Bioorganic & Medicinal Chemistry 19 (2011) 588–594

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Peripheral site ligand–oxime conjugates: A novel concept towards reactivation of nerve agent-inhibited human acetylcholinesterase

M. C. de Koning *, M. J. A. Joosen, D. Noort, A. van Zuylen, M. C. Tromp

TNO Defence, Security and Safety, PO Box 45, 2280 AA Rijswijk, The Netherlands

ARTICLE INFO

Article history:
Received 24 August 2010
Revised 14 October 2010
Accepted 28 October 2010
Available online 31 October 2010

Keywords:
Acetylcholinesterase
Oxime
Reactivation
Nerve agent
Peripheral site

ABSTRACT

A conceptually novel approach to the design of reactivators of nerve agent-inhibited acetylcholinesterase (AChE) is presented. The concept comprises the linkage of a peripheral site ligand via a spacer to a reactivating moiety with the eventual goal to develop non-ionic reactivators with sufficient affinity for AChE to induce reactivation and potentially improved blood–brain barrier penetration. Herein, the first step towards that goal—the synthesis and biological evaluation of a peripheral site ligand conjugated to a charged pyridinium oxime is discussed. It was found, that the introduction of the peripheral site ligand not only increased affinity of the construct for AChE but also enhanced reactivation of nerve agent-inhibited AChE.

1. Introduction

Acetylcholinesterase (AChE) is a serine hydrolase which has an active site (or A-site) at the bottom of a long narrow gorge and a second substrate binding site at the entrance of the gorge, called peripheral anionic site (PAS or P-site).[1–3] The A-site catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses at a rate close to the diffusion-controlled limit.[4] Certain organophosphate (OP) compounds, such as insecticides (e.g., chlorpyrifos) and nerve agents (e.g., sarin, soman, tabun, VX, etc.) arrest ACh hydrolysis by irreversibly blocking the key serine residue in the A-site by phosphorylation.[5] The resulting accumulation of ACh levels leads to failure of the cholinergic synaptic transmission and, as a consequence, flaccid muscle paralysis and seizures in the central nervous system. The toxic effects of OP-inhibited AChE can be attenuated by the timely administration of certain oximes,[6] such as HI-6, Obidoxime and 2-PAM (Fig. 1) that mediate reactivation of the enzyme by breaking the covalent bond between the OP-inhibitor and the serine residue of the enzyme. One drawback of the current oximes is that there is no broad spectrum reactivator that sufficiently reactivates AChE inhibited by the various types of OPs.[7] The mono-quaternary oxime 2-PAM is the drug of choice for a number of countries.[8] On the other hand, the bis-quaternary oximes (i.e., HI-6, Obidoxime) are generally considered more effective than mono-quaternary oximes, however, whereas HI-6 reactivates sarin- and VX-inhibited AChE it is outperformed by obidoxime for

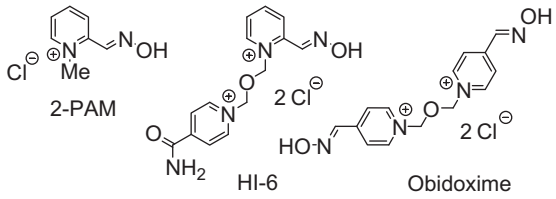


Figure 1. Currently employed oximes in the treatment of patients intoxicated by nerve agents or OP-pesticides.

tabun-poisoning.[9] A second major drawback of these (bis-)quaternary oximes is their low penetration of the blood brain barrier (BBB), which is the most impermeable lipoidal membrane.[10] As a result, the uneven distribution of the oximes between the tissues leads to only limited activity in central nervous system (CNS), more rapid excretion and shorter biological half lives. The limited penetration of oximes through the BBB (e.g., about 10% for 2-PAM)[10] is mainly due to the presence of permanent positive charge(s) in the oximes. Thus, much effort has been directed in the past to the development of non-quaternary (uncharged) oximes to improve BBB penetration.[11–18] However, the majority of the compounds tested showed unsatisfactory reactivation of OP-inhibited AChE. One of the reasons underlying the loss in reactivation potency is the absence of charge, which not only affects the reactivity of the nucleophilic oxime moiety, but also reduces the affinity of the oximes for the active site of AChE.[19] In more recent contributions, attempts were made to improve the BBB penetration of the charged PAM-, HI-6- and obidoxime derivatives by increasing their

* Corresponding author. Tel.: +31 15 2843516; fax: +31 15 2843963.
E-mail address: m.dekoning@tno.nl (M.C. de Koning).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.10.059

lipohilicity.[20,21] For instance, Ohta et al. synthesized over 40 2-, 3- and 4-PAM analogues replacing the methyl-group with various more lipophilic alkyl-groups.[21] Contrary to the 2- and 3-PAM derivatives, the 4-PAM analogues showed increasing reactivating capacity toward OP-inhibited AChE with increasing alkylchain size, albeit lower than that of the standard 2-PAM. In addition, from that series mainly the 4-PAM derivatives showed improved BBB penetration (e.g., 30% for 4-octyl-PAM) compared to 2-PAM.[22]

We reasoned that the covalent linkage of a neutral ligand with affinity for the P-site to a reactivating oxime (i.e., **1** in Fig. 2) could result in an increased affinity of the total construct for the enzyme by a dual site binding mode. The extra contribution in affinity may compensate for the loss in affinity when a non-quaternary oxime in future constructs would be used. In this report we describe the synthesis and in vitro evaluation of 4-pyridinealdoxime conjugated to a non-ionic piperidine derived P-site ligand (PSL) via ethyleneglycol linkers (compounds **1a–c** in Fig. 2). This type of constructs, comprising a lipophilic tail and a charged oxime, may thus complement those of Ohta et al.[21] except that our molecules contain the extra feature of a P-site binding moiety. We demonstrate here that the affinity of the constructs for AChE increases with the presence of the P-site ligand. In addition, we also show an enhancement of reactivating capacity of the constructs compared to the parent oximes.

2. Results and discussion

The choice for the non-ionic P-site ligand in our design of PSL–oxime conjugates **1a–c** (Fig. 2) was based on a recent report by Kwon et al.[23] They described the synthesis of dual site inhibitors of AChE comprising of two moieties with affinity for the A-site and the P-site, respectively, as potential drugs for the treatment of Alzheimer disease. More specifically, the A-site binding moieties in these constructs were connected to benzhydryl-piperidine derivative **4** (Scheme 1) via N-alkylation. Modelling studies confirmed that the benzhydryl-piperidine part of these constructs was located at the P-site of the enzyme. Another consideration for us was the non-ionic character of this P-site ligand which should increase the lipophilicity of the construct (thus increasing the potential for BBB penetration) and facilitate its synthetic accessibility. The ethyleneglycol linkers were selected over aliphatic linkers for solubility reasons.

2.1. Chemistry

The synthesis of conjugates **1a–c**, as outlined in Scheme 1, commenced with the alkylation of 4-pyridinealdoxime **2** with bis-chloroethyleneglycol linkers of varying length to give, after silica gel column chromatography, 4-pyridiniumaldoximes **3a–c** in reasonable to good yields. The subsequent condensation of **3a–c** with piperidine derivative **4** to give the target conjugates **1a–c** was accompanied with the formation of several byproducts in which the aldoxime moieties (–CH=NOH) in **1a–c** were substituted with chloride, hydroxide and the piperidine derivative **4** itself. HPLC purification of **1a** proceeded uneventfully. However, following HPLC-purification of **1b** and **1c**, evaporation of the solvents in vacuo under slight heating resulted in partial cleavage of the ethyleneglycol spacers to give, as judged by mass spectrometry, 1-(2-hydroxyethyl)-4-pyridiniumaldoxime and the corresponding *O*-vinyl fragments. These undesired reactions were overcome by lyophilisation of the HPLC fractions, affording pure **1b** and **1c**. The identity and homogeneity of the conjugates **1a–c** were firmly established by HPLC, mass spectrometry and NMR.

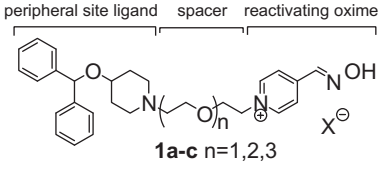
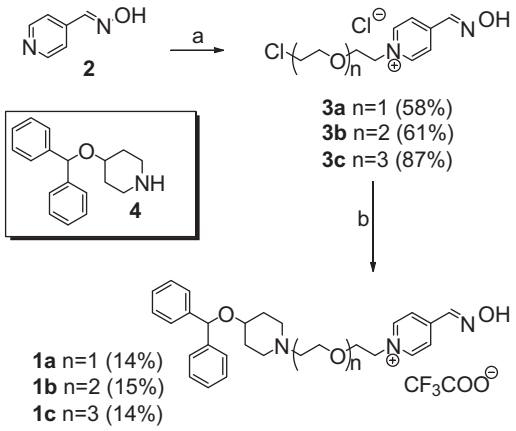


Figure 2. Designed P-site ligand–oxime conjugates with varying spacer length.



Scheme 1. Synthetic route towards conjugates **1a–c**. Reagents and conditions: (a) $Cl(CH_2CH_2O)_nCH_2CH_2Cl$ (neat or in ACN), ΔT, 48 h; (b) **4** (1 equiv), $NaHCO_3$ (5 equiv), KI (cat.), DMF, 90 °C/24 h ($n = 1$) or 50 °C/48 h ($n = 2$, 3). Compounds **1a–c** were isolated as the TFA salts following purification by HPLC. Compound **4** was prepared according to a published route.[23]

2.2. Biological evaluation

2.2.1. Evaluation of binding affinity

The relative binding affinity of conjugates **1a–c** was evaluated using purified human AChE by measuring their enzyme inhibiting potency. These measurements were executed to provide insight into the inhibitory activity of the compounds to the free enzyme, which is a measure of toxicity. A concern with the covalent linkage of ligands targeting different binding sites of an enzyme is that the resulting hybrid molecules may form high-affinity inhibitors[24,25] of the enzyme and may thus exert their toxicity by reversible inhibition. Thus, in a typical experiment, hAChE (3 nM) was incubated for 30 min with a series of 10-fold dilutions (10^{-3}–10^{-6} M) of **1a–c** and the enzyme activity was measured using the method of Ellman et al.[26] For comparison, the enzyme inhibition by the oximes lacking the PSL (**3a–c**) and 4-pyridinealdoxime methiodide (4-PAM)[27] was measured in a similar way.

The results of the inhibition experiments are depicted in Figure 3.[28] Inhibition is given relative to the control (non-inhibited enzyme ~100% activity). From Figure 3A it is immediately clear that the conjugates **1a–c** show the highest affinity for the enzyme of the compounds tested. At 10^{-4} M enzyme inhibition by **1a–c** is 51%, 30% and 37%, respectively. At 10^{-3} M the enzyme activity is almost completely suppressed (>75%). Contrary, 4-PAM shows almost no inhibitory power at the various concentrations and the compounds lacking the PSL (**3a–c**) only show inhibition at 10^{-3} M (44% for **3a** and 19% for **3b**). From the latter results it was concluded that the ethyleneglycol spacers in **3a–c** contribute to enzyme affinity somewhat but that only becomes significant at pharmacologically less relevant ($\geqslant 10^{-3}$ M) concentrations. To probe the effect of the PSL in conjugates **1a–c** in more detail some experiments were carried out using mixtures of oxime **3c** and the PSL **4** (Fig. 3B). First of all, it became apparent that **4** showed low inhibitory power even at high concentrations. It should be noted however, that **4** is supposed to bind the P-site whereas the oximes **1a–c**, **3a–c**, and 4-PAM bind the A-site. It is therefore not excluded (nor confirmed) that **4** binds the P-site

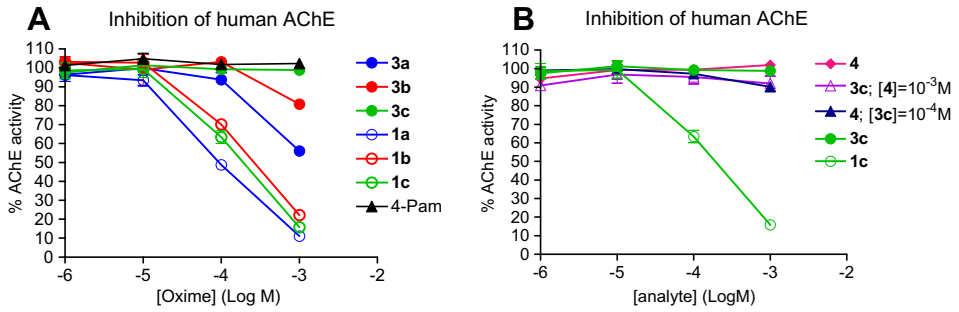


Figure 3. Reversible inhibition of human AChE by (A) conjugates **1a–c**, oximes **3a–c** and 4-PAM and (B) combinations of **4** and oxime **3c** at varying concentrations.

without affecting the activity of the enzyme. As a consequence, comparison of the result of this experiment with those of the oximes is difficult. To circumvent this issue, another experiment was undertaken in which hAChE was simultaneously subjected to a high concentration of **4** (10^{-3} M) and varying concentrations of **3c** and in a separate experiment hAChE was treated with a fixed concentration of oxime **3c** (10^{-4} M) and varying concentrations of **4**. However, in both experiments only at the highest concentrations a marginal change in inhibition of the enzyme by the various combinations of oxime and PSL concentrations compared to the inhibition by **3c** alone was observed. These results clearly show that the conjugation of the PSL to the oxime via a spacer is absolutely required to obtain a molecule with enhanced affinity for AChE like **1c** (inhibition by **1c** is added in Fig. 3B for comparison). It is further concluded that **1a–c** are not high-affinity inhibitors given the low inhibition at the pharmacologically relevant concentration of 10^{-5} M.

2.2.2. AChE reactivation experiments

Having established the affinity of the constructs **1a–c** for AChE, attention was turned to their reactivating capacity. The in vitro reactivating capacity of conjugates **1a–c** was first evaluated against sarin-inhibited human acetylcholinesterase (hAChE). Briefly, hAChE (3 nM) was treated for 30 min with the minimum amount of sarin to achieve at least 85% inhibition of the enzyme. Next, the inhibited enzyme was subjected to a series of 10-fold dilutions of oximes **1a–c** and incubated for 30 min. The amount of active enzyme present after that period of time was measured using the method of Ellman.[26]

The results of the reactivation experiments carried out with 10^{-5} M of **1a–c** are depicted in Figure 4 (left series).[29] In addition, the results of similar experiments with fragments of the conjugate (i.e., the parent oxime 4-PAM, the compounds lacking the PSL (**3a–c**) and PSL **4** alone) as well as the reactivation by HI-6, one of the best reactivators known, are also displayed for comparison. Reactivation is given relative to the control (no inhibited enzyme ~100% activity) and the values were corrected for spontaneous reactivation and residual activity after inhibition (measured from inhibited enzyme without oxime). Comparison of the graphs in Figure 4 led to several conclusions. From the graph it is immediately clear that HI-6 outperforms all oximes used in this study, including **1a–c**, in the reactivation of sarin-inhibited human AChE. However, it was not the goal of this study to develop a better reactivator than HI-6.[30] More importantly, it was very gratifying to find that all conjugates **1a–c** showed a dramatically higher reactivation capacity than the parent oxime 4-PAM. Further, the conjugates **1a–c** showed increasing reactivating power with increasing spacer length

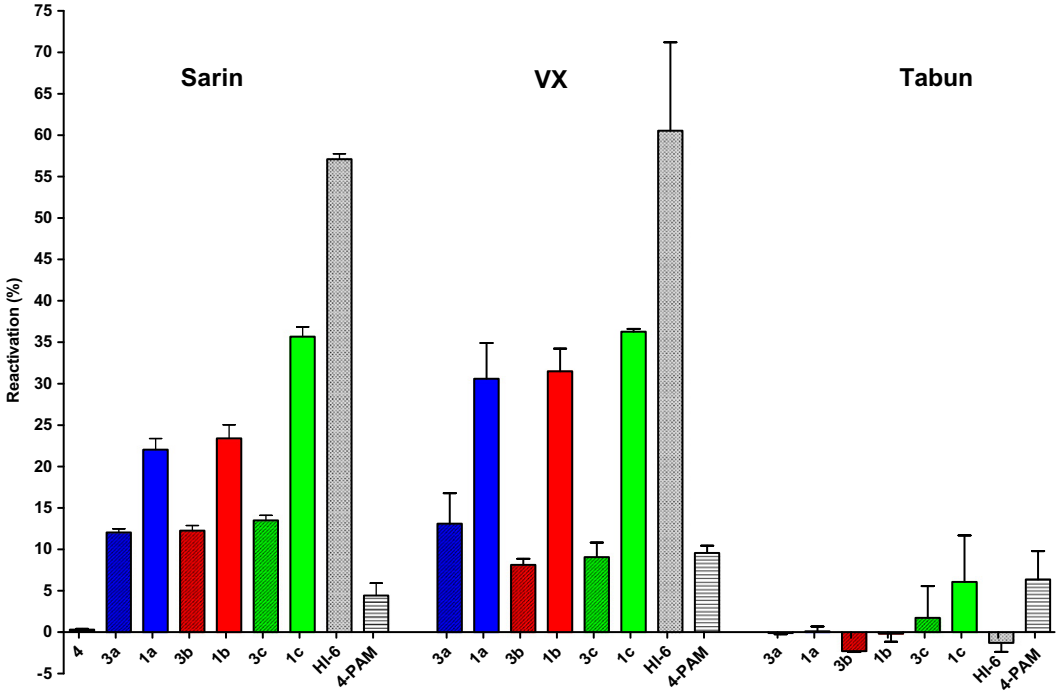


Figure 4. Reactivation of sarin, VX and tabun-inhibited human AChE by conjugates **1a–c**, oximes **3a–c**, PSL **4**, HI-6 and 4-PAM.

(i.e., 22%, 23% and 36%, respectively, at 10^{-5} M). This is in agreement with the finding by Ohta et al. that an increasing number of atoms attached to 4-PAM increases reactivating capacity (contrary to 2-PAM and 3-PAM analogues).[21] More interestingly, all conjugates also show an improved reactivation compared to compounds **3a–c**, which is most pronounced for **1c**/**3c** at 10^{-5} M (36%/14%, respectively). It is also of interest to note that **1c**, performing best in this study, does not show the highest affinity for the enzyme (which is **1a**). This may indicate an optimum between reactivation capacity and affinity for the enzyme with this particular linker length.

Similar reactivation experiments were carried out for VX- and tabun-inhibited hAChE (Fig. 4: middle and right side, respectively).[31] In the case of VX, the compounds **1a–c** also show enhanced reactivation compared to the parent compounds **3a–c**, which is even more pronounced. The overall performance of the compounds is similar as measured for sarin. However, no satisfying results were obtained with tabun-inhibited hAChE, which is notoriously difficult to reactivate (<5% in all cases).[32]

Several reports in the literature demonstrate that in some cases an allosteric enhancement of reactivation of carbamoylated or phosphorylated acetylcholinesterases occurred by the event of P-site occupation by several other PSL's.[33–38] Moreover, it was also shown that oxime-mediated dephosphorylation was accelerated in the presence of a ligand with affinity for the P-site.[39] In order investigate whether such a cooperative mechanism of reactivation was also possible with our compounds a reactivation experiment was carried out using mixtures of **3c** and **4** (data not shown). However, when sarin-inhibited hAChE was subjected to a mixture of a high concentration of **4** (10^{-3} M) and varying concentrations of **3c** and, in a separate experiment, with a fixed concentration of oxime **3c** (10^{-4} M) and varying concentrations of **4** no enhancement in reactivating power was observed compared to **3c** alone. These results confirm the necessity of conjugating the PSL and the oxime via a spacer to obtain a molecule (**1c**) with enhanced reactivating capacity. It can further be concluded from these experiments that enhancement of reactivation is probably affinity-related rather than via an allosteric mechanism.

In line with the experiments described above, some reactivation experiments were carried out using mixtures of conjugate **1c** (10^{-5} M) and PSL **4** (Fig. 5). In these experiments the presence of competing PSL **4** in the reactivating mixture may reveal information about the binding mode of the construct to the enzyme. As mentioned earlier the inhibitory power of **4** could not unambiguously be ascertained in the inhibition experiments (Fig. 3). Nevertheless, it was expected that if **4** binds the P-site it would compete with conjugate **1c** at higher concentrations and result in lower reactivation. Unexpectedly, with increasing concentration of **4** a slight increase in reactivation potency was observed (Fig. 5).

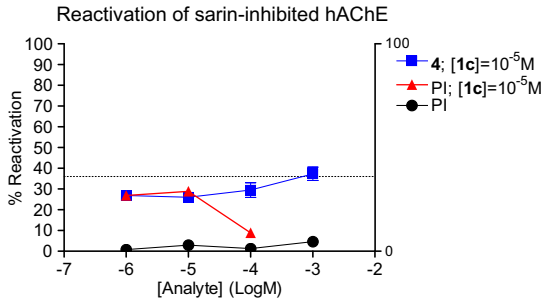


Figure 5. Reactivation of sarin-inhibited human AChE by mixtures of PSL **4** and propidium iodide with conjugate **1c**. The dotted line indicates the level of reactivation by **1c** alone at 10^{-5} M.

As **4** does not have any reactivating capacity for sarin-inhibited hAChE (Fig. 4) the results either indicate a different binding mode of the construct than anticipated or a low affinity of the non-conjugated **4** for the enzyme combined with an experimental error in the reactivation experiment. It is also tempting to attribute the marginal enhancement of reactivation to an allosteric enhancement of reactivation by the event of P-site occupation. However, that explanation is highly speculative at this stage and obviously requires further investigations.

As a control, a similar experiment was carried out using mixtures of **1c** and the well-known PSL propidiumiodide (PI). In this case the enzyme activity is lowered, as expected, at higher concentrations of PI because of the high affinity of propidium for the P-site.[40]

3. Conclusions

We have presented here a conceptually novel approach to the design of AChE reactivators.[41] The concept comprises the linkage of a PSL via a spacer to a reactivating moiety with the eventual goal to develop non-ionic reactivators with sufficient affinity for AChE to induce reactivation. As a first step in this approach, we here described the first example of the synthesis and biological evaluation of a P-site ligand conjugated to a charged pyridinium oxime via ethyleneglycol linkers. The P-site ligand of choice should be neutral and have a relatively weak affinity for the P-site to prevent the formation of high-affinity constructs as such compounds would be toxic.[24,25] The conjugates showed higher affinity for the enzyme than all parent compounds and combinations of parent PSL and oxime compounds, which demonstrated the necessity of linking the PSL to the oxime via a spacer. In addition, the conjugates were able to reactivate sarin- and VX-inhibited hAChE[31] and showed higher reactivation potency than their parent compounds at pharmacologically relevant concentration. Competition experiments using the PSL from this study as well as the well-known PSL propidium iodide could not unambiguously confirm the binding mode of the constructs, which will be subject of further investigation. Nevertheless, the results reported here justify the synthesis of non-ionic conjugates with potentially increased BBB uptake comprising a P-site ligand and a reactivating moiety, which is the focus of ongoing research.

4. Experimental

4.1. General information

All compounds and solvents were used as received from the suppliers (Aldrich). Analytical LC was conducted on an AKTA system using an Alltima C18 analytical column (5 μ particle size, flow: 1.0 mL/min). Absorbance was measured at 214 and 254 nm. Solvent system: A: 5% ACN, 0.1% TFA; B: 80% ACN, 0.1% TFA. Gradients of B were applied over 20 min unless otherwise stated. HPLC purifications were conducted on the AKTA system supplied with a semi-preparative Alltima C18 column (5 μ particle size, running at 5 mL/min).

LC/electrospray tandem mass spectrometric analyses for obtaining structural information were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Accurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 μL injection loop mounted and a PepMap C_{18} (LC Packings) column (15 cm × 1 mm ID, 3 μm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid)

and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The following gradient was applied: 0–5 min, 100% solution A, flow 0.1 mL/min; 5–60 min, 100% A to 30% A, flow 0.6 mL/min. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 μL/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 15 and 30 V and a collision energy between 11 and 25 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Other mass spectrometric analyses were carried out on a TSQ Quantum Ultra mass spectrometer (Finnigan, Thermo Electron Corporations, San Jose, USA) equipped with an Acquity Sample Manager and Binary Solvent Manager (Waters, Milford, USA). For LC–MS experiments, the liquid chromatograph was connected to the mass spectrometer source via the Sample Manager equipped with a 10 μL loop and an Acquity BEH C18 column (1.7 μ particles, 1 × 100 mm; Waters, Milford, USA). The liquid chromatography system was run with a 25 min linear gradient from 100% A to A/B 55.5:45.5 v/v (A: 0.2% formic acid in water; B: 0.2% formic acid in acetonitrile) at a flow rate of 0.09 mL/min. The TSQ Quantum Ultra mass spectrometer was operated with a spray voltage of 3 kV, a sheath gas pressure of 41 AU, aux gas pressure of 2 AU and a capillary temperature of 350 °C. Positive electrospray product ion spectra were recorded at an indicated collision energy of 15–20 eV, using argon as the collision gas at a pressure of 1.5 mTorr.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA, USA) Mercury*plus* spectrometer operating at 400 MHz and 100 MHz respectively. Chemical shifts (δ) are given in ppm relative to tetramethyl silane (δ 0 ppm).

4.2. Synthetic procedures

4.2.1. 1-(4-Pyridiniumaldoxime)-2-(2-chloroethoxy)ethane (3a)

A solution of 4-pyridinealdoxime (400 mg, 3 mmol) in dichloroethyl ether (20 mL) was stirred at 80 °C for 48 h. A brown, oily residue separated from the solution. Most of the solution was discarded and the brown oily residue was washed with DCM which was also separated from the oily residue. The crude **3a** was purified using silica gel column chromatography using a gradient of CH_3OH in CH_2Cl_2 (5→30%) and was isolated as a yellow oil. Yield: 399 mg, 1.7 mmol (58%). ^{1}H NMR (CD_3OD): δ 3.62 (t, 2H, J = 5.5 Hz), 3.75 (t, 2H, J = 5.5 Hz), 4.03 (t, 2H, J = 4.7 Hz), 4.86 (t, 2H, J = 4.7 Hz), 8.24 (d, 2H, J = 6.3 Hz), 8.35 (s, 1H), 8.98 (d, 2H, J = 6.6 Hz). ^{13}C NMR (CD_3OD): δ 43, 61, 69, 71, 124, 145, 146, 150. [M+H$^+$]: 229.0760 (theoretical value: 229.0744).

4.2.2. 1-(4-Pyridiniumaldoxime)-2-((2-chloroethoxy)-2-ethoxy)ethane (3b)

A solution of 4-pyridinealdoxime (500 mg, 4 mmol) and 1,2-bis-(2-chloroethoxy)ethane (7.5 g, 40 mmol) in acetonitrile (20 mL) was heated to reflux temperature and stirred for 48 h. A brown, oily residue separated from the solution. Most of the solution was discarded and the brown oily residue washed with DCM. The DCM was discarded and crude **3b** was purified over silica gel using a gradient of CH_3OH in CH_2Cl_2 (10→50%) and isolated as a yellow oil. Yield: 663 mg, 2.4 mmol (61%). ^{1}H NMR (CD_3OD): δ 3.63 (m, 8H), 4.05 (t, 2H, J = 4.7 Hz), 4.76 (t, 2H, J = 4.7 Hz), 8.22 (d, 2H, J = 6.6 Hz), 8.32 (s, 1H), 8.88 (d, 2H, J = 6.6 Hz). ^{13}C NMR (CD_3OD): δ 43, 61, 69, 70, 71, 72, 124, 145, 146, 150. [M+H$^+$]: 273.098 (theoretical value: 273.1006).

4.2.3. 1-(4-Pyridiniumaldoxime)-2-(((2-chloroethoxy)-2-ethoxy)ethoxy)ethane (3c)

A solution of 4-Pyridinealdoxime (500 mg, 4 mmol) and bis[2-(2-chloroethoxy)ethyl] ether (9.2 g, 40 mmol) in acetonitrile (20 mL) was heated to reflux temperature and stirred for 48 h. A brown, oily residue separated from the solution. Most of the solution was discarded and the brown oily residue washed with DCM. The DCM was discarded and crude **3c** was purified over silica gel using a gradient CH_3OH in CH_2Cl_2 (10→30%) and isolated as a yellow oil. Yield: 1.115 g, 3.5 mmol (87%). ^{1}H NMR (CD_3OD): δ 3.62 (m, 10H), 3.72 (t, 2H, J = 5.5 Hz), 3.98 (t, 2H, J = 4.7 Hz), 4.76 (t, 2H, J = 4.7 Hz), 8.22 (d, 2H, J = 6.6 Hz), 8.32 (s, 1H), 8.90 (d, 2H, J = 6.3 Hz). ^{13}C NMR (CD_3OD): δ 43, 61, 69, 70, 71, 71, 72, 124, 145, 146, 150. [M+H$^+$]: 317.1240 (theoretical value: 317.1268).

4.2.4. Conjugate 1a

To a solution of **3a** (43 mg, 0.2 mmol) and 4-*O*-(benzhydryl)-hydroxypiperidine (50 mg, 0.2 mmol) in DMF (2 mL) was added $NaHCO_3$ (80 mg, 1 mmol) and a catalytic amount of KI. This mixture was stirred at 90 °C for 24 h. The mixture was filtrated and the filtrate was concentrated under reduced pressure. Crude **1a** was purified by preparative HPLC (5–80% B) and isolated as the TFA–salt after lyophilisation. Yield: 12.6 mg, 0.03 mmol (14%). ^{1}H NMR (CD_3OD): δ 8.9 (d, 2H), 8.3 (ma/mi, 1H), 8.2 (m, 2H), 7.2–7.4 (m, 10H), 5.6 (ma/mi, 1H), 4.8 (bs, 2H), 4.0 (br s, 2H), 3.8 (br s, 2H), 3.7–3.2 multiple signals (6H), 2.95 (t, 1H, J = 11.7 Hz), 2.25–1.65 (m, 4H). [M+H$^+$]: 460.2588 (theoretical value: 460.2600).

4.2.5. Conjugate 1b

To a solution of **3b** (55 mg, 0.2 mmol) and 4-*O*-(benzhydryl)-hydroxypiperidine (50 mg, 0.2 mmol) in DMF (2 mL) was added $NaHCO_3$ (80 mg, 1 mmol) and a catalytic amount of KI. This mixture was stirred at 50 °C for 48 h. The mixture was filtrated and the filtrate was concentrated under reduced pressure. Purification by preparative HPLC (5–80% B) and freeze drying of the correct fractions yielded 15 mg (15%) of compound **1b** as the TFA-salt. ^{1}H NMR (CD_3OD): δ 8.8 (d, 2H, J = 5.5 Hz), 8.25 (s, 1H), 8.15 (d, 2H, J = 5.5 Hz), 7.4–7.1 (m, 10H), 5.6/5.5 mi/ma (s, 1H), 4.65 (br s, 2H), 3.9 (br s, 2H), 3.8–3.2 multiple signals (10H), 2.95 (t, 1H, J = 11.7 Hz), 2.2–1.6 (m, 4H). [M+H]$^+$: 504.284 (theoretical value: 504.2862).

4.2.6. Conjugate 1c

To a solution of **3c** (55 mg, 0.2 mmol) and 4-*O*-(benzhydryl)-hydroxypiperidine (50 mg, 0.2 mmol) in DMF (2 mL) was added $NaHCO_3$ (80 mg, 1 mmol) and a catalytic amount of KI. This mixture was stirred at 50 °C for 48 h. The mixture was filtrated and the filtrate was concentrated under reduced pressure. Purification by preparative HPLC (5–80% B) and freeze drying of the correct fractions yielded 15 mg (14%) of compound **1c** as the TFA-salt. ^{1}H NMR (CD_3OD): δ 8.85 (d, 2H, J = 6.3 Hz), 8.35 (s, 1H), 8.2 (d, 2H, J = 6.3 Hz), 7.4–7.2 (m, 10H), 5.65 (mi), 5.60 (ma) (s, 1H), 4.75 (br s, 2H), 3.95 (br s, 2H), 3.9–3.2 multiple signals (16H), 3.0 (t, 1H, J = 11.7 Hz), 2.3 (d, 1H, J = 12.5 Hz), 2.15 (d, 1H, J = 14.5 Hz), 2.0–1.9 (m, 2H). [M+H]$^+$: 548.310 (theoretical value: 548.3124).

4.3. Sample preparation for biological tests

As some of the isolated test compounds (from HPLC) tend to contain some water, or exist as the TFA salt, the weighing of the amounts necessary for biological testing could be quite inaccurate. As an alternative, UV measurements (absorption max at 270 nm) were used to calibrate all the solutions. The reference solution (1×10^{-2} M) was prepared from 4-PAM taken into account the purity, which was accurately determined to be 93.5% (w/w) by quantitative ^{1}H NMR. Thus, dilution of the stock solution of 4-PAM to 1.0×10^{-5} and subsequent UV measurements gave an absorption of 0.363 at 270 nm (ε = 36,300 L mol^{-1} cm^{-1}). Next, solutions of approx. 2×10^{-2} M were prepared by weighing a

sufficient amount (typically 5 mg) of the test substances and dissolve them in water, except for **4**, which was dissolved in isopropylalcohol (IPA). Aliquots of these stock solutions were diluted 1000 times and UV absorption of the resulting dilutions were compared to that of the reference 4-PAM solution. Next, the test substance stock solutions were diluted correcting for the difference in UV absorption and, after preparing a new 10^{-5} M dilution rechecked with UV. This method assumes an equal extinction coefficient for all of the test substances. This is justified, because the extinction coefficient is not expected to change dramatically on variation of the linker length. The UV-measurement of the PAS ligand (4-*O*-(benzhydryl)-hydroxypiperidine) showed no absorption in the 250–350 nm region at similar concentrations, and thus did not disturb the measurements involving the complete constructs **1a–c**.

4.4. AChE inhibition experiments

To 50 μL enzyme (hAChE, 3nM in 100 mM phosphate buffer pH 7.4) 5 μL oxime solution (end concentrations: 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M) was added and the mixture was incubated for 30 min at 37 °C. A blank experiment (positive control) was run in parallel and prepared by adding 5 μL of buffer instead of oxime solution to the enzyme. From each sample 20 μL was added to 500 μL DTNB (0.6 mM in 100 mM phosphate buffer pH 7.4) and from the DTNB solutions 4 × 100 μL was transferred to a 96well plate. To each well 100 μL ATCh (0.9 mM in MilliQ water) was added and the absorption at 415 nM was measured every 5 min for 15 min (abs <0.8).

Enzyme activity was calculated using the formula:

$$\% \text{ AChE activity} = S \times 100/P$$

S = absorption value of test substance; P = absorption value of positive control (~100% activity).

4.5. AChE reactivation experiments

4.5.1. Determination of the amount of nerve agent necessary to inhibit the enzyme

To 50 μL enzyme solution (hAChE, 3 nM in 100 mM phosphate buffer pH 7.4) 5 μL of nerve agent solution (concentrations ranging from 10^{-9} to 10^{-5} M) was added and incubated for 30 min at 37 °C.

From each of these samples 20 μL was diluted in 500 μL DTNB (0.6 mM in 100 mM phosphate buffer pH 7.4) and from the resulting DTNB solutions 4 × 100 μL was transferred to a 96 well plate. Then, to each sample another 50 μL of hAChE (3 nM) was added and incubated for 30 min, after which 20 μL was transferred into 500 μL DTNB. To the DTNB solutions (100 μL/well), 100 μL of ATCh was added and absorption was measured at 415 nM every 5 min for 15 min (abs <0.8). From these experiments the nerve concentration was calculated (5×10^{-8} M) that maximally inhibited the initial sample, but did not additionally inhibit the added 3 nM of AChE. Consequently, no nerve agent capable of inhibition is left in the sample at the time of oxime addition.

4.5.2. Reactivation experiments

To 50 μL enzyme solution (hAChE, 3 nM in 100 mM phosphate buffer pH 7.4) 5 μL of 5×10^{-8} M nerve agent (VX, Sarin or Tabun) in water was added and incubated for 30 min at 37 °C. Blank samples were run in parallel and consisted of: (1) a positive control (P): 5 μL buffer was added to the enzyme instead of nerve agent solution and (2) a negative control (N): 5 μL buffer was added to the inhibited enzyme instead of an oxime solution. From this point the procedure described under Section 4.4 was followed.

Reactivation was calculated using the formula:

$$\% \text{ Reactivation} = (S - N) \times 100/(P - N)$$

S = absorption value of test substance; P = absorption value of positive control (~100% activity); N = absorption value of negative control (~0% AChE activity ~100% inhibition).

Acknowledgements

The authors wish to acknowledge the funding from the Dutch Ministry of Defence, the generous gift of purified human acetylcholinesterase by Dr. Florian Nachon from IRBA/CRSSA (France) and COL Dr. F. Worek from IPT (Germany) for performing additional reactivation experiments using erythrocyte acetylcholinesterase inhibited with VX, tabun, sarin, paraoxon-ethyl and cyclosarin.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.059.

References and notes

1. Sussman, J. L.; Harel, M.; Frolow, F.; Oefner, C.; Goldman, A.; Toker, L.; Silman, I. *Science* **1991**, *253*, 872.
2. Masson, P.; Legrand, P.; Bartels, C. F.; Froment, M.-T.; Schopger, L. M.; Lockridge, O. *Biochemistry* **1997**, *36*, 2266.
3. Szegletes, T.; Mallender, W. D.; Thomas, P. J.; Rosenberry, T. L. *Biochemistry* **1999**, *38*, 122.
4. Quinn, D. M. *Chem. Rev.* **1987**, *87*, 955.
5. Marrs, T. C. *Pharmacol. Therap.* **1993**, *58*, 51.
6. Worek, F.; Eyer, P.; Aurbek, N.; Szinicz, L.; Thiermann, H. *Toxicol. Appl. Pharmacol.* **2007**, *219*, 226.
7. Bajgar, J.; Fusek, J.; Kuca, K.; Bartosova, L.; Jun, D. *Mini-Rev. Med. Chem.* **2007**, *7*, 461.
8. Luo, C.; Tong, M.; Chilukuri, N.; Brecht, K.; Maxwell, D. M.; Saxena, A. *Biochemistry* **2007**, *46*, 11771.
9. Lundy, P. M.; Raveh, L.; Amitai, G. *Toxicol. Rev.* **2006**, *25*, 231.
10. Sakurada, K.; Matsubara, K.; Shimizu, K.; Shiono, H.; Seto, Y.; Tsuge, K.; Yoshino, M.; Sakai, I.; Mukoyama, H.; Takatori, T. *Neurochem. Res.* **2003**, *28*, 1401.
11. Benschop, H. P.; van Oosten, A. M.; Platenburg, D. H. J. M.; van Hooidonk, C. *J. Med. Chem.* **1970**, *13*, 1208.
12. Benschop, H. P.; van den Berg, G. R.; Van Hooidonk, C.; De Jong, L. P. A.; Kientz, C. E. *J. Med. Chem.* **1979**, *22*, 1306.
13. Kenley, R. A.; Howd, R. A.; Mosher, C. W.; Winterle, J. S. *J. Med. Chem.* **1981**, *24*, 1124.
14. Kenley, R. A.; Bedford, C. D.; Dailey, O. D.; Howd, R. A.; Miller, A. *J. Med. Chem.* **1984**, *27*, 1201.
15. Kenley, R. A.; Bedford, C. D.; Howd, R. A.; Jackson, S. E. *Biochem. Pharm.* **1985**, *34*, 3606.
16. Bedford, C. D.; Howd, R. A.; Dailey, O. D.; Miller, A.; Nolen, H. W., III; Kenley, R. A.; Kern, J. R.; Winterle, J. S. *J. Med. Chem.* **1986**, *29*, 2174.
17. Bedford, C. D.; Miura, M.; Bottaro, J. C.; Howd, R. A.; Nolen, H. W., III *J. Med. Chem.* **1986**, *29*, 1689.
18. Degorre, F.; Kiffer, D.; Terrier, F. *J. Med. Chem.* **1988**, *31*, 757.
19. Kuca, K.; Jun, D.; Musilek, K. *Mini-Rev. Med. Chem.* **2006**, *6*, 269.
20. Jeong, H. C.; Kang, N. S.; Park, N.-J.; Yum, E. K.; Jung, Y.-S. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1214.
21. Ohta, H.; Ohmori, T.; Suzuki, S.; Ikegaya, H.; Sakurada, K.; Takatori, T. *Pharm. Res.* **2006**, *23*, 2827.
22. Okuno, S.; Sakurada, K.; Ohta, H.; Ikegaya, H.; Kazui, Y.; Akutsu, T.; Takatori, T.; Iwadate, K. *Toxicol. Appl. Pharmacol.* **2008**, *227*, 8.
23. Kwon, Y. E.; Park, J. Y.; No, K. T.; Shin, J. H.; Lee, S. K.; Eun, J. S.; Yang, J. H.; Shin, T. Y.; Kim, D. K.; Chae, B. S.; Leem, J.-Y.; Kim, K. H. *Bioorg. Med. Chem.* **2007**, *15*, 6596.
24. Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radić, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem.* **2002**, *41*, 1053.
25. Manetsch, R.; Kransinski, A.; Radić, Z.; Raushel, J.; Taylor, P.; Sharpless, K. B.; Kolb, H. C. *J. Am. Chem. Soc.* **2004**, *126*, 12809.
26. Ellman, G. L.; Courtney, K. D.; Anders, V., Jr.; Feather-Stone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.
27. Ginsburg, S.; Wilson, I. B. *J. Am. Chem. Soc.* **1957**, *79*, 481.
28. All biological tests described in this manuscript were performed in triplicate.
29. The results of reactivation at other concentrations are depicted in Figure S3 in Supplementary data.
30. Interestingly, when sarin-inhibited electric eel AChE was used instead of human AChE, reactivation by HI-6 was poor, whereas the conjugates **1a–c** performed comparably as with human AChE, thus dominating HI-6. Graphs of inhibition/reactivation experiments using electric eel AChE are given in Figure S4 in Supplementary data.

31. More reactivation experiments with varying inhibitors were carried out by Dr. F. Worek (IPT, Germany). The results of these experiments are given in the supporting information (Fig. S5). These results show the same trends (enhanced reactivation of the total construct compared to the parent compounds), but the percentage reactivation is generally somewhat lower. These differences can be attributed to the use of a slightly different method employing erythrocyte hAChE (ghosts).
32. Ashani, Y.; Radic, Z.; Tsigelny, I.; Vellom, D. C.; Pickering, N. A.; Quinn, D. M.; Doctor, B. P.; Taylor, P. *J. Biol. Chem.* **1995**, *270*, 6370.
33. Rosenberry, T. L.; Johnson, J. L.; Cusack, B.; Thomas, J. L.; Emani, S.; Venkatasubban, K. S. *Chem. Biol. Interact.* **2005**, *157–158*, 181.
34. Kitz, R. J.; Braswell, L. M.; Ginsburg, S. *Mol. Pharmacol.* **1969**, *6*, 108.
35. Tomlinson, G.; Mutus, B.; McLennan, I. *Mol. Pharamacol.* **1980**, *18*, 33.
36. Brochier, L.; Pontié, Y.; Willson, M.; Estrada-Mondaca, S.; Czaplicki, J.; Klaébé, A.; Fournier, D. *J. Biol. Chem.* **2001**, *276*, 18296.
37. Rosenberry, T. L.; Sonoda, L. K.; Dekat, S. E.; Cusack, B.; Johnson, J. L. *Chem. Biol. Interact.* **2008**, *175*, 235.
38. Crone, H. D. *J. Neurochem.* **1973**, *20*, 225.
39. Harris, L. W.; Heyl, W. C.; Stitcher, D. L.; Broomfield, C. A. *Biochem. Pharmacol.* **1978**, *27*, 757.
40. The inhibition curve of propidium iodide is given in Figure S1 in Supplementary data.
41. The idea for conjugation of a peripheral site ligand and a reactivating oxime as a dual site binding reactivator was recently mentioned in the literature. Taylor, P.; Kovarik, Z.; Reiner, E.; Radić, Z. *Toxicology* **2007**, *233*, 70.