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Kinetic interactions of a homologous series of bispyridinium monooximes (HGG oximes) with native and phosphonylated human acetylcholinesterase☆

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ABSTRACT

Inhibition of acetylcholinesterase (AChE) is the main toxic mechanism of organophosphorus compounds (OP) and reactivation of OP-inhibited AChE by oximes is a mainstay of antidotal treatment. The inadequate efficacy of clinically used oximes led to the synthesis of numerous new compounds in the past decades to identify more effective reactivators. Despite of extensive in vitro reactivation studies the structural features for the development of effective oximes are not well understood. In the present study we investigated the kinetic interactions of a homologous series of bispyridinium monoximes bearing C1 to C12 alkylketone groups on the second pyridinium ring with native and cyclosarin-inhibited human AChE. We observed a correlation of the length of the alkyl side chain with an up to 20-fold increased affinity towards native AChE. The effect of the alkyl side chain on the affinity and reactivity towards phosphonylated AChE was moderate, except of a markedly reduced reactivity of C10 and C12 oximes. In comparison to the reference oxime HI-6 all HGG oximes had a lower reactivating potency and these oximes are not considered as promising compounds for the reactivation of cyclosarin-inhibited AChE.

1. Introduction

Oxime-induced reactivation of acetylcholinesterase (AChE) inhibited by organophosphorus (OP) compounds, i.e. nerve agents and pesticides, constitutes the only available causal treatment of OP poisoning (Eyer and Worek, 2007). Numerous oximes have been synthetized since the first clinical use of pralidoxime (2-PAM) in the 1950ies and obidoxime in the 1960ies (Worek et al., 2007; Bismuth et al., 1992). Extensive in vitro reactivation studies with isolated AChE and therapeutic animal studies gave insight into the potential and limitations of oximes and revealed that AChE inhibited by various OP, e.g. soman, tabun and different pesticides, is hardly reactivatable by oximes (Eyer and Worek, 2007; Jokanovic, 2009). Further comprehension into structural determinants of effective reactivators was provided by the investigation of crystal structures of AChE in complex with OP and oximes and by studies using AChE mutants (Wong et al., 2000; Ekström et al., 2006).

Structural studies and the investigation of in vitro reactivation kinetics gave evidence that the ability of oximes to reactivate OP-inhibited AChE is determined by the structure of the OP and by the AChE source, e.g. human, guinea pig, swine (Luo et al., 2007, 2008; Worek et al., 2002, 2011). Hence, the rational evaluation of the susceptibility of OP-AChE-complexes towards reactivation by oximes requires the determination of the various reactivation constants with numerous OP-AChE-oxime combinations at identical conditions (Eyer and Worek, 2007).

Recently, we determined the reactivation kinetics of a homologous series of bis-ortho-pyridiniumaldoximes tethered with C4–C9 linkers, designated as ortho-4–ortho-9, with OP-inhibited human AChE and revealed a decisive effect of the length of the linker on the affinity towards native and inhibited AChE (Wille et al., 2010). In fact, the length of the linker was positively correlated with affinity which was in the nanomolar range with ortho-9. In addition, linkage of peripheral side ligands to quaternary and non-quaternary oxime moieties resulted in a substantial increase of affinity and reactivity towards human AChE (de Koning et al., 2011a,b).

Now, it was tempting to investigate the kinetic interactions of a homologous series of bispyridinium monoximes ("HGG oximes") bearing alkylketone groups on the second pyridinium ring (Table 1) with native and cyclosarin-inhibited human AChE, a phosphonylated AChE which is rather resistant towards reactivation by clinically used oximes (Worek et al., 2004). HGG oximes, bearing an oxime function at position 2 of the pyridinium ring, were originally developed as potential soman antidotes in the laboratory of Prof. Hagedorn, University of Freiburg, Germany (Gross, 1980; Schoene et al., 1983) but were never investigated systematically in respect to their kinetic properties. Hence, the present study should provide further insight into the impact of side ligands on affinity and reactivity towards native and phosphonylated human AChE and should extend the available data on structure–activity relationships of oximes.

☆ This article is devoted to Prof. Dr. Peter Eyer on the occasion of his 70th birthday.

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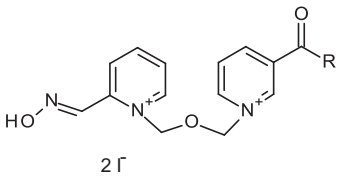
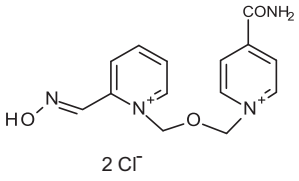
Table 1
Chemical structure of oximes.

Code	R	Structure
HGG21	methyl	
HGG73	ethyl	
HGG58	propyl	
HGG60	butyl	
HGG69	pentyl	
HGG70	hexyl	
HGG93	heptyl	
HGG82	octyl	
HGG81	decyl	
HGG62	dodecyl	
HI-6		

2. Materials and methods

2.1. Materials

The tested HGG oximes (≥95% by ^{1}H NMR; Table 1) were synthetized by Dr. Gross and Prof. Hagedorn at the University of Freiburg, Freiburg, Germany (Gross, 1980) and were kindly donated by Prof. Eyer (University of Munich, Munich, Germany). Cyclosarin (>98% by GC–MS, ^{1}H NMR and ^{31}P NMR, Fig. 1) was made available by the German Ministry of Defence. 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ATCh) were supplied by Sigma–Aldrich. All other chemicals were from Merck (Darmstadt, Germany).

Oxime stock solutions (100 mM in DMSO) were prepared at the day of the experiment, were further diluted in DMSO to the required concentrations and were stored at ambient temperatures. Cyclosarin stock solutions (0.1%, v/v) were prepared in acetonitrile, appropriately diluted in distilled water just before the experiment and kept on ice until use.

Heparinized human whole blood was centrifuged at 3000 rpm and 4 °C for 10 min, the plasma was discarded and the erythrocytes were washed five times with an approximately three-fold volume phosphate buffer. Then, the packed erythrocytes were used for preparation of hemoglobin-free erythrocyte ghosts as AChE source (Worek et al., 2002). Aliquots of the erythrocyte ghosts with an AChE activity adjusted to that found in whole blood were stored at −80 °C, and prior to use aliquots were homogenized on ice to achieve a homogeneous matrix for the kinetic studies.

2.2. AChE assay

AChE activities were determined with a modified, spectrophotometric Ellman assay (Worek et al., 1999; Eyer et al., 2003) using polystyrol cuvettes, 0.45 mM ATCh as substrate and 0.3 mM DTNB as a chromogen in 0.1 M phosphate buffer (pH 7.4).

All experiments were performed at 37 °C and pH 7.4. Stated concentrations refer to final concentrations.

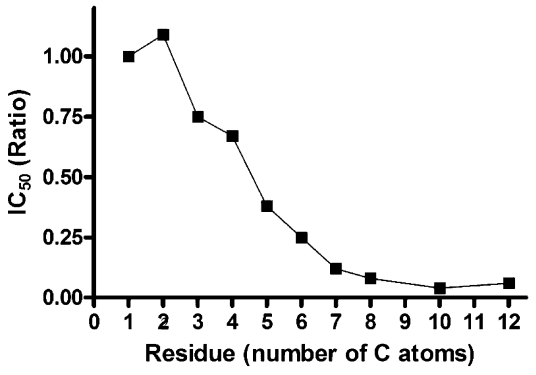


Fig. 1. Inhibition of human AChE by HGG oximes. The IC_{50} values of HGG oximes were related to the respective value of HGG21, bearing a methylketone group.

2.3. Inhibition of human AChE by HGG oximes

20 μl HGG oximes (1–1000 μM final concentration) or 20 μl DMSO (control) were added to pre-warmed cuvettes (37 °C) containing 1860 μl phosphate buffer (0.1 M, pH 7.4), 60 μl DTNB and 10 μl AChE. The mixture was incubated for 2 min at 37 °C and finally 50 μl ATCh was added. AChE activity was measured for 1 min and analysed by linear regression analysis. In addition, the potential reaction of HGG oximes with DTNB and ATCh was quantified by measuring the blank reaction in absence of AChE. AChE activities were corrected for the concentration dependent oxime blank and were referred to control activity.

The IC_{50} values of duplicate runs were calculated from semi-logarithmic plots of the oxime concentration versus the AChE activity.

2.4. Reactivation of cyclosarin-inhibited human AChE by HGG oximes

Human erythrocyte ghosts were incubated for 15 min at 37 °C with a small volume (1%, v/v) cyclosarin (20 nM final concentration) to achieve an AChE inhibition of >95%. Then, residual inhibitor was removed by overnight dialysis (phosphate buffer, 0.1 M, pH 7.4) at 4 °C (Worek et al., 2011). Aliquots were stored at −80 °C until use.

150 μl inhibited AChE were mixed with 150 μl phosphate buffer containing 0.2% gelatine in order to stabilize AChE activity during prolonged incubation at 37 °C (Worek et al., 2011). 5 μl oxime was added at $t = 0$ to initiate reactivation. After specified time intervals (2–60 min; 2–120 min with HGG62) 20 μl aliquots were transferred to tempered cuvettes containing 3000 μl phosphate buffer and 100 μl DTNB for the measurement of AChE activity after addition of 50 μl ATCh. This protocol allowed the use of rather high oxime concentrations during incubation with inhibited AChE but minimized a potential inhibition of reactivated AChE by oximes during enzyme assay.

8–10 different oxime concentrations (10–1000 μM) were used for the determination of the reactivation rate constants in duplicate experiments. AChE activity in the presence and absence of oximes was referred to control AChE and % reactivation was calculated thereof.

The time-dependent % reactivation of individual data sets was analyzed by non-linear regression analysis (Worek et al., 2010) to obtain K_D, which approximates the dissociation constant being inversely proportional to the affinity of the oxime for the inhibited enzyme, and k_r, indicating the reactivity of the oxime. The hybrid reactivation rate constant k_{r2} was calculated from the ratio of k_r and K_D.

2.5. Data analysis

Data processing for the determination of the different kinetic constants was performed by non-linear regression analysis using curve fitting programs provided by Prism™ Vers. 4.0 (GraphPad Software, San Diego, CA).

3. Results

The solubility of HGG oximes in water was generally low and decreased with the length of the alkyl residue at the keto group. In order to enable identical conditions for the investigation of the properties of the oximes, stock and working solutions of the compounds were prepared in DMSO. Therefore, control activities were determined with identical DMSO concentrations.

3.1. Inhibition of human AChE by HGG oximes

The determination of AChE activity in the presence of HGG oximes resulted in a concentration-dependent inhibition of the enzyme. The calculated IC_{50} values correlated with the length of the alkyl residue (Table 2) and HGG oximes having a decyl or dodecyl residue had an approximately 20-fold higher inhibitory potency compared to HGG21 bearing a methyl residue (Fig. 1).

3.2. Reactivation of cyclosarin-inhibited human AChE by HGG oximes

The incubation of cyclosarin-inhibited AChE with HGG oximes resulted in a mono-exponential increase of AChE activity and in complete reactivation of the enzyme at various concentrations. The reactivity of HGG oximes bearing a C1–C8 group at the keto function was comparably high but decreased substantially with C10 and C12 oximes (Table 3). In contrast, the affinity of C1–C4 oximes was lower compared to C5–C12 oximes (Table 3). The different affinities and reactivities resulted in a gradual increase of the bimolecular