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Coordination of Pt(II) as a strategy to enhance the oxime reactivation of acetylcholinesterase inhibited by nerve agent surrogates

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The activity of brain or erythrocyte acetylcholinesterase (AChE) was inhibited by two nerve agent surrogates at 70% causing a severe intoxication state. The organophosphate pesticides methyl parathion or paraoxon were effective at 10^{-5}-10^{-6} M and 10^{-7}-10^{-8} M, respectively. The *in vitro* experiments revealed that the etiological antidotes 2-PAM, Obidoxime, BT-07, BT-08 and BT-07-4M (quaternary pyridinium aldoximes) possess low to moderate reactivation potential against the effects of methyl parathion. All studied oximes were effective against paraoxon poisoning at 4×10^{-5} M leading to 40-60% recovery of brain-AChE and 20-60% of erythrocyte-AChE activity. The binding of Pt(II) ions to oxime reactivators led to the *in situ* formation of mononuclear coordination species which in some cases exhibit increased recovery ability compared to the starting antidotes. Attention should be paid to the Pt(II)-containing Obidoxime, BT-07 and BT-07-4M, where the presence of the metal(II) ion enhances the reactivation potential of aldoximes by 10-13%. The strategy of modifying the biologically active compounds by complexation reactions may overcome some shortcomings in the therapy of intoxications caused by organophosphorus compounds.

Key words: Organophosphorus compounds, nerve agents, cholinesterase reactivators, quaternary pyridinium aldoximes, Pt(II) complexes, *in vitro* AChE assay

INTRODUCTION

Organophosphorus compounds (OPCs) are used as nerve agents (NAs) and represent the most toxic class of Chemical Warfare Agents. They pose a significant threat to the civilian population in the event of a terrorist attack due to their high toxicity degree. Some OPCs are also commonly applied in agriculture as effective pesticides (insecticides). Despite the structural diversity of these chemicals used at domestic or military level, all OPCs cause an irreversible inactivation of the enzyme acetylcholinesterase (AChE) which breaks down the neurotransmitter acetylcholine (ACh) responsible for the transmission of nerve impulses. The abnormal accumulation of ACh in the neuromuscular synaptic cleft leads to muscle spasms and death due to oxygen deprivation [1, 2].

The standard treatment for OPCs intoxication consists of administration of atropine and oxime reactivator of AChE (RChE), assisted by intensive care. Despite the significant benefits of this worldwide adopted therapy, we still face certain challenges, especially the lack of universal antidote(s) effective against all OPCs [3-5]. An approach to overcome some disadvantages of the currently accepted protocols in case of OPCs intoxications, can be seen at the level of coordination chemistry, since the modification of biologically active compounds upon metal complexation often leads to their enhanced bioactivity. The focus of the present research is pointed to the evaluation of the *in vitro* reactivation potential of selected RChE (Fig. 1) and their coordination species with Pt(II) ions [6, 7] against brain or erythrocyte AChE inhibited by the NAs surrogates methyl parathion (MPT) and paraoxon (PO) [8, 9].

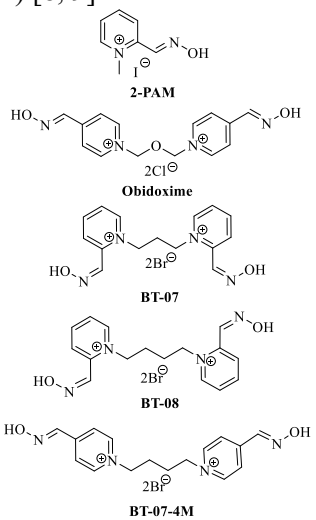

Fig. 1. Structures of the oxime reactivators of AChE (RChE).

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MATERIALS AND METHODS

Reagents and chemicals

The AChE reactivators 2-PAM (HL^+, HLI) and Obidoxime, BT-07, BT-08, BT-07-4M (H_2L^{2+}, H_2LX_2, X = Cl^-, Br^-), as well as methyl parathion (MPT) were provided by the Research Laboratory of Military Toxicology at the Military Medical Academy, Sofia, Bulgaria. Paraoxon (PO), ammonium tetrachloroplatinate(II) ($(NH_4)_2PtCl_4$), sodium hydroxide (NaOH), orthophosphoric acid (H_3PO_4), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), anhydrous potassium dihydrogen phosphate (KH_2PO_4), potassium bicarbonate ($KHCO_3$), Triton X-100, acetylthiocholine iodide (ATChI), 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB) and abs. ethanol (EtOH) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All reagents and solvents were of „p.a." grade. Deionized water was used in all experiments (18.2 MΩ.cm).

Determination of the purity of OPCs

The purity of MPT and PO was determined using an Agilent 6890N gas chromatograph with flame-ionization detector (GC-FID). The solutions of MPT (M_W = 263.2 g/mol, ρ = 1.36 g/cm^3) and PO (M_W = 275.2 g/mol, ρ = 1.27 g/cm^3) in abs. EtOH at 1×10^{-2} M were analyzed (Agilent J&W HP-5 GC column, 30 m × 0.32 mm × 0.25 μm; 2.0 mL/min flow rate for helium gas carrier; 250 °C injector temperature; splitless mode with temperature program starting at 40 °C, hold for 1 min, followed by an increase at a rate of 10 °C/min to 280 °C and hold for 5 min). The volume of the injected sample was 1.0 μL. The amount of each OPC was calculated as a percentage of the corresponding total peak area.

Preparation of stock solutions of the tested compounds

The stock solutions of Pt(II) salt and studied oximes were made at 4×10^{-3} M concentration in Britton-Robinson buffer (8×10^{-2} M, pH 7.4). The corresponding complex species ($[PtL]^{2+}$/$[PtHL]^{3+}$ [6, 7]) were prepared *in situ* 24 hours before experiments by mixing appropriate volumes of the solutions of Pt(II) ion, the given oxime and Britton-Robinson buffer at metal-to-ligand molar ratio of 1:1 and constant total ligand concentration (4×10^{-4} M).

Biological samples

Dissected brains from male Wistar rats (reached sexual maturity, weighing 180-220 g) were provided by the Institute of Neurobiology at the Bulgarian Academy of Sciences. Brain homogenate (2% (w/v) in H_2O) prepared using a mechanical tissue homogenizer was used as a source of brain-AChE. Erythrocytes (RBCs) were isolated from whole blood (healthy volunteers) containing heparin as an anticoagulant by repeatedly washing the cell mass with freshly prepared phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and centrifugation (5 min, 3 000 rpm). They were suspended in 2.5% Triton X-100 to obtain 10% hemolysate which served as a source of erythrocyte AChE (RBC-AChE). Prior to the experiments, the resulting brain homogenates and RBCs hemolysates were stored at the temperature range from –16 to –20 °C.

Enzyme activity inhibition assay

For measuring the enzyme activity inhibition, 200 μL of 2% brain homogenate or 10% RBCs hemolysate were mixed with 25 μL of the inhibitor solution at different concentrations and 25 μL deionized H_2O (final volume 250 μL). The sample was vortexed, incubated at 37 °C for 30 min, and stored in an ice bath until measurement. For the control sample (100% enzyme activity), 25 μL of deionized H_2O were used instead of OPC.

Reactivation assay of enzyme activity

To evaluate the reactivation of enzyme activity, 25 μL of inhibitor solution were added to 200 μL of 2% brain homogenate or 10% erythrocyte hemolysate at the appropriate concentration to achieve 60-80% inhibition after incubation at 37 °C for 30 min. Then 25 μL of the tested solution containing RChE, metal salt or complex were added. The resulting mixture (final volume 250 μL) was homogenized and incubated again at 37 °C for another 30 min. The mixture was then placed on an ice bath until the measurement.

Equipment

The determination of brain acetylcholinesterase (brain-AChE) and erythrocyte acetylcholinesterase (RBC-AChE) activity was performed according to the classical Ellman assay [10], using a Shenzhen Mindray Bio-Medical Electronics BA-88A (semi-automated biochemical analyzer). The reaction mixture consisted of 650 μL of 0.1 M phosphate buffer pH 8.0, 50 μL of 0.01 M DTNB and 10 μL of 0.075 M ATChI. The measurement was started by adding 25 μL of sample. After mixing, the initial absorbance at 420 nm (1 cm optical path) was read after 60 s and every 60 s up to 4 min of the reaction at 37 ºC. The mean absorbance change per minute (ΔA/min) is proportional to the AChE activity (EA)

in the examined sample and is expressed in AU/min. All experiments were performed in triplicate.

Calculations

The inhibitory effect of the insecticides MPT and PO was calculated as % Inhibition = (1 – EA_{INH} / EA_{CONTR}) × 100, where EA_{CONTR} is the enzyme activity of the control sample and EA_{INH} is the enzyme activity of the inhibited sample.

The reactivation potential (recovery ability) of the studied oximes and their Pt(II)-containing coordination species was calculated as % Reactivation = (EA_{REACT} – EA_{INH}) / (EA_{CONTR} – EA_{INH}) × 100, where EA_{REACT} is the enzyme activity of the reactivated sample.

RESULTS AND DISCUSSION

The studied AChE-reactivators (RChE) at present are well-known antidotes for OPCs intoxications in civil clinical practice and military toxicology (2-PAM and Obidoxime) or representatives of a new generation of oximes (BT-07, BT-08, BT-07-4M). Their binding to Pt(II) as new coordination species [6, 7] requires an additional assessment of the effect that the metal(II) ion may render on the reactivation potential of the parent ligands. We therefore carried out *in vitro* studies to evaluate the recovery of brain- or RBC-AChE, inhibited separately by the organophosphate pesticides MPT and PO. The latter are widely used as surrogates (replacements) for NAs in experimental toxicology.

Initially, the purity of MPT and PO pesticides was determined by GC-FID to find that it is 65.3% for MPT and 93.4% for PO, respectively. In subsequent experiments, the concentration of OPCs in the stock solutions was recalculated as 6.6×10^{-3} M for MPT and 1×10^{-2} M for PO. These solutions were further used for the *in vitro* inhibition assay of cholinesterase activity by preparing 10-fold diluted series up to 6.6×10^{-8} M (MPT) and 1×10^{-8} M (PO). The obtained solutions were assayed to determine the concentration of OPCs inducing 60-80% inhibition of acetylcholinesterase, which is associated with the severe state of intoxication where the enzyme reactivation is still possible.

In experiments with brain-AChE, MPT (at 6.6×10^{-6} M) and PO (at 1×10^{-8} M) caused 77.8% and 71.5% inhibition of the enzyme activity, respectively. The activity of RBC-AChE was inhibited by 73.0% using MPT at 2.6×10^{-5} M and by 76.5% in the case of PO applied at 8×10^{-8} M. These OPCs concentrations were used for the next determination of the reactivation potential of the target aldoximes and their complexes with Pt(II) ions. The total ligand concentration was kept constant at 4×10^{-5} M in all final reaction mixtures. Pt(II) salt was also tested at 4×10^{-5} M and no AChE reactivation was observed.

Reactivation of brain-AChE

The classical aldoximes 2-PAM and Obidoxime exhibit very weak effects against MPT-poisoning of brain-AChE but are suitable reactivators when it is inhibited by PO (Figs. 2, 3). The reactivation ability of the corresponding Pt(II) complexes is similar to that of the parent ligands. It should be noted that the efficacy of Pt(II)-containing Obidoxime against MPT-inhibited brain-AChE, although insufficient, significantly increases the potential of the uncoordinated oxime.

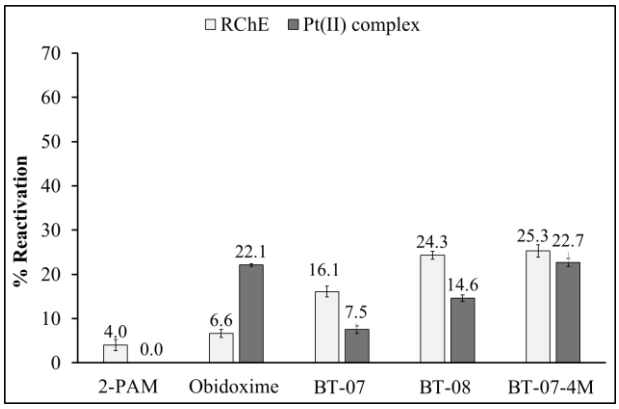

Fig. 2. Reactivation potential of RChE and their Pt(II) complexes against MPT-inhibited brain-AChE.

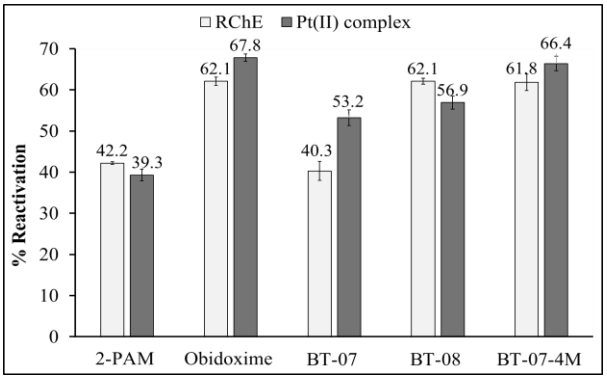

Fig. 3. Reactivation of PO-inhibited brain-AChE by RChE and their Pt(II) complexes.

Compared to the clinically approved antidotes, the new generation oxime reactivators BT-07, BT-08 and BT-07-4M possess an increased activity when the brain-AChE is inhibited by MPT and manifest high reactivation potential towards the PO-effect. In the case of MPT, the Pt(II) derivatives of these aldoximes are less effective than the initial RChE. In contrast, the coordination species of BT-07, BT-08 and BT-07-4M exhibit significant recovery of enzyme activity upon PO inhibition, and even the binding of Pt(II) enhances the reactivation by BT-07

with more than 10%. Within this series the Pt(II) complex of BT-07-4M is the strongest reactivator.

Reactivation of RBC-AChE

The reactivation potential of the classical antidotes and their complexes on OPCs-inhibited RBC-AChE (Figs. 4, 5) follows the same tendency as that observed for brain-AChE. The approved antidotes 2-PAM, Obidoxime and their Pt(II) derivatives are ineffective against MPT and restore the PO-inhibition at lower levels compared to brain-AChE. It is noteworthy that Obidoxime in the presence of Pt(II) exhibits better potential against PO-inhibited RBC-AChE than administered alone.

Although relatively effective against MPT-inhibited RBC-AChE, the new generation oximes BT-07, BT-08 and BT-07-4M express less noticeable recovery ability compared to their efficacy on brain-AChE reactivation, and the complexation with Pt(II) ions retains the properties of the parent RChE. The aldoximes BT-08 and BT-07-4M express the most significant recovery of PO-inhibited RBC-AChE, while BT-07 is a weaker reactivator in contrast to its effect on brain-AChE. The presence of Pt(II) ions plays a diverse effect on the antidote potential of aldoximes – it is negative for BT-07 (no recovery ability), lower by *ca.* 30% for BT-08 and higher by *ca.* 10% for BT-07-4M.

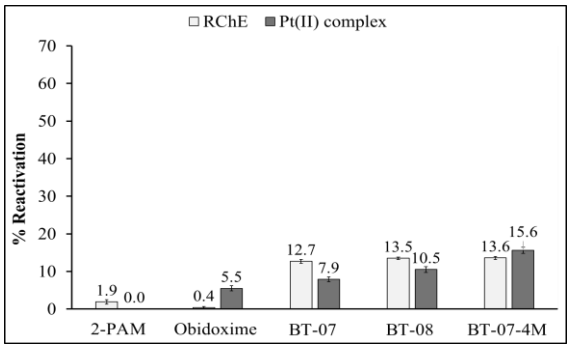

Fig. 4. Reactivation of MPT-inhibited RBC-AChE by RChE and their Pt(II) complexes.

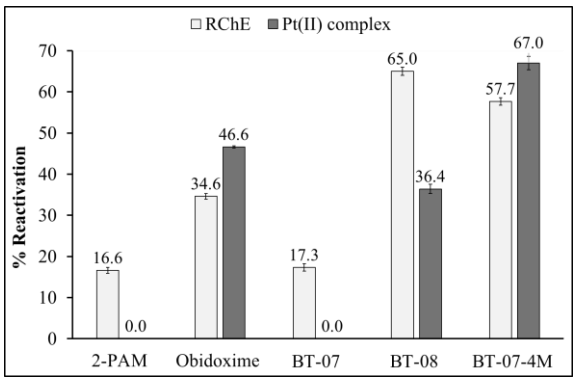

Fig. 5. Reactivation potential of RChE and their Pt(II) complexes against PO-inhibited RBC-AChE.

CONCLUSIONS

The reactivation potential of selected classical (2-PAM and Obidoxime) and new generation (BT-07, BT-08 and BT-07-4M) aldoximes together with that of their Pt(II) derivatives was determined under *in vitro* conditions. The results reveal that, in general, all studied compounds are weak reactivators against the MPT-inhibition of brain- and RBC-AChE but exhibit a pronounced potential towards the action of PO. According to the data obtained, the studied compounds can be ranked in the following hierarchy in the case of PO intoxication, starting from the most potent:

i) *brain-AChE*

aldoximes: Obidoxime ≈ BT-08 ≈ BT-07-4M > 2-PAM > BT-07

Pt(II) species: Obidoxime ≈ BT-07-4M > BT-08 > BT-07 > 2-PAM

ii) *RBC-AChE*

aldoximes: BT-08 > BT-07-4M > Obidoxime > BT-07 ≈ 2-PAM

Pt(II) species: BT-07-4M > Obidoxime > BT-08.

It can be summarized that the coordination of Pt(II) ions influences the potential of the recently studied oxime reactivators in different directions. Thus, Obidoxime exhibits improved recovery ability in the presence of Pt(II) against brain- and RBC-AChE, both inhibited by MPT and PO. Except brain-AChE in the case of MPT-intoxication, the Pt(II) complex of BT-07-4M is more effective than the parent ligand, especially regarding the effect of PO on RBC-AChE activity. In addition, Pt(II)-bound BT-07 is a better reactivator towards PO-inhibited brain-AChE compared to the unmodified oxime.

At this stage of research, it is difficult to provide a reliable explanation for the observed results, but attention should be paid to those particular cases where Pt(II) positively affects (improves) the recovery ability of parent aldoximes. The obtained data can serve as a starting point for future studies, since some of the target coordination compounds are promising chemotherapeutics against OPCs intoxications.

Compliance with ethical standards: *The ethical review and approval were waived for this study due to the use of leftover tissue from untreated (control) animals handled in independent experiments at the Institute of Neurobiology (Bulgarian Academy of Sciences). Informed consent was obtained from all volunteers involved in the study.*

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