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The estimation of oxime efficiency is affected by the experimental design of phosphylated acetylcholinesterase reactivation

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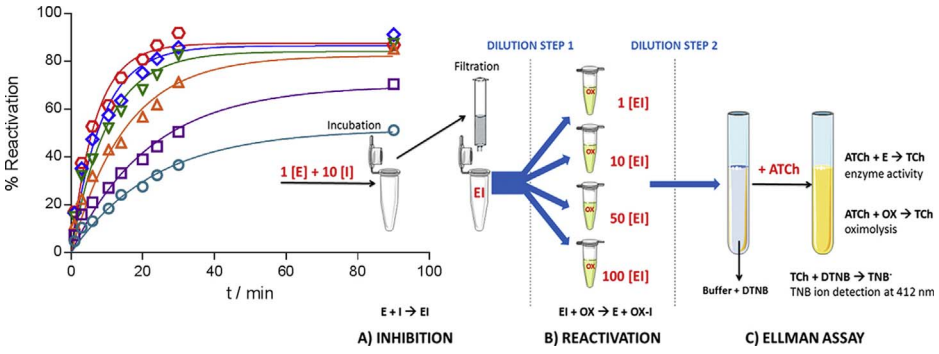
GRAPHICAL ABSTRACT



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ABSTRACT

Reactivation of acetylcholinesterase (AChE), an essential enzyme in neurotransmission, is a key point in the treatment of acute poisoning by nerve agents and pesticides, which structurally belong to organophosphorus compounds (OP). Due to the high diversity of substituents on the phosphorous atom, there is a variety of OP-AChE conjugates deriving from AChE inhibition, and therefore not only is there no universal reactivator efficient enough for the most toxic OPs, but for some nerve agents there is still a lack of any reactivator at all. The endeavor of many chemists to find more efficient reactivators resulted in thousands of newly-designed and synthesized oximes – potential reactivators of AChE. For an evaluation of the oximés reactivation efficiency, many research groups employ a simple spectrophotometric Ellman method. Since parameters that describe reactivator efficiency are often incomparable among laboratories, we tried to emphasize the critical steps in the determination of reactivation parameters as well as in the experimental design of a reactivation assay. We highlighted the important points in evaluation of reactivation kinetic parameters with an aim to achieve better agreement and comparability between the results obtained by different laboratories and overall, a more efficient evaluation of *in vitro* reactivation potency.

1. Introduction

Recent events in Syria regarding the use of the nerve agent sarin against civilians once again pushed the focus of attention on therapy against organophosphorus compounds (OP). In the event of OP poisoning, immediate medical intervention is vital due to the deadly effects caused by irreversible inhibition of acetylcholinesterase (AChE, EC 3.1.1.7), an enzyme essential in cholinergic neurotransmission. The currently applied therapy includes the anticholinergic drug atropine and an oxime reactivator of inhibited AChE, along with anti-seizure

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agents like diazepam (Dawson, 1994; Gray, 1984). Since there is neither a universal reactivator nor one with abundant efficiency, the search for more effective treatments of OP exposure than the currently used standard oximes (2-PAM, obidoxime or HI-6) is still ongoing.

The basic tool for the evaluation of oxime efficiency as a reactivator of OP-inhibited AChE is an assay based on the spectrometric Ellman method (Ellman et al., 1961). Nevertheless, even though the assay itself is simple, the kinetic parameters of reactivation are hardly comparable among laboratories due to various experimental approaches and data analysis. Over the past decades, several hundred compounds were tested in search for an effective reactivator of AChE inhibited by different OPs. The kinetic parameters obtained by these studies differed not only in the chemical differences of the tested compounds (*i.e.* counter ions), AChE species differences (Luo et al., 2007; Worek et al., 2002), the use of recombinant enzymes as opposed to native AChE (Kovarik et al., 2004; Lucić Vrdoljak et al., 2006; Maček Hrvat et al., 2016; Worek et al., 2012a), or experimentation at different temperatures, but also due to the differences in the experimental design and data analysis (Ashani et al., 1995; Kovarik et al., 2004; Luo et al., 2007; Maxwell et al., 2008; Musilek et al., 2011; Renou et al., 2016; Worek and Thiermann, 2011). Taking this into account, the search for an effective oxime reactivator is often difficult when one compares literature data for the structure activity relationship. Therefore, this study aimed to address the issue, mainly focusing on the experimental design and data analysis and on the example of sarin-inhibited human recombinant AChE reactivated by the standard oxime 2-PAM. Moreover, we singled out the key differences in the reactivation assay protocol found in the literature and emphasized experimental parameters that would help systematize the reactivation assay together with data analysis. This should enable interlaboratory comparisons of kinetic parameters determined by the evaluation of oxime-assisted reactivation of OP-inhibited AChE.

2. Materials and methods

2.1. Chemicals and enzyme

Sarin (NC Laboratory, Spiez, Switzerland) was diluted in isopropyl alcohol and further dilutions were made in water before use. 2-PAM (2-[(hydroxyimino)methyl]-1-methylpyridin-1-ium chloride), acetylthiocholine iodide (ATCh), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Recombinant human AChE, wild type (Cochran et al., 2011) was a kind gift from Professor Palmer Taylor, Skaggs School of Pharmacy and Pharmaceutical Sciences, UCSD, La Jolla, SAD.

2.2. Reactivation assay and evaluation of kinetics

AChE was incubated with a ten-fold excess of sarin until inhibition was greater than 95%. The inhibited enzyme was passed through a Sephadex G-50 spin column (Roche Diagnostic GmbH, Mannheim, Germany) to remove the excess of unconjugated sarin. After filtration and dilution in 0.1 M sodium phosphate buffer pH 7.4 containing 0.01% BSA, the inhibited enzyme was incubated with 2-PAM oxime in phosphate buffer (reactivation mixture, R_{mix}). At specified time intervals, an aliquot of R_{mix} was diluted in phosphate buffer containing DTNB and upon ATCh addition, enzyme activity was measured according to Ellman method (Ellman et al., 1961). Final concentrations of ATCh and DTNB were 1.0 and 0.3 mM, respectively. An equivalent sample of uninhibited enzyme was passed through a parallel column, diluted to the same extent as the inhibition mixture, and control activity was measured in the presence of an oxime at concentrations used for reactivation. The concentration of the inhibited AChE in the R_{mix} ranged from 0.2 to 22.2 nM, while 2-PAM concentrations were in the 4.4 to 1750 μM range.

Oxime-induced reactivation of the OP-AChE conjugate can be described through a two-step reaction (Kovarik et al., 2004) and proceeds according to Scheme 1:

$$EI + OX \underset{}{\overset{K_{ox}}{\rightleftharpoons}} [EI]\,[OX] \xrightarrow{k_2} E + I\text{-}OX \qquad k_r$$

where EI stands for phosphylated enzyme and OX for oxime, [EI][OX] is the reversible complex, E is the active enzyme and I-OX the phosphylated oxime. The K_{ox} constant approximates the dissociation constant which is the reciprocal of the affinity of the [EI] to oxime, k_2 is the maximal first-order reactivation rate constant and k_r is the overall second-order reactivation rate constant. Scheme 1 is defined by the equation:

$$\ln\frac{[EI]_0}{[EI]_t} = \frac{k_2\cdot[OX]}{K_{OX} + [OX]}\cdot t = k_{obs}\cdot t \tag{1}$$

where $[EI]_0$ and $[EI]_t$ represent the concentration of the phosphylated enzyme at time zero and at time t. k_{obs} is the observed first order rate constant of reactivation at any given oxime concentration. The overall reactivation rate k_r is the ratio:

$$k_r = \frac{k_2}{K_{OX}} \tag{2}$$

Experimental data were presented as percentage of reactivation (React) in time:

$$React = \frac{v(EI+OX)_t}{v(E+OX)}\cdot 100 \tag{3}$$

where $v_{(EI+OX)t}$ denotes the activity of the reactivated enzyme at time t and $v_{(E+OX)}$ stands for activity of uninhibited enzyme incubated with oxime (control). Since (100 − React) is equal to $100\cdot[EI]_t/[EI]_0$, one can relate the experimental data to Eq. (1), and k_2 and K_{ox} were obtained from the plot k_{obs} *vs.* [OX]. Control and reactivated AChE activities were corrected for oxime-induced degradation of ATCh (oximolysis).

At each oxime concentration, k_{obs} was calculated by two methods: (a) non-linear regression (method A) utilized by one phase exponential increase in enzyme activity given in Eq. (4):

$$React = React_{max}\cdot(1 - e^{-k_{obs}\cdot t}) \tag{4}$$

where React stands for percentage of reactivation at given time, $React_{max}$ is the maximal percentage of reactivation and t is time of reactivation; and (b) linear regression of an initial part of the reactivation (method B).

Enzyme activity measurements were performed at 25 °C (or 37 °C) and at 412 nm, on the CARY 300 spectrophotometer (Varian Inc., Mulgrave, Australia) with a temperature controller. Prism software (Version 6, GraphPad Software, San Diego, CA, USA) was used for statistical analyses of results.

2.3. Acetylcholinesterase thermal stability

The enzyme incubated with OP to achieve at least 95% of inhibition was passed through a Sephadex G-50 spin column (Roche Diagnostic GmbH, Mannheim, Germany) and diluted in phosphate buffer pH 7.4 to a 0.7 μg/mL concentration. The uninhibited enzyme used as control was treated in the same way as the inhibited sample. Samples were divided in three sets and stored overnight at room temperature, 4 °C, and −16 °C. The sample's thermal stability was measured using a Prometheus NT.48 nanoDSF (NanoTemper technologies GmbH, Munich, Germany). The results are presented as fluorescence change against temperature.