aldoximes were good reactivators of OP-*h*BChE conjugates. The group tried to identify the optimal uncharged reactivators of OP-*h*AChE, unfortunately the synthesized imidazole aldoxime didn't surface as optimal candidates. However, the reactivation results of present investigation didn't follow any regular trend in case of (**5a-5e**), but the insertion of pyridinium ring (**9a-9c**) thoroughly enhanced reactivation ability as well as affinity of synthesized oximes towards OP-AChE conjugates.

5.1 Structure activity relationship (SAR)

The evaluation of physicochemical properties along with reactivation kinetics may facilitate suitable structure-activity-relationships for the synthesized oximes. An increase in carbon chain length for quaternary imidazolium oximes (**5a-5e**) didn't display any notable change in acid dissociation constant (pK_a) value (Table 1), while insertion of pyridinium ring connected to various, alkyl chains considerably affected the pK_a (Table 1). All the synthesized charged aldoximes (**5a-5e** and **9a-9c**) exhibited negative log*P* values (Table 2). The reactivation strength of synthesized oximes significantly improved with the incorporation of pyridinium oxime (**9a-9c**) on the other end of the alkyl chain of imidazolium ring. Presence of pyridinium ring is an important factor for cation-π interactions with aromatic amino acid residues of the peripheral anionic site (PAS) of AChE. A plausible explanation of the enhanced reactivation percentage of **9a-9c** is the presence of additional π -π interactions and thereby results in strong binding with AChE active site. The order of reactivity of compound **9a** was 1.5 folds higher as compare to 2-PAM against sarin-inhibited *h*AChE. The recognized optimal chain length for the moderate

reactivity of examined reactivator was 3-4 carbon atoms. The findings are in well agreement with the earlier reported trend [5, 7, 16]. Compounds **9a-9c** showed moderate to significant reactivation potency against tested nerve agents.

Reactivation potency of investigated oximes was also influenced by structure of nerve agents. Out of the synthesized oximes, compounds **9a-9c** showed comparable reactivation ability for VX-inhibited *h*AChE. This may be accounted on the basis of least occupied gorge of VX-inhibited AChE due to the removal of bulkier leaving group: *N*, *N*-diisopropyl-S-ethyl, hence facilitated more space for oximes to attack phosphorylated active site with minimum steric hindrance as compared to sarin [11].

6.0 Conclusion

We have synthesized a series of mono- and bis-quaternary imidazolium aldoximes (**5a-5e** and **9a-9c**) from imidazol-2-carboxaldehyde. All the synthesized oximes were assayed for their *in-vitro* reactivation efficacy against sarin- and VX- inhibited-*h*AChE. Effect of presence and absence of pyridinium ring along with imidazole ring was also studied. Apart from reactivation kinetic studies, physicochemical studies were also performed. Based upon the study it was found that all the oximes have pK_a with in the range of 7.65-8.92 and compound **9a** showed best interaction with AChE sites and resulted into good reactivation potential against both sarin- and VX- inhibited *h*AChE. Overall assessment led to the conclusion that compounds **5a**, **9a-9c** may provide useful therapeutic potential for the reactivation of *h*AChE inhibited by toxic organophosphorus nerve agents. The detail study of antidotal efficacy of synthesized compounds along with their *in-vivo* reactivation potential will be reported in due course of time.

7.0 Conflict of interest statement

Authors declare that there are no conflicts of interest.

8.0 Acknowledgment

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Highlights

- A series of 1,3-disubstituted imidazolium aldoximes were synthesized.
- Analysis of physico-chemical properties of prepared oximes were also studied.
- Synthesized oximes were evaluated against sarin and VX inhibited human AChE.
- Insertion of pyridinium ring to imidazolium oxime resulted in better reactivity.