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Counteracting tabun inhibition by reactivation by pyridinium aldoximes that interact with active center gorge mutants of acetylcholinesterase

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Abstract

Tabun represents the phosphoramidate class of organophosphates that are covalent inhibitors of acetylcholinesterase (AChE), an essential enzyme in neurotransmission. Currently used therapy in counteracting excessive cholinergic stimulation consists of a muscarinic antagonist (atropine) and an oxime reactivator of inhibited AChE, but the classical oximes are particularly ineffective in counteracting tabun exposure. In a recent publication (Kovarik et al. 2019), we showed that several oximes prepared by the Huisgen 1,3 dipolar cycloaddition and related precursors efficiently reactivate the tabun−AChE conjugate. Herein, we pursue the antidotal question further and examine a series of lead precursor molecules, along with triazole compounds, as reactivators of two AChE mutant enzymes. Such studies should reveal structural subtleties that reside within the architecture of the active center gorge of AChE and uncover intimate mechanisms of reactivation of alkylphosphate conjugates of AChE. The designated mutations appear to minimize steric constraints of the reactivating oximes within the impacted active center gorge. Indeed, after initial screening of the triazole oxime library and its precursors for the reactivation efficacy on Y337A and Y337A/F338A human AChE mutants, we found potentially active oxime-mutant enzyme pairs capable of degrading tabun in cycles of inhibition and reactivation. Surprisingly, the most sensitive *ex vivo* reactivation of mutant AChEs occurred with the alkylpyridinium aldoximes. Hence, although the use of mutant enzyme bio-scavengers in humans may be limited in practicality, bioscavenging and efficient neutralization of tabun conjugates might be achieved with mutant AChE-oxime combinations.

Keywords: organophosphates, acetylcholinesterase reactivation, antidotes to nerve agents, 2PAM, bioscavengers, phosphoramidates

Introduction

Acetylcholinesterase (AChE) is a pivotal enzyme responsible for the rapid degradation of neurotransmitter acetylcholine. Irreversible inhibition of AChE by organophosphates (OPs), such as pesticides and nerve agents, leads to the neurotoxic accumulation of acetylcholine. The symptoms reveal cardiovascular and respiratory compromise that, along with CNS excitation and associated seizures, can eventually lead to fatal outcomes. Antidotal treatment of nerve agent exposure has remained relatively unchanged through the decades. Typically, therapy couples administration of an anticholinergic drug, usually atropine, and an oxime reactivator of inhibited AChE (Gray 1984; Dawson 1994; Taylor 2018). Furthermore, anticonvulsant drugs may be indicated, when necessary, to minimize seizures during the initial cholinergic crisis (Jokanović and Prostan 2009). Since medical assistance in case of nerve agent exposure is restricted by the post-exposure interval and involves standard quaternary antidotes (2-PAM, HI-6, obidoxime, TMB-4), seeking an optimal reactivator remains an ongoing challenge. This is particularly true for tabun poisoning, because tabun conjugation of the active center serine is difficult to reverse due to the electron pair located on the phosphoramide group (Eto 1976). In addition, formation of the AChE-tabun conjugate leads to additional steric hindrance within the active center gorge (Ekström et al. 2006a,b). Therefore, other modes of protection and therapy avenues are also being intensively sought (Masson and Nachon 2017).

Current research in the scavenging arena is directed to administering exogenous enzymes or plasma capable of inactivating OP compounds before they react with the target AChE (Ashani et al. 1991; Maxwell et al.1993; Lenz et al. 2007; Vučinić et al. 2013; Wille et al. 2014). These enzymes may be stoichiometric bio-scavengers, such as plasma butyrylcholinesterase (BChE), that covalently link with OPs (Doctor and Saxena 2005), pseudo-catalytic, engineered AChE mutants or BChE capable of OP degradation when assisted by oximes (Kovarik et al. 2007, 2015; Taylor et al. 2007; Mazor et al. 2008; Radić et al. 2013; Noy-Porat et al. 2015; Maček Hrvat et al. 2016; Katalinić et al. 2018) and other phosphoesterases that catalyze the hydrolysis of OP compounds (Harel et al. 2004; Amitai et al. 2006; diTargiani et al. 2010; Gupta et al. 2011; Goldsmith et al. 2012; Melzer et al. 2012).

The orientations of the oxime and conjugated OP within the narrow active center gorge, as well as the angle of nucleophilic attack by oxime at the conjugated phosphorus atom, are critical determinants of the reactivation mechanism (Millard et al. 1999; Wong et al. 2000; Kovarik et al. 2004, 2006, 2008; Ekström et al. 2006a). Moreover, a crystallographic study of tabun-inhibited mouse AChE revealed that conformational changes of certain residues may result in decreased accessibility in the active-site gorge to reactivators (Ekström et al. 2006b). Accordingly, site-directed mutagenesis has been used to optimize reactivation of phosphorylated AChE assisted by oximes (Artursson et al. 2009; Katalinić and Kovarik 2012; Kovarik et al. 2013; Katalinić et al. 2018) as well as production of AChE mutants with slower aging rates (Cochran et al. 2011). Moreover, our recent studies reported the first *in vivo* example of effective oxime-assisted catalytic soman and VX bioscavenging based on combined administration of sub-stoichiometric amounts of a choline-binding site AChE mutant and an oxime reactivator (Kovarik et al. 2015; Maček Hrvat et al. 2016). Two mutations in the AChE choline binding site Y337A/F338A were shown to be the most important for reducing the aging rate of soman (Cochran et al. 2011; Maček Hrvat et al. 2016).

In this paper we report the search for potent reactivators of two AChE mutants inhibited by tabun to find candidates for the oxime-assisted catalytic bioscavenging of tabun and its conjugate with AChE. For reactivators we used a library of 111 novel oximes, mainly those with triazole ring synthesized using the copper-catalyzed azide-alkyne cycloaddition to form a linking triazole (Kovarik et al., 2019). The mutants were selected to accommodate a larger active center area at the base of the gorge in order to allow reactivators with a more extended *N*-pyridinium substitution access to the gorge base. Screening and detailed reactivation kinetics enabled us to detect oxime-enzyme pairs with scavenging potential to counteract tabun. A lead oxime-enzyme pair was tested in *ex vivo* conditions, revealing the potential and limitations in further development of bioscavengers using AChE mutants.

Materials and methods

Chemicals and enzymes

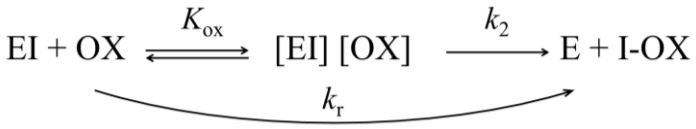
A library of oximes were synthesized as *N*-alkylpyridinium analogues and by Cu (I) catalyzed azide-alkyne cycloaddition to form the *anti*-triazoles using a trove of small molecule building blocks (Cochran et al. 2011; Kovarik et al. 2019). Stock solutions of oximes were prepared in water or dimethyl sulfoxide (DMSO; Kemika, Zagreb, Croatia) and diluted in sodium phosphate buffer (0.1 M, pH 7.4) just before use. Tabun [ethyl *N*,*N*-dimethylphosphoramidocyanidate] was purchased from NC Laboratory, Spiez, Switzerland. Stock solutions of tabun were prepared in isopropyl alcohol, while further solutions were diluted in water immediately before administration. Cholinesterase substrate, acetylthiocholine iodide (ATCh), pralidoxime (2-PAM) and thiol reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Recombinant DNA expressed human AChE, wild-type and its mutants (Y337A and Y337A/F338A), were prepared as described in (Cochran et al. 2011). Purified human plasma BChE was a gift from the late Dr Douglas Cerasoli, USAMRICD, Edgewood, USA. Human whole blood (hWB) was drawn from a female donor at the Institute for Medical Research and Occupational Health, Zagreb, Croatia in accord with approval of the institutional Ethics Committee.

Enzyme activity measurements

For reactivation experiments, AChE was incubated with a ten-fold excess of tabun for an hour to achieve inhibition greater than 95%, fractionated on a Sephadex G-50 spin column (Roche Diagnostics GmbH, Mannheim, Germany), and then incubated with oxime in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.01% BSA at 25 °C. At specified time intervals, aliquots were diluted in phosphate buffer containing DTNB, and upon addition of ATCh, residual enzyme activity was measured as described by Ellman et al. 1961. Final concentrations of ATCh and DTNB were 1.0 and 0.3 mM, respectively. Equivalent samples of uninhibited enzyme were passed through parallel columns, diluted to the same extent as the inhibition mixture, and control activity was measured in the presence of oxime at concentrations used for reactivation. Activities of

control and reactivation mixtures were corrected for the oxime-induced hydrolysis of ATCh. Reactivation is a two-step reaction between phosphylated enzyme (EI) and oxime (OX) that, through the reversible Michaelis-type complex [EI][OX], results with the active enzyme (E) and the phosphylated oxime (I-OX), as described by Scheme 1:



Reactivation rates were measured at a given oxime concentration (1 mM), wherefrom the observed first-order reactivation rate constant (k_{obs}) and maximal percentage of reactivation ($React_{max}$) were determined as previously described (Maček Hrvat et al. 2018). More detailed reactivation kinetics using a wider oxime concentration range of leads enabled determination of reactivation constants: k_2 (maximal first-order reactivation rate constant), K_{OX} (apparent phosphylated enzyme-oxime dissociation constant) and k_r (second-order reactivation rate constant) as described previously (Kovarik et al. 2004). Reactivation parameters were determined using Equation 1:

$$\ln \frac{[EI]_0}{[EI]_t} = \frac{k_2 \cdot [OX]}{K_{OX} + [OX]} \cdot t = k_{obs} \cdot t$$

where $[EI]_0$ and $[EI]_t$ represent the concentrations of the phosphylated enzyme at time zero and at time t. The overall second-order reactivation rate is determined in the following Equation 2:

$$k_r = \frac{k_2}{K_{OX}}$$

Ex vivo detoxification of tabun was conducted in human whole blood samples, alone or supplemented with the AChE mutant, and incubated with parallel 10- or 50-fold excesses of tabun. After 1 h of incubation (achieving 95–100% inhibition), oxime (0.10 or 1.0 mM) was added to the mixture. At specified time intervals, aliquot samples were taken for enzyme activity measurements. Loss of tabun activity in human whole blood was determined as percentage of total cholinesterase activity.

Inhibition of cholinesterases by tabun was measured at designated times (up to 30 min) with tabun (1–100 nM). Second-order inhibition rate constants (k_i) were calculated as described previously (Kovarik et al. 2003a, b). All cholinesterase activity measurements were performed in 0.1 M sodium phosphate buffer containing 0.01% BSA, pH 7.4 at 25 °C using the Ellman spectrophotometric method at 412 nm (or 436 nm with whole blood) on a CARY 300 spectrophotometer (Varian Inc., Australia) with a temperature controller. Data presentation and kinetics evaluation employed GraphPad Prism program (San Diego, USA).

Results and Discussion

In our recent study, out of a large oxime library that was screened and assayed for the reactivation of human recombinant AChE wild type inhibited by tabun, VX, sarin, cyclosarin and paraoxon, several of the lead oximes were ranked as potent antidotes in tabun poisoning (Kovarik et al. 2019). Herein we tested the same oxime library as reactivators of two AChE mutants of the choline binding sub-site inhibited by tabun (**Fig. S1**). Oximes were initially monitored for reactivation up to 24 h at 1 mM concentration. The most efficacious oxime reactivators of Y337A and Y337A/F338A mutants (**Table S1**) were ordered in terms of the observed reactivation rate constant (k_{obs}) as shown in **Fig. 1**, and obtained results were compared to 2-PAM and other standard oximes. Out of 111 tested oximes, only 13 and 18 oximes restored 80% or more of Y337A and Y337A/F338A activity, respectively. Surprisingly, reactivation rates higher than 0.1 min^{-1} for the tabun-Y337A conjugate with two lead oximes, **5B** and **4B,** (**Fig. 1** and **Fig. S1**) present up to 10-fold accelerations when compared to the reactivation of the wild type AChE with the same oxime library (Kovarik et al. 2019) or K-oximes previously shown as the most active tabun reactivators (Čalić et al. 2006, 2008; Kovarik et al. 2009; Katalinić et al. 2018; Zorbaz et al. 2018a). Moderate enhancements of reactivation with rate constants exceeding k_{obs} of 0.01 min^{-1} were achieved by 15 oximes in reactivation of the Y337A mutant and only by 3 oximes in reactivation of the Y337A/F338A mutant. The assays established that mutations introduced a

marked departure in reactivation kinetics, compared to wild type AChE (Kovarik et al. 2019) and no single oxime was an efficient reactivator of both mutants and the wild type (**Fig S1**). For instance, the reactivation rate (k_{obs}) for the standard oxime 2-PAM was 3.5 and 10 times slower for Y337A and Y337A/F338A mutants, respectively (**Fig. 1**), than the rate for the wild type AChE (k_{obs} = 0.0026 min^{-1}; Kovarik et al. 2019).

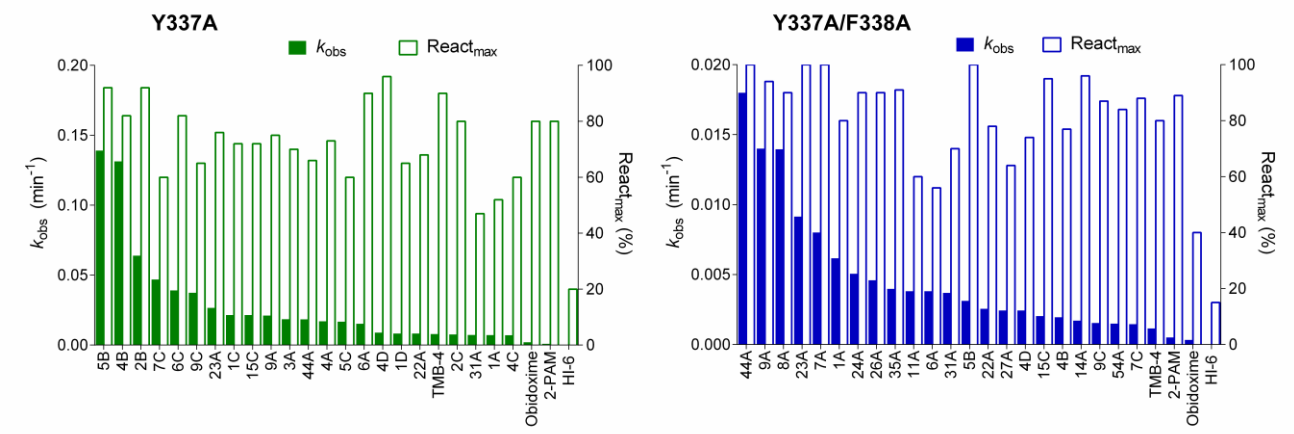
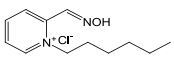
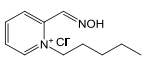
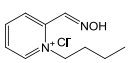


Figure 1. Reactivation of tabun-inhibited human AChE mutants (Y337A and Y337A/F338A) by the most active oximes (**Table S1**) at 1 mM concentration. Results are expressed as means of the observed first-order reactivation rate constants (k_{obs}) and maximal percentages of reactivation (React$_{max}$) determined from at least three experiments (deviations were less than 10%).

The most efficient reactivators were selected for further kinetic analysis with several concentrations of oximes and evaluation of reactivation constants. The highlighted oximes **5B**, **4B**, and **2B**, as the most effective reactivators of tabun-inhibited Y337A mutant, restored enzyme activity approaching 90% or greater in short time (**Table 1**). Interestingly, the three most efficient reactivators are 2-PAM analogues with extended alkyl chains butyl (**2B**), pentyl (**4B**) and hexyl (**5B**), while *N*-propyl (**1B**), *N*-ethyl (**3B**), and 2-PAM itself, the *N*-methyl analogue, were relatively poor reactivators of tabun-Y337A conjugate. Although hydrophobicity increases with **5B**, removal of the aromatic portion of the tyrosine in the mutant enzyme allows the compound to be accommodated in the active center presumably with a distinctive binding pose. One should note that the overall reactivation rate (k_r) correlates with the length of the alkyl substitution on the pyridinium ring (**Table 1**). However, further extension of the alkyl or other hydrophobic chain may create cationic detergent-like properties in the oxime with a hydrophobic tail and a cationic moiety.

Table 1. Reactivation kinetics of tabun-inhibited human AChE Y337A mutant by three selected oximes. Kinetic parameters (± S.E.): the maximal first-order reactivation rate constant (k_2), the dissociation constant of the phosphylated enzyme-oxime reversible complex (K_{OX}), overall second-order reactivation rate constant (k_r), maximal percentage of reactivation (React$_{max}$) and time in which maximal reactivation was approached (t) were determined from at least 3 experiments at 25 °C.

Oxime		k_2 (min^{-1})	K_{OX} (mM)	k_r (M^{-1}min^{-1})	React$_{max}$ (%)	t (min)
5B		0.21 ± 0.01	0.032 ± 0.005	6450 ± 910	90	20
4B		0.16 ± 0.01	0.069 ± 0.019	2300 ± 650	90	20
2B		0.056 ± 0.005	0.21 ± 0.06	260 ± 70	90	60

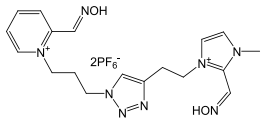
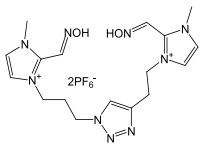
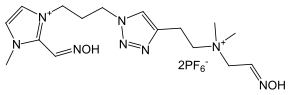
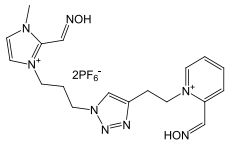
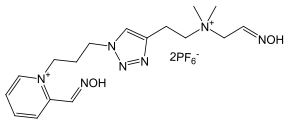
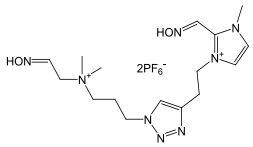
To understand the enhancement of rates of oxime nucleophilic attack, we compared reactivation of wild type AChE with the reactivation of tabun-inhibited BChE, since mutation Y337A defines a residue found in BChE – at the presumed position of the choline binding sub-site. Reactivation kinetics for **5B** are tabulated in **Table 2**, while representative experiments on three enzymes are presented in **Fig. S2**. As much as 90% of mutant Y337A activity was restored by oxime **5B** within 20 min. However, for wild type AChE and BChE, the maximal reactivation was limited to 50% in 10 hours, even though reactivation was monitored up to 24 h. In comparison to wild type AChE, we observed a 95-fold enhancement of the maximal reactivation rate of **5B** in case of the Y337A mutation. Therefore, our results highlighted the positive effect of a single mutation in the choline binding sub-site to enable oxime embedding and adopting an orientation for a productive nucleophilic interaction of the presumed oximate with the phosphorylated active site serine. A similar positive effect of the single mutation was observed in previous studies with OPs, where the Y337A mutation enhanced the dephosphylation rate and/or overall reactivation rate relative to wild type AChE (Kovarik et al. 2004, 2006, 2007; Katalinić and Kovarik 2012, Katalinić et al. 2018). Although the enhancement in reactivation with **5B** seen for the mutant was not observed for BChE reactivation, it is important to note that the reactivation of tabun-inhibited BChE with **5B** is one of the most efficient reported so far (Lucić Vrdoljak et al. 2006; Čalić et al. 2008; Kovarik et al. 2010; Radić et al. 2013; Horn et al. 2015; Katalinić et al. 2017; Zorbaz et al. 2018b). This was primarily due to 5-50- fold increase in affinity: e.g. a lower K_{OX} for the apparent oxime-conjugated AChE complex. Additionally it is noteworthy that oxime **5B** shares a similar pK_a (**Fig. S3**) with standard oximes 2-PAM and HI-6 (Radić et al. 2013; Šinko et al. 2006), and shows a non-cytotoxic profile *in vitro* (**Fig. S4**).

Table 2. Reactivation of tabun-inhibited human AChE mutant Y337A, wild type AChE and BChE by the oxime **5B**. Kinetic parameters (±S.E.): the maximal first-order reactivation rate constant (k_2), the dissociation constant of the phosphylated enzyme-oxime reversible complex (K_{OX}), an overall second-order reactivation rate constant, the (k_r), maximal percentage of reactivation ($React_{max}$) and time (t) in which maximal reactivation was approached were determined from at least 3 experiments at 25 °C.

Enzyme	k_2 (min^{-1})	K_{OX} (mM)	k_r ($M^{-1}min^{-1}$)	$React_{max}$ (%)	t (h)
Y337A	0.210 ± 0.008	0.032 ± 0.005	6450 ± 915	90	0.33
AChE	0.0022 ± 0.0005	0.24 ± 0.16	8.9 ± 4.2	50	10
BChE	0.0022 ± 0.0001	0.049 ± 0.019	45 ± 18	50	10

Based on initial screening (**Fig. 1**), we selected six oximes for an evaluation of detailed reactivation kinetics of the double AChE mutant shown in **Table 3**. All of the six oximes reactivated the tabun-inhibited Y337A/F338A approaching completion in 2 to 10 hours, but k_2 was about 6 times slower than that for **5B** and reactivation of the Y337A-tabun conjugate. The most efficient reactivators of tabun-phosphorylated Y337A/F338A had the equivalent distance of eight methylenes between two quaternary nitrogens (**Table 3**), as was found for wild-type AChE previously (Kovarik et al. 2019). Moreover, oxime **9A** and **1A** were among the best reactivators of the wild-type AChE reactivators (Kovarik et al. 2019). However, if one compares the absolute values of constants, the reactivation of tabun-inhibited Y337A/F338A was not enhanced for these oximes with respect to the wild type, because the maximal 2-fold increase in k_2 was compensated by a 6-fold increase of K_{OX} (cf. **Table 3** and Kovarik et al. 2019). Indeed, the comparison of the overall reactivation for leading reactivators of tabun-inhibited AChE and its tabun-inhibited mutants, shown in **Fig. 2**, implies that the most sensitive parameter for reactivation is the maximal reactivation rate (k_2). When compared to the wild type AChE, the single mutation increased k_2 about 20-fold and maintained the molecular recognition capacity reflected in similar K_{OX} constants.

Table 3. Reactivation of tabun-inhibited human AChE Y337A/F338A mutant by selected oximes. Kinetic parameters (± S.E.): the maximal first-order reactivation rate constant (k_2), the dissociation constant of the phosphylated enzyme-oxime reversible complex (K_{OX}), an overall second-order reactivation rate constant (k_r), the maximal percentage of reactivation ($React_{max}$) and time (t) in which maximal reactivation was approached were determined from at least 3 experiments at 25°C.

Oxime		k_2 (min^{-1})	K_{OX} (mM)	k_r ($M^{-1}min^{-1}$)	$React_{max}$ (%)	t (h)
9A		0.016 ± 0.008	0.024 ± 0.006	650 ± 180	100	2.5
44A		0.033 ± 0.003	0.068 ± 0.018	480 ± 130	100	3.5
8A		0.024 ± 0.002	0.059 ± 0.021	400 ± 140	100	2
23A		0.015 ± 0.002	0.082 ± 0.034	180 ± 79	90	4
7A		0.0054 ± 0.0004	0.042 ± 0.017	130 ± 50	90	10
1A		0.013 ± 0.002	0.16 ± 0.11	85 ± 60	100	5

In addition, since mutations can modify the general mechanism of enzyme reactivity, we analyzed kinetics of phosphorylation of Y337A and Y337A/F338A by tabun, and the determined second order rate constants are shown in **Table 4.** The inserted single mutation did not reduce significantly the rate of inhibition, whereas the phosphorylation rate of the Y337A/F338A mutant was 10-fold faster than that of the wild type AChE. Similar results were obtained with the mouse enzymes (Katalinić et al. 2018). It seems that the double mutation of the choline binding sub-site, Y337A/F338A, enables more direct access of bulky tabun to the active serine by eliminating the steric hindrance and results in a faster rate of inhibition. These results nicely correspond to an increase in the AChE inhibition rates achieved by decreasing the size of the amino-substituent of tabun analogues shown in several other studies (Artursson et al. 2009; Aurbek et al. 2009; Gray 1984) or with other organophosphates (Kovarik et al. 2003b; Kovarik et al. 2007). BChE also reacts readily with tabun forming the characteristic conjugate on the active site serine.

Table 4 Progressive inhibition of human cholinesterases with tabun determined at 25 °C. The second order rate constants (± SD) were mean values determined from three experiments.

Enzyme	k_i (10^6 M^{-1} min^{-1})
AChE w.t.	6.8 ± 0.2
BChE w.t.	6.4 ± 0.1
Y337A	5.9 ± 0.1
Y337A/F338A	76 ± 1.0

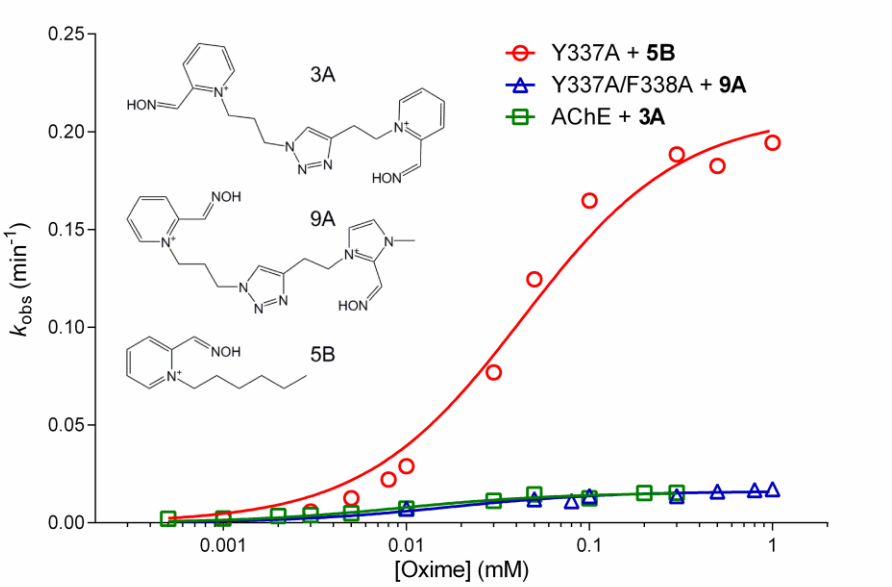


Figure 2. Oxime concentration dependent reactivation rates of tabun-inhibited human AChE and its two mutants represented by their best reactivators with distinctive substitutions on the pyridinium nitrogens (mean values from at least three experiments are presented). For comparable data on wild type AChE, see Kovarik et al., 2019.

Phosphorylation rates and reactivation rates of tabun-inhibited cholinesterases by the most potent reactivators directed our interest to the AChE mutant Y337A and oxime **5B** as a potential oxime-assisted catalytic bioscavenger. The oxime and mutant enzyme could generate a catalytic cycle of tabun hydrolysis through rapid conjugation of tabun with the mutant that is followed by enhanced oxime-assisted hydrolysis of the phosphoramidate-enzyme conjugate. Hence, we tested the Y337A−**5B** pair for *ex vivo* degradation of tabun in human whole blood supplemented with Y337A. Tabun was in a 10- and 50-fold excess of the mutant. As shown in **Fig. 3**, tabun degradation occurred within a few minutes after adding 1.0 mM **5B** and resulted in a 100% recovery (80−95% recovery in case of adding 0.1 mM **5B**) of total cholinesterase activity with respect to tabun excess. Therefore, regardless of the concentration of tabun and the oxime, tabun was counteracted within the initial 15 min of oxime-assisted catalysis, indicating successful reduction of tabun concentration by cycles of re-inhibition and reactivation as the Y337A activity increased to maximum, resulting from total tabun degradation. This is the first report of such successful *ex vivo* tabun degradation. On the other hand, without supplemental mutant enzyme in the presence of 1 mM **5B** with human whole blood, the maximal activity recovery was only ~40%. The kinetics are in accordance with data presented in **Table 2** (and **Fig. S2**) wherefrom oxime **5B** was a poor reactivator of tabun-inhibited AChE and BChE. In cases of rapid AChE reactivation, complete degradation of OPs is possible without the administration of exogenous cholinesterases as it was recently reported with hydroxypyridine aldoximes, i.e., total degradation of VX and sarin was a consequence of efficient reactivation of erythrocyte AChE (Zorbaz et al. 2018b).

We have shown here that a mutant form of human AChE, Y337A, with a mutation located in the vicinity of the choline binding sub-site, allows rapid reactivation of human AChE *in vitro* and *ex vivo*. Such a mutant may prove useful in the detection of tabun or related toxic phosphoramidates, such as the Novichok compounds (Vale et al. 2018), when distributed as admixtures *in vitro* in areas of potential toxicity. The compounds studied here have intermediate alkyl chain lengths connected to the pyridinium nitrogen. Extending beyond an alkyl chain length of six carbons in a series of congeneric compounds may create cationic detergent-associated properties and separate issues of toxicity *in vivo*. Chambers and colleagues (2016) have studied other *N*-alkyl substituted analogues of 2-PAM and noted that they appear to cross the blood-brain barrier, suggesting the potential for reactivation in the CNS. However, shorter chain alkyl pyridinium and imidazolium aldoximes are not likely to cross the blood-brain barrier rapidly (Katalinić et al. 2015), in contrast to zwitterionic, non-quaternary oximes, such as RS194B (Sit et al. 2018). Oximes, when

directly applied to the ventral (rostral) surface of the medulla oblongata in cats, reverse the central effects of organophosphate inhibition of blood pressure (Edery et al. 1986).

Investigation of the combination of a reactivating small molecule and a cholinesterase-simulating catalytic scavenger will require a careful match of plasma levels of the small molecule and the catalytic scavenger in order to achieve sufficient scavenging activity *in vivo*. Such formulations also constitute challenges for counteracting terrorism in field settings during or post-exposure. Decontamination of admixed sources of organophosphates containing phosphoramidates *in vitro* may prove to be a useful alternative application of the mutant cholinesterases identified here.

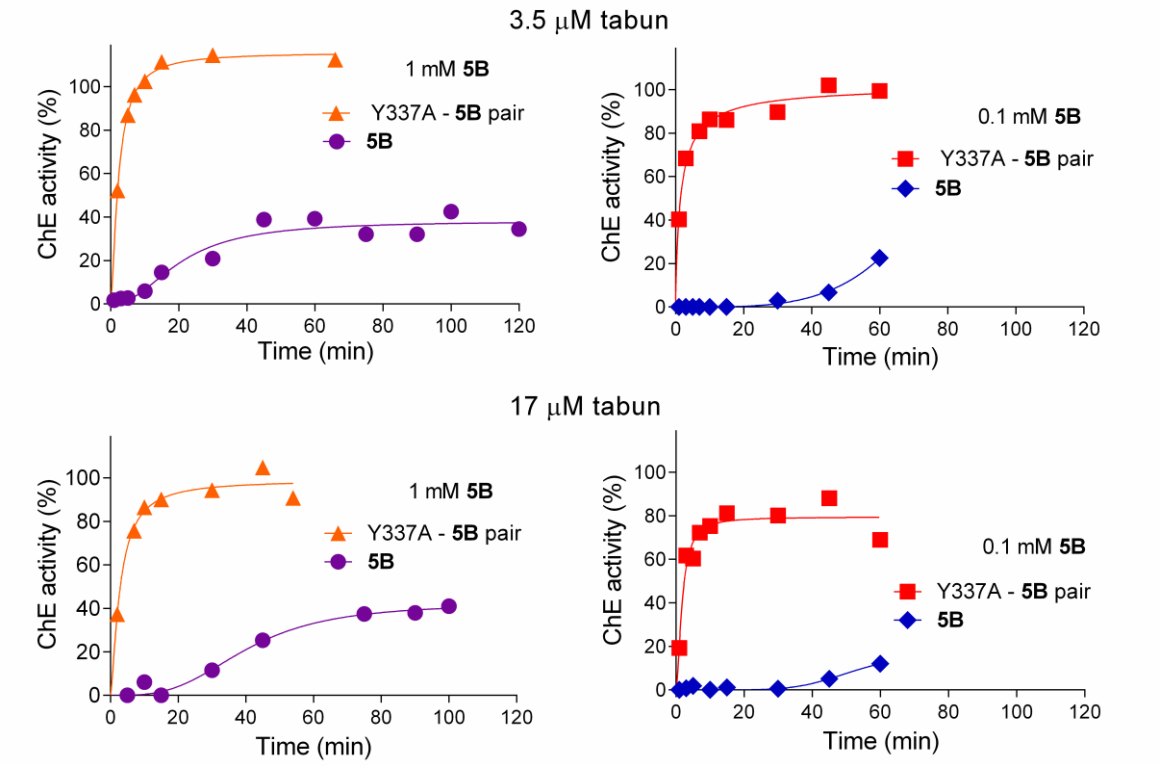


Figure 3. *Ex vivo* detoxification of tabun in whole human blood supplemented by human AChE mutant Y337A (330 nM) and oxime **5B** at 25 °C. Absorbances of controls were ΔA~0.0650 (hWB) *vs* ~0.3600 (Y337A, 330 nM).

Conclusion

Among the new oximes tested as reactivators of tabun-inhibited Y337A and Y337A/F338A human AChE mutants, oxime **5B**, a lengthened alkyl chain congener of 2-PAM, stood out as candidate for tabun scavenging when paired with the Y337A mutant of AChE. The capacity of the pair to detoxify tabun was established *ex vivo* in whole human blood. Whether such mutants will be an adjunct to small molecule oxime administration *in vivo* remains to be established and may be limiting by practicality.

Conflict of interest: The authors declare that they have no conflicts of interest.

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SUPPLEMENTAL MATERIAL

Table S1. Structure of selected aldoximes (Kovarik et al. 2019).

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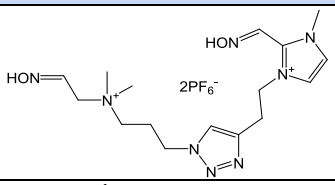
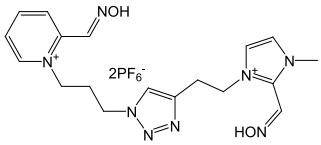
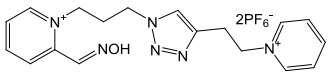
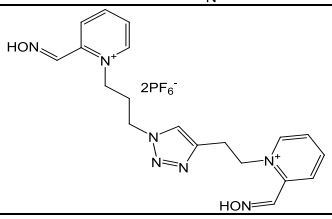
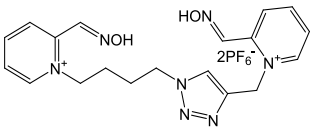
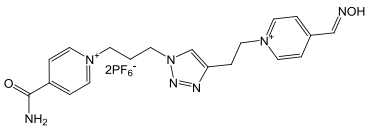
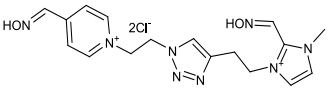
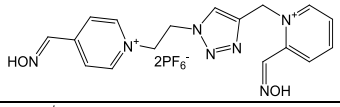
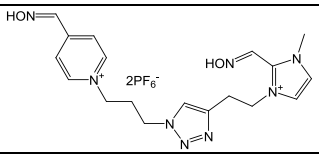
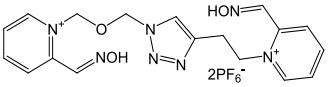
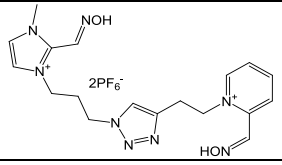
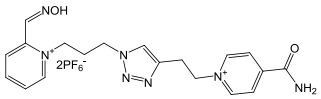
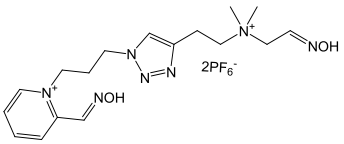
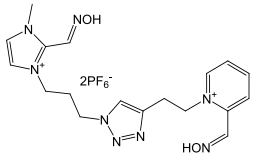
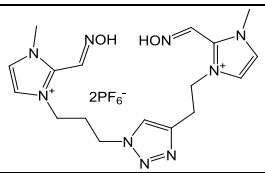
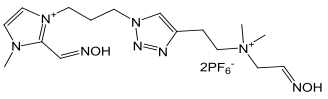
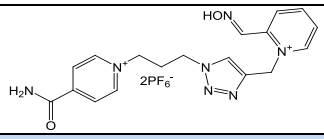
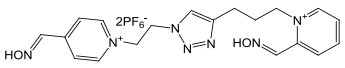
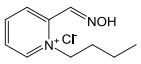
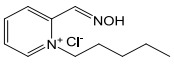
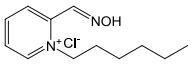
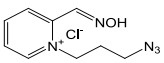
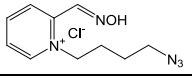
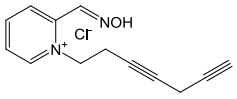
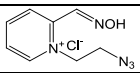
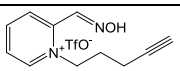
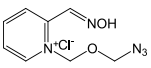
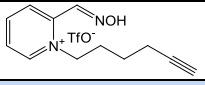
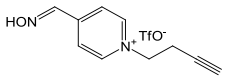
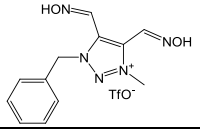
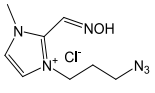
1A 	**9A** 	**26A** 
3A 	**11A** 	**27A** 
4A 	**14A** 	**31A** 
6A 	**22A** 	**35A** 
7A 	**23A** 	**44A** 
8A 	**24A** 	**54A** 
N-alkyl-2 pyridinium compounds		
2B 	**4B** 	**5B** 
Compounds with more complex *N*-alkyl substitutions		
1C 	**5C** 	**9C** 
2C 	**6C** 	
4C 	**7C** 	**15C** 
Miscellaneous compounds		
1D 	**4D** 	

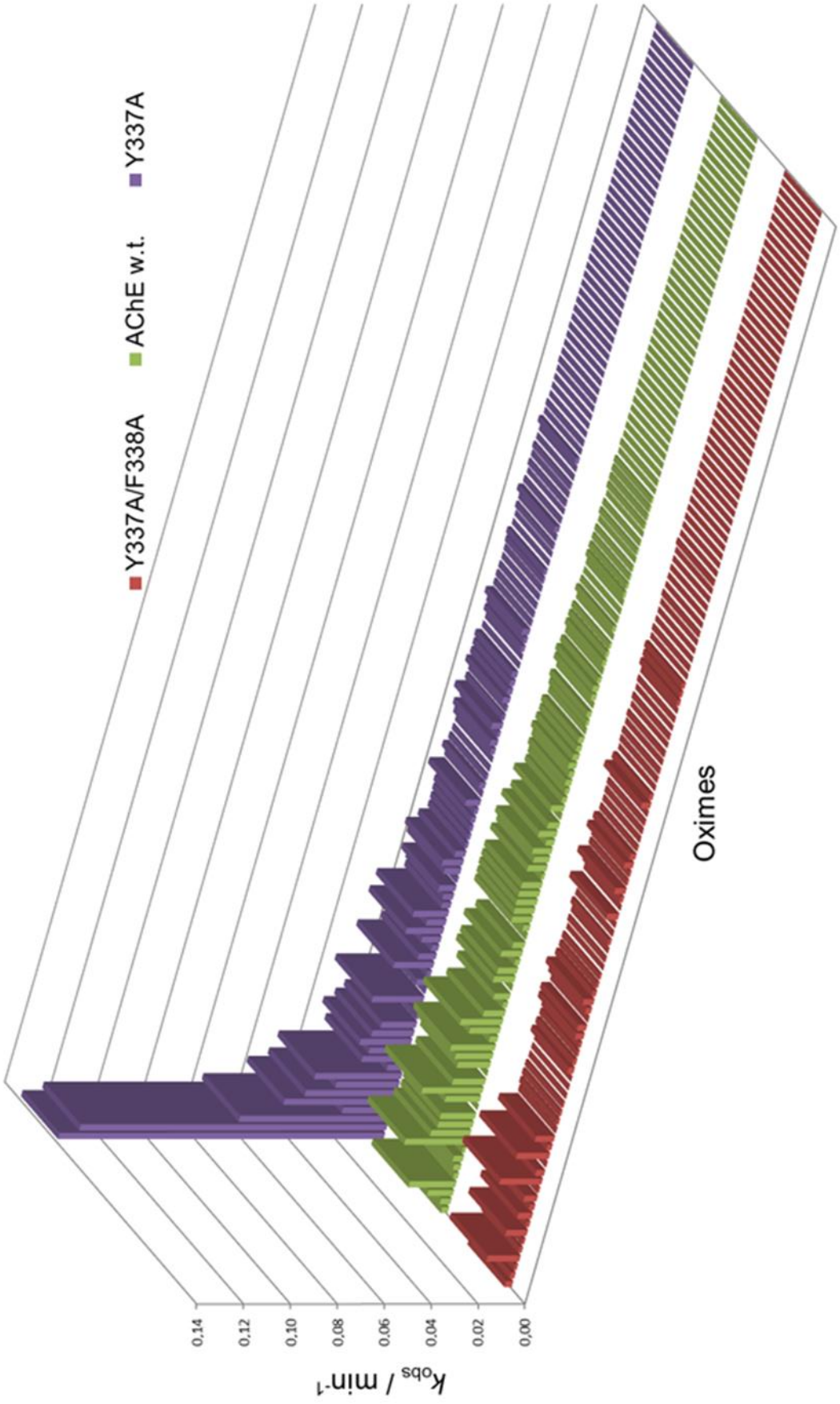


Figure S1. Reactivation rates (kobs) of tabun-inhibited human recombinant AChE w.t., and its two mutants by 1 mM oximes (Kovarik et al. 2019) recorded within 22 h. Oximes are ordered by average of their reactivity.

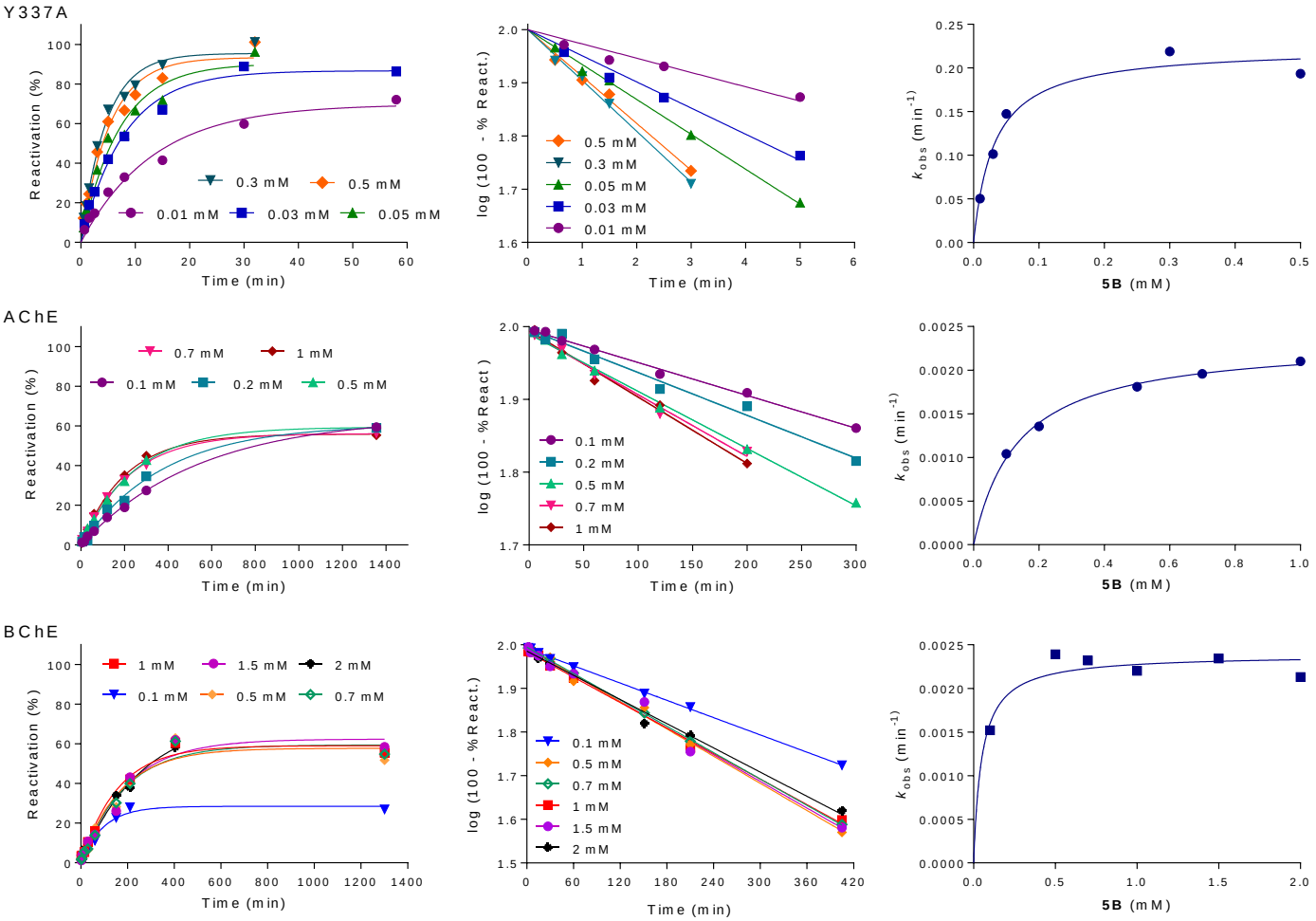


Figure S2. Representative experiments of reactivation tabun-inhibited human AChE mutant Y337A, AChE and BChE with **5B**, and evaluation of the kinetics parameters: k_{obs},.K_{OX}, k_{max}, and k_r as described previously (Maček Hrvat et al. 2018).

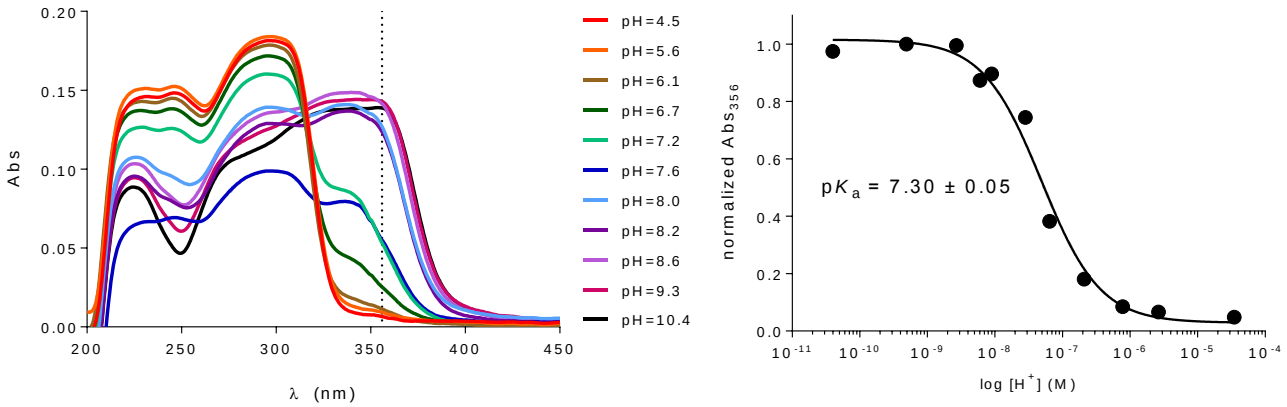


Figure S3. Spectrophotometric determination of the dissociation constant pK_a of oxime **5B** at 25°C evaluated as described previously (Zorbaz et al. 2018)

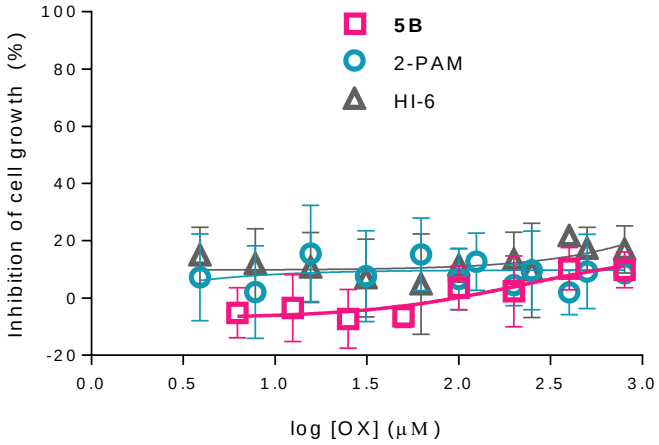


Figure S4. Cytotoxicity of oxime **5B** on SH-SY5Y cells determined by MTS assay (Promega, USA) and plotted as IC_{50} curves (IC_{50} > 800 μM). The results were expressed as a percentage of inhibition in 24 h compared to the values of non-treated cells, and evaluated as described previously (Katalinić et al. 2017)

References:

Katalinić M, Zandona A, Ramić A, Zorbaz T, Primožič I, Kovarik Z (2017) New cinchona oximes evaluated as reactivators of acetylcholinesterase and butyrylcholinesterase inhibited by organophosphorus compounds. Molecules 22(7):1234

Kovarik Z, Kalisiak J, Maček Hrvat N, Katalinić M, Zorbaz T, Žunec S, Green C, Radić Z, Fokin VV, Sharpless KB, Taylor P (2019) Reversal of tabun toxicity enabled by a triazole-annulated oxime library-reactivators of acetylcholinesterase. Chem Eur J. 25(16):4100-4114

Maček Hrvat N, Zorbaz T, Šinko G, Kovarik Z (2018) The estimation of oxime efficiency is affected by the experimental design of phosphylated acetylcholinesterase reactivation. Toxicol Letters 293:222-228

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