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ORIGINAL ARTICLE

Reactivation study of pyridinium oximes for acetylcholinesterases inhibited by paraoxon or DFP

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Summary
We tested the potency to reactivate AChE inhibited by diisopropyl fluorophosphates (DFP) by using bis-pyridinium oxime reactivators connected with $CH_2CH_2OCH_2CH_2$ linkers between two pyridinium rings. The potency was strongly dependent on oxime functional groups, and the bis-oxime derivatives 1,1-[Oxybis(ethylene)]-bis[4-(hydroxyimino)methyl]pyridinium dibromide (1) and 1,1-[Oxybis(ethylene)]-bis[2-(hydroxyimino)methyl]pyridinium dichloride (2) are more potent than mono-oxime compounds 1-(4-hydroxyiminomethyl-1-pyridino)-5-(4-carbamoyl-1-pyridino)-3-oxapentane dibromide (3) and 1-(3-hydroxyiminomethyl-1-pyridino)-5-(4-carbamoyl-1-pyridino)-3-oxapentane dibromide (4). Not only is the number of oxime groups an important structural factor, but also their position. The *in vitro* reactivation ability of the most potent bis-pyridinium oxime 2 was further evaluated for the housefly (HF) AChE inhibited by DFP and the bovine red blood cell (RBC) AChE inhibited by paraoxon. The reactivation ability of oxime 2 at $5x10^{-3}$M concentration was almost 80% for HF-AChE inhibited by DFP and 82.1% for RBC-AChE inhibited by paraoxon.

Keywords: Paraoxon – DFP – Organophosphorus agents – Bis-pyridinium oxime reactivators – Acetylcholinesterase

INTRODUCTION

Organophosphorus nerve agents such as sarin, soman, and cyclosarin are extremely toxic chemicals that were developed in secrecy for military use. It is well known that these organophosphorus agents exert their biological effects by inhibition of the enzyme acetylcholinesterase (AChE), which the active center serine hydroxyl group can attack the phosphorus atom of the organophosphorus agents to form a strong P-O bond (Scheme 1) (Marrs 1993). The inhibition of AChE increases the amount of acetylcholine (ACh) at central and peripheral sites of the nerve system. High doses of organophosphorus agents cause convulsions and paralysis of the respiratory muscles.

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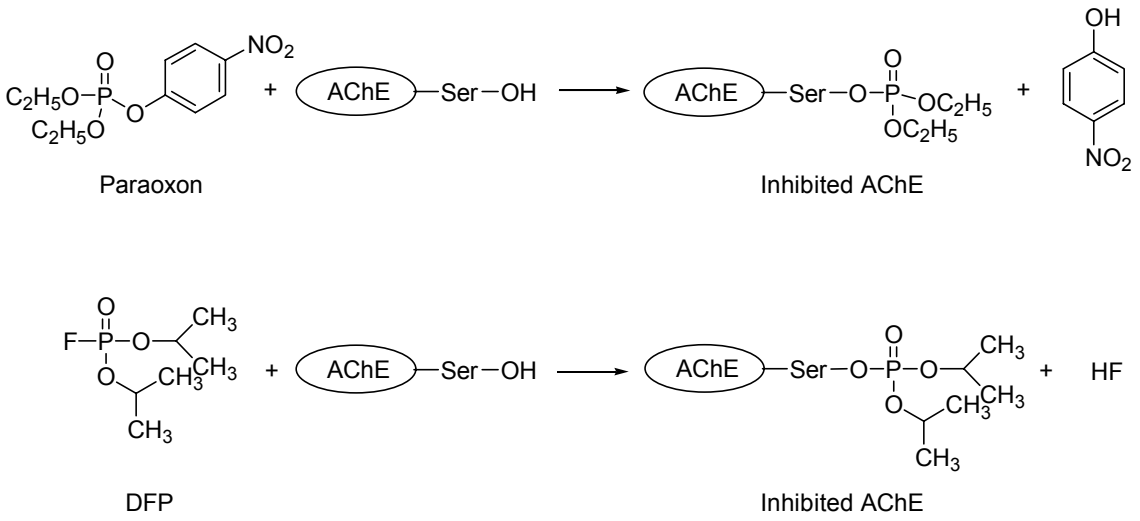

Scheme 1. **Inhibition of AChE by paraoxon and DFP**

Several other organophosphorus agents such as parathion, malathion, and diazinon have also been developed (Figure 1), and became widely used as insecticides because of their low volatility and stability in aqueous solution (Taylor 1994). Among these organophosphorus insecticides, parathion probably has been responsible for more cases of accidental poisoning and death than any other organophosphous insecticide (London and Myers 1995, Yacoub et. al. 1981).

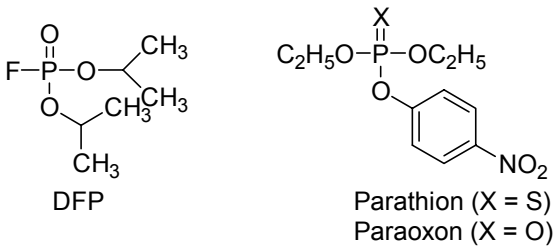

Fig. 1. **Structures of organophosphorus agents**

After the organophosphorus compounds attach to AChE to inhibit it, AChE can be reactivated by oxime reactivators, and 2-PAM is the best well-known reactivator. After thorough study of many of the oxime reactivators, bis-pyridinium oximes such as TMB, Toxogonin, and HI-6 (Kassa et al. 1997, Kassa 1998, Kassa 2002) have been developed, and are used currently in many countries (Figure 2). In a previous paper (Kim et al. 2005), new bis-pyridinium oxime reactivators connected with a $CH_2CH_2OCH_2CH_2$ linker between two pyridinium rings were designed and synthesized. During tests of their potency to reactivate AChE inhibited by cyclosarin, the bis-pyridinium oxime achieved reactivation potency higher than 10% at the lower concentration 10^{-4}M.

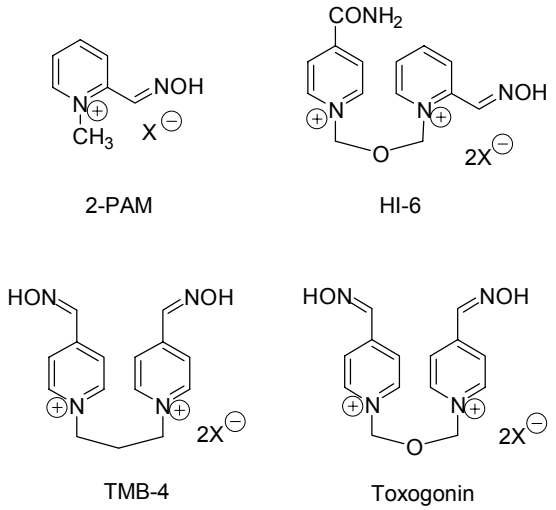

Fig. 2. **Currently used oxime reactivators**

Although these compounds were not extraordinarily potent reactivators for AChE inhibited by cyclosarin, they could be effective in reactivation for AChE inhibited by other nerve agents or pesticides, because the reactivation potency of AChE reactivators depends on the organophosphorus agent used (Bajgar 2004, Dohnal et al. 2005, Kuca and Kassa 2003, Kuca et al. 2003).

In continuing our efforts to develop new oxime reactivators for AChE inhibited by organophosphorus agents, we are interested in the development of antidotes that have potent reactivation activity. Therefore, we tested the reactivation efficiency of the new oximes for

diisopropyl fluorophosphates (DFP)- or paraoxon-inhibited AChE (Figure 3).

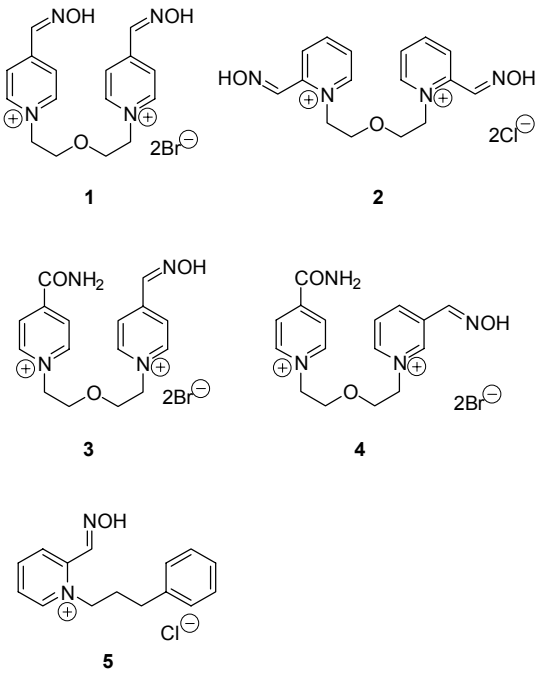

Fig. 3. **Pyridinium oxime compounds**

MATERIAL AND METHODS

All new pyridinium oximes and HI-6 were prepared in our laboratory, and 2-PAM was purchased from Sigma-Aldrich. DFP and paraoxon are commercially available from Fluka and Sigma-Aldrich, respectively. Two kinds of AChE were used in this experiment; the first was extract from housefly (HF) head (Central Research Center, National Agricultural Cooperative Federation, Korea), and the other was bovine red blood cells (RBC) AChE, which were purchased from Sigma-Aldrich.

Determination of AChE activity

The enzyme activity was measured in a 96-well Microplate using a microplate reader (Benchmark Microplate Reader, BioRad) at 415 nm and 37 ^{o}C with acetylthiocholine (1 mM) as substrate and DTNB (1 mM) as chromogen in 0.05 M Tris-HCl buffer, pH 7.8 (Park and Kamble 2001) with a slight modification of Ellman's AChE assay method (Ellman et al. 1961). For RBC AChE, 1% of Triton X-100 (Sigma-Aldrich), we added a Tris-HCl buffer to preserve enzyme activity (Rosenberry and Scoggin 1984). The percentage reactivation of AChE activity was measured by the change of optical density per minute (OD/min) after correction for the control reaction.

AChE Inhibition and reactivation

AChE was inhibited with enough of the inhibitor to inactivate 99% for 10 minutes at room temperature. The concentration of DFP was 12.5 μM for HF AChE and 25 μM for RBC AChE, respectively, and 20 μM of paraoxon for both AChEs. To remove surplus inhibitor molecules after inhibition, the aqueous phase was separated by centrifugation after partitioning with two volumes of hexane (Worek et al. 1998). The collected solution containing phosphorylated AChE was incubated with various concentrations of 2-PAM or HI-6 for various reactivation times, respectively. Small molecules such as the reactivator and phosphorylated oxime were removed by filtration through a micro spin-column packed with Sephadex-G50 (Bio-Rad) (Luo et al. 1998). The AChE activity of the filtrate was measured in a 96-well microplate, and the percentage reactivation of AChE activity was calculated as previously described.

AChE Reactivation with newly synthesized oxime compounds

The reactivating capability of newly synthesized oxime compounds was evaluated against DFP or paraoxon-inhibited HF or RBC AChE, respectively, as previously described, and 5 mM of each oxime compound for 30 minutes for DFP-inhibited AChE and for 1 hour for paraoxon-inhibited AChE, respectively. The percentage reactivation of AChE was calculated as previously described.

RESULTS AND DISCUSSION

It is well known that 3 types of AChEs are generated after the post-transcriptional process of alternative splicing from the same origin (Taylor and Radic 1994). Each type of AChE exists in multiple forms, multimeric for nerve and muscle, dimeric for red blood cell, and monomeric for embryonic and tumor cells, respectively, and their structural difference appears only in the C-terminal extension with 40 residue peptides in contrast to the well-preserved functional subsites such as catalytic triad, acyl pocket, and hydrophobic subsite (Grisaru et. al. 1999). House fly brain AChE and bovine red blood cells AChE were selected for this study as alternative forms of multimeric and dimeric AChEs. DFP has been primarily chosen for this study because of its close structural property to nerve gas (Taylor 2001). Parathion itself is inactive in inhibiting AChE *in vitro* in contrast to its metabolite paraoxon which is active. The sulfur-for-oxygen substitution is carried out predominantly in the liver by the mixed-function oxidases (Dauterman 1971, Butler and Murray

1997). This reaction is also carried out in insects, typically with more efficiency (O'Brien 1960, Oppenoorth 1972, Prestwich 1990). These two organophosphorus compounds have been used as representatives for the organophosphorus AChE inhibitor by many researchers (Gearhart et. al. 1994, Schwarz et. al. 1995, Krummer et. al. 2002, Luo et. al. 2003). We have compared the reactivation potency of oximes (1-5) with two currently used AChE reactivators, 2-PAM and HI-6. As it is shown in Figure 4, the reactivation test was carried out for the DFT-inhibited housefly (HF).

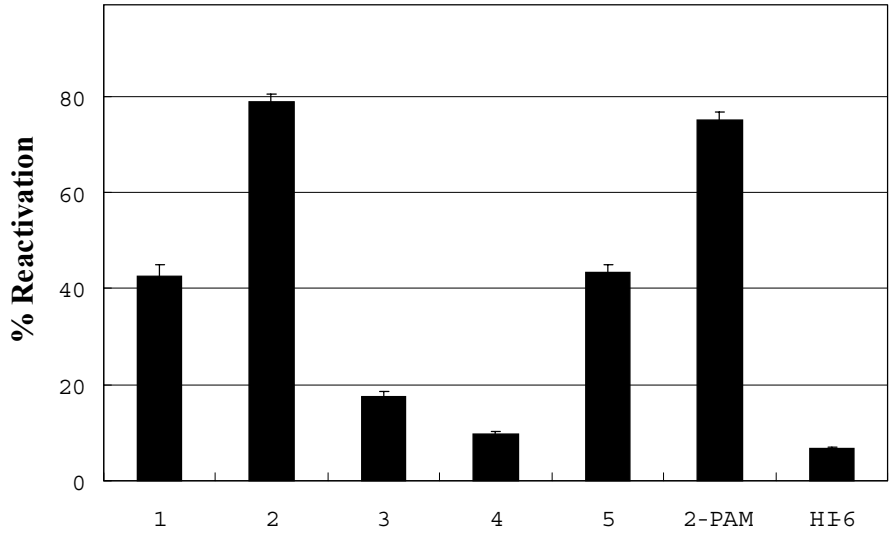

Fig. 4. **Reactivation potency of tested oximes for DFP-inhibited HF AChE**

The potency was strongly dependent upon oxime functional groups, and bis-oxime derivatives (1, 2) are the most potent compared to mono-oxime compounds (3, 4). The position of the oxime group is also an important factor influencing the reactivation process, and compounds with the oxime group at position 2 on the pyridinium ring are the most potent reactivators for DFP-inhibited AChE. The potency of oxime 5 is same as that of oxime 1. From this preliminary result, bis-pyridinium oxime 2 is the most active among the prepared compounds and moreover oxime 2 is more potent than 2-PAM. Even though HI-6 is one of the most active reactivators against many organophosphorus nerve agents, it is no longer active against DFP at $5x10^{-3}$M concentration. Therefore oxime 2 was selected for further evaluation of its reactivation activities. Figure 5 shows the reactivation potency of the oxime 2 for HF and RBC-AChE inhibited by DFP or paraoxon, and compared with the potency of 2-PAM and HI-6. 2-PAM is quite potent for HF-AChE inhibited by DFP and RBC-AChE inhibited by paraoxon, whereas HI-6 shows very weak potency from all tests. Oxime 2 is more potent than 2-PAM, and is especially potent for HF-AChE inhibited by DFP and RBC-AChE inhibited by paraoxon.

In summary, in this *in vitro* reactivation evaluation of the oxime compounds, we found the bis-pyridinium oxime 2 is a strong reactivator for HF-AChE inhibited by DFP and RBC-AChE inhibited by paraoxon.

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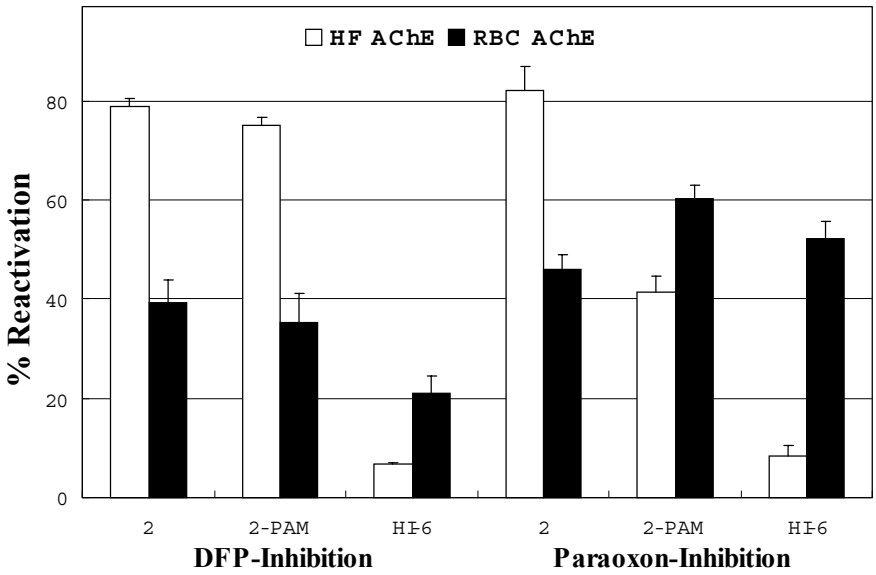

Fig. 5. **Reactivation potency of oxime 2 for DFP- and paraoxon-inhibited AChE**

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