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Bisquaternary pyridinium oximes: Comparison of in vitro reactivation potency of compounds bearing aliphatic linkers and heteroaromatic linkers for paraoxon-inhibited electric eel and recombinant human acetylcholinesterase

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ABSTRACT

Oxime reactivators are the drugs of choice for the post-treatment of OP (organophosphorus) intoxication and used widely for mechanistic and kinetic studies of OP-inhibited cholinesterases. The purpose of the present study was to evaluate new oxime compounds to reactivate acetylcholinesterase (AChE) inhibited by the OP paraoxon. Several new bisquaternary pyridinium oximes with heterocyclic linkers along with some known bisquaternary pyridinium oximes bearing aliphatic linkers were synthesized and evaluated for their in vitro reactivation potency against paraoxon-inhibited electric eel acetylcholinesterase (EeAChE) and recombinant human acetylcholinesterase (rHuAChE). Results herein indicate that most of the compounds are better reactivators of EeAChE than of rHuAChE. The reactivation potency of two different classes of compounds with varying linker chains was compared and observed that the structure of the connecting chain is an important factor for the activity of the reactivators. At a higher concentration (10^{-3} M), compounds bearing aliphatic linker showed better reactivation than compounds with heterocyclic linkers. Interestingly, oximes with a heterocyclic linker inhibited AChE at higher concentration (10^{-3} M), whereas their ability to reactivate was increased at lower concentrations (10^{-4} M and 10^{-5} M). Compounds bearing either a thiophene linker **26**, **46** or a furan linker **31** showed 59%, 49% and 52% reactivation of EeAChE, respectively, at 10^{-5} M. These compounds showed 14%, 6% and 15% reactivation of rHuAChE at 10^{-4} M. Amongst newly synthesized analogs with heterocyclic linkers (**26**–**35** and **45**–**46**), compound **31**, bearing furan linker chain, was found to be the most effective reactivator with a k_r 0.042 min^{-1}, which is better than obidoxime (**3**) for paraoxon-inhibited EeAChE. Compound **31** showed a k_r 0.0041 min^{-1} that is near equal to pralidoxime (**1**) for paraoxon-inhibited rHuAChE.

1. Introduction

Exposure to organophosphorus compounds (OPs) such as sarin, paraoxon, soman, and VX causes acute intoxication inclusive of convulsions and paralysis of the respiratory muscles. The toxic action of organophosphorus compounds is generally attributed to the inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) thus blocking its physiologic action—hydrolyzing the neurotransmitter acetylcholine at central and peripheral synapses. Acetylcholine accumulation results in an over-stimulation of cholinergic receptors and, depending on the type and dose of the OP can lead to respiratory arrest and death.[1,2] Reactivation of AChE is the primary therapeutic approach to reverse the ill effects of OP poisoning and oximes are the universal choice for this therapeutic intervention. Oximes are also important tools in vitro serving in kinetic, mechanistic and biochemical studies. Oximes reactivate phosphorylated cholinesterases by cleaving the phosphoester bond between the active site serine and the phosphorus atom. By virtue of their high affinity for AChE and their inherent nucleophilicity, oxime-based reactivation is relatively efficient forming a dephosphorylated, restored enzyme and a transient phosphorylated oxime.[3]

The search for oxime-based reactivators dates back to the early 1950s and led to the discovery of 2-pyridine aldoxime methiodide (2-PAM, **1**) as an effective reactivator used to treat human OP poisonings.[4,5] The next compound of interest was trimedoxime (TMB-4, **2**) which was synthesized and tested by Poziomek et al.[6] and independently by Hobbiger et al.[7] This bispyridinium derivative was superior to 2-PAM, particularly in tabun poisoning. Obidoxime (**3**) was synthesized by Luttringhaus and Hagedorn in the early 1960s[8] and studied in pesticide-poisoned patients by Erdmann and von Clarmann.[9] In the following decades, Hagedorn et al. synthesized numerous oximes in order to increase the efficacy against soman, resulting in the development of HI-6 (**4**).[10–12] HI-6 is considered to be superior to 2-PAM, obi-

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doxime and TMB-4 in reactivating AChE after nerve agent poisoning. Apart from these well known oxime reactivators, several bisquaternary pyridinium oximes bearing varying linker chains with π-electron functionalities are reported in literature viz. (*E*)-but-2-ene linker **5** (55% reactivation of tabun-inhibited AChE),[13,14] $CH_2CH_2OCH_2CH_2$ linker **6** (17% reactivation of cyclosarin-inhibited AChE),[15] $CH_2O(CH_2)_2OCH_2$ linker **7** (63% reactivation of DFP-inhibited AChE)[16,17] and xylene linker **8** (44% reactivation of paraoxon-inhibited AChE).[18] Structures of pyridinium oximes **1**-**8** are shown in Figure 1. Overall, reactivators differ mainly in the number of quaternary pyridinium rings (1 or 2), length and structure of connecting chain and/or in the number and position of functional oxime groups.

From a toxicological standpoint, the reactivating efficacy of oximes has been mainly investigated in rodents.[19] However, owing to its ready availability, an extensive number of in vitro investigations have been conducted over the years with electric eel AChE (EeAChE). As a result, it is generally necessary to conduct the screening of new oximes with EeAChE for comparison purposes. Nevertheless, the structural and functional differences between human, animal and electric eel AChE may result in a different affinity and reactivity of oximes. Several studies indicate substantial species-dependent marked differences in the ability of oximes to reactivate OP-inhibited AChE.[20–22] Overall, measurements of species differences can be crucial for the assessment of oxime efficacy in humans. Experiments were undertaken to determine percentage of reactivation and reactivation rate constant of several newly synthesized as well as some known bisquaternary oximes for recombinant human and electric eel AChE inhibited by paraoxon.

Paraoxon has been responsible for more cases of poisoning than any other OP insecticide,[23] so it is vitally important to determine the potency of new oximes for this OPI. Recently, we reported the reactivation potency of a series of newly synthesized monoquaternary pyridinium oximes against paraoxon-inhibited electric eel AChE (EeAChE) and recombinant human AChE (rHuAChE).[24] In the present paper, a series of bisquaternary pyridinium oximes bearing heterocyclic linkers along with several known bisquaternary oximes bearing aliphatic 3–4 atom chain linkers were synthesized and evaluated for their reactivation potency against paraoxon-inhibited electric eel AChE (EeAChE) and recombinant human AChE (rHuAChE). A comparison of the reactivation profile of two different classes of oxime reactivators against rHuACHE and EeAChE is also discussed.

2. Results and discussion

2.1. Synthesis of bisquaternary pyridinium oximes

Bisquaternary reactivators were prepared as shown in Schemes 1 and 2. A series of known bisquaternary pyridinium oximes bearing 3–4 atom aliphatic linkers (**2**, **3**, **19**–**25**, **39**–**41**, **44**)[25–31] along with two new structural analogs (**42** and **43**) were synthesized. Furthermore, a series of new bisquaternary pyridinium oximes bearing the heterocyclic linkers viz. thiophene (**26**–**28**, **45**–**46**), furan (**29**–**32**) and isoxazole (**33**–**35**) were synthesized. Heterocyclic linkers were used to alter the lipophilicity and include a π-electron characteristic capable of interacting with the AChE active site residues via π–π or cation-π interactions. Additionally, various positions of the oxime functional groups on the pyridinium ring were used to optimize any angular dependence between cation and nucleophile to afford the effective reactivation of AChE. All compounds were characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS and IR data and have purity of >95% from NMR spectra.

2.2. In vitro reactivation screening

All compounds were assayed for their capacity to reactivate rHuAChE and EeAChE using a 96-well format modified Ellman assay.[32] Substrate and reporter reagents (AtChI at 1 mM and DTNB at 0.3 mM) were added in 0.1 M phosphate buffer solution *p*H 7.2. The OP paraoxon was used to inhibit both AChEs, and pralidoxime (**1**), trimedoxime (**2**) and obidoxime (**3**) were used as the reference oxime reactivators. Reactivation results are summarized in Table 1. Among compounds bearing aliphatic linkers, **25**, **41** and **44** at 10^{-5} M promoted reactivation of EeAChE (68%, 56% and 52%, respectively) at levels equal to or greater than that of pralidoxime (**1**), but less than that of trimedoxime (**2**). These compounds, along with newly synthesized compound **42**, showed 58%, 80%, 71% and 74% reactivation of EeAChE, respectively at 10^{-4} M. Compound **42** also reactivated rHuAChE to 20% at 10^{-4} M. For rHuAChE, compounds **41** and **44** were the most active with 64% and 73% reactivation at 10^{-3} M, respectively. Compounds with heterocyclic linker chains (thiophene, furan and isoxazole) displayed a reverse concentration-activity relationship. These compounds (**26**–**35** and **45**), with the exception of **46**, showed ⩽ 7% reactivation of EeAChE and no reactivation (⩽3%) of rHuAChE at the highest concentration (10^{-3} M). Lower concentrations of oxime (10^{-4}–10^{-5} M) **26**–**35** and **45**–**46** were better reactivators of both enzymes suggesting that these compounds may inhibit AChE at higher concentrations and explain, in part, the inability to reactivate inhibited AChE at 10^{-3} M.[18,33] To confirm this change in mechanism, compounds **2**, **3**, **26**, **28**, **31** and **46** were tested as inhibitors against rHuAChE and EeAChE. The results indicated that the compounds (except obidoxime, **3**) gave 23–87% inhibition of both AChE's at 10^{-3} M while <3% inhibition was observed at 10^{-5} M (Table 2). Pyridinium oximes bearing heterocyclic linkers **26**, **28**, **31** and **46** were better inhibitors than those oximes bearing aliphatic linkers **2** and **3**. These results explain, in part, the inability of pyridinium oximes with heterocyclic linkers to reactivate paraoxon-inhibited AChE at 10^{-3} M. The lower reactivation potency of bisquaternary oximes with heterocyclic linkers compared with oximes bearing aliphatic linkers might also be due to the restricted flexibility of the heterocycles in the AChE active site cavity. Since the concentration 10^{-3} M is not attainable with in vivo experiments,[34] 10^{-5} M is more appropriate and attainable for clinical use.[1]

At 10^{-5} M, compounds **26**, **31** and **46** were the best reactivators of both enzymes (59%, 52% and 49% reactivation of EeAChE, and 57%, 77% and 7% reactivation of rHuAChE). The efficacy of each reactivator varied with concentration, and four trends were identified from the data (Table 1) (Fig. 2). First, percent reactivation correlated with increasing concentration of oximes **20**, **22**, and **40**. Second, we observed that an increasing reactivation correlated with a decreasing concentration of oximes **2**, **21**, **23**, **25**, **26**–**28**, **31**, and **35**.

Third, oximes **3**, **19**, **24**, **29**, **30**, **39**, **41**–**46** showed the best percent reactivation at midrange concentrations 10^{-4} M. This might be explained by a combination of inefficient reactivation at low concentrations coupled with inhibitory activity at high concentrations.[33] Last, 2- and 4-oximes were better reactivators than 3-oximes. Control compound **32**, which does not possess any oxime functionality, did not reactivate either enzyme.

All synthesized pyridinium oximes were also tested for their cytotoxic effects towards mammalian kidney fibroblasts (Vero cells). None of the compounds showed cytotoxicity up to the concentration of 4.67 μg/ml (unpublished results).[35]

2.3. Determination of reactivation rate constants (k_r)

Owing to their greater activity (Table 1) amongst newly synthesized compounds (**26**–**35**, **42**, **45**–**46**), compounds **26**, **31**, **42** and **46** were selected for analysis of the oxime-mediated reactivation rate constants (k_r; characterizes the dissociation of the enzyme and the phosphorylated-oxime). The k_r was calculated using the equation from the linear portion of activity curves (0–15 min).[36]

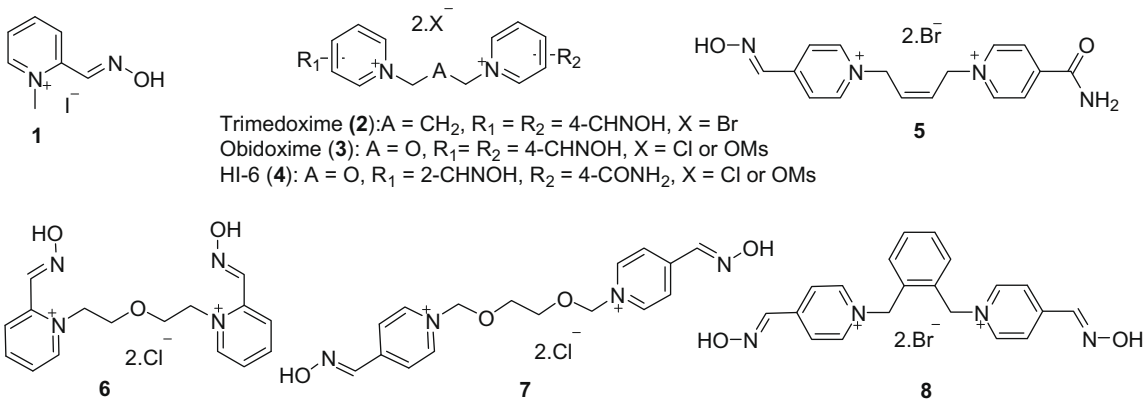
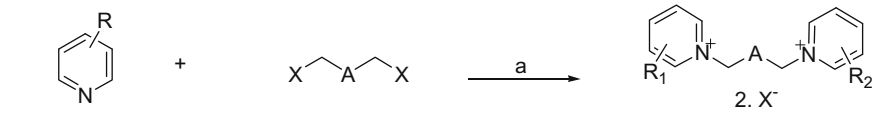


Figure 1. Examples of mono- and bisquaternary pyridinium oximes.

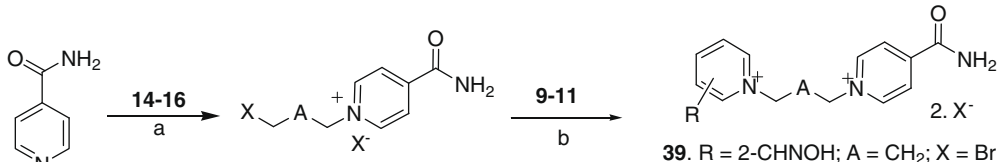


9. R = 2-CHNOH
10. R = 3-CHNOH
11. R = 4-CHNOH
12. R = 4-$CONH_2$

13. A = O; X = OSO_2CH_3
14. A = CH_2; X = Br
15. A = CH_2CH_2; X = Br
16. A = Thiophen-2,5-yl; X = Cl
17. A = Furan-2,5-yl; X = Br
18. A = Isoxazol-3,5-yl; X = Br

19. R_1= R_2 = 2-CHNOH, A = O, X = OSO_2CH_3
20. R_1= R_2 = 3-CHNOH, A = O, X = OSO_2CH_3
3. R_1= R_2 = 4-CHNOH, A = O, X = OSO_2CH_3
21. R_1= R_2 = 2-CHNOH, A = CH_2, X = Br
22. R_1= R_2 = 3-CHNOH, A = CH_2, X = Br
2. R_1= R_2 = 4-CHNOH, A = CH_2, X = Br
23. R_1= R_2 = 2-CHNOH, A = CH_2CH_2, X = Br
24. R_1= R_2 = 3-CHNOH, A = CH_2CH_2, X = Br
25. R_1= R_2 = 4-CHNOH, A = CH_2CH_2, X = Br
26. R_1= R_2 = 2-CHNOH, A = Thiophen-2,5-yl; X = Cl
27. R_1= R_2 = 3-CHNOH, A = Thiophen-2,5-yl; X = Cl
28. R_1= R_2 = 4-CHNOH, A = Thiophen-2,5-yl; X = Cl
29. R_1= R_2 = 2-CHNOH, A = Furan-2,5-yl; X = Br
30. R_1= R_2 = 3-CHNOH, A = Furan-2,5-yl; X = Br
31. R_1= R_2 = 4-CHNOH, A = Furan-2,5-yl; X = Br
32. R_1= R_2 = 4-$CONH_2$, A = Furan-2,5-yl; X = Br
33. R_1= R_2 = 2-CHNOH, A = Isoxazol-3,5-yl; X = Br
34. R_1= R_2 = 3-CHNOH, A = Isoxazol-3,5-yl; X = Br
35. R_1= R_2 = 4-CHNOH, A = Isoxazol-3,5-yl; X = Br

Scheme 1. Reagents and conditions: (a) DMF, 80 °C, 2–24 h, 24–89%.



36. A = CH_2; X = Br
37. A = CH_2CH_2; X = Br
38. A = Thiophen-2,5-yl; X = Cl

39. R = 2-CHNOH; A = CH_2; X = Br
40. R = 3-CHNOH; A = CH_2; X = Br
41. R = 4-CHNOH; A = CH_2; X = Br
42. R = 2-CHNOH; A = CH_2CH_2; X = Br
43. R = 3-CHNOH; A = CH_2CH_2; X = Br
44. R = 4-CHNOH; A = CH_2CH_2; X = Br
45. R = 3-CHNOH; A = Thiophen-2,5-yl; X = Cl
46. R = 4-CHNOH; A = Thiophen-2,5-yl; X = Cl

Scheme 2. Reagents and conditions: (a) MeCN, 70–80 °C, 2–3 h, 60–75%; (b) DMF, 80 °C, 2–24 h, 55–75%.

In agreement with the preliminary data (Table 1), these oximes (**26**, **31**, **42** and **46**) showed 10–26-fold greater k_r values for reactivation of paraoxon-inhibited EeAChE than rHuAChE (Table 3). Compounds **26**, **31**, **42** and **46** showed k_r values (0.034, 0.042, 0.064 and 0.041 min^{-1}, respectively) better than obidoxime (0.033 min^{-1}) for EeAChE. In case of rHuAChE, **26**, **31** and **42** showed k_r values (0.0035, 0.0041 and 0.0045 min^{-1}, respectively) comparable to that of pralidoxime (0.0041 min^{-1}). None of

Table 1
Reactivation potency of bisquaternary pyridinium oximes for paraoxon-inhibited acetylcholinesterase

AChE	% Reactivation = $[(A_r - A_i)/(A_o - A_i)] \times 100$					
	EeAChE (%R ± SEM)			rHuAChE (%R ± SEM)		
Reactivator	10^{-3} M[a]	10^{-4} M[a]	10^{-5} M[a]	10^{-3} M[a]	10^{-4} M[a]	10^{-5} M[a]
Pralidoxime (**1**)	67 ± 1	67 ± 3	63 ± 3	42 ± 2	22 ± 2	7 ± 1
Trimedoxime (**2**)	43 ± 2	60 ± 3	74 ± 4	61 ± 3	38 ± 1	18 ± 1
Obidoxime (**3**)	78 ± 2	80 ± 4	57 ± 3	75 ± 2	36 ± 1	10 ± 1
19	6 ± 1	21 ± 1	12 ± 1	0	3 ± 0	3 ± 1
20	49 ± 3	19 ± 0	3 ± 1	1 ± 0	4 ± 0	2 ± 0
21	0	5 ± 0	7 ± 2	1 ± 2	1 ± 0	1 ± 1
22	56 ± 2	50 ± 1	15 ± 2	1 ± 0	10 ± 1	2 ± 0
23	0	6 ± 0	33 ± 2	0 ± 1	1 ± 0	3 ± 0
24	21 ± 1	45 ± 2	22 ± 0	1 ± 0	11 ± 1	3 ± 0
25	28 ± 2	58 ± 2	68 ± 4	0	26 ± 1	14 ± 1
26	2 ± 1	42 ± 3	59 ± 5	0	14 ± 2	5 ± 1
27	0	2 ± 1	14 ± 2	0	2 ± 0	4 ± 0
28	3 ± 1	24 ± 2	47 ± 3	0	5 ± 0	11 ± 1
29	3 ± 0	10 ± 0	2 ± 2	3 ± 1	1 ± 0	1 ± 0
30	7 ± 1	14 ± 0	8 ± 1	0	5 ± 1	1 ± 0
31	6 ± 2	38 ± 1	52 ± 3	0	15 ± 1	7 ± 0
32	0	1 ± 1	0	0	0	0
33	0	0	0	0	1 ± 0	1 ± 0
34	0	0	1 ± 1	1 ± 0	0 ± 0	1 ± 0
35	4 ± 1	29 ± 2	31 ± 2	0 ± 1	7 ± 1	4 ± 0
39	16 ± 2	18 ± 1	3 ± 1	1 ± 0	4 ± 2	1 ± 0
40	38 ± 3	28 ± 2	4 ± 1	19 ± 1	12 ± 1	2 ± 1
41	77 ± 4	80 ± 3	56 ± 2	64 ± 2	35 ± 2	10 ± 2
42	59 ± 4	74 ± 3	25 ± 3	12 ± 1	20 ± 1	4 ± 1
43	38 ± 2	40 ± 2	9 ± 0	20 ± 1	13 ± 1	2 ± 1
44	61 ± 2	71 ± 1	52 ± 2	73 ± 1	40 ± 3	8 ± 1
45	5 ± 1	13 ± 1	3 ± 1	0	2 ± 0	2 ± 0
46	17 ± 1	52 ± 3	49 ± 4	1 ± 0	6 ± 1	7 ± 1

[a] Reactivator concentration.

Table 2
Inhibition of AChE by pyridinium oximes

AChE	% Inhibition ± SEM					
	EeAChE			rHuAChE		
Oxime/paraoxon	10^{-3} M[a]	10^{-4} M[a]	10^{-5} M[a]	10^{-3} M[a]	10^{-4} M[a]	10^{-5} M[a]
Trimedoxime (**2**)	29 ± 2	0	0	23 ± 4	13 ± 4	0
Obidoxime (**3**)	0	0	0	16 ± 4	5 ± 3	0
26	62 ± 1	0	0	42 ± 1	0	0
28	87 ± 1	28 ± 1	3 ± 1	85 ± 1	31 ± 2	0
31	74 ± 1	16 ± 2	0	78 ± 0	16 ± 4	0
46	55 ± 1	5 ± 2	0	82 ± 1	15 ± 4	0
Paraoxon	100	100	100	100	100	100

[a] Oxime/paraoxon concentration.

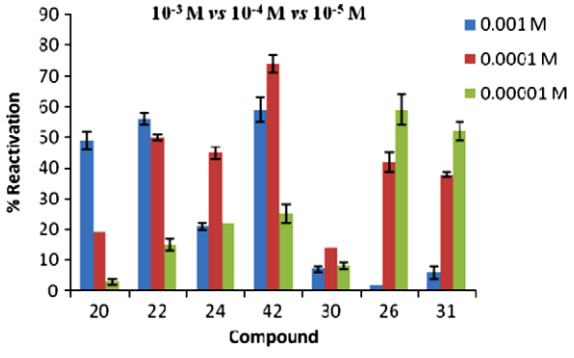


Figure 2. Comparison of reactivation potency at three different concentrations (0.001–0.00001 M) for selected compounds with aliphatic linker (**20**, **22**, **24** and **42**) and heterocyclic linkers (**30**, **26** and **31**) at three different concentrations (0.001–0.00001 M) for EeAChE. Three different trends [(i) **20** and **22**; (ii) **24**, **42** and **30**; (iii) **26** and **31**] of % reactivation with increasing concentration of oxime are also shown. Bars are represented as % reactivation ±SEM.

Table 3
Reactivation rate constant (k_r) values for **26**, **31**, **42** and **46**

Reactivator[a]	k_r (min^{-1})[b]	
	EeAChE	rHuAChE
Pralidoxime (**1**)	0.1766 ± 0.0134	0.0041 ± 0.0003
Trimedoxime (**2**)	0.1070 ± 0.0059	0.0182 ± 0.0018
Obidoxime (**3**)	0.0330 ± 0.0037	0.0083 ± 0.0013
26	0.0347 ± 0.0022	0.0035 ± 0.0002
31	0.0423 ± 0.0051	0.0041 ± 0.0004
42	0.0642 ± 0.0024	0.0045 ± 0.0001
46	0.0417 ± 0.0004	0.0016 ± 0.0001

[a] 10^{-5} M oxime concentration used.
[b] k_r—First order rate constant for reactivation.

compounds have k_r values greater than that of trimedoxime with either enzyme. The trend of k_r values was **1** > **2** > **42** > **31** > **46** > **26** > **3** for EeAChE and **2** > **3** > **42** > **31** > **26** > **1** > **46** for rHuAChE (Table 3).

2.4. Reactivation activity of pyridinium oximes bearing aliphatic linkers versus heterocyclic linkers

Linker structure influences the reactivation potential of bispyridinium oximes. At 10^{-3} M, compounds bearing aliphatic linkers showed better reactivation potential than compounds with heterocyclic linkers (Fig. 3). None of the compounds bearing heterocyclic linkers showed >17% reactivation of EeAChE (**46**: 17%) at 10^{-3} M, while those with aliphatic chains (**22**, **40**–**42** and **44**) showed >50% reactivation of EeAChE at this concentration. At a lower concentration (10^{-5} M), compounds bearing both heterocyclic linkers (**26**, **31**) and aliphatic linkers (**25**, **41**, **44**) showed >50% reactivation of EeAChE. Compounds **26**, **31**, **42** and **46** were found to be faster reactivators compared with obidoxime **3** for EeAChE. Amongst compounds with heterocyclic linkers, compounds **31**, a 4,4′-bisoxime with a furan linker was found to be a fast reactivator (k_r 0.0423 and 0.0041 min^{-1} for EeAChE and rHuAChE, respectively) for both enzymes. This rate is better than obidoxime **3** (k_r 0.033 min^{-1}) for EeAChE and equal to pralidoxime (**1**) for rHuAChE (0.0041 min^{-1}). Compound **31** is a structural analog of obidoxime (**3**) differing in the linker portion by the presence of furan ring in lieu of the central dimethyl ether unit. The comparison of reactivation potency of four 4,4′-bisoximes differing from each other by linker chain is depicted in Figure 3.

In general, it was noted that 2,2′ and 4,4′-bisoximes were more efficient reactivators of AChE than 3,3′-bisoximes. When bisquaternary pyridinium bisoximes bearing two different types of linkers were compared, the following differences were observed: (i) among 2,2′-bisoximes, compounds containing a heterocyclic linker (e.g., **26**) showed better reactivation activity (59%) than aliphatic chain linked compounds for EeAChE (e.g., **21** and **23**: 7% and 33%) at 10^{-5} M; (ii) among 4,4′-bisoximes, aliphatic chain linked compounds (**2** and **25**: 74% and 68% reactivation) were better than heterocycle linked compounds (**28** and **31**: 47% and 52% reactivation) for EeAChE at 10^{-5} M. The bisquaternary pyridinium monooximes **44** (aliphatic linker with 4-oxime) and **46** (thiophene linker with 4-oxime) had similar reactivation potencies (52% and 49% reactivation for EeAChE and 8% and 7% for rHuAChE, respectively) at 10^{-5} M. For rHuAChE, compounds with aliphatic linkers had greater reactivation activity than compounds with heterocyclic linkers. Compounds **41** and **44**, bearing aliphatic linkers, resulted in 64% and 73% reactivation of rHuAChE at 10^{-3} M, while compound **35** (with an isoxazole linker) had 19% reactivation at 10^{-3} M.

2.5. Reactivation of EeAChE versus rHuAChE

Results of the reactivation studies indicate that most of the compounds are better reactivators of EeAChE than of rHuAChE. The comparison of reactivation potency of selected compounds for EeAChE and rHuAChE at 10^{-5} M is depicted in Figure 4. For rHuAChE, among heterocyclic linked compounds, the greatest reactivation was obtained with compounds **26**, **31** (14% and 15% at 10^{-4} M) and **35** (19% at 10^{-3} M). Compounds **41** and **44**, which are both aliphatic chain linked compounds, displayed 64% and 73% reactivation of rHuAChE when present at 10^{-3} M. Compounds **26** and **31** showed 59% and 52% reactivation of EeAChE at 10^{-5} M, while aliphatic chain linked compounds such as **25** (68%, 10^{-5} M), **41** (80%, 10^{-4} M), **44** (71%, 10^{-4} M) were even more effective. Thus, in general, most compounds showed better reactivation of EeAChE than of rHuAChE, with the exception of compound **39** (which showed only 16% reactivation of EeAChE and 68% reactivation for rHuAChE at 10^{-3} M).

Paraoxon-inhibited EeAChE undergoes greater than 57% reactivation with obidoxime (**3**) from 10^{-3} M to 10^{-5} M (Table 1). Conversely, paraoxon-inhibited rHuAChE is barely reactivatable by obidoxime (**3**) at 10^{-5} M (10%). This suggests that over time rHuAChE will eventually become unable to reactivate at all following inhibition by paraoxon. The diethylphosphoryl hAChE adduct is known to undergo aging slowly with a rate constant of $k_a = 0.022\ h^{-1}$,[37] therefore, the low reactivatibility of diethyl-rHuAChE adducts may be due to one or more non-reactivation processes including denaturation that occur to a greater extent with rHuAChE. The reason for this 10–26-fold greater rate of reactivation observed for EeAChE is not obvious from the amino acid sequences of rHuAChE[38] and EeAChE[39] that are 89% homologous.

3. Experimental

3.1. General

Chemicals were purchased from Sigma–Aldrich and were used without further purification. Electric eel AChE (EeAChE) and human recombinant AChE (rHuAChE) were purchased from Sigma. Paraoxon was synthesized in our laboratory by reaction of *p*-nitrophenol with diethyl chlorophosphate using a method reported by Ghanem et al.[40] *Para*-nitrophenol and diethyl chlorophosphate were purchased from Aldrich. ^{1}H NMR spectra were recorded on a Varian 400 MHz NMR spectrometer with TMS as the internal standard. Mass spectra were obtained on a Waters LCT Premier time-of-flight mass spectrometer and IR spectra on Nexus 670 FTIR spectrometer. Reactivation assay readings were measured using $VERSA_{max}$ microplate reader. Melting points are uncorrected.

3.2. Synthesis of bisquaternary pyridinium bis-oximes 2, 3, 19–31, 33–35 and bis-amide 32

A mixture of pyridine aldoxime **9**–**11** or isonicotinamide (**12**) (1 mmol) and halo-methyl compounds **13**–**18** (0.6 mmol) in DMF

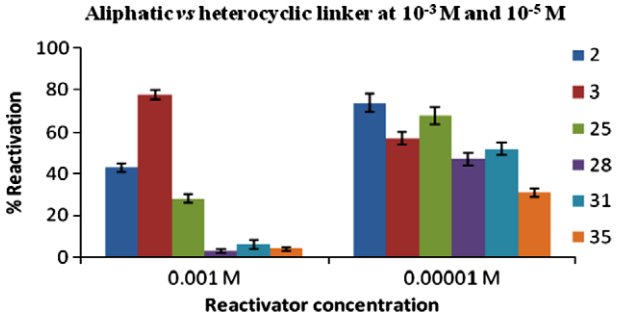


Figure 3. Comparison of reactivation potency of 4,4′-bisoximes differing by a linker chain (aliphatic—**2**, **3**, **25**; heterocyclic—**28**, **32**, **35**) for EeAChE at 10^{-3} M and 10^{-5} M. Bars are represented as % reactivation ±SEM.

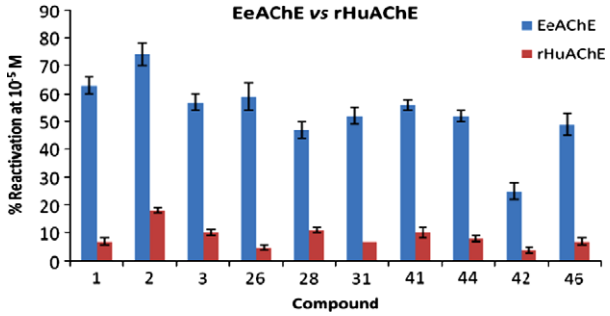


Figure 4. Comparison of reactivation potency for selected compounds at 10^{-5} M for EeAChE and rHuAChE. Bars are represented as % reactivation ±SEM.

was refluxed for 2–24 h. The reaction mixture was allowed to cool to room temperature and acetone was added to precipitate the product. The precipitate was filtered and the residue was washed with acetone (3 × 20 mL). The solid residue was dried under vacuum to obtain the desired bisquaternary pyridinium compounds **2**, **3** and **19**–**35** in 24–89% yield. Compounds **2**, **3**, and **19**–**25** were characterized by comparison of their spectral data with literature values.[25,28,29,31]

3.2.1. 2,2′-Bis(hydroxyiminomethyl)-1,1′-(2,5-thiophenediyldimethylene)-bispyridinium dichloride (26)

Dark green solid; yield: 59%; mp 185–187 °C; ^{1}H NMR (D_2O, 400 MHz): δ 8.75 (d, J = 6.0 Hz, 2H), 8.55 (s, 2H), 8.39 (t, J = 8.0 Hz, 2H), 8.20 (d, J = 8.4 Hz, 2H), 7.84 (t, J = 8.0 Hz, 2H), 7.01 (s, 2H), 5.98 (s, 4H); ^{13}C NMR (D_2O, 100 MHz): δ 147.2, 146.52, 145.57, 142.25, 136.93, 130.40, 128.37, 127.88, 56.66; IR (KBr): ν_{max} 3295, 3070, 2951, 2816, 2721, 1508, 1145, 1023 cm^{-1}; ESI-MS: m/z 353.17 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_2S]^{2+}$ 353.11).

3.2.2. 3,3′-Bis(hydroxyiminomethyl)-1,1′-(2,5-thiophenediyldimethylene)-bispyridinium dichloride (27)

Cream colored solid; yield: 68%; mp 245–247 °C; ^{1}H NMR (D_2O, 400 MHz): δ 8.97 (s, 2H), 8.75 (m, 2H), 8.57 (m, 2H), 8.15 (s, 2H), 7.91 (m, 2H), 7.25 (s, 2H), 5.83 (s, 4H); ^{13}C NMR (100 MHz, D_2O): δ 144.75, 144.03, 143.12, 142.15, 136.93, 134.01, 131.98, 128.63, 58.92; IR (Neat): ν_{max} 3098, 3037, 2974, 2870, 2762, 1511 cm^{-1}; ESI-MS: m/z 388.17 (calcd for $[C_{18}H_{17}N_4O_2SCl]^+$ 388.08); 353.12 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_2S]^{2+}$ 353.11).

3.2.3. 4,4′-Bis(hydroxyiminomethyl)-1,1′-(2,5-thiophenediyldimethylene)-bispyridinium dichloride (28)

Light brown solid; yield: 65%; mp 244–246 °C; ^{1}H NMR (D_2O, 400 MHz): δ 8.71 (d, J = 6.8 Hz, 4H), 8.18 (s, 2H), 8.02 (d, J = 6.4 Hz, 4H), 7.23 (s, 2H), 5.82 (s, 4H); ^{13}C NMR (D_2O, 100 MHz): δ 149.46, 146.29, 144.25, 137.08, 131.80, 125.11, 58.22; IR (Neat): ν_{max} 3345, 3117, 3040, 2824, 2721, 1689, 1660, 1640, 1606, 1567, 1445, 1297, 1150 cm^{-1}; ESI-MS: m/z 388.19 (calcd for $[C_{18}H_{17}N_4O_2SCl]^+$ 388.08); 353.11 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_2S]^{2+}$ 353.11).

3.2.4. 2,2′-Bis(hydroxyiminomethyl)-1,1′-(2,5-furandiyldimethylene)-bispyridinium dibromide (29)

Black solid; yield: 28%; mp 73–75 °C; ^{1}H NMR (D_2O, 400 MHz): δ 8.71 (d, J = 6.4 Hz, 2H), 8.52 (s, 2H), 8.38 (t, J = 8.0 Hz, 2H), 8.19 (d, J = 6.8 Hz, 2H), 7.87 (dd, J = 1.6, 6.4 Hz, 2H), 6.60 (s, 2H), 5.78 (s, 4H); ^{13}C NMR (D_2O, 100 MHz,): δ 150.92, 147.40, 146.48, 145.91, 141.93, 128.28, 127.17, 113.35, 54.50; IR (Neat): ν_{max} 3421, 3107, 3031, 2967, 2829, 1660, 1638, 1601, 1458, 1419, 1389, 1288, 1151 cm^{-1}; ESI-MS: m/z 337.21 $[M]^+$ (calcd for $[C_{18}H_{18}N_4O_3]^{2+}$ 337.13).

3.2.5. 3,3′-Bis(hydroxyiminomethyl)-1,1′-(2,5-furandiyldimethylene)-bispyridinium dibromide (30)

Light brown solid; yield: 89%; mp 234–236 °C; yield: 54%; ^{1}H NMR (D_2O, 400 MHz): δ 8.91 (s, 2H), 8.72 (d, J = 6.0 Hz, 2H), 8.55 (d, J = 8.4 Hz, 2H), 8.13 (s, 2H), 7.91 (t, J = 6.0 Hz, 2H), 6.72 (s, 2H), 5.70 (s, 4H); ^{13}C NMR (D_2O, 100 MHz): δ 147.70, 144.65, 144.35, 143.22, 142.28, 133.89, 128.60, 114.77, 57.11; IR (Neat): ν_{max} 3073, 3018, 2964, 2867, 2773, 2733, 1505, 1300 cm^{-1}; ESI-MS: m/z 416.97 $[M]^+$ (calcd for $[C_{18}H_{18}BrN_4O_3]^+$ 417.06); 337.23 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_3]^{2+}$ 337.13).

3.2.6. 4,4′-Bis(hydroxyiminomethyl)-1,1′-(2,5-furandiyl dimethylene)-bispyridinium dibromide (31)

Light brown solid; yield: 61%; mp 163–166 °C; ^{1}H NMR (D_2O, 400 MHz): δ 8.67 (d, J = 6.4 Hz, 4H), 8.16 (s, 2H), 8.01 (d, J = 6.4 Hz, 4H), 6.70 (s, 2H), 5.64 (s, 4H); ^{13}C NMR (D_2O, 100 MHz): δ 149.47, 147.87, 146.20, 144.54, 124.98, 114.50, 56.44; IR (Neat): ν_{max} 3073, 3018, 2963, 2963, 2867, 2734, 1504, 1300 cm^{-1}; ESI-MS: m/z 416.97 $[M]^+$ (calcd for $[C_{18}H_{18}N_4O_3Br]^+$ 417.06); 337.22 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_3]^{2+}$ 337.14)

3.2.7. 4,4′-Bis(carbamoyl)-1,1′-(2,5-furandiyldimethylene)-bispyridinium dibromide (32)

Light brown solid; yield: 70%; mp 222–224 °C; ^{1}H NMR (D_2O, 400 MHz): δ 8.92 (d, J = 6.8 Hz, 4H), 8.21 (J = 6.8 Hz, 4H), 6.76 (s, 2H), 5.76 (s, 4H); ^{13}C NMR (D_2O, 100 MHz): δ 163.07, 149.59, 147.59, 145.60, 126.70, 115.15, 57.02; IR (Neat): ν_{max} 3354, 3120, 3041, 2908, 2829, 1663, 1644, 1615, 1567, 1452, 1403 cm^{-1}; ESI-MS: m/z 416.98 $[M]^+$ (calcd for $[C_{18}H_{18}N_4O_3Br]^+$ 417.06).

3.2.8. 2,2′-Bis(hydroxyiminomethyl)-1,1′-(3,5-isoxazoldiyldimethylene)-bispyridinium dibromide (33)

Brown viscous oil; yield: 24%; ^{1}H NMR (CD_3OD, 400 MHz): δ 9.14 (d, J = 6.4 Hz, 1H), 9.02 (d, J = 6.3 Hz, 1H), 8.76 (s, 1H), 8.61 (m, 3H), 8.52 (t, J = 4.0 Hz, 2H), 8.10 (m, 2H), 6.94 (s, 1H), 6.34 (s, 2H), 6.24 (s, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 165.56, 158.87, 148.98, 148.78, 148.61, 146.67, 146.54, 146.44, 140.78, 140.69, 127.84, 127.59, 127.11, 126.46, 104.71, 53.82, 52.65; IR (Neat): ν_{max} 3322, 3032, 2900, 2726, 1646, 1604, 1557, 1412, 1203 cm^{-1}; ESI-MS: m/z 338.04 $[M]^+$ (calcd for $[C_{17}H_{16}N_5O_3]^{2+}$ 338.12).

3.2.9. 3,3′-Bis(hydroxyiminomethyl)-1,1′-(3,5-isoxazoldiyldimethylene)-bispyridinium dibromide (34)

Brown viscous oil; yield: 24%; ^{1}H NMR (CD_3OD, 400 MHz): δ 9.38 (d, J = 9.6 Hz, 2H), 9.11 (m, 2H), 8.84 (d, J = 8.4 Hz, 2H), 8.30 (d, J = 3.6 Hz, 2H), 8.17 (m, 2H), 7.13 (s, 1H), 6.22 (s, 2H), 6.13 (s, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 165.36, 158.84, 145.03, 144.72, 143.31, 142.96, 142.81, 142.40, 135.29, 128.73, 128.50, 106.18, 56.04, 54.88; IR (Neat): ν_{max} 3324, 3100, 2900, 2728, 1653, 1605, 1527, 1300 cm^{-1}; ESI-MS: m/z 417.79 $[M]^+$ (calcd for $[C_{17}H_{17}BrN_5O_3]^+$ 418.05); 338.06 $[M]^+$ (calcd for $[C_{17}H_{16}N_5O_3]^{2+}$ 338.12).

3.2.10. 4,4′-Bis(hydroxyiminomethyl)-1,1′-(3,5-isoxazoldiyldimethylene)-bispyridinium dibromide (35)

Dark brown sticky mass; yield: 26%; ^{1}H NMR (CD_3OD, 400 MHz): δ 9.07 (d, J = 6.4 Hz, 2H), 9.03 (d, J = 6.4 Hz, 2H), 8.66 (s, 2H), 8.37 (d, J = 5.2 Hz, 2H), 8.27 (d, J = 6.0 Hz, 2H), 7.07 (s, 1H), 6.13 (s, 2H), 6.04 (s, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 165.63, 158.97, 151.28, 151.11, 145.73, 145.65, 144.48, 124.89, 124.63, 122.40, 105.82, 55.22, 54.11; IR (Neat): ν_{max} 3372, 3100, 2958, 2739, 1653, 1640, 1537, 1303 cm^{-1}; ESI-MS: m/z 338.04 $[M]^+$ (calcd for $[C_{17}H_{16}N_5O_3]^{2+}$ 338.12).

3.3. Synthesis of bisquaternary pyridinium mono-oximes 39–46

A mixture of isonicotinamide (**12**, 1 mmol) and halo-methyl compound **14**–**16** (3 mmol) in acetonitrile was stirred at 70–80 °C for 2–3 h. The reaction mixture was cooled to room temperature and the precipitated product was filtered, washed with acetone (3 × 20 mL), and then dried under vacuum to produce monoquaternary compounds **36**–**38** in 60–75% yield. Compounds **36**–**38** (1 mmol) were further treated with pyridine aldoximes **9**–**11** (1.5 mmol) in DMF at 80 °C for 2–24 h. The reaction mixture was cooled to room temperature and acetone was added to precipitate the product. The precipitate was filtered and the residue was washed with acetone (3 × 20 mL). Finally, the solid residue was dried under vacuum to obtain the desired bisquaternary pyridinium compounds **39**–**46** in 60–80% yield. Compounds **39**–**41** and **44** were characterized by comparison of their spectral data with literature values.[26,27,30]

3.3.1. 2-(Hydroxyiminomethyl)-4′-(carbamoyl)-1,1′-(1,4-butylene)-bispyridinium dibromide (42)

Cream colored solid; yield: 55%; mp 196–198 °C; ^{1}H NMR (CD_3OD, 400 MHz): δ 9.19 (d, J = 6.0 Hz, 2H), 9.00 (d, J = 6.4 Hz, 1H), 8.73 (d, J = 6.8 Hz, 2H), 8.52–8.43 (m, 2H), 8.02 (t, J = 8.0 Hz, 1H), 7.85 (d, J = 6.0 Hz, 1H), 4.84–4.75 (m, 4H), 2.24–2.18 (m, 2H), 2.11–2.06 (m, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 166.82, 149.11. 145.80, 145.42, 145.01, 143.02, 134.03, 128.41, 126.84, 58.22, 55.21, 32.12; IR (Neat): ν_{max} 3354, 3120, 3041, 2908, 2829, 1663, 1644, 1615, 1567, 1452, 1403 cm^{-1}; ESI-MS: m/z 379.06 $[M]^+$ (calcd for $[C_{16}H_{19}N_4O_2Br]^+$ 379.08); 299.13 $[M]^+$ (calcd for $[C_{16}H_{19}N_4O_2]^{2+}$ 299.15).

3.3.2. 3-(Hydroxyiminomethyl)-4′-(carbamoyl)-1,1′-(1,4-butylene)-bispyridinium dibromide (43)

White solid; yield: 75%; mp 238–240 °C; ^{1}H NMR (CD_3OD, 400 MHz): δ 9.24 (s, 1H), 9.22 (d, J = 6.0 Hz, 2H), 9.01 (d, J = 6.4 Hz, 1H), 8.78 (d, J = 6.5 Hz, 1H), 8.64 (d, J = 6.8 Hz, 2H), 8.30 (s, 1H), 8.12 (t, J = 8.0 Hz, 1H), 4.00 (m, 4H), 2.22 (m, 4H); ^{13}C NMR (D_2O, 100 MHz): δ 166.83, 148.81, 145.70, 144.91, 144.42, 142.78, 142.52, 133.92, 128.63, 126.73, 61.27, 27.45, 27.37; IR (Neat): ν_{max} 3361, 3114, 3054, 2900, 2823, 1655, 1634, 1610, 1563, 1444, 1200 cm^{-1}; ESI-MS: m/z 378.99 $[M]^+$ (calcd for $[C_{16}H_{19}N_4O_2Br]^+$ 379.08); 299.10 $[M]^+$ (calcd for $[C_{16}H_{19}N_4O_2]^{2+}$ 299.15).

3.3.3. 3-(Hydroxyiminomethyl)-4′-(carbamoyl)-1,1′-(2,5-thiophenediyldimethylene)-bispyridinium dichloride (45)

Cream colored solid; yield: 60%; mp 220–222 °C; ^{1}H NMR (CD_3OD, 400 MHz,): δ 9.30 (s, 1H), 9.22 (d, J = 6.0 Hz, 2H), 9.01 (d, J = 6.8 Hz, 1H), 8.78 (d, J = 6.0 Hz, 1H), 8.42 (d, J = 6.0 Hz, 2H), 9.26 (s, 1H), 8.11 (t, J = 8.0 Hz, 1H), 7.48 (d, J = 2.0 Hz, 2H), 6.18 (s, 2H), 6.10 (s, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 149.88, 145.54, 143.80, 142.43, 142.10, 137.70, 137.60, 131.93, 131.87, 131.69, 128.56, 126.59, 58.61, 58.45; IR (Neat): ν_{max} 3400, 3071, 2951, 2810, 2721, 1574, 1508, 1145, 1022 cm^{-1}; ESI-MS: m/z 353.11 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_2S]^{2+}$ 353.11).

3.3.4. 4-(Hydroxyiminomethyl)-4′-(carbamoyl)-1,1′-(2,5-thiophenediyl dimethylene)-bispyridinium dichloride (46)

Light yellow solid; yield: 64%; mp 214–216 °C; ^{1}H NMR (D_2O, 400 MHz): δ 8.92 (d, J = 5.2 Hz, 2H), 8.71 (d, J = 6.8 Hz, 2H), 8.20 (d, J = 6.0 Hz, 2H), 8.18 (s, 1H), 8.03 (d, J = 6.8 Hz, 2H), 7.27 (m, 2H), 5.94 (s, 2H), 5.83 (s, 2H); ^{13}C NMR (D_2O, 100 MHz): δ 146.26, 145.27, 144.25, 137.41, 136.38, 132.42, 132.27, 131.83, 126.70, 125.09, 58.84, 58.19; IR (Neat): ν_{max} 3070, 2949, 2909, 2816, 2727, 1508, 1145, 1022 cm^{-1}; ESI-MS: m/z 388.90 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_2SCl]^+$ 389.08); 353.01 $[M]^+$ (calcd for $[C_{18}H_{17}N_4O_2S]^{2+}$ 353.11).

3.4. In vitro reactivation screening

An AChE stock solution (0.2 mg/mL in 0.1 M sodium phosphate buffer, pH 7.2) was treated with ethanol (1% v/v) and paraoxon (1% v/v of 100 μM in ethanol; final concentration of paraoxon is 1 μM) as control and experiment vessels. After 20–50 min, 90% AChE inhibition was achieved and halted with a 32-fold dilution (PBS). A 16 μL aliquot from control and experiment vessels was diluted to 20 μL with PBS, and incubated for 30 min at 25 °C as activity control and inhibition control. The initial activity (A_0) or inhibition (A_i) was analyzed by adding DTNB (final concentration of 0.3 mM, 200 μL total volume) and ATChI (final concentration of 1 mM, 200 μL total volume). Oxime reactivator (4 μL, 1–0.01 mM final conc) was added to a 16 μL aliquot and after 30 min incubation, the reactivated activity was determined by Ellman assay as A_r. The % reactivation for each reactivator is reported as an average with $n \geqslant 3$:

$$\%\ \text{reactivation} = [(A_r - A_i)/(A_o - A_i)] \times 100$$

A control experiment (without inhibitor) was performed to determine the AChE inhibition ability of oximes at different concentrations (1–0.01 mM). Paraoxon was used as reference inhibitor. The % AChE inhibition for each oxime is reported as an average with $n \geqslant 3$.

3.5. Determination of the reactivation rate constant (k_r)

Reactivation rate constants (k_r) were determined for compounds **1**, **2**, **3**, **26**, **31**, **42** and **46** using a similar experimental protocol to that indicated for the in vitro reactivation screening except using different reactivation time period (0–15 min). A 10^{-5} M oxime concentration was used in this experiment. By plotting ln(reactivation % − age) versus reactivation time (t), k_r is presented as the negative slope of the plot according to the equation given below.[36] Each experiment was repeated with $n \geqslant 3$. Plots with R^2 >0.9

$$2.3 \log \left[100 \cdot \frac{A_o - A_t}{A_o - A_o'}\right] = k_r \cdot t$$

were chosen for k_r calculations.

4. Conclusion

The results demonstrate key differences in the reactivation profile of paraoxon-inhibited AChE depending on the type of oxime, concentration of oxime and species. Oximes bearing aliphatic linker were better reactivators at higher concentration (10^{-3} M) compared with oximes bearing heterocyclic linkers while at a lower concentration (10^{-5} M), both class of oximes exhibited comparable reactivation potency. Another important finding from this study is that most of the oximes are better reactivators of EeAChE than of rHuAChE which may be due to one or more non-reactivation processes including denaturation or aging that occur to a greater extent with rHuAChE. Amongst newly synthesized oximes, thiophene-containing analogs **26** and **46** and furan containing analog **31** were the better reactivators with 59%, 52% and 49% reactivation of EeAChE at 10^{-5} M and 14%, 15% and 6% reactivation of rHuAChE at 10^{-4} M. Compounds **26**, **31**, **42** also showed a higher k_r value than obidoxime (**3**) for paraoxon-inhibited EeAChE and equal to pralidoxime (**1**) for paraoxon-inhibited rHuAChE. Although the newly synthesized compounds showed slower reactivation rate, interesting structure–activity dependence was found. Introduction of a highly electron rich heterocycle in linker resulted in oximes that were most effective at lower concentrations. Among these, compounds bearing a thiophene and furan linker exhibited better reactivation compared to compounds with the isoxazole ring.

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