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Pyridoxal oxime derivative potency to reactivate cholinesterases inhibited by organophosphorus compounds

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ABSTRACT

Organophosphorus (OP) nerve agents (sarin, tabun VX and soman) inhibit the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) by binding to its active site while preventing neurotransmission in the cholinergic synapses. The protection and treatment of this kind of poisoning are still a challenge as we are yet to discover an antidote that would be effective in all cases of poisoning. To aid the search for more efficient antidotes, we evaluated the ability of nine pyridoxal oxime derivatives, prepared by a novel synthetic pathway, to reactivate recombinant human AChE and the related purified human plasma butyrylcholinesterase (BChE, EC 3.1.1.8) inhibited by VX, tabun and paraoxon. Oximes are derivatives of vitamin B6 bearing a phenacyl moiety attached to the quaternary nitrogen atom and having various substituents on the phenyl ring. As the results have shown, the tested oximes were in general more efficient in the reactivation of OP-inhibited BChE than AChE. The highest observed rate was in the case of VX-inhibited BChE reactivation, where k_{obs} was 0.0087 min^{-1} and the reactivation maximum of 90% was achieved within 5 h. The cholinesterases displayed a binding affinity for these derivatives in a μmolar range no matter the substituent on their rings which was in accordance with the molecular modelling results showing a similar binding pattern for all oximes within the active site of both AChE and BChE. Such a positioning reveals also that hydroxy and a metoxy substituents at the vicinity of the oxime moiety present a possible steric hindrance explaining the reactivation results.

1. Introduction

Numerous, structurally different organophosphorus compounds (OPs) constitute a heterogeneous category of chemicals specifically designed for the control of pests, weeds or plant diseases (Delfino et al., 2009). Though they are highly toxic to humans, their application is still an accepted means for the plant protection, and has contributed significantly to enhanced agricultural productivity and crop yields (Bolognesi, 2003). OPs are also used as plasticizers, stabilizers in lubricating and hydraulic oils, flame retardants, and gasoline additives (Kumar et al., 2010). In addition to these beneficial uses, some highly potent OPs, including tabun, sarin, and VX have been used as nerve agents in chemical warfare.

OP nerve agents inhibit acetylcholinesterase (AChE 3.1.1.7) forming a covalent bond with the active site catalytic serine. The inhibition prevents AChE's essential role, a hydrolysis of the neurotransmitter acetylcholine (ACh), which causes its accumulation, resulting in overstimulation of nicotinic and muscarinic receptors in both the central and peripheral nervous systems leading to severe symptoms in the victim (including death). The phosphylated AChE is relatively stable, and depending on the groups attached to the phosphorus atom, becomes irreversibly inhibited. Modern therapy utilises compounds bearing the oxime group capable of reactivating inhibited enzymes by a nucleophilic attack on the phosphylated serine. Unfortunately, none of the currently used oximes is sufficiently effective against all nerve agents and pesticides, meaning that they are not able to reactivate inhibited enzymes regardless of the structure of the conjugated phosphorus moiety (Kumar et al., 2010; Dawson, 1994; Žunec et al., 2015). For example, one of the most problematic nerve agents to counteract is tabun, since the existence of a lone electron pair located on its amino group makes the nucleophilic attack required for reactivation very difficult (Eto, 1976; Koplovitz and Stewart, 1994; Čalić et al., 2006; Carletti et al., 2009). Furthermore, oximes used in medical practice today lack the ability to cross the blood-brain barrier and reactivate AChE in the central nervous system in order to diminish the life threatening symptoms (Kalász et al., 2015; Katalinić et al., 2015). In this field of research, certain progress has been made by the synthesis of novel oximes demonstrating the entry to the brain and up to 35% reactivation of VX or sarin inhibited AChE (Chambers et al., 2013, 2016a, 2016b).

On the other hand, OP pesticides present a somewhat different problem. Although less lethal than chemical weapons, OP pesticides are more accessible to general population both in greater amounts and at higher concentrations. The World Health Organization reports about three million cases per year of intoxication with OP pesticides in the world. These intoxications usually result from pesticides misuses or suicidal attempts (Carlton et al., 1998; Bardin et al., 1994).

In the search for more efficient OP poisoning treatments, we synthesized nine oxime derivatives of vitamin B6 continuing our previous study (Gašo-Sokač et al., 2010), and tested them as reactivators

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of human AChE and BChE inhibited by tabun, VX and paraoxon (Fig. 1). The vitamin B6 scaffold was chosen since it shares a similar structure to classical monopyridinium oximes and contains a template to facilitate an optimum binding and efficient attack on the phosphylated active site serine. To be more precise, the neighbouring substituents to feasible oxime group, like −OH and $-CH_2OH$, should prevent reinhibition of the reactivated enzyme by phosphorylated oxime due to the accelerated decomposition of phosphorylated oximes via elimination of the phosphoric group, and may improve enzyme affinity through additional bonding (Kliachyna et al., 2014). Several previous studies employed a similar hypothesis as well (Kliachyna et al., 2014; Radić et al., 2013a).

We approached the quaternary salts of pyridoxal oxime derivatives synthesized here through a novel environmentally friendly and facile method. Pyridoxal oxime derivatives contain quaternary nitrogen on the pyridinium ring; the oxime group at the position 4 on the heteroaromatic ring; and acyl moiety for accommodation at the acyl pocket of the enzyme (Fig. 1).

2. Materials and methods

2.1. Chemicals

The general procedure for the synthesis of compounds **1**–**9** has been given in our previous study (Gašo-Sokač et al., 2014; Bušić et al., 2013). The structure of the compounds is given in Fig. 1, which were named accordingly: **1** (R = 4-H), **2** (R = 4-F), **3** (R = 4-Cl), **4** (R = 4-Br), **5** (R = 4-NO_2), **6** (R = 4-CH_3), **7** (R = 2-OCH_3), **8** (R = 4-OCH_3) and **9** (R = 4-Ph). Purity of compounds was higher than 95% according to their spectral analysis (Gašo-Sokač et al., 2014; Bušić et al., 2013). The synthesized oximes were dissolved in DMSO as 100 mM solutions. Paraoxon (*O*,*O*-diethyl-*O*-(4-nitrophenyl)phosphate; CAS number 311-45-5; (Pestanal®) was purchased from Sigma-Aldrich, St. Louis, MO, USA (purity 90%). VX (*S*-[2-(diisopropylamino)ethyl]-*O*-ethyl methylphosphonothioate; CAS number 50782-69-9) and tabun (*O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate; CAS number 77-81-6) were purchased from NC Laboratory, Spiez, Switzerland (purity >95%). The stock solution of paraoxon was prepared in ethanol while VX and tabun in isopropyl alcohol. Further dilutions were done in distilled water. Acetylthiocholine iodide (ATCh) and thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Stock solutions were prepared in water or 0.1 M sodium phosphate buffer pH 7.4, respectively. The final concentration of DTNB was 0.33 mM in all of the experiments. All of the experiments were done in 0.1 M sodium phosphate buffer pH 7.4, at 25 °C.

2.2. Oxime-assisted reactivation of AChE and BChE inhibited by OPs

Recombinant human AChE and purified human plasma BChE were a gift from Dr Florian Nachon (Institut de Recherche Biomédicale des Armées, Brétigny-sur-Orge, France). The enzymes were in-

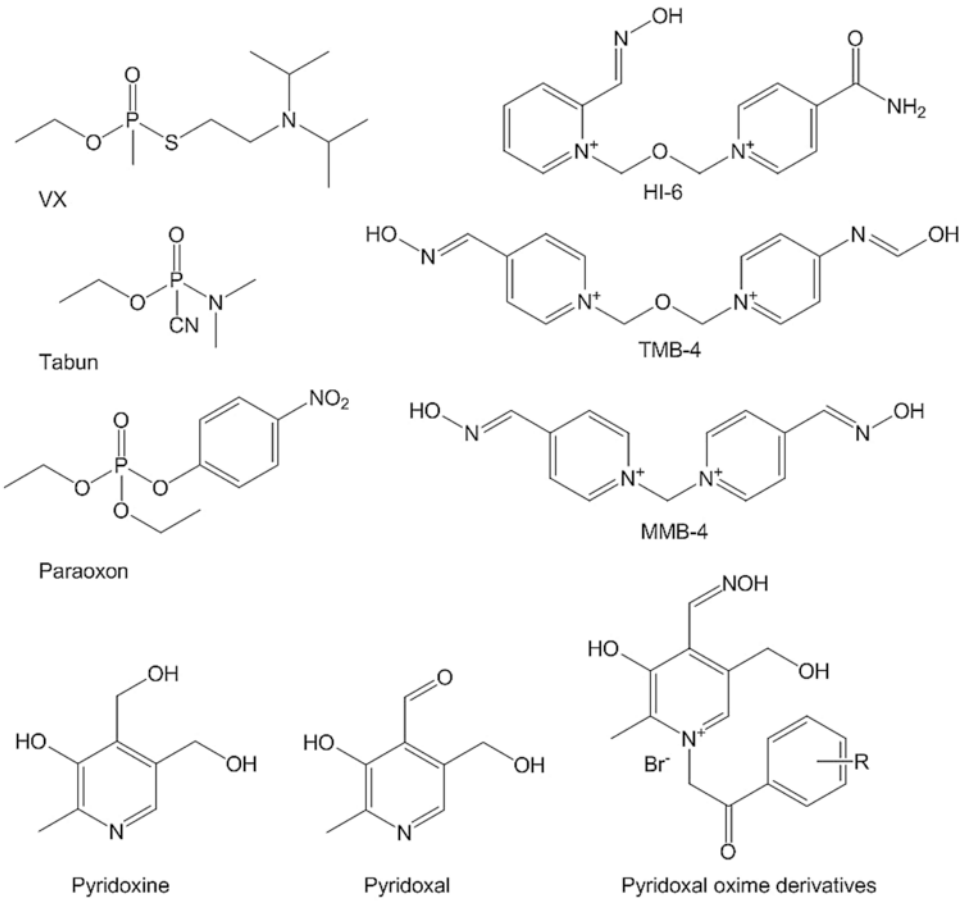

Fig. 1. Structure of organophosphorus compounds (VX, tabun and paraoxon), conventional oximes (MMB-4, TMB-4, HI-6), vitamin B6 forms (pyridoxine, pyridoxal) and nine pyridoxal oxime derivatives as potential antidotes. R denotes substituent and its position on the phenyl ring: **1** (R = 4-H), **2** (R = 4-F), **3** (R = 4-Cl), **4** (R = 4-Br), **5** (R = 4-NO_2), **6** (R = 4-CH_3), **7** (R = 2-OCH_3), **8** (R = 4-OCH_3) and **9** (R = 4-Ph).

cubated with 3.5×10^{-7} M VX, 5×10^{-6} M tabun or 10^{-6} M paraoxon up to 60 min, achieving 95–100% inhibition. The excess of OP was removed by filtration through the Strata® C18-E column (Phenomenex, Torrance, USA). The incubation mixture was diluted 5-times with phosphate buffer containing 1 mM oxime to start the reactivation. After a given time of reactivation, aliquots were diluted 40 times in buffer (containing DTNB) for the activity measurement. By addition of ATCh (1 mM, final), the enzyme activity was measured spectrophotometrically according to the Ellman procedure (Ellman et al., 1961) at 412 nm. An equivalent sample of the uninhibited enzyme was passed through the column, diluted to the same extent as the inhibited enzyme, and the control activity was measured in the presence of the oxime used for reactivation. Both activities of the control and reactivation mixture were corrected for the oxime-induced hydrolysis of ATCh. Under these conditions, the enzyme activity in the absence of the oxime was stable and no spontaneous reactivation of the phosphorylated enzyme took place. The reactivation for a single oxime concentration followed first-order kinetics and the observed first-order reactivation rate constant (k_{obs}) for the studied oxime concentration was determined from at least two experiments according to the previously described procedure and equation (Čalić et al., 2006; Kovarik et al., 2004):

$$\ln \frac{[EP]_0}{[EP]_t} = \frac{k_{+2} \cdot [OX]}{K_{OX} + [OX]} \cdot t = k_{obs} \cdot t \quad (1)$$

where EP_0 and EP_t denote the concentrations of the phosphylated enzyme at time zero and *t*, respectively; OX is a given oxime concentration, k_{+2} is the maximum first-order reactivation rate constant and K_{OX} is a dissociation constant of the phosphylated enzyme-oxime complex. The experimentally obtained percentage of reactivation in time was related to Eq. (1), wherefrom k_{obs} was obtained using the Prism6 software (Graph Pad Software, San Diego, USA). The maximal percentage of reactivation ($React_{max}$/%), as the maximal experimentally obtained reactivation in time, was also evaluated.

2.3. Reversible inhibition of AChE and BChE by oximes

The reversible inhibition of AChE and BChE by oximes was determined by a previously published procedure (Čalić et al., 2006; Katalinić et al., 2010). The enzyme-oxime dissociation constant K_i was evaluated from the effect of substrate concentration (ATCh) on the degree of oxime inhibition. For each substrate concentration, enzyme activity was measured with at least three concentrations of the oxime. Dissociation constants of the reversible inhibition were determined from at least three experiments by the Hunter-Downs equation as described previously (Čalić et al., 2006; Katalinić et al., 2010):

$$K_{i,app} = \frac{v_i \cdot [OX]}{v_0 - v_i} = K_i + \frac{K_i}{K_S} \cdot [ATCh] \quad (2)$$

where $K_{i,app}$ is the apparent enzyme-oxime dissociation constant at a given substrate concentration [ATCh], and v_0 and v_i enzyme activities measured in the absence or in the presence of the oxime concentration ([OX], *i*), respectively; K_i is the enzyme-oxime dissociation constant and K_S is the enzyme-substrate dissociation constant. The K_i values were determined from plotting $K_{i,app}$ *vs.* substrate concentration using the Prism6 software (Graph Pad Software, San Diego, USA).

2.4. Non-enzymatic reaction of oximes and substrate acetylthiocholine

The reaction of the substrate ATCh with the oximes was measured and calculated as described earlier (Kovarik et al., 2008; Šinko et al., 2006, 2007). For each substrate concentration, a range of oxime concentrations was tested. In general, the reaction mixture contained DTNB and an oxime, and the reaction was started by adding the ATCh. Activity was measured at 412 nm. Two to four measurements were done with each ATCh/oxime concentration pair. The second-order rate constant of the non-enzymatic reaction (k_{NE}) was calculated according to the previously published equation (Kovarik et al., 2008; Šinko et al., 2006, 2007):

$$\frac{\Delta[TCh]}{\Delta t} = k_{NE} \cdot [ATCh] \cdot [OX] \quad (3)$$

where $\Delta TCh/\Delta t$ denotes the released thiocholine concentration in time *t* as a product of substrate degradation, [ATCh] and [OX] are the initial substrate or oxime concentrations, respectively. The k_{NE} values were determined from plotting the experimentally obtained data using the Prism6 software (Graph Pad Software, San Diego, USA). Thiocholine concentration was calculated using the standard Lambert-Beer law ($\varepsilon = 14\,220\ M^{-1}\ cm^{-1}$).

2.5. Molecular modelling experiments

The three-dimensional structures of human AChE PDB code 4PQE (Kryger et al., 2000) and human BChE PDB code 2PM8 (Ngamelue et al., 2007) were used for molecular modelling. For each oxime, 20 poses in the active site gorge of AChE or BChE were generated by CDOCKER docking protocol with a CHARMm force field (Accelrys, USA). Poses were ranked according to the CDOCKER interaction energy calculated from interactions between oxime and cholinesterase active site residues (i.e. hydrogen bonds, π–π interactions, cation–π interactions and hydrophobic interactions).

3. Results and discussion

Since pralidoxime (2-PAM), a simple pyridinium oxime mimicking the structural features of the substrate acetylcholine, has been introduced as reactivator, various pyridinium oximes were created modifying its chemical structure to improve antidotal properties. Although an improvement has been made in the treatment of poisoning with certain OP pesticides, the challenge is still present not only in the search for new more potent reactivators but also in the synthesis of the desired new compounds. In respect to this, we prepared nine structurally similar monopyridinium oximes with a systematic change in the acyl moiety by a novel eco-friendly synthetic pathway (Gašo-Sokač et al., 2014; Bušić et al., 2013). The benefits of a novel synthetic pathway are purity and yields higher than those of the conventional method by which several of these oximes were synthesised previously (Gašo-Sokač et al., 2014). The microwave irradiation provided a remarkable rate of acceleration for *N*-alkylation, reaction times decreased dramatically, the consumed energy decreased considerably. We have also demonstrated a rapid and extremely simple mechanochemical method, by which strategy we employed LAG (*The Liquid-Assisted Grinding*) for the conversion of pyridoxal oxime and substituted phenacyl bromides into quaternary pyridinium salts (Gašo-Sokač et al., 2014; Bušić et al., 2013). Therefore, our strategy

to engage the microwave chemistry for conversion of pyridoxal oxime and substituted phenacyl bromides into quaternary pyridinium salts is a valuable technique in respect to the green chemistry.

The ability of the studied oximes to reactivate hAChE inhibited by VX, tabun, and paraoxon is summarised in Table 1. The obtained results indicate low reactivation rates (max. k_{obs} of 0.0013 min^{-1}) although inhibited hAChE was reactivated up to 80% within 24 h. One representative experiment showing the obtained percentage of reactivation followed in time is given in Fig. 2A. Compared to recent studies on the oximes with a novel structure, our results obtained on hAChE reactivation are rather limited (Mercey et al., 2012; Radić et al., 2012, 2013b; Renou et al., 2013, 2016). To be specific, studies done by Radić et al. pointed to specific *N*-substituted 2-hydroxyiminoacetamido alkylamines (RS194B, RS41A) as the potent reactivators having k_{obs} values for reactivating paraoxon or VX inhibited hAChE in the range of 0.05 min^{-1}–1.0 min^{-1} (Radić et al., 2012, 2013b). Furthermore, Mercey et al. and Renou et al. showed that reactivators designed with moieties for binding to the AChE's peripheral site are efficient as well (Mercey et al., 2012; Renou et al., 2013, 2016). These oximes had k_{obs} values for reactivating the inhibited hAChE up to 0.7 min^{-1}. In the case of tabun reactivation, none of these novel oximes exceeded the capacities of bispyridinium oximes like K048 or K203 (Čalić et al., 2006; Kovarik et al., 2009). The inefficiency of oximes **1**–**9** to reactivate tabun-inhibited hAChE is in accordance with our results from the previous study on several of these oximes with substituent -R: -H, -F, -Cl, $-CH_3$, and 4-OCH_3 (Gašo-Sokač et al., 2010).

The results of VX-, tabun- and paraoxon-inhibited hBChE reactivation are presented in Table 2 and a representative experiment is given in Fig. 2B. VX-inhibited hBChE showed to be the most susceptible to reactivation and the highest observed rates were in the case of oximes **6** and **8**, where the reactivation maximum of 90% was achieved within 5–8 h (k_{obs} was 0.0051 min^{-1} and 0.0087 min^{-1}, respectively). As for hAChE reactivation, these results are far from being considered encouraging according to the data from the literature (Kovarik et al., 2010; Lucić Vrdoljak et al., 2006; Sit et al., 2014; Horn et al., 2015). Specifically, structurally different bispyridinium oximes as well as novel *N*-alkyl-substituted imidazole and imidazolium oximes reactivated hBChE even 100 times faster than oximes **1**–**9** (Kovarik et al., 2010; Lucić Vrdoljak et al., 2006; Sit et al., 2014; Horn et al., 2015).

Since binding affinity plays an important factor in reactivation, we analysed the reversible inhibition of hAChE and hBChE by the tested pyridoxal oxime derivatives. The results in terms of the dissociation constants, K_i, are presented in Table 3 and Table 4, respectively. All of the oximes reversibly inhibited both enzymes in a micromolar range similar to other monopyridinium compounds (Simeon et al., 1981; Odžak et al., 2007) or bispyridinium oximes (Kovarik et al., 2008), without displaying any significant selectivity in binding. This similarity in affinity between AChE and BChE for these oximes may be due to a network of conserved aromatic residues in the active site of both hAChE and hBChE (Kryger et al., 2000; Ngamelue et al., 2007). This network rich in π-electrons includes Trp86 in AChE (Trp82 in BChE), the residue involved in the mechanism of substrate hydrolysis, and surrounding 4 additional residues Tyr128, Tyr440, Trp430 and Tyr332 (in BChE Tyr133, Tyr449, Trp439, Tyr341). Close to this network two also relevant aromatic residues are present: Phe329 and Trp231 in AChE and matching Phe338 and Trp236 in BChE. These conserved residues may stabilize the tested oximes forming analogue interactions with the heteroaromatic pyridinium ring and hydrophobic aromatic acyl moiety.

The most potent inhibitor of both enzymes was oxime **4**. It seems that a lone electron pair of oxygen in residues like glutamine, histidine or serine form an electrostatic interaction with the electron-withdrawing bromine atom (Lewis acid) as it was shown in other studies like the one of the enzyme aldose reductase complex with a halogenated inhibitor (Howard et al., 2004). Nonetheless, even though the determined binding affinities assume binding of the oximes to the active site, they did not correlate with the reactivation efficiencies. This confirms the premise that the efficient reactivation is primarily based on a reactivatorś capability to attack phosphorus conjugated to the catalytic serine, and not solely on the binding affinity (Čalić et al., 2006; Kovarik et al., 2004).

The lack of efficiency in oximes **1**–**9** to reactivate inhibited hAChE and hBChE could perhaps be explained by a steric hindrance of the oxime group caused by the neighbouring substituents –OH and $–CH_2OH$ on the ring. Though the intent of introducing these two groups was somewhat justifiable, it seems that the combination of the two led to a decreased accessibility of the oxime group itself. This indication was observed when we analysed the ability of these oximes to lyse the substrate ATCh, or in other words, oximolysis reaction. The mechanism of this reaction is analogous to the proposed nucle-

Table 1
Reactivation of OP-inhibited human AChE by 1 mM oximes **1**–**9** recorded within 24 h. Constants were determined from at least two experiments fitting the experimental data to Eq. (1). Standard error was within 10%. Data for conventional oximes is given for comparison.

		VX-inhibited AChE			Tabun-inhibited AChE			Paraoxon-inhibited AChE		
Oxime	-R	k_{obs} (min^{-1})	React.$_{max}$ (%)	Time of React.$_{max}$ (h)	k_{obs} (min^{-1})	React.$_{max}$ (%)	Time of React.$_{max}$ (h)	k_{obs} (min^{-1})	React.$_{max}$ (%)	Time of React.$_{max}$ (h)
1	4-H	0.0008	75	23	–	<15	24	0.0013	60	24
2	4-F	0.0008	80	23	–	<10	24	0.0011	40	24
3	4-Cl	0.0004	60	23	–	<15	24	0.0006	40	24
4	4-Br	0.0003	50	23	–	<10	24	0.0008	50	24
5	4-NO_2	0.0004	40	23	–	<10	24	0.0008	40	24
6	4-CH_3	0.0005	70	23	–	<10	24	0.0009	50	24
7	2-OCH_3	0.0010	40	12	–	<10	24	0.0013	75	24
8	4-OCH_3	0.0008	70	23	–	<10	24	0.0009	60	24
9	4-Ph	0.0010	85	23	–	<10	24	0.0011	40	24
Obidoxime	–	0.957[a]	/	/	0.0364[a]	/	/	0.785[a]	/	/
TMB-4	–	/	/	/	0.100[b]	/	/	0.650[c]	/	/
HI-6	–	0.239[a]	/	/	n.d.[b]	<10[b]	24[b]	0.129[a]	/	/

[a] From (Worek et al., 2004).

[b] From (Čalić et al., 2006).

[c] From (Kovarik et al., 2010).

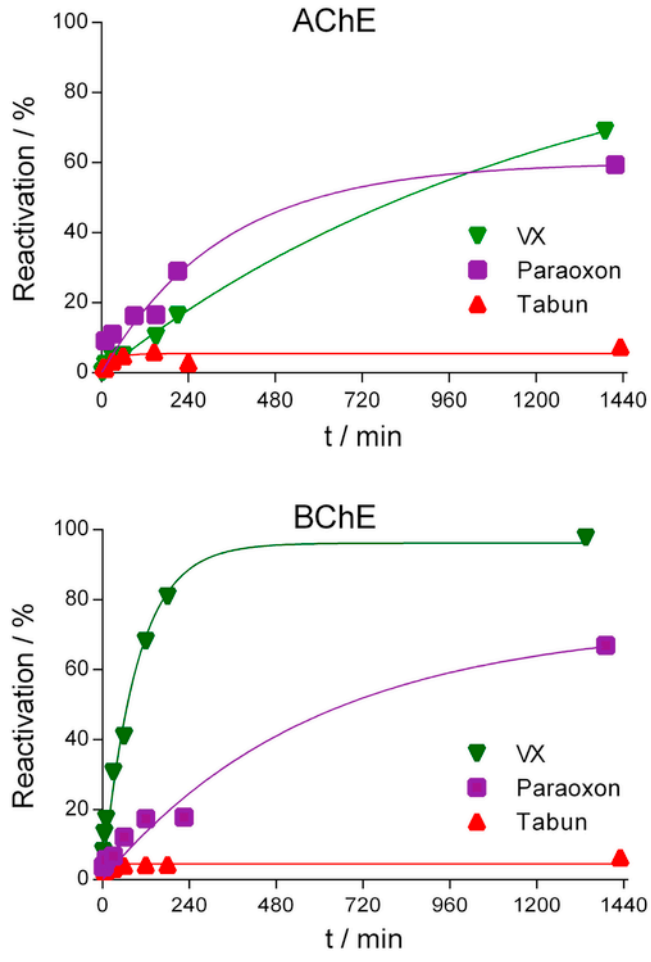

Fig. 2. Reactivation of OP-inhibited human recombinant AChE and human plasma BChE by 1 mM oxime **8**. Data presents one representative experiment with the obtained percentage of reactivation followed in time, from which the observed first-order reactivation rate constant (k_{obs}) was evaluated.

ophilic displacement of the phosphorus moiety from the active site serine during the reactivation and therefore could be used as a prediction of the nucleophile potency of the oxime molecule (Kovarik et al., 2008; Šinko et al., 2006, 2007). The reaction constants, k_{NE}, summarised in Table 5, ranged from 1 to 5 M^{-1} min^{-1} for the tested oximes, which is much lower than the ones determined for mono-oxime reactivator of AChE like HI-6 or bis-oxime reactivator TMB-4 (Kovarik et al., 2008; Šinko et al., 2006, 2007).

To visualise the interactions within the active site, we performed molecular docking. All of the oximes displayed similar results. Representative data is given in Fig. 3 and Fig. 4 for oxime **8** in the active site of hAChE and hBChE, respectively. This oxime showed the highest potency in reactivation as well as somewhat selectivity between bindings to the enzymes (K_i values). Looking in details, in the case of hAChE, poses of all oximes (20 poses for each oxime or 180 in total) formed interactions with the AChE choline binding site (Trp86, Tyr332) and peripheral binding site (Trp286, Tyr124). The majority of poses, 102, showed orientation of the oxime group toward the peripheral binding site. Only 15 of 180 poses evaluated were positioned toward the AChE catalytic serine Ser203 which is considered a favourable orientation required for the nucleophilic attack during a reactivation reaction. This 15 poses refer only to oximes **2**, **3**, **4**, **5** and **8** (Fig. 3). Resuming poses showed that the oxime group is directed toward Tyr133 and pyridinium ring creating π–π or cation–π interactions with Trp86. This is a stable binding for the oxime molecule but unfavourable for nucleophilic attack during a reactivation reaction.

In the case of hBChE, analysis of all oximes (20 poses for each oxime or 180 poses total) showed that the volume occupied by the poses was 1203 $Å^3$ (for AChE 976 Å3), which is a consequence of a larger BChE active site. This leads to a higher number of oxime poses, 20 in total positioned toward BChE catalytic serine Ser198. Here the results refer to oximes **1**, **5**, **6** and **8** (Fig. 4). Some of the poses showed an oxime group directed toward Tyr128 and pyridinium ring creating π–π or cation–π interactions with Trp82. This is an analogue mode of unfavourable oxime binding with Trp86 and Tyr133 observed in AChE. However, oximes in BChE formed the π-π interaction with Trp231 in the proximity of the catalytic Ser198. In AChE, this interaction was negligible due to the two phenylalanines of the acyl pocket, Phe295 and Phe297, which constrained the inter-

Table 2
Reactivation of OP-inhibited human plasma BChE by 1 mM oximes **1–9** recorded within 23 h. Constants were determined from at least two experiments fitting the experimental data to Eq. (1). Standard error was within 10%. Data for conventional oximes is given for comparison.

		VX-inhibited BChE			Tabun-inhibited BChE			Paraoxon-inhibited BChE		
Oxime	-R	k_{obs} (min^{-1})	React.$_{max}$ (%)	Time of React.$_{max}$ (h)	k_{obs} (min^{-1})	React.$_{max}$ (%)	Time of React.$_{max}$ (h)	k_{obs} (min^{-1})	React.$_{max}$ (%)	Time of React.$_{max}$ (h)
1	4-H	0.0034	90	12	–	<10	24	0.0011	60	23
2	4-F	0.0027	85	12	–	<10	24	0.0013	65	23
3	4-Cl	0.0017	75	23	–	<10	24	0.0004	35	23
4	4-Br	0.0009	60	23	–	<10	24	0.0004	35	23
5	4-NO_2	0.0007	35	10	–	<10	24	0.0010	20	8
6	4-CH_3	0.0051	90	8	–	<10	24	0.0013	70	23
7	2-OCH_3	0.0040	95	12	–	<10	24	0.0005	30	23
8	4-OCH_3	0.0087	95	5	–	<10	24	0.0013	65	23
9	4-Ph	0.0026	100	23	–	<10	24	0.0009	80	23
Obidoxime	–	0.069[a]	/	/	0.0096[b]	100[b]	8[b]	0.0059[b]	90[b]	12[b]
TMB-4	–	0.083[a]	/	/	0.0029[c]	60[c]	12[c]	0.0049[b]	90[b]	12[b]
HI-6	–	0.11[a]	/	/	0.0017[d]	/	/	0.0126[d]	/	/

[a] From (Sit et al., 2014).

[b] From (Kovarik et al., 2010).

[c] From (Lucić Vrdoljak et al., 2006).

[d] From (Horn et al., 2015).

Table 3
Reversible inhibition of human recombinant AChE by tested oximes **1–9**. Dissociation constants ($K_i \pm$ S.E.) were calculated by Eq. (2) from $K_{i,app}$ obtained in at least three experiments. Data for conventional oximes is given for comparison.

Oxime	-R	Oxime concentration (μM)	ATCh (mM)	$K_i \pm$ SE (μM)
1	4-H	100–300	0.05–0.5	231 ± 34
2	4-F	100–300	0.05–0.5	436 ± 85
3	4-Cl	100–330	0.05–0.5	127 ± 23
4	4-Br	83–125	0.05–0.5	76 ± 17
5	4-NO_2	200–330	0.05–0.5	167 ± 26
6	4-CH_3	100–300	0.05–0.5	104 ± 22
7	2-OCH_3	200–330	0.05–0.5	89 ± 45
8	4-OCH_3	100–300	0.05–0.5	126 ± 11
9	4-Ph	200–330	0.05–0.5	127 ± 36
TMB-4[a]	–	100–700	0.05–0.7	180 ± 10
HI-6[b]	–	/	/	20 ± 4

[a] From (Čalić et al., 2006).

[b] From (Šinko et al., 2010).

Table 4
Reversible inhibition of human plasma BChE by tested oximes **1–9**. Dissociation constants ($K_i \pm$ S.E.) were calculated by Eq. (2) from $K_{i,app}$ obtained in at least three experiments. Data for conventional oximes is given for comparison.

Oxime	-R	Oxime concentration (μM)	ATCh (mM)	$K_i \pm$ SE (μM)
1	4-H	100–300	0.05–0.5	204 ± 53
2	4-F	100–300	0.05–0.5	222 ± 56
3	4-Cl	50–300	0.05–0.5	69 ± 17
4	4-Br	17–167	0.05–0.5	24 ± 10
5	4-NO_2	100–500	0.05–0.5	112 ± 53
6	4-CH_3	50–300	0.05–0.5	164 ± 56
7	2-OCH_3	100–670	0.05–0.5	228 ± 72
8	4-OCH_3	100–670	0.05–0.5	469 ± 121
9	4-Ph	100–500	0.05–0.5	168 ± 99
TMB-4[a]	–	100–800	0.05–0.3	190 ± 40
HI-6[b]	–	500–1000	0.02–10	230 ± 20

[a] From (Lucić Vrdoljak et al., 2006).

[b] From (Škrinjarić-Špoljar and Simeon, 1993).

Table 5
Non-enzymatic reaction between the substrate acetylthiocholine and the tested oximes **1–9**. Constants ($k_{NE} \pm$ S.E.) were calculated by Eq. (3) from data obtained in at least three experiments. Data for conventional oximes is given for comparison.

Oxime	-R	Oxime concentration (μM)	ATCh (mM)	$k_{NE} \pm$ SE (M^{-1} min^{-1})
1	4-H	100–700	0.05–1.0	3.7 ± 0.09
2	4-F	100–700	0.05–1.0	5.5 ± 0.15
3	4-Cl	300–700	0.05–1.0	3.7 ± 0.09
4	4-Br	83–500	0.05–1.0	2.8 ± 0.11
5	4-NO_2	100–500	0.05–1.0	1.1 ± 0.08
6	4-CH_3	100–700	0.05–1.0	4.4 ± 0.07
7	2-OCH_3	200–700	0.05–1.0	4.8 ± 0.18
8	4-OCH_3	100–700	0.05–1.0	3.8 ± 0.08
9	4-Ph	100–500	0.05–1.0	5.1 ± 0.26
TMB-4[a]	–	100–700	0.05–0.7	31 ± 0.5
HI-6[b]	–	25–600	0.05–1.0	10.7 ± 0.1

[a] From (Kovarik et al., 2007).

[b] From (Kovarik et al., 2008).

action with analogues Trp236. This difference in allowed binding conformations between AChE and BChE may contribute to differences observed in the oxime reactivation potencies, especially a noticeable difference if comparing overall VX-inhibited AChE and BChE reactivation results.

Interestingly, such binding was also observed with the conventional oxime MMB-4 that can be related to our oximes **1–9** (Fig. 5) by somewhat similar structural characteristics including two heteroaromatic pyridinium rings, a short interconnecting linker and a presence of oxime groups that are *para*-positioned at each pyridinium ring. MMB-4 showed 5-times higher energy barrier for rotation around a single bond in the linker between two pyridinium rings and 20-times less efficient reactivation if compared to longer-linker analogues (Kovarik et al., 2008). However, MMB4 showed a superior reactivation profile to oximes **1–9** in the case of VX and paraoxon treatment (Kovarik et al., 2008; Horn et al., 2015; Luo et al., 2010), confirming the importance of accessibility of the oxime group that had not been present in our oximes.

4. Conclusion

This study presented reactivation of human recombinant AChE and purified human plasma BChE inhibited by tabun, VX and paraoxon by nine oximes, derivatives of vitamin B6, synthesized by novel eco-friendly method. Oximes showed to be the most potent in the reactivation of VX-inhibited hAChE and hBChE, which confirms that the structure of conjugate formed between cholinesterase and OP governs the reactivation efficiency. Even though the tested oximes showed to be better reactivators of hBChE than hAChE, the low observed reactivation rate compared to the available literature data on reactivation placed these oximes at a lower priority end concerning development as potential antidotes. Despite our limited reactivation results, they open a wider view on the investigation of structural features of the oximes that could enhance or impede reactivation. The proposed improvement in the structure should avoid the steric hindrance of the oxime group and a short linker between two aromatic rings which makes the structure less flexible and therefore inappropriate for a desired accommodation within the active site. However, one should keep in mind that a positive correlation between an enzyme's affinity for an oxime and its reactivation efficacy is not always present.

Disclosure

The authors declare that they have no conflict of interest. All authors approved the manuscript and its submission to the journal.

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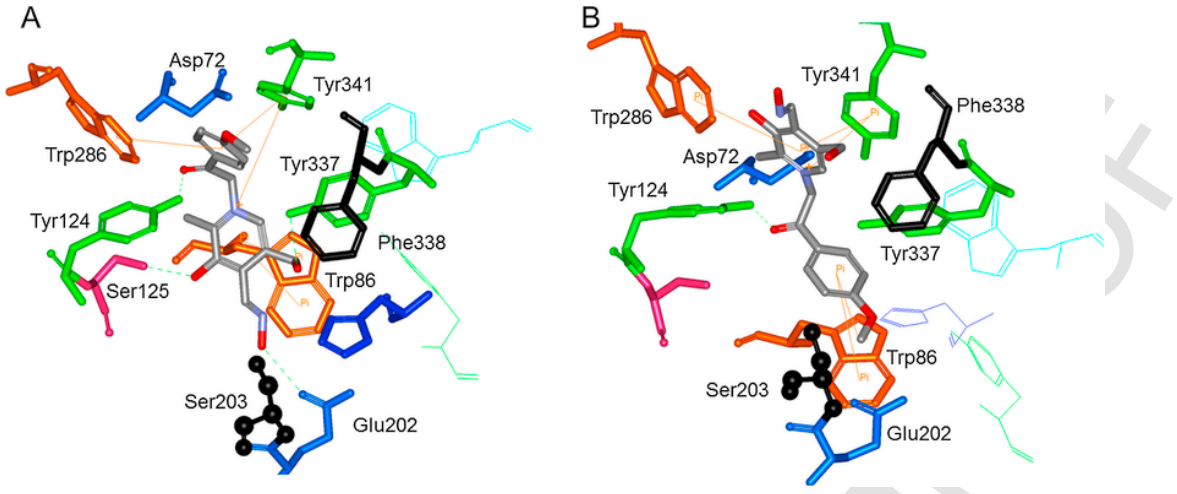

Fig. 3. Docked pose of oxime **8** in the active site of AChE. Orientation of oxime that can reactivate phosphorylated active site serine 203 (A). Oxime **8** crates H-bonds with residues Tyr124, Ser125, Glu202 and Tyr337 (green dashed lines). Hydrophobic interactions like π-π and cation-π interactions with aromatic residues are important for additional stabilisation of oxime in the active site (orange line). Alternative orientation of **8** in the active site (B). Oxime is stabilised via the same type of interactions including H-bond and hydrophobic interactions. Oxime group is far away from Ser203 to reactivate phosphorylated AChE. Hydrogen atoms are omitted for better visibility. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

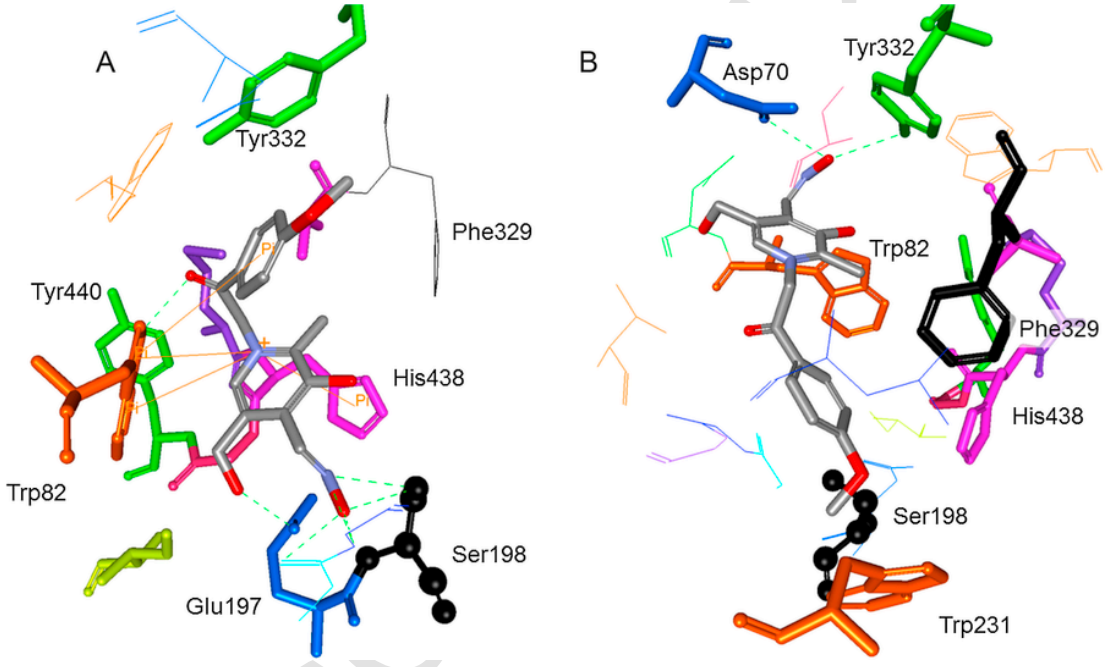

Fig. 4. Docked pose of oxime **8** in the active site of BChE. Orientation of oxime that can reactivate phosphorylated active site serine 198 (A). Oxime **8** crates H-bonds with residues Trp82, Glu197 and Ser198 (green dashed lines). Hydrophobic π-π and cation-π interactions with aromatic residues are between oxime and Trp82 and His438 (orange line). Alternative orientation of **8** in the active site (B). Oxime is stabilised via H-bonds between oxime group and Asp70, and Tyr332. Hydrophobic interaction is between methoxy group of **8** and Trp231. Oxime group is positioned away from Ser198 therefore it is not likely to reactivate phosphorylated BChE. Hydrogen atoms are omitted for better visibility. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

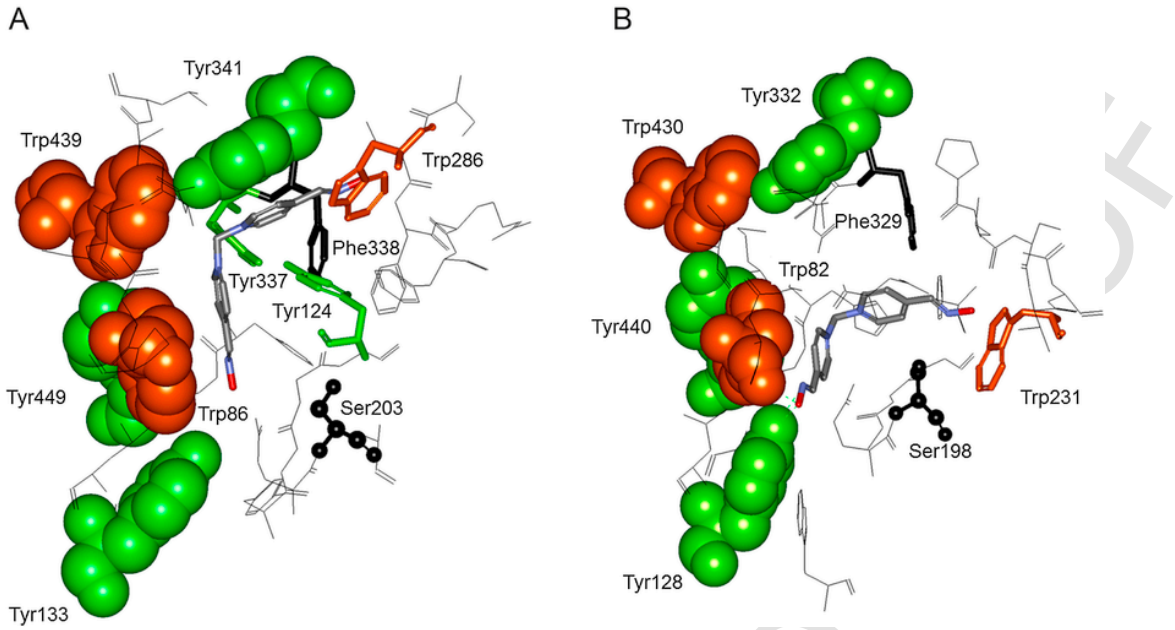

Fig. 5. Docked structure of oxime MMB-4 in to active site of hAChE (A) and hBChE (B). Conserved aromatic residues, constituents of aromatic network rich in π-electrons, are represented as space fill atoms. MMB-4 is represented as sticks, carbon (grey), oxygen (red) and nitrogen (blue). Hydrogen atoms are omitted for better visibility. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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