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Reactions of methylphosphonic difluoride with human acetylcholinesterase and oximes – Possible therapeutic implications

Franz Worek *, Paul Elsinghorst, Marianne Koller, Horst Thiermann

Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany

HIGHLIGHTS

- We investigated the interaction of methylphosphonic difluoride (DF) and human AChE in vitro.
- DF and its main metabolite MF are extremely weak AChE inhibitors but form an aged AChE.
- The oximes obidoxime and HI-6 increased the inhibition of AChE by DF and MF.

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ABSTRACT

Highly toxic organophosphorus (OP) nerve agents are well characterized regarding chemical, biological and toxicological properties and the effectiveness of standard atropine and oxime therapy. Open literature data on the key nerve agent precursor methylphosphonic difluoride (DF) are scarce. To fill this gap the reactions of DF and its main degradation product methylphosphonofluoridic acid (MF) with human acetylcholinesterase (AChE) and the oximes obidoxime, HI-6 and 2-PAM were investigated in vitro. DF and MF were found to be weak inhibitors of human AChE being at least five orders less potent compared to the nerve agent sarin. Incubation of human AChE with millimolar DF and MF and subsequent addition of obidoxime and HI-6 resulted in a concentration-dependent decrease of AChE activity. This effect was not observed when incubating highly diluted AChE with oximes. The most likely explanation for this phenomenon is an inhibitory effect of phosphonyloximes formed by direct reaction of DF or MF with obidoxime and HI-6. These data indicate that high DF doses, resulting in millimolar blood and tissue DF/MF concentrations, are necessary to induce cholinergic signs and that under these conditions treatment with obidoxime and HI-6 may even worsen the poisoning.

1. Introduction

Highly toxic organophosphorus compounds (nerve agents) act primarily by inhibition of the pivotal enzyme acetylcholinesterase (AChE) resulting in an endogenous overflow of acetylcholine at cholinergic synapses (Holmstedt, 1959; Aldridge and Reiner, 1972). This leads to an overstimulation of nicotinic and muscarinic receptors, to a disturbance of multiple organ functions and, finally, to respiratory arrest and death (Grob, 1956; Krieger, 2001). The standard treatment of nerve agent poisoning consists of an antimuscarinic, e.g., atropine, and an AChE reactivator (oxime), e.g., obidoxime, pralidoxime (2-PAM) or HI-6 (Eyer and Worek, 2007; Jokanovic, 2009).

The recent homicidal use of the nerve agent sarin in Syria again demonstrated the toxic potential of such agents and their threat to the civilian population (Eisenkraft et al., 2014). The chemical and biological properties of nerve agents as well as the effectiveness of medical countermeasures are well characterized. However, data on nerve agent precursors are limited in open literature (Sidell, 1997).

A key precursor for the synthesis of sarin and related methylfluorophosphonates is methylphosphonic difluoride (DF; CAS-No. 676-99-3; Fig. 1; Bryant et al., 1960; Monard and Quinchon, 1961; Black and Harrison, 1996). DF hydrolyses extraordinarily fast in aqueous solutions to methylphosphonofluoridic acid (MF; CAS-No. 1511-67-7; Fig. 1; Beach and Sass, 1961). Animal studies indicated a substantially lower toxicity of DF compared to sarin and a weak inhibition of cholinesterases (Crook et al., 1969; Dahl et al., 1986). However, data on the potency of DF to inhibit human AChE as well as the ability of oximes to reactivate DF-inhibited AChE are not available. In order to fill this data gap we

* Corresponding author. Tel.: +49 89 992692 2930; fax: +49 89 992692 2333.
E-mail address: franzworek@bundeswehr.org (F. Worek).

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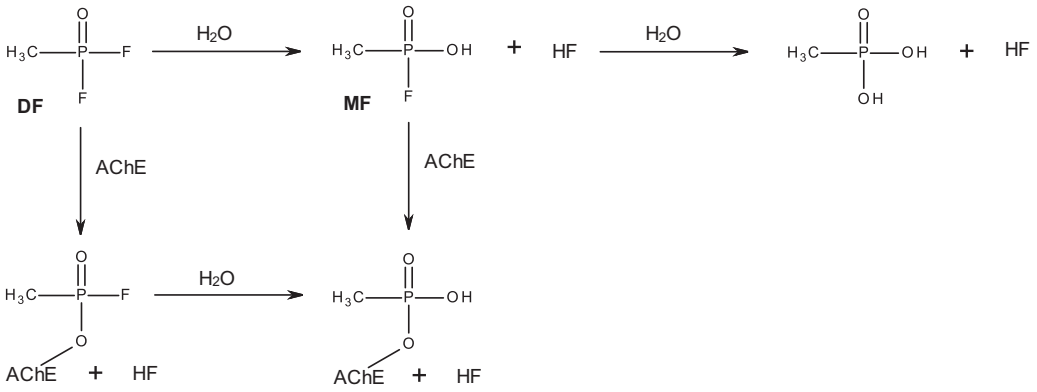

Fig. 1. Chemical structures of methylphosphonic difluoride (DF), its main degradation product methylphosphonofluoridic acid (MF) and the proposed reactions of DF and MF with AChE.

investigated the interaction of DF, human AChE and oximes in vitro.

2. Materials and methods

2.1. Chemicals

The organophosphorus compound methylphosphonic difluoride (DF; 95% by ^{1}H NMR, ^{19}F NMR and ^{31}P NMR, Fig. 1) was supplied by the German Ministry of Defence. DF stock solutions (10% v/v) were prepared in acetonitrile and stored at 20 °C.

Obidoxime (1,1′-[oxybis(methylene)]bis[4-(hydroxyimino) methyl] pyridinium dichloride) was purchased from Merck (Darmstadt, Germany), pralidoxime (2-PAM; 2-(hydroxyimino-methyl)-1-methylpyridinium chloride) from Sigma–Aldrich (Taufkirchen, Germany) and HI-6 (1-[[[4-(aminocarbonyl) pyridinio] methoxy]methyl]-2-[(hydroxyimino) methyl]pyridinium dichloride monohydrate) was made available by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada).

Oxime stock solutions (200 mM) were prepared in distilled water and stored at −80 °C. At the day of experiment the oxime stock solutions were diluted appropriately with distilled water and were kept on ice until use.

The chemicals acetylthiocholine iodide (ATCh) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma–Aldrich. All other substances were purchased from Merck (Darmstadt, Germany).

Hemoglobin-free human erythrocyte ghosts served as AChE source and were prepared as described from heparinized human blood (Worek et al., 2002). Aliquots in phosphate buffer (0.1 M, pH 7.4) were stored at −80 °C and were homogenized prior to use.

2.2. AChE assay

AChE activities were measured with a modified Ellman assay (Worek et al., 1999) at 412 nm (Cary 3Bio Varian, Darmstadt, Germany) using polystyrol cuvettes and 0.45 mM ATCh (substrate) and 0.3 mM DTNB (chromogen).

All experiments were performed at pH 7.4 and 37 °C.

2.3. Inhibition of human AChE by DF

200 μl human AChE were incubated with 2 μl 300 mM DF (in acetonitrile) and aliquots were taken after 1–60 min to determine the residual AChE activity. In addition, DF was diluted in distilled water (500 mM final concentration) and after 15 min an aliquot was removed for incubation with human AChE and determination of residual AChE activity after 1–90 min. Enzyme activities were referred to control AChE activity and the first order inhibition rate constant, k_1, was determined by non-linear regression analysis. The second order inhibition rate constant, k_i, was derived from Eq. (1):

$$k_i = \frac{k_1}{[I]} \quad (1)$$

with [I] resembling the inhibitor concentration.

2.4. Reactivation of DF-inhibited human AChE by oximes

Reactivation of DF-inhibited human AChE by oximes was tested with different protocols:

1 Human AChE was incubated with 3 mM DF for 15 min followed by addition of 10, 100 or 1000 μM obidoxime, HI-6 or 2-PAM. After 15 min the AChE activity was determined.
2 Human AChE was incubated with 0.3 mM DF for 60 min followed by addition of 10, 100 or 1000 μM obidoxime, HI-6 or 2-PAM. After 15 min the AChE activity was determined.
3 Human AChE was incubated with 3 mM DF for 15 min followed by 100 fold dilution in phosphate buffer and addition of 10, 100 or 1000 μM obidoxime, HI-6 or 2-PAM. After 15 min the AChE activity was determined.
4 DF was diluted in distilled water (500 mM final concentration) and after 15 min an aliquot was removed for 60 min incubation with human AChE (anticipated final concentration of 5 mM MF). Then, 10, 100 or 1000 μM obidoxime or 2-PAM were added and after 15 min the AChE activity was determined.

2.5. Data analysis

Data processing was performed with Microsoft Excel 2010 and GraphPad Prism™ Version 4.03.

3. Results and discussion

Initially, the purity of the used DF was analysed by ^{1}H NMR, ^{19}F NMR and ^{31}P NMR and the sample was found to be 95% pure (Fig. 2A). The described ultra-rapid degradation of DF in aqueous media to MF (Beach and Sass, 1961) was verified by ^{31}P NMR analysis (Fig. 2). When incubating 1 μl neat DF in 600 μl D_2O in a NMR tube no DF was observed after 5 min as the original triplet resulting from ^{19}F–^{31}P coupling (Fig. 2A) was completely converted to a doublet (Fig. 2B). The generated MF remained stable over prolonged time (tested up to 220 min; Fig. 2C), which corresponds to literature data showing a hydrolysis half-time in the range of days in aqueous solutions (Bechtold and Dahl, 1987).