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# New series of monoquaternary pyridinium oximes: Synthesis and reactivation potency for paraoxon-inhibited electric eel and recombinant human acetylcholinesterase

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## ABSTRACT

The preparation of a series of monoquaternary pyridinium oximes bearing either a heterocyclic side chain or a functionalized aliphatic side chain and the corresponding in vitro evaluation for reactivation of paraoxon-inhibited electric eel acetylcholinesterase (EeAChE) and recombinant human acetylcholinesterase (rHuAChE) are reported. Several newly synthesized compounds efficiently reactivated inhibited EeAChE, but were poor reactivators of inhibited rHuAChE. Compounds bearing a thiophene ring in the side chain (**20**, **23**, **26** and **29**) showed better reactivation (24–37% for EeAChE and 5–9% for rHuAChE) compared to compounds with furan and isoxazole heterocycles (0–8% for EeAChE and 2–3% for rHuAChE) at $10^{-5}$ M. The N-pyridyl-CH$_2$COOH analog **8** reactivated EeAChE (36%) and rHuAChE (15%) at $10^{-4}$ M with a $k_r$ value better than 2-pyridine aldoxime methiodide (2-PAM) for rHuAChE.

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Reactivators of acetylcholinesterase (AChE; EC 3.1.1.7) are important therapeutic agents for the treatment of toxicity resulting from organophosphorus inhibitors (OPI) such as sarin, paraoxon, tabun, chlorpyrifos, cyclosarin, VX, and DFP. Some of these OPIs have been used as warfare agents (such as sarin, soman, tabun, VX) and are some of the most toxic man made compounds known.[1] OPIs inhibit AChE via formation of a covalent bond at a serine hydroxyl group within the enzyme's active site. Once inhibited, AChE is not able to hydrolyze acetylcholine and accumulates in the synaptic cleft leading to cholinergic over-stimulation, compromised respiration and sometimes death.[1] AChE inhibited by OPI can be reactivated by 2-pyridine aldoxime methiodide (2-PAM, pralidoxime; **1**)[2] that nucleophilically attacks the phosphoryl moiety displacing it from the active site serine (Fig. 1). In addition to 2-PAM,[3–6] the bis-oximes trimedoxime (TMB-4, **2**),[7,8] obidoxime (**3**)[9,10] and HI-6 (**4**)[11,12] show efficacy as reactivators in vivo and are widely used for the treatment of OPI poisoning.[13,14] Recently, several new pyridinium oxime reactivators were reported including bisquaternary pyridinium oximes connected with different linker chains viz. alkylene linker,[15–18] but-2-ene linker,[19–22] aromatic ring containing linkers[23–25] and oxygenated linker chain.[26–28] Monoquaternary pyridinium oximes include 2-PAM analogs with

varying side chain[29,30] and 4-PAM analogs with modified oximes.[31] Amongst these, but-2-ene linked bisquaternary 4,4'-bisoxime[22] and 2-PAM analogs with a propyl side chain[29] showed reactivation of paraoxon-inhibited AChE comparable to clinically known reactivators.

Although bis-quaternary oximes are considered more effective AChE reactivators, many countries use 2-PAM as the therapeutic agent.[32,33] The efficacy of the oxime used is also dependent on the AChE type. Certain AChE types undergo rapid reactivation
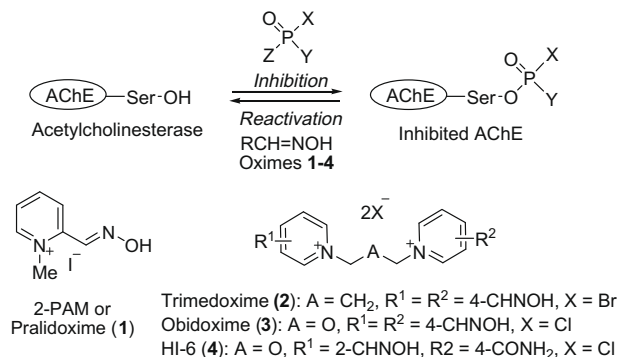


**Figure 1.** Equation representing inhibition and reactivation of AChE by OPI and oximes. Selected examples of oximes are shown.

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whereas others show little to no appreciable activity after oxime treatment. This and other limitations highlight that there has been no single broad-spectrum oxime reactivator identified to date.[1,34] Therefore, the development and selection of new, more effective AChE oxime reactivators is very important. The focus of this work was to determine the effect of different heterocyclic and aliphatic side chains (capable of hydrogen bonding) on the ability of mono-quaternary pyridinium oximes to reactivate two forms of AChE inhibited by paraoxon.

Monoquaternary pyridinium oximes **5–35** were prepared in 40–92% yield via reaction of 2-, 3- or 4-pyridine aldoximes with different substituted alkyl halides in acetonitrile or acetone at 60–80 °C for 1–24 h.[35] Monoquaternary pyridinium oximes **5–16** were designed to contain an aliphatic side chain with a group capable of hydrogen bonding viz. carbamoyl, carboxyl and hydroxyl. Furthermore, highly electron rich heterocycles, viz. thiophene, furan and isoxazole were used as side chains **17–35** to explore interactions with cation-containing (cation–π bonding) or aromatic residues (π–π bonding) at or near the AChE active site. The oxime group was positioned at 2, 3 and 4 on the pyridinium ring to determine the best position for reactivation of AChE using the N-derivatized groups. All compounds were characterized by ${}^1$H NMR, ${}^{13}$C NMR, ESI-MS and IR prior to assay.[35] Compounds **10, 11** and **13** have been reported as reactivators but not against EeAChE or rHu-AChE.[7,36] A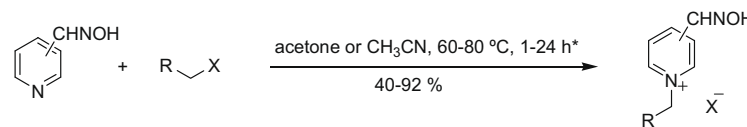ll compounds were assayed for their capacity to reacti-vate EeAChE and rHuAChE using a modified Ellman assay.[37,38] 2-PAM and paraoxon were chosen as a reference reactivator and as representative OPI.

Reactivation results are summarized in Table 1. Oximes with aliphatic side chains mostly showed concentration-dependent reactivation of AChE. Compounds containing carboxamide or car-boxylic acid groups paired with the 2 or 4-oxime position (**5, 7** and **8**) gave 40–45% reactivation of EeAChE while compounds with hydroxyethyl side chain **11** and **13** showed 50% and 70% reactiva-tion of EeAChE at $10^{-3}$ M comparable to 2-PAM **1**. Compound **8** also showed promise as a reactivator of rHuAChE (15%) at $10^{-3}$ M. Compounds with aromatic side chains (thiophene, furan and isoxazole) displayed a reverse concentration–activity relation-ship. These compounds, with the exception of **19** and **35**, showed ⩽8% reactivation of EeAChE and no reactivation of rHuAChE at the highest concentration ($10^{-3}$ M). Lower concentrations of oxime ($10^{-4}$–$10^{-5}$ M) **17–29** were better reactivators of both enzymes suggesting that these compounds may inhibit AChE at higher con-centrations and explain, in part, the inability to reactivate inhibited AChE at $10^{-3}$ M.[19,23]

Each reactivator varied with respect to the optimal concentra-tion used, and four trends were identified from the data (Table 1). First, a correlation was found between percent reactivation and increasing concentration of oxime (**5, 7, 8, 11–16**). A second trend was found that inversely correlates increasing reactivation

**Table 1**
Synthesis and in vitro reactivation potency of monoquaternary pyridinium oximes **5–35** for paraoxon-inhibited EeAChE and rHuAChE



| Entry | R | Oxime position | X | Reaction time (h) | % Yield | % Reactivation = $[(A_r - A_i)/(A_o - A_i)] \times 100$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | EeAChE (% R ± SEM) | | | rHuAChE (% R ± SEM) | | |
| | | | | | | $10^{-3}$ M[a] | $10^{-4}$ M[a] | $10^{-5}$ M[a] | $10^{-3}$ M[a] | $10^{-4}$ M[a] | $10^{-5}$ M[a] |
| 1 | H | 2 | I | — | — | 67 ± 1 | 67 ± 3 | 63 ± 3 | 42 ± 2 | 22 ± 2 | 7 ± 1 |
| 5 | CONH$_2$ | 2 | I | 24 | 62 | 41 ± 3 | 9 ± 0 | 1 ± 0 | 2 ± 1 | 2 ± 0 | 1 ± 0 |
| 6 | CONH$_2$ | 3 | I | 2 | 76 | 1 ± 1 | 0 | 0 | 0 | 1 ± 0 | 2 ± 0 |
| 7 | CONH$_2$ | 4 | I | 2 | 84 | 44 ± 2 | 6 ± 1 | 0 | 0 | 1 ± 0 | 1 ± 0 |
| 8 | COOH | 2 | I | 24 | 61 | 45 ± 3 | 36 ± 3 | 34 ± 2 | 15 ± 1 | 15 ± 2 | 5 ± 1 |
| 9 | COOH | 3 | I | 2 | 71 | 1 ± 0 | 0 | 0 | 0 | 1 ± 0 | 1 ± 0 |
| 10 | COOH | 4 | I | 2 | 78 | 4 ± 0 | 0 | 0 | 0 | 1 ± 0 | 2 ± 0 |
| 11 | CH$_2$OH | 2 | Br | 24 | 58 | 50 ± 4 | 37 ± 3 | 13 ± 1 | 2 ± 2 | 5 ± 0 | 2 ± 0 |
| 12 | CH$_2$OH | 3 | Br | 2 | 60 | 8 ± 1 | 0 | 0 ± 0 | 0 | 1 ± 0 | 1 ± 0 |
| 13 | CH$_2$OH | 4 | Br | 2 | 68 | 70 ± 2 | 2 ± 2 | 3 ± 1 | 6 ± 1 | 3 ± 0 | 2 ± 0 |
| 14 | CH$_2$COOH | 2 | Br | 24 | 48 | 10 ± 1 | 0 | 0 | 0 | 1 ± 0 | 1 ± 0 |
| 15 | CH$_2$COOH | 3 | Br | 2 | 56 | 5 ± 0 | 0 | 0 | 0 | 1 ± 0 | 1 ± 0 |
| 16 | CH$_2$COOH | 4 | Br | 2 | 68 | 15 ± 1 | 0 | 0 | 0 | 2 ± 0 | 1 ± 0 |
| 17 | 4-Methoxybenzen-1-yl | 2 | Cl | 12 | 70 | 8 ± 1 | 19 ± 2 | 18 ± 2 | 0 | 7 ± 1 | 3 ± 0 |
| 18 | 4-Methoxybenzen-1-yl | 3 | Cl | 1 | 84 | 1 ± 1 | 0 | 0 | 0 | 3 ± 0 | 2 ± 0 |
| 19 | 4-Methoxybenzen-1-yl | 4 | Cl | 1 | 92 | 19 ± 1 | 11 ± 1 | 3 ± 1 | 0 | 3 ± 1 | 2 ± 0 |
| 20 | Thiophen-2-yl | 2 | Cl | 24 | 58 | 1 ± 0 | 24 ± 2 | 34 ± 2 | 0 | 9 ± 2 | 9 ± 1 |
| 21 | Thiophen-2-yl | 3 | Cl | 2 | 65 | 0 | 3 ± 1 | 1 ± 0 | 0 | 4 ± 0 | 5 ± 0 |
| 22 | Thiophen-2-yl | 4 | Cl | 2 | 78 | 3 ± 0 | 12 ± 2 | 12 ± 0 | 0 | 4 ± 1 | 5 ± 0 |
| 23 | 5-Bromothiophen-2-yl | 2 | Cl | 12 | 74 | 0 | 29 ± 2 | 37 ± 2 | 0 | 7 ± 0 | 8 ± 1 |
| 24 | 5-Bromothiophen-2-yl | 3 | Cl | 1 | 84 | 4 ± 1 | 7 ± 0 | 8 ± 1 | 0 | 5 ± 1 | 6 ± 0 |
| 25 | 5-Bromothiophen-2-yl | 4 | Cl | 1 | 90 | 3 ± 1 | 14 ± 1 | 10 ± 1 | 0 | 5 ± 1 | 4 ± 0 |
| 26 | Benzthiophen-3-yl | 2 | Cl | 24 | 67 | 0 | 28 ± 2 | 24 ± 3 | 0 | 7 ± 1 | 8 ± 1 |
| 27 | Benzthiophen-3-yl | 3 | Cl | 2 | 79 | 0 | 5 ± 1 | 8 ± 1 | 0 | 2 ± 0 | 5 ± 1 |
| 28 | Benzthiophen-3-yl | 4 | Cl | 2 | 85 | 0 | 0 | 1 ± 1 | 0 | 2 ± 0 | 2 ± 0 |
| 29 | Thiophen-3-yl | 2 | Cl | 24 | 79 | 0 | 8 ± 1 | 28 ± 2 | 0 | 2 ± 0 | 5 ± 1 |
| 30 | Furan-2-yl | 3 | Cl | 1 | 62 | 0 | 0 | 0 | 0 | 2 ± 0 | 2 ± 0 |
| 31 | Furan-2-yl | 4 | Cl | 1 | 60 | 8 ± 1 | 14 ± 2 | 1 ± 1 | 0 | 3 ± 0 | 2 ± 0 |
| 32 | Furan-3-yl | 3 | Cl | 1 | 46 | 0 | 0 | 0 | 0 | 3 ± 0 | 2 ± 0 |
| 33 | 3-Methyl-isoxazol-5-yl | 2 | Br | 24 | 40 | 7 ± 1 | 25 ± 3 | 8 ± 1 | 0 | 7 ± 1 | 3 ± 0 |
| 34 | 3-Methyl-isoxazol-5-yl | 3 | Br | 2 | 64 | 0 | 0 | 0 | 0 | 3 ± 1 | 2 ± 0 |
| 35 | 3-Methyl-isoxazol-5-yl | 4 | Br | 2 | 67 | 20 ± 2 | 24 ± 2 | 6 ± 0 | 0 | 2 ± 1 | 2 ± 0 |

* Compounds **5–16**: acetone, 60–70 °C, 2–24 h; **17–35**: MeCN, 70–80 °C, 2–24 h.
  a Reactivator concentration.

**Table 2**
Reactivation rate constant ($k_r$) for **8**, **13**, **20**, **23** and **26**

| Entry | $k_r$ (min$^{-1}$) | |
|---|---|---|
| | EeAChE | rHuAChE |
| 2-PAM (**1**) | 0.1766 ± 0.0134 | 0.0041 ± 0.0003 |
| **8** | 0.0406 ± 0.0021 | 0.0048 ± 0.0004 |
| **13** | 0.0108 ± 0.0002 | 0.0010 ± 0.0002 |
| **20** | 0.0339 ± 0.0026 | 0.0016 ± 0.0001 |
| **23** | 0.0189 ± 0.0004 | 0.0012 ± 0.0001 |
| **26** | 0.0138 ± 0.0008 | 0.0010 ± 0.0001 |

with decreasing concentration of oxime (**20**, **22–24**, **27–29**). Third, compounds **17**, **21**, **25**, **26**, **31**, **33**, **35** showed the best percent reactivation at mid-range concentrations of oxime.[19] Last, analogs bearing the oxime in the 2-position were better reactivators than those with oximes in the 3- or 4-position. Of the compounds with heteroaromatic side chains, thiophene analogs **20**, **23**, **26** and **29** with the 2-oxime group reactivated paraoxon-inhibited EeAChE 34%, 37%, 24% and 28% at 10$^{-5}$ M, respectively. These thiophene analogs also reactivated rHuAChE but only ~5% at 10$^{-5}$ M. Oximes with a thiophen-2-yl side chain (e.g., **20** and **23**) were slightly better reactivators than the corresponding thiophen-3-yl structures (**26** and **29**). The furan analogs **30–32** were poor reactivators but the 3-methylisoxazole analogs **33** and **35** showed 24–25% reactivation of EeAChE at 10$^{-4}$ M. The 4-methoxybenzyl analogs **17–19** were poor reactivators of paraoxon-inhibited EeAChE and rHuAChE although the 2- and 4-positional oximes **17** and **19** reactivated AChE up to 19% of its activity.

Owing to their greater activity, compounds **8**, **13**, **20**, **23** and **26** were selected for analysis of the oxime-mediated reactivation rate constants ($k_r$; characterizes the dissociation of the enzyme and the phosphorylated-oxime).[39] The $k_r$ was calculated using the equation from the linear portion of activity curves (0–15 min).[40]

As expected from the preliminary data (Table 1), these oximes showed 8–21-fold greater $k_r$ values for reactivation of paraoxon-inhibited EeAChE than rHuAChE. Compound **8** showed the highest $k_r$ values among the analogs tested against for both EeAChE and rHuAChE, and **8** was superior to 2-PAM for rHuAChE reactivation. Also interesting was the trend of $k_r$ values **8** > **20** > **23** > **26** ⩾ **13** that was the same for both EeAChE and rHuAChE (Table 2).

Paraoxon-inhibited EeAChE undergoes greater than 60% reactivation with 2-PAM from 10$^{-3}$ M to 10$^{-5}$ M (Table 1). Conversely, 2-PAM reactivates paraoxon-inhibited rHuAChE to a far lesser extent at 10$^{-3}$ M (42%) and is barely reactivatable at 10$^{-5}$ M (7%). This suggests that rHuAChE will eventually become unable to reactivate at all following inhibition by paraoxon. This may be due to one or more non-reactivation processes including denaturation or aging. The amino acid sequences of rHuAChE[41] and EeAChE[42] are 89% homologous and do not reveal any obvious differences to account for the 8–21-fold greater rate of reactivation observed for EeAChE. However, species-specific differences in reactivation rates following inhibition by a variety of OP agents are known[43,44] and any data comparisons should consider these differences.

In conclusion, a new series of monoquaternary pyridinium oximes with heteroaromatic side chains have been reported of which thiophene-containing analogs **20**, **23** and **26** were the better reactivators. Compound **8**, bearing an acetic acid side chain, was found to be the most effective reactivator of the 31 novel compounds tested with a higher $k_r$ value than pralidoxime (**1**) for paraoxon-inhibited rHuAChE.

## Acknowledgments

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35. Typical procedure for synthesis of monoquaternary pyridinium salts 5–35: Hydroxyiminomethylpyridine (1 mmol) and the alkylating agent (1.5 mmol) in solvent (20 mL) were stirred at 60–80 °C for 1–24 h. The mixture was cooled to rt, the precipitate collected, washed with acetone (3 × 20 mL), dried under vacuum, and characterized by $^1$H NMR, $^{13}$C NMR, ESI-MS and IR. Representative compounds as follows: 1-Carboxymethyl-2-hydroxyimino methylpyridinium iodide (8): light green solid; yield: 61%; mp 138–140 °C; $^1$H NMR (400 MHz, CD$_3$OD): δ 8.80 (d, J = 5.2 Hz, 1H), 8.65 (s, 1H), 8.58–8.42 (m, 2H), 8.01 (t, J = 8.0 Hz, 1H), 4.41 (s, 2H); $^{13}$C NMR (100 MHz, CD$_3$OD): δ 169.21, 147.01, 146.53, 145.36, 141.25, 127.52, 125.99, 46.46; IR (Neat): ν$_{max}$ 3059, 3098, 3048, 2965, 2880, 2743, 1692, 1628, 1508, 1480, 1288, 1160, 1018 cm$^{-1}$; ESI-MS: m/z 181.06 [M]$^+$ (calcd for [C$_8$H$_9$N$_2$O$_3$]$^+$ 181.06). 1-(2-Hydroxy)-ethyl-2-hydroxyimino methyl pyridinium bromide (11): brown solid; yield: 58%; mp 184–186 °C; $^1$H NMR (400 MHz, D$_2$O): δ 8.64 (d, J = 6.4 Hz, 1H), 8.57 (s, 1H), 8.37 (t, J = 8.0 Hz, 1H), 8.26 (d, J = 8.4 Hz, 1H), 7.87 (t, J = 6.8 Hz, 1H), 4.71 (t, J = 4.4 Hz, 2H), 3.89 (t, J = 4.4 Hz, 2H); $^{13}$C NMR (100 MHz, D$_2$O): δ 147.20, 146.38, 145.85, 142.49, 127.78, 127.03, 60.33, 60.07; IR (Neat): ν$_{max}$ 3377, 3072, 2991, 2865, 2734, 2620, 1627, 1590, 1504, 1430, 1313, 1152, 1072, 1000 cm$^{-1}$; ESI-MS: m/z 167.08 [M]$^+$ (calcd for [C$_8$H$_{11}$N$_2$O$_2$]$^+$ 167.08). 1-(Thiophen-2-yl)-methyl-2-hydroxyiminomethyl-pyridinium chloride (20): off white solid; yield: 58%; mp 150–152 °C; $^1$H NMR (400 MHz, D$_2$O): δ 8.73 (d, J = 6.4 Hz, 1H), 8.63 (s, 1H), 8.38 (t, J = 8.0 Hz, 1H), 8.20 (d, J = 8.0 Hz, 1H), 7.86 (t, J = 6.4 Hz, 1H), 7.40 (d, J = 5.2 Hz, 1H), 7.09 (d, J = 3.6 Hz, 1H), 6.94 (t,

$J$ = 4.0 Hz, 1H), 5.98 (s, 2H); $^{13}$C NMR (100 MHz, D$_2$O): $\delta$ 146.15, 145.23, 142.28, 139.01, 133.52, 130.28, 129.20, 128.25, 127.94, 127.50, 56.61; IR (Neat): $\nu_{max}$ 3069, 3013, 2945, 2831, 2735, 1711, 1628, 1577, 1514, 1474, 1321, 1253, 1020 cm$^{-1}$; ESI-MS: *m/z* 219.21 [M]$^+$ (calcd for [C$_{11}$H$_{11}$N$_2$OS]$^+$ 219.06).

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