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A comprehensive evaluation of novel oximes in creation of butyrylcholinesterase-based nerve agent bioscavengers

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ABSTRACT

A well-considered treatment of acute nerve agents poisoning involves the exogenous administration of butyrylcholinesterase (BChE, EC 3.1.1.8) as a stoichiometric bioscavenger efficient in preventing cholinergic crises caused by acetylcholinesterase (AChE, EC 3.1.1.7) inhibition. An additional improvement in medical countermeasures would be to use oximes that could reactivate BChE as well to upgrade bioscavenging from stoichiometric to oxime-assisted catalytic. Therefore, in this paper we investigated the potency of 39 imidazolium and benzimidazolium oximes (36 compounds synthesized for the first time) to be considered as the reactivators specifically designed for reactivation of phosphorylated human BChE. Their efficiency in the reactivation of paraoxon-, VX-, and tabun-inhibited human BChE, as well as human AChE was tested and compared with the efficiencies of HI-6 and obidoxime, used in medical practice today. A comprehensive analysis was performed for the most promising oximes defining kinetic parameters of reactivation as well as interactions with uninhibited BChE. Furthermore, experimental data were compared with computational studies (docking, QSAR analysis) as a starting point in future oxime structure refinement. Considering the strict criteria set for *in vivo* applications, we determined the cytotoxicity of lead oximes on two cell lines. Among the tested oxime library, one imidazolium compound was selected for preliminary *in vivo* antidotal study in mice. The obtained protection in VX poisoning outlines its potential in development oxime-assisted OP-bioscavenging with BChE.

1. Introduction

Butyrylcholinesterase (BChE, EC 3.1.1.8) is a serine hydrolase, related to the neurotransmission-essential acetylcholinesterase (AChE, EC 3.1.1.7) (Darvesh et al., 2003). The two enzymes differ genetically, structurally and in their substrate specificities and sensitivities to a wide range of inhibitors. Though BChE is not physiologically essential for humans, it has been shown that BChE is widely distributed in the organism (Chatonnet and Lockridge, 1989; Darvesh et al., 2003; Çokuğras, 2003) and can serve as a first line of defence in the case of poisoning by highly toxic anti-acetylcholinesterase compounds like organophosphorus (OP) nerve agents and pesticides. Consequently, BChE has been explored as a possible stoichiometric bioscavenger in such a poisoning (Raveh et al., 1997; Lenz et al., 2010; Doctor and Saxena, 2005; Saxena et al., 2011). Nowadays, a newly considered treatment of acute OP poisoning involves the exogenous administration of BChE as a stoichiometric OP bioscavenger efficient in preventing inhibition of native cholinesterases and death (Cerasoli et al., 2005; Huang et al., 2007; Vučinić et al., 2013). Further, joint administration of an efficient oxime reactivator of the OP–BChE conjugate and purified BChE protein to assist catalysis and turnover of the OP, should, in theory, reduce the amount of BChE needed for efficient protection by establishing a so called "oxime-assisted catalytic bioscavenger" system (Kovarik et al., 2010; Sit et al., 2011, 2014). The feasibility of such an approach was demonstrated recently by a cationic non-pyridinium aldoxime, TAB2OH and BChE pair as well as by combination of AChE mutants and pyridinium oximes (Radić et al., 2013a; Kovarik et al., 2015; Maček Hrvat et al., 2016).

Reactivation of OP-BChE conjugate by imidazole-based oximes has been recently described in the literature as a promising step forward (Sit et al., 2011, 2014). The absence of a charge in some of these imidazole oximes bears the promise of reasonable oral bioavailability and retention in tissue as well as potential for central nervous system (CNS) penetration despite some agents having limited solubility. The quaternary analogues are expected to have smaller volumes of distribution and higher initial plasma concentrations. Quaternary imidazolium oximes were extensively studied as mono-oxime OP-AChE reactivators (Grifantini et al., 1972, 1973; Franchetti et al., 1974; Bedford et al., 1984, 1989a, 1989b; Goff et al., 1991; Koolpe et al., 1991). The attention was turned to the imidazole heterocyclic system because it was isosteric with the pyridine ring in known pyri-

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dinium oxime antidotes. Numerous derivatives were prepared and valuable structure-activity relationships found for the reactivation of OP-inhibited AChE. Some of these compounds were reactivators as good as the oxime 2-PAM, and usually showed strong affinity toward the enzyme. Spatial aryl groups were shown to enhance reactivation of the enzyme in *in vitro* experiments. Furthermore, bisquaternary imidazolium oximes in combination with pyridinium (Galoši et al., 1988; Mesić et al., 1991, 1992; Deljac et al., 1992) and quinuclidinium oximes (Simeon-Rudolf et al., 1998; Primožič and Tomić, 2004; Reiner and Simeon-Rudolf, 2006) were synthesized in order to determine whether such conjugates can have better antidotal properties compared to compounds with only one heterocyclic moiety. Some of these compounds were characterized as promising for reactivation of tabun-, VX- and soman-inhibited AChE, both *in vitro* and *in vivo* (Simeon-Rudolf et al., 1998; Reiner and Simeon-Rudolf, 2006).

Herein, we report the synthesis of a series of 39 derivatives of imidazole and benzimidazole oximes and the thorough evaluation of their reactivation efficacy of OP-BChE conjugate and the potential to be used as oximes of choice in the creation of the BChE-based OP bioscavengers.

2. Materials and methods

2.1. Synthesis of derivatives of imidazole and benzimidazole oximes

The synthesis of the desired oximes was done according to the scheme given in Fig. 1. Commercially available imidazole and benzimidazole were alkylated with different substituted haloalkanes. *N*-alkylations of commercially available heterocyclic azoles were carried out with appropriate electrophile in 50% aqueous NaOH and presence of DMSO (Simeon-Rudolf et al., 1998). *N*-alkyl/aryl imidazoles and benzimidazoles were converted to the appropriate 2-carbaldehydes by *n*-butyllithium and DMF (Iversen and Lund, 1966) and then with hydroxylamine into 2-aldoximes. Reaction with alkyl/aryl halides resulted in the desired quaternary salts (Bedford et al., 1989a, 1989b; Goff et al., 1991).

Melting points were determined in open capillaries using a Büchi B-540 melting point apparatus and are uncorrected. Elemental analyses were performed with a PerkinElmer PE 2400 Series II CHNS/O Analyser. IR spectra were recorded with a PerkinElmer FTIR 1725 X spectrometer. 1H and 13C 1D and 2D NMR spectra were recorded with a Varian XL-GEM 300 spectrometer at room temperature. Chemical shifts are given in ppm downfield from TMS as internal standard. Analysis for all compounds is given in the Supporting information to this manuscript.

2.2. pK_a determination of imidazole and benzimidazole oximes

Oxime UV spectra were recorded at different pHs on the *Analytik Jena* Specord 200 spectrophotometer in the spectral range 600–200 nm applying a 2 cm^{-1} of nominal resolution and 128 scans. The spectra were recorded at 22 °C.

2.2.1. Multivariate data analysis

Numerical analyses were performed using the second order tensor analysis tool principal component analysis PCA where data matrix (or two-way array) $\boldsymbol{X}$ of rank r is decomposed as a sum of r matrices $\boldsymbol{t}_i\boldsymbol{p}_i^{\tau}$ of rank 1

$$\boldsymbol{X} = \sum_{i=1}^{r} \boldsymbol{t}_i \boldsymbol{p}_i^{\tau}$$

where $\boldsymbol{t}_i$ stands for score and $\boldsymbol{p}_i^{\tau}$ for loading vectors. PCA finds the best linear projections for a high dimensional set of data in the least squares sense. Scores represent projections of the original points on the principal component (PC) direction and can be used for classification, whereas loadings represent eigenvectors of data covariance (or correlation) matrix and can be used for the identification of variability among the data. Development of PCA goes back to Beltrami (1873) and Pearson (1901) while the name was introduced by Hotelling (1933). More details can be found in recent literature (Jolliffe, 2002; Smilde et al., 2004).

Data obtained by UV spectral measurements of compounds were exported to ASCII format and arranged in a matrix (numbers written in a free format). Subsets of spectral data in the range from 230 to 400 nm were selected providing two-way data matrices. Data were mean-centred and PCA on the covariance matrix was carried out using our own FORTRAN code ***moonee*** (Hrenar, 2016a) based on the NIPALS algorithm (Geladi and Kowalski, 1986). Most of the calculated eigenvectors converged within a small number of iterations which took only a few seconds of computational time on an average workstation.

2.3. Chemicals for cholinesterase assays

The synthesized oximes were dissolved in DMSO or in water as 100 mM, 10 mM or 1 mM solutions. Paraoxon (Pestanal®) was purchased from Sigma-Aldrich®, St. Louis, MO, USA while tabun and VX from NC Laboratory, Spiez, Switzerland. Stock solutions of tabun and VX (5000 μg/ml) were prepared in isopropyl alcohol and further dilutions were made in water before use. Paraoxon was diluted in ethanol at 10^{-4} M just before use. 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. The final concentration of ATCh in the IC_{50} determination was 0.2 mM while in the reactivation experiments 1.0 mM. The final concentration of DTNB was 0.3 mM for all activity measurements at 25 °C.

The source of AChE and BChE was human erythrocytes and human plasma, respectively. Human whole blood (hWB) was collected from a young female donor at the Institute for Medical Research and Occupational Health, Zagreb, Croatia (IMROH). This study was reviewed and approved by the Ethics Committee of IMROH.

2.4. Oxime-assisted reactivation of OP-inhibited cholinesterases

Human plasma BChE or human erythrocyte AChE was incubated with OPs: 10^{-6} M paraoxon, 5×10^{-6} M tabun or 3×10^{-7} M VX, up to 1 h, achieving 95–100% inhibition. Such inhibited enzymes were passed through a Strata® C18-E column (Phenomenex®, Torrance, USA) to remove the excess of unconjugated OP. After filtration, the enzyme was incubated with 0.1 mM or 1 mM oximes for screening their reactivation potency. At specified time intervals (up to 22 h), an aliquot was taken and diluted 40-fold in buffer containing DTNB. The recovered enzyme activity was measured upon addition of the substrate ATCh (1 mM) by the Ellman spectrophotometric method (Ellman et al., 1961). An equivalent sample of uninhibited enzyme

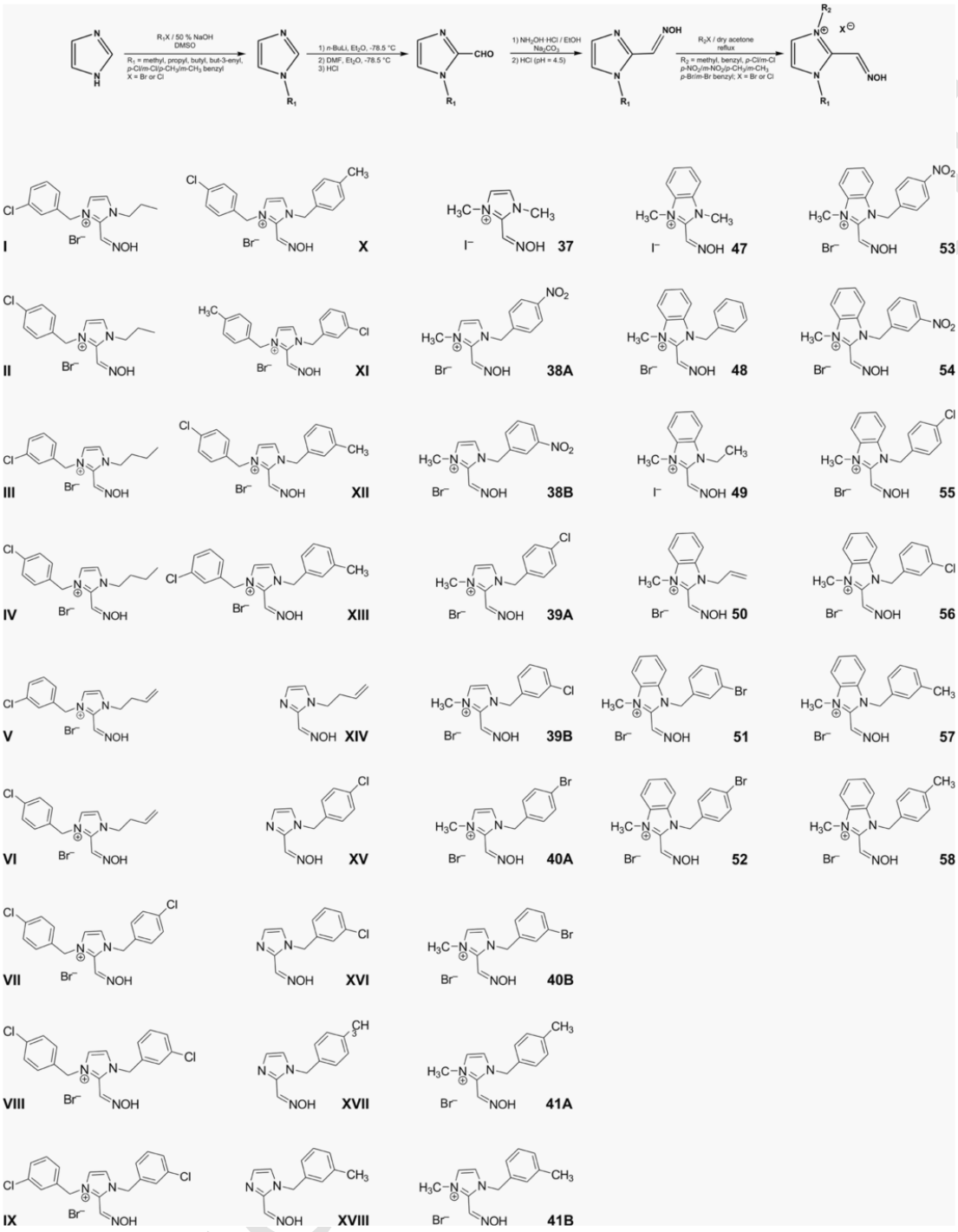

Fig. 1. General procedure for the syntheses of imidazole and benzimidazole derivatives and their structures: imidazole (I–XVIII and 37–41B) and benzimidazole (47–58) 2-aldoximes.

was passed through a parallel column, diluted to the same extent as the inhibition mixture, and control activity was measured in the presence of oxime at concentrations used for reactivation. Both activities of control and reactivation mixture were corrected for oxime-induced hydrolysis of ATCh. Enzyme activity measurements were performed at 25 °C and 412 nm, on the CARY 300 spectrophotometer (Varian Inc., Australia) with a temperature controller.

The reactivation screening was done at a given oxime concentration wherefrom the observed first-order reactivation rate constant (k_{obs}) and maximal percentage of reactivation (React.$_{max}$/%) were determined from at least two experiments as previously described (Kovarik et al., 2004). The detailed reactivation kinetics using wider oxime concentration range (from 0.02 up to 1 mM) resulted in the determination of reactivation constants: k_{max} (maximal first-order reactivation rate constant), K_{ox} (phosphorylated enzyme-oxime disso-

ciation constant) and k_r as described previously (Kovarik et al., 2004).

2.5. Determination of oxime inhibition potency for BChE, IC_{50} values

To determine IC_{50} values (the concentration of an oxime at which it inhibits 50% of enzyme activity) we measured human plasma BChE activity in the presence of a wide range of oximes´ concentrations ensuring 0–100% inhibition compared to the control activity. The assay was performed in the 96 well-plates on the Tecan Infinite M200PRO plate reader. The inhibition mixture (300 μL final) contained a buffer, BChE, oxime and DTNB (0.3 mM final), and by the addition of the ATCh (0.2 mM final), the activity was assayed by Ellman's method (Ellman et al., 1961). For oximes dissolved in DMSO, the final DMSO concentration was kept under 0.5% to eliminate its influence on BChE activity. The measured activity was corrected for oxime-induced hydrolysis of ATCh.

The IC_{50} values were determined from at least two experiments by a nonlinear fit of the oxime concentration logarithm values *vs.* % of BChE activity using Prism6 software (Graph Pad Software, San Diego, USA).

2.6. Quantitative structure-activity relationship (QSAR) regression models

In order to find the best molecular descriptor for the estimation of IC_{50} oxime value, more than a 1000 molecular descriptors were calculated using the e-DRAGON software (VCCLAB, Virtual Computational Chemistry Laboratory, http://www.vcclab.org, 2005) and correlated with experimentally obtained IC_{50}. All regression calculations, including the leave-one-out procedure (LOO) of cross validation, were done using the CROMRsel program (Lučić and Trinajstić, 1999). The standard error of the cross validation estimate ($S.E._{cv}$) is defined as:

$$S.E._{cv} = \sqrt{\sum_i \frac{\Delta X_i^2}{N}}$$

where ΔX and N denotes cv residuals and the number of reference points, respectively. For all data sets, the most consisting models for the prediction of IC_{50} were obtained with the Eccentric Connectivity Index, ξ^c (Sharma et al., 1997).

2.7. Molecular modelling studies

The coordinates of BChE enzyme inhibited with VX (2xqf) (Wandhammer et al., 2011) were obtained from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) and the model of the active site was built. Quantum mechanical docking calculation was performed by systematic scanning of the BChE active site using a search algorithm implemented in our program code ***qcc*** (Hrenar, 2016b). Three molecular translational degrees of freedom were scanned with a step of 1.0 Å whereas three molecular rotational degrees of freedom were scanned with increments of 45°. Besides these 6 molecular degrees of freedom, rotations around 7 single bonds that correspond to the torsional movements of compounds were also investigated. Single point calculation for the model of active site and investigated compound was performed using the PM6 method from Gaussian 09 program package (Frisch et al., 2013). All energy values from single point calculations were arranged in the 13-way array and search for all local minima was performed by using a combinatorial algorithm built in our program for multivariate analysis ***moonee*** (Hrenar, 2016a). Selected local minima were subjected to geometry optimization procedure using the semiempirical PM6 method and subsequent clustering of geometries was performed and classified on the basis of the energy values. The results were inspected visually and on the basis of the energy values one structure was selected and reoptimized using the QM/QM 2-layer ONIOM approach with semiempirical PM6 method for the outer layer, and density functional theory B3LYP/6-31G(d) method, for the inner layer of the system (Lee et al., 1988; Becke, 1993).

2.8. Cytotoxicity screening

Human Caucasian hepatocyte carcinoma (HepG2) and Human monocytic leukaemia (THP-1) were used for oxime cytotoxicity profiling. Cells were cultured 24 h in the presence of the oximes (0.5–500 μM) selected on the basis of structural differences. Cytotoxicity was determined using the standard MTS detection reagent assay measuring the succinate dehydrogenase mitochondrial activity of living cells (Mosmann, 1983). Procedure followed a previously described protocol (Katalinić et al., 2010). Cells and oximes were incubated for 24 h at 37 °C. 5 μL of MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) was added in each well, incubated 0.5–3 h (different cell lines) after which the absorbance was read at 492 nm on an EnVision (Perkin Elmer) spectrophotometer. Total percentage of DMSO in cytotoxicity assay was 0.5%. The assay was performed in 384-well in 50 μL/well media volume (Corning flat clear bottom (black) with 20,000 or 30,000 cells (for THP-1) and 10,000 or 20,000 cells (for HepG2). Pipetting was performed using Janus (Perkin Elmer) and Mosquito (TTP Labtech) instruments. Data was evaluated using predefined IC_{50} eqn. from GraphPad Prism program.

2.9. Preliminary ex vivo and in vivo studies

For the VX detoxification *ex vivo*, hWB was supplemented with the 0.3 μM BChE and incubated with 1.5 μM VX. After 30 min of inhibition (achieving 95–100% inhibition), oxime VI (0.1 or 0.05 mM) was added to the mixture. At specified time intervals, an aliquot was taken for enzyme activity measurements by a previously described method. Preliminary results were expressed as percentage of control activity. Obtained results are given in the Supporting information to this manuscript.

For the *in vivo* study we used male CD-1 mice of 25–30 g body weight (purchased from Ruđer Bošković Institute, Zagreb, Croatia). Mice were fed on a standard diet, had free access to water and were kept in Macrolone cages at 21 °C, exchanging light and dark cycles every 12 h. Nerve agent stock solutions were prepared in isopropyl alcohol. Immediately before use, further dilutions were made in physiological saline. Oxime VI was dissolved in saline. An estimation of acute intravenous toxicity of oxime VI was based on the administration of fixed dose to two mice and observation of clear signs of toxicity including 24 hour mortality (Chinedu et al., 2013). The result of this test was used for selecting the subsequent dose in testing its antidotal properties. Further on, mice were pretreated intravenously with oxime VI (2 mg/kg) and BChE (0.4 mg/kg) 5 min prior to VX *s.c.* exposure, and then treated by atropine (10 mg/kg, *i.m.*). Four doses of VX were used (5, 6.3, 7.9 and 10 × LD_{50}; LD_{50} of VX was 28.3 μg/kg) and four animals were injected per dose. The antidotal efficacy of treatment was expressed as a protective index (PI) with 95% confidence limits and maximal dose of poison (MDP). The PI was the ratio of LD_{50} between VX with antidote treatments and VX

given alone. The MDP was the maximal dose of the VX LD_{50}, which was fully counteracted by the treatment applied. The mice were treated in accordance with the permission of the Ethics Committee of the Institute for Medical Research and Occupational Health in Zagreb, Croatia.

3. Results and discussion

Recent studies distinguished imidazolium oximes as potential oximes of choice in the creation of a BChE-based OP-catalytic scavenging system (Sit et al., 2011, 2014). According to these findings, we synthesized and profiled 39 differently substituted imidazole and benzimidazole derivatives as reactivators of the OP-BChE conjugate. The structure of the studied oximes is given in Fig. 1.

N-alkylated imidazoles and benzimidazoles, which were not commercially available, were synthesized in a reaction with differently substituted alkyl halides. Prepared *N*-substituted imidazole and benzimidazole derivatives were converted to the appropriate 2-carbaldehydes, followed by the conversion to the appropriate aldoximes. Quaternization was carried out with methyl, ethyl, allyl iodide, as well as with differently substituted *meta* and *para* benzyl bromides. A synthesis of imidazole derivatives is outlined in Fig. 1. Benzimidazole derivatives were prepared in the same manner. All of the compounds were prepared in good yields and were characterized by standard analytical spectroscopy methods (1D and 2D NMR, IR, MS, elemental analysis; presented in the Supplemental information to this paper). Such a synthesis enables easier scale-up if any oxime shows potency for further investigations. Novel oximes were designed to comply with the structural characteristics of the enzyme's active site or finding that the hydrophobic moiety positioned several methylene groups from the imidazole ring favours reactivation (Sit et al., 2014). Previously, we focused our studies mainly on the charged oximes (Čalić et al., 2006; Kovarik et al., 2008; Žunec et al., 2015), however the uncharged oximes emerged as leads in recent studies (Sit et al., 2011; Radić et al., 2012, 2013a, 2013b; Kovarik et al., 2013). Although uncharged oximes should possess better characteristics for crossing the biological barriers, their solubility and *in vivo* administration still present a challenge. Furthermore, considering that plasma is the primary site of BChE's OP-scavenging based action, charged oximes could present a better choice due to their preferable bioavailability (Katalinić et al., 2015).

We started our investigation by determining the pK_a values for the oximes to evaluate oximate concentrations at physiological pH. 2-PAM was used as the oxime standard. Obtained results are summarised in Fig. 2 and Table 1.

pK_a values of neutral derivatives ranged between 10.3 and 10.4 for measured imidazole derivatives, and 9.7–9.8 for chosen benzimidazole derivatives. The impact of the 2-aldoxime group on the basicity of the imidazole and benzimidazole ring free nitrogen atom are noticeable in the decrease of pK_a values, and the same effect, only smaller, is shown by replacing the *N*-substituent with the more electron withdrawing group. On the other hand, pK_a values for the oxime group of quaternary imidazoles ranged from 8.0–8.3 (similar to that of 2-PAM; Fig. 2b) and for the tested quaternary benzimidazole compounds 7.4 and 7.6. Therefore, regardless of the moieties added to the main ring, lower pK_a values were obtained for benzimidazolium oximes and were similar to the classical *ortho*-pyridinium oximes like HI-6, while imidazolium oximes had pK_as similar to the classical *para*-pyridinium oximes like TMB-4 or obidoxime (Gray, 1984; Šinko et al., 2006). Such a result gives stronger influence to the benzimidazolium group of oximes in terms of dissociated oxime group concentration and nucleophilicity.

Further on, oximes were screened for their efficiency to reactivate tabun-, VX and POX-inhibited BChE. The obtained results are given in Fig. 3 and presented as k_{obs}, the reactivation rate constants observed for the studied oxime concentration.

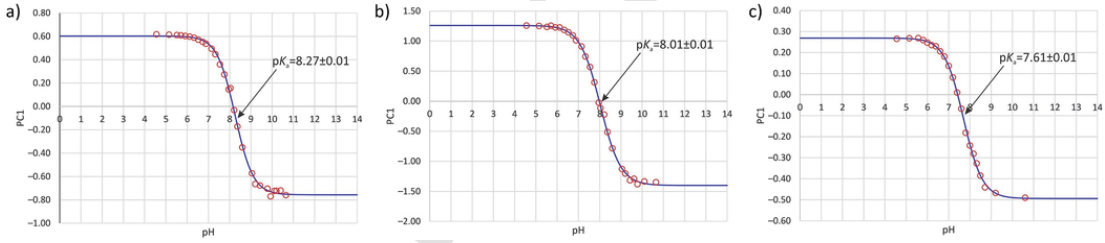

Fig. 2. pK_a determined by principal component analysis conducted on a set of pH dependent UV spectra (nonlinear regression carried out on sigmoidal function) for compounds: a) 37, b) 2-PAM and c) 47.

Table 1
pK_a (± S.D.) determined by principal component analysis conducted on a set of pH dependent UV spectra for imidazole and benzimidazole derivatives.

Compound	$pK_{a,1}$ (ring *N* atom)	$pK_{a,2}$ (oxime)	R_1	R_2	Anion
Imidazole	7.33 ± 0.02				
VI		8.01 ± 0.02	*p*-$ClC_6H_5CH_2$	But-3-enyl	Br^-
X		8.13 ± 0.02	*p*-$CH_3C_6H_5CH_2$	*p*-$ClC_6H_5CH_2$	Br^-
XIV	5.502 ± 0.003	10.37 ± 0.01	But-3-enyl	–	–
XV	4.99 ± 0.01	10.24 ± 0.01	*p*-$ClC_6H_5CH_2$	–	–
XVII	5.131 ± 0.003	10.31 ± 0.01	*p*-$CH_3C_6H_5CH_2$	–	–
37		8.27 ± 0.01	Methyl	Methyl	I^-
41A		8.24 ± 0.01	*p*-$CH_3C_6H_5CH_2$	Methyl	Br^-
Benzimidazole	5.502 ± 0.003				
N-methylbenzimidazole-2-aldoxime	4.597 ± 0.004	9.77 ± 0.01	Methyl	–	–
47		7.61 ± 0.01	Methyl	Methyl	I^-
N-benzylbenzimidazole-2-aldoxime	4.04 ± 0.02	9.76 ± 0.01	$C_6H_5CH_2$	–	–
N,*N*-dibenzyl-2-hydroxyiminomethylbenzimidazolium bromide		7.37 ± 0.01	$C_6H_5CH_2$	$C_6H_5CH_2$	Br^-

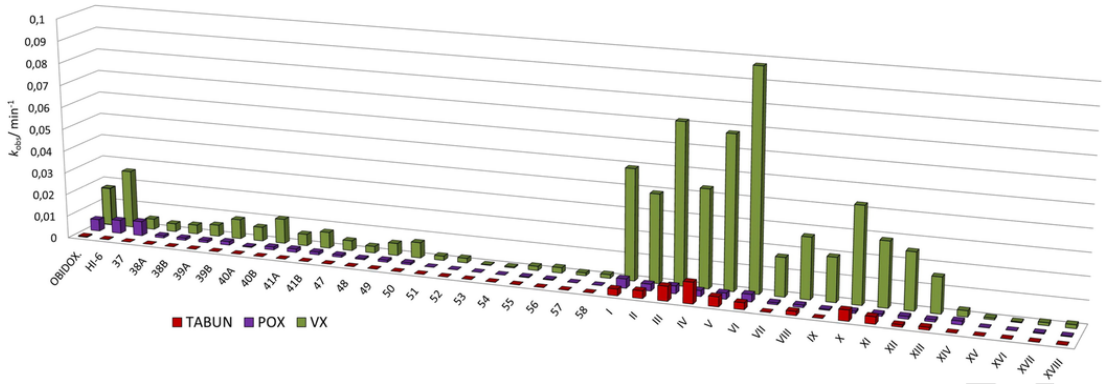

Fig. 3. Observed reactivation rate constant (k_{obs}) of OP-inhibited human plasma BChE by 0.1 mM oximes 37–58, I–XVIII recorded within 22 h. Constants were determined from at least two experiments with the standard error within 10%. Obidoxime and HI-6 were 1.0 mM for tabun, 1.0 mM and 0.1 mM for VX, and 0.1 mM and 1.0 mM for paraoxon, respectively (Katalinić, 2011; Horn et al., 2015).

As can be seen, imidazolium and benzimidazolium oximes fell short of reactivating tabun- or POX-inhibited BChE efficiently. However, a group of imidazolium oximes I-XIII, carrying two substituents on position 1 (aromatic) and 3 (aliphatic or aromatic) on the imidazolium ring, surfaced in reactivation of VX-inhibited BChE. Five of the tested uncharged imidazolium oximes XIV–XVIII did not reactivate OP-inhibited BChE in tested concentration, though based on the Sit et al. (2014), we anticipated a different result especially for oxime XVI as a structural analogue of favourable oximes from that study. It could be that the used concentration of the enzyme *vs.* the concentration of the oxime (almost 7 times lower than in Sit et al. (2014)) was too low to assure efficient oxime binding to the OP-BChE conjugate. Next, in order to quantify the reactivating potencies for the most promising oximes, we performed detailed kinetic studies determining all relevant kinetic constants (Table 2). Neither for tabun-inhibited nor for POX-inhibited BChE, the evaluated oximes did not reach effectiveness of bispyridinium oximes like K117 (Kovarik et al., 2010) or obidoxime (Horn et al., 2015), respectively, though the OP-inhibited BChE showed a binding in μM range (K_{OX}) for most of them. For VX-inhibited BChE, some promising imidazolium oxime leads were detected with reactivation rate constant, k_{max}, higher than 0.1 min^{-1} and reactivation maximum of 90% reached within 30 min. Their structure is similar to the structure of the efficient imidazolium oximes from a previous study by Sit et al. (2014). However, since in that study the k_{obs} values were calculated using a different approach, we cannot directly compare the efficiency values. Among our oximes, we selected imidazolium oxime VI (K_{OX} = 0.021 mM, k_{max} = 0.15 min^{-1}) for further studies of BChE based VX-scavenger and as the starting structure for future refinement. One representative experiment showing the reactivation profile of the oxime VI is given in Fig. 4.

Table 2
Reactivation kinetic studies of tabun-, paraoxon- or VX-inhibited human BChE with the most efficient oximes. The kinetic parameters (± S.E.): first order reactivation rate constant (k_{max}), phosphorylated enzyme-oxime dissociation constant (K_{OX}), the second order reactivation rate constant (k_r), reactivation maximum (React.$_{max}$), time of reaching the reactivation maximum (t) were determined from at least 3 experiments.

Compound (mM)	k_{max} (min^{-1})	K_{OX} (mM)	k_r (M^{-1} min^{-1})	React.$_{max}$ (%)
BChE + tabun				
IV (0.02–0.5)	0.0092 ± 0.0007	0.025 ± 0.0085	362 ± 124	80
BChE + POX				
37 (0.02–1.0)	0.029 ± 0.0067	0.74 ± 0.32	39 ± 19	100
I (0.01–1.0)	0.0042 ± 0.0001	0.018 ± 0.0028	230 ± 36	60
III (0.01–1.0)	0.0055 ± 0.0005	0.069 ± 0.024	80 ± 29	65
VI (0.005–0.5)	0.0037 ± 0.0001	0.0083 ± 0.0014	440 ± 76	70
Obidoxime[a] (0.02–1.0)	–	–	72 ± 5.5	100
BChE + VX				
I (0.01–1.0)	0.12 ± 0.02	0.12 ± 0.06	975 ± 500	95
III (0.005–0.5)	0.059 ± 0.0035	0.012 ± 0.0036	5100 ± 1600	85
V (0.002–0.5)	0.12 ± 0.008	0.020 ± 0.006	6200 ± 1900	95
VI (0.005–0.5)	0.15 ± 0.01	0.021 ± 0.006	6900 ± 2200	90
X (0.002–0.3)	0.063 ± 0.011	0.060 ± 0.029	1050 ± 540	85
HI-6 (0.005–1.0)	0.12 ± 0.03	0.37 ± 0.23	330 ± 230	85
Obidoxime[a] (0.005–1.0)	–	–	230 ± 16	90

[a] Due to a linear dependence of k_{obs} *vs.* oxime, k_{max} and K_{OX} could not be calculated separately.

Based on the knowledge of the mechanism of BChE reactivation inhibited by VX, we have chosen the most probable orientation of the reactivator from the obtained docked structures. The best possible orientation of compound VI, which can result in an attack on the phosphorus atom, is displayed in Fig. 5. Subsequent ONIOM calculation positioned the oxime group in such a way that the oxygen atom was pointed toward the phosphorus VX atom, the distance being 3.68 Å. That position is optimal for the reaction to occur (Fig. 5). The double bond of the *N*-but-3-enyl group is positioned in the vicinity of the Pro285 carbonyl group (2.3 Å) and Ser287 (≈ 3.1 Å), also having close contact with Leu286. The aromatic moieties of the compound are positioned halfway down the gorge interacting mainly with aromatic amino acids. Benzyl ring are in the choline binding site and have close contacts with the indole Trp82 ring, as well as Tyr332, which is close to the imidazole ring as well. The chlorine atom of the benzyl moiety is pointed toward Met437, having close contact with Tyr332, Ala328, and Tyr440. The results obtained from molecular modelling gave us better insight into BChE–VX–oxime interactions and structural requirements for good reactivation properties of imidazole oximes.

Furthermore, we estimated the binding affinities of BChE for oximes in terms of IC_{50} values. The obtained results are summarised in Fig. 6. Most of the tested oximes showed an IC_{50} smaller than 20 μM. If compared to BChE's affinity for classical pyridinium oximes (inhibition constants, K_i, in mM range; Čalić et al., 2008; Kovarik et al., 2009), it is clear that imidazolium oximes fit the active site more efficiently. Such a result confirms the concept applied for our oximes' structure design. The highest affinity was observed for oximes VII–XIII with a 1,3-aromatic substituent on the imidazolium ring demonstrating the importance of interaction with the BChE active site aromatic residues, just as it is for AChE (Ekström et al.,

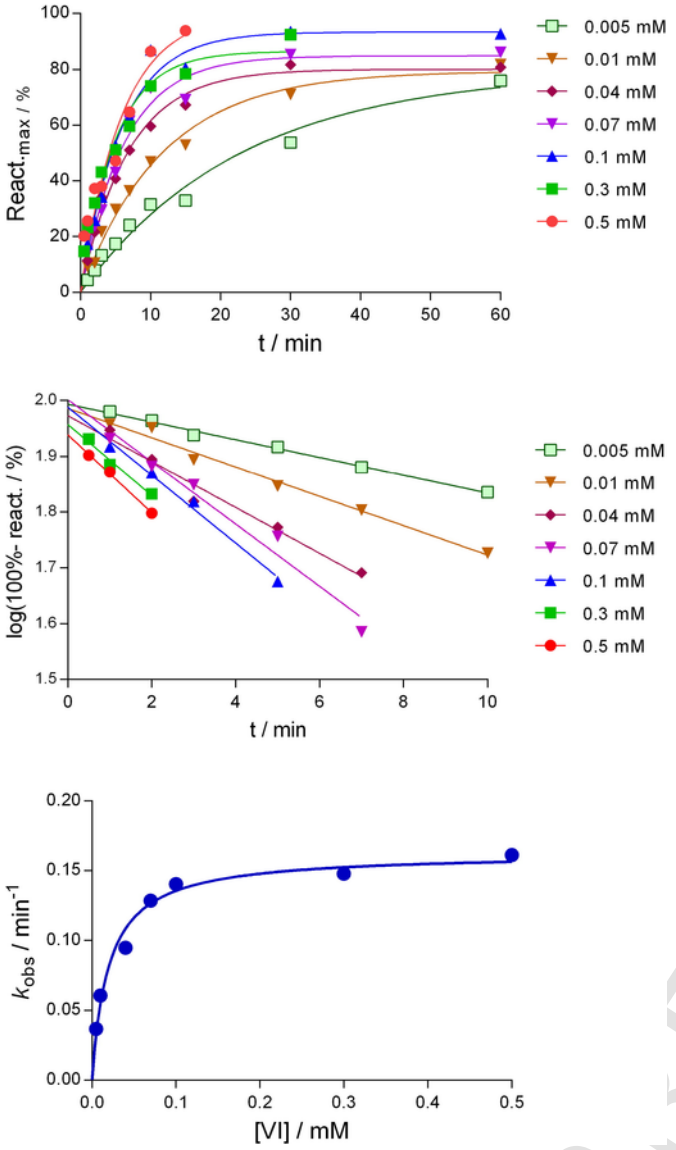

Fig. 4. Representative experiment showing the reactivation profile of oxime VI assisted reactivation of VX-inhibited BChE.

2006). In addition, looking at our lead oxime VI, the affinity was not affected by the presence of the OP moiety bound to the catalytic serine (K_{OX} *vs.* IC_{50}) indicating an overall favourable positioning of this oxime in the active site. This also confirms the positioning predicted by molecular docking studies.

We also performed a QSAR analysis to obtain models for the prediction on experimental pIC_{50} (negative logarithm of IC_{50}) values of oximes. The initial set (34 oximes with measured IC_{50} values) was divided into three subsets based on the oxime's general structural characteristics: imidazolium (37–41B), benzimidazolium (47–58) and benzyl imidazolium (I–XIII). For all data sets the most consistent models, both in regression *a* (slope) and *b* (intercept) and statistic parameters (*r*, S.E., and $S.E._{cv}$) were obtained with the Eccentric Connectivity Index, ξ^c (Fig. 7, Table 3) (Sharma et al., 1997). The almost equal *a* and *b* in separate models enabled us to develop a common model for all three subsets. As all ξ^c models are equally good in connecting structure and activity of oximes, this emphasises the potency of this index for designing a new oxime with the desired properties.

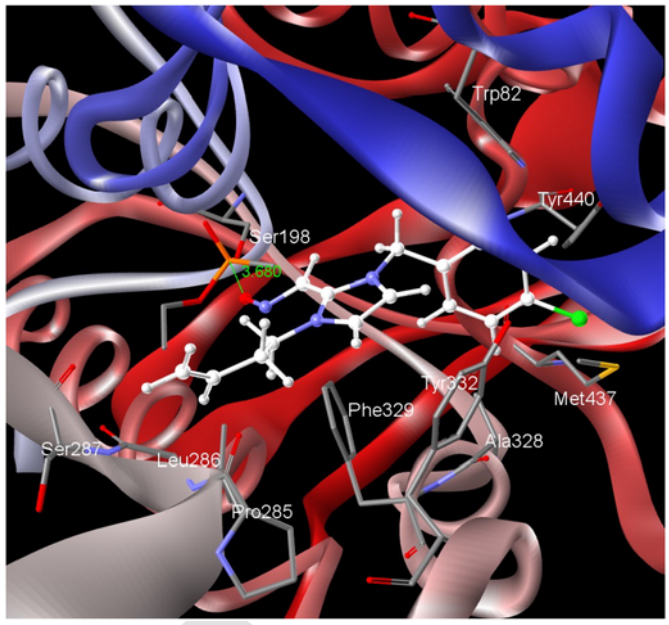

Fig. 5. Geometry of the compound VI in the active site of human BChE inhibited with VX obtained by ONIOM calculations. Oxime oxygen (NO^-) is pointed toward the phosphorus VX atom, distance being 3.68 Å (green line). Only amino acids in close contacts (< 4 Å) with the compound and Ser198 are displayed. Hydrogen atoms of amino acid are omitted for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ξ^c is an adjacency-cum-distance based topological descriptor that was successfully used in modelling various biological activities (Sardana and Madan, 2000, 2002a, 2002b). Generally, the advantage of topological indices over other molecular descriptors is their simplicity, and they can usually be calculated by using only paper and pencil (Trinajstić, 1983; Todeschini and Consonni, 2000).

Though a limitation of QSAR analysis in general is the inefficiency to extrapolate data beyond the structures used for the model, the 34 oximes used to build up this one differ enough to ensure a good source for a lead oxime structure refinement. The goal is to search for potential substituents on the imidazolium ring that would result in oximes with the desired IC_{50} values (like oxime VI). Such oximes could result with the similar binding pattern, and therefore, efficient reactivation of OP-BChE conjugate.

We also screened oxime potency to reactivate tabun-, VX- and POX-inhibited AChE, and the obtained results in terms of k_{obs} values are given in Fig. 8. None of the 18 oximes at 1 mM concentration reactivated POX- or tabun-inhibited AChE efficiently as it was for BChE. Reactivation of VX-inhibited AChE revealed a different profile. Oximes IX, X and XIII emerged as the most potent ones with k_{obs} around 0.1 min^{-1} and reactivation completed within an hour. The lead oxime VI was five times less potent in restoring VX-inhibited AChE than VX-inhibited BChE activity, thus fulfilling one of the requests for the BChE-based scavenging system. Although none of the oximes showed improvement in VX-inhibited AChE reactivation compared to standards like HI-6 and obidoxime, we performed detailed kinetic studies to evaluate their overall activity (Table 4).

Moreover, we performed cytotoxicity screening for our oximes to asses any influence on the cell viability that they might display. All drugs have to pass such a test before being considered for *in vivo* use. For this screening we selected several oximes based on the most prominent structural differences. Oxime effect was tested up to 500 μM concentration on two representative cell lines HepG2 (human Caucasian hepatocyte carcinoma, epithelial) and THP-1

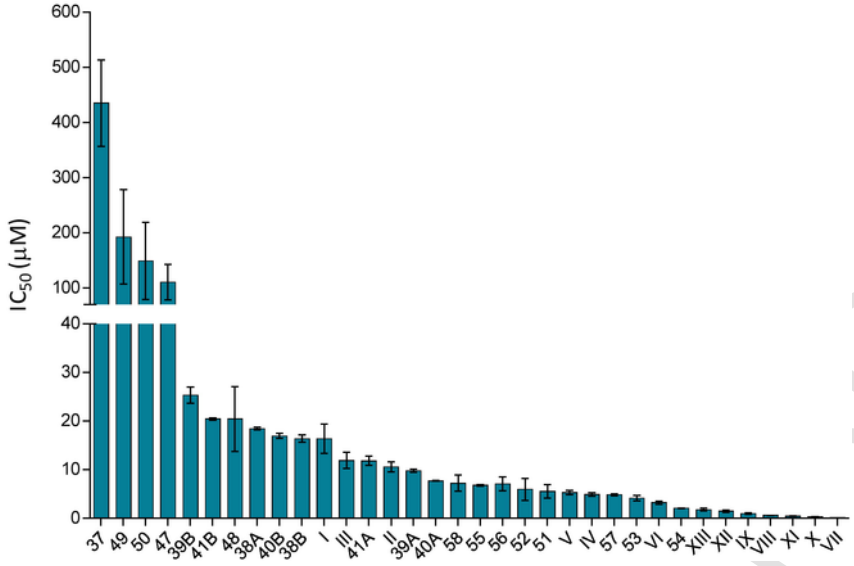

Fig. 6. Inhibition of human plasma BChE by oximes, expressed as IC_{50} (± S.E.). Inhibition was evaluated at 0.2 mM substrate ATCh from at least two experiments.

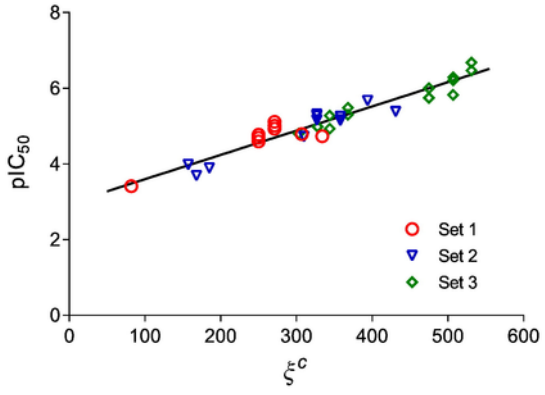

Fig. 7. QSAR regression model. Dependence of negative logarithm of IC_{50} (pIC_{50}) on ξ^c (Eccentric Connectivity Index) for 34 oximes (Set 1 (oximes 37–41B), Set 2 (oximes 47–58) and Set 3 (I–XIII)).

Table 3
Regression models for the estimation of pIC_{50} of 34 oximes divided in three subsets; Set 1 (oximes 37–41B), Set 2 (oximes 47–58) and Set 3 (I–XIII). Equation used for the model: $IC_{50} = a\xi^c + b$; where *a* denotes slope and *b* intercept. Values *r*, S.E. and $S.E._{cv}$ denote coefficient of correlation, standard error and standard error of cross-validation.

Set	*N*	Regression coefficients		*r*	S.E.	$S.E._{cv}$
		a (S.E.)	*b* (S.E.)			
1	9	0.0062 (13)	3.10 (35)	0.870	0.23	0.54
2	12	0.00700 (73)	2.73 (23)	0.950	0.20	0.25
3	13	0.00675 (65)	2.79 (29)	0.952	0.18	0.21
All	34	0.00641 (34)	2.96 (12)	0.957	0.21	0.23

(human monocyte leukaemia) by the standard MTS assay. Results are summarised in Table 5.

As the results indicate, many of our lead group imidazolium oximes (I–XIII) were deadly to the cells in the studied concentration range. Compared to pyridinium oximes like TMB-4 and HI-6, our oximes seemed to be much more cytotoxic (Čalić et al., 2006). The strongest effect was displayed by oximes VII-XII with 1,3-aromatic substituents on the imidazolium ring for which we also observed the highest affinity for BChE. It seems that such a three-ring system interferes with the other biological targets present at the cell level and it would be of an interest to investigate the mechanism behind their cytotoxicity. However, such an effect limits their consideration as potential antidotes. On the other hand, our lead imidazolium oxime VI, differing in the substituents on the imidazolium ring from the mentioned oximes, was far less toxic. Since it was effective in VX-BChE conjugate reactivation in concentrations well below the cytotoxicity IC_{50} of 250 μM, we could continue investigating its scavenging capacity and structural refinements.

Therefore, preliminary *ex vivo* and *in vivo* tests were performed resulting in a promising starting point for further investigation (Supplemental information). *Ex vivo* VX detoxification studies showed that in the human whole blood (hWB) combination of 0.1 or 0.5 mM VI and 0.3 μM BChE will degrade 1.5 μM VX within 25 min (Fig. S1). Further, the acute toxicity of oxime VI after intravenous administration was estimated by a sequential dosing of two mice (Chinedu et al., 2013). After the administration of the first dose, 100 mg/kg, mortality was recorded so the subsequent dose was adjusted downward. After the administration of the second dose, 10 mg/kg and 5 mg/kg, behavioural signs of toxicity were noted (strong tremor for 10 mg/kg, light tremor for 5 mg/kg) but no mortality occurred. This result was used as a basis for selecting a dose of 2 mg/kg of oxime VI in testing its antidotal properties according to the previously described protocols (Radić et al., 2013a) including several VX doses and four animals per each dose. Preliminary *in vivo* results revealed that the application of 2 mg/kg VI and 0.4 mg/kg BChE (*i.v.*) as a pretreatment protected animals from 6.3 × LD_{50} of VX (Table S1). If compared to some previously published results on the BChE-based reactivator TAB2OH done by our group (Radić et al., 2013a), this is an improvement in the overall outcome of the therapy. Namely, pretreatment and treatment with a combination of hBChE and a non-pyridinium oxime TAB2OH ensured survival up to 3.4 × LD_{50} of VX. hBChE alone ensured survival of only 1 × LD_{50} of VX in concentration up to 1 mg/kg (Radić et al., 2013a).

4. Conclusions

We synthesized and profiled 39 differently substituted imidazole and benzimidazole derivatives as reactivators of OP-inhibited BChE.

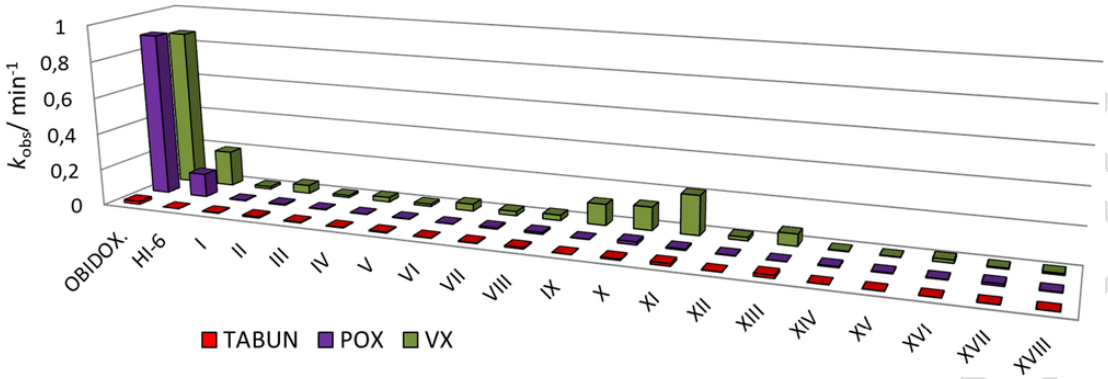

Fig. 8. Observed reactivation rate constant (k_{obs}) of OP-inhibited human erythrocyte AChE by oximes I-XVIII recorded within 22 h. Oximes were tested in 1 mM concentration or 0.5 mM for oximes VIII–XIII in VX/tabun reactivation. Constants were determined from at least two experiments with the standard error within 10%. Obidoxime and HI-6 were 1.0 mM for tabun, 1.0 mM and 0.1 mM for VX, and 0.1 mM and 1.0 mM for paraoxon, respectively (Katalinić, 2011; Worek et al., 2004).

Table 4
Reactivation kinetic studies of tabun- and VX-inhibited human AChE with the most efficient oximes. The kinetic parameters (± S.E.): first order reactivation rate constant (k_{max}), phosphorylated enzyme-oxime dissociation constant (K_{OX}), the second order reactivation rate constant (k_r), reactivation maximum (React.$_{max}$), time of reaching the reactivation maximum (t) were determined from at least 3 experiments.

Compound (mM)	k_{max} (min^{-1})	K_{OX} (mM)	k_r (M^{-1} min^{-1})	React.$_{max}$ (%)	t (h)
AChE + tabun					
X (0.02–0.5)	0.0049 ± 0.0007	0.025 ± 0.018	196 ± 140	80	12
AChE + VX					
VIII (0.01–0.5)	0.030 ± 0.0035	0.043 ± 0.018	703 ± 298	90	2
IX (0.02–1.0)	–	–	286 ± 15	90	0.8
X (0.02–0.5)	0.14 ± 0.026	0.18 ± 0.079	809 ± 386	90	0.8
XI (0.–0.5)	0.047 ± 0.0066	0.038 ± 0.023	1240 ± 758	80	0.8
XIII (0.–0.5)	0.081 ± 0.018	0.22 ± 0.12	372 ± 222	80	0.8

Table 5
Cytotoxicity of selected oximes on two cell lines (HepG2, human Caucasian hepatocyte carcinoma, epithelial; THP-1 human monocyte leukaemia) determined by measuring the succinate dehydrogenase mitochondrial activity of living cells (MTS assay; Mosmann et al., 1983). IC_{50} values (± S.D.) were evaluated from at least two experiments.

Compound	IC_{50} (μM)	
	HepG2 cells	THP-1 cells
37	> 500	> 500
40 A	> 500	264 ± 4
47	> 500	> 500
49	> 500	> 500
58	> 500	241 ± 2
I	> 500	> 500
VI	> 500	259 ± 9
VII	189 ± 48	67 ± 11
IX	305 ± 9	75 ± 1
X	227 ± 15	66 ± 6
XII	282 ± 30	75 ± 3

Synthesis was performed in a way that would enable an easier scale-up if any oxime shows potency for further investigations. From all of the oximes tested here, we selected oxime VI as the lead for further investigations and structure refinements, based on the overall obtained promising reactivation profile and showing selectivity toward BChE reactivation. Moreover, preliminary *ex vivo* and *in vivo* tests support further investigation.

If looking at the overall results, the differences of the OP-enzyme conjugate structure for the reactivation came forward, showing that a formation of equally good or universal antidote is a tough challenge to conquer. However, the difference between AChE and BChE conjugates enables focused research on selective reactivators fulfilling the requests for the BChE-based scavenging system. Furthermore, the observed cytotoxicity of some of the synthesized oximes revealed the importance for such studies at the very beginning of antidote development. Any unwanted effect on the viability of cells presents a shortcoming in the consideration of these oximes in medical practice.

Ethical approval

All procedures performed in studies involving human blood and mice were in accordance with the approval obtained by Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia at which the studies were conducted.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Supplementary data

Preparation of tested oximes. *Ex vivo* and *in vivo* experimental results. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.taap.2016.09.015.

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