Nano-Intercalated Organophosphorus Hydrolyzing Enzymes in Organophosphorus Antagonism

Running title: Nano-Intercalated Hydrolyzing Enzymes

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Abstract

A dendritic poly(2-alkyloxazoline) based polymer was studied as a new carrier system for the organophosphorus-hydrolyzing recombinant enzymes, organophosphorus acid anhydrolase and organophosphorus hydrolase. Paraoxon and diisopropylfluorophosphate were used as model organophosphorus compounds. Changes in plasma cholinesterase activity were monitored. The cholinesterase activity was proportional to the concentrations of DFP or paraoxon. Plasma cholinesterase activity was higher in animals receiving enzyme and oxime before the organophosphates than in the oxime-only pre-treated groups. These studies suggest that cholinesterase activity can serve as an indicator for the *in vivo* protection by the nano-intercalated organophosphorus acid anhydrolase or organophosphorus hydrolase against organophosphorus intoxications. These studies represent a practical application of polymeric nano-delivery systems as enzyme carriers in drug antidotal therapy.

Keywords: Organophosphorus acid anhydrolase (OPAA), 2-PAM, Paraoxon, Dendritic polymer, Diisopropylfluorophosphate (DFP)

Introduction

The toxic effects of organophosphorus (OP) compounds are largely attributable to the inhibition of the enzyme acetylcholinesterase (AChE, EC. 3.1.1.7). AChE mediates the degradation of acetylcholine (ACh), a neurotransmitter of both the peripheral and the central nervous system. When an electrical impulse reaches the presynaptic neuron, ACh is released and subsequently binds to a receptor (muscarinic or nicotinic). After binding, ACh is degraded by AChE, thus regenerating the receptor and rendering it active again. OP compounds inhibit AChE by phosphorylation (organophosponates by phosphonylation) of a serine hydroxyl located in the active site. Thus, ACh is not hydrolyzed and continues to interact with the receptor, resulting in persistent and uncontrolled stimulation. Clinical effects of OP compounds, which include common pesticides and nerve agents, are the result of this persistent stimulation and subsequent fatigue at the ACh receptor. The inactivation of AChE eventually becomes irreversible, a phenomenon known as aging. Once aging occurs, the AChE enzyme cannot be reactivated. For the clinical effect to be reversed, new enzyme has to be produced.

Currently, the standard treatment for OP poisoning is the co-administration of the AChE reactivator pralidoxime (2-PAM) and the anticholinergic atropine (Tong et al., 1962). Atropine antagonizes the pharmacologic effects of acetylcholine by occupying the muscarinic receptors, thus blocking the effect of the accumulated acetylcholine. 2-PAM reactivates the inhibited AChE by nucleophilic displacement of the phosphate moiety. Although 2-PAM + atropine combination therapy can effectively treat the symptoms of OP poisoning, this is accomplished without protecting AChE from subsequent inhibition by the OP persistent in

the body. If health effects of chemical agent exposure are to be minimized, a treatment should be developed to reduce or eliminate the OPs from the body, thus protecting AChE from inhibition.

The ability of enzymes to detoxify OPs has long been recognized. Enzymes capable of detoxifying OPs include organophosphorus acid anhydrolase (OPAA) and organophosphorus hydrolase (OPH). OPAA is a recombinant OP hydrolyzing enzyme (Aldridge et al., 1989) which demonstrates substrate specificity for the P-F bond of sarin, soman, and diisopropylfluorophosphate (DFP) (Cheng et al., 1993), while OPH has a broader substrate specificity and is capable of hydrolyzing P-O (paraoxon), P-F (DFP, soman and sarin), P-S (demeton-S and VX) and P-CN (tabun) bonds. The application of these enzymes for protection against OP poisoning is a concept with exciting potential in the treatment of both pesticide and chemical warfare agent (CWA) exposure.

One of the earliest studies demonstrating the utility of enzymatic prophylaxis used resealed and annealed red blood cells (RBC) prepared by hypotonic dialysis as a carrier platform (DeLoach et al., 1980; Ihler et al., 1973). Subsequently, carrier red blood cells (CRBC) were evaluated as enzyme carriers, originally in cyanide antagonism (Cannon et al., 1992; Leung et al., 1986; Petrikovics et al., 1995; Way et al., 1985), and more recently in OP antagonism (Pei et al., 1994, 1995). In addition to the CRBC, these enzymes have been deployed with other enzyme delivery platforms, including sterically stabilized liposomes (SL) (Allen 1994; Lasic and Papahadjopoulos, 1998; Szoka and Papahadjopoulos, 1980; Woodle and Lasic, 1992). Encapsulation of OPAA and OPH within sterically stabilized liposomes (SL-OPAA

and SL-OPH, respectively) and the successful prophylactic/therapeutic application in OP antagonism has been reported (Petrikovics et al., 1999, 2000a, 2000b, 2004).

Dendritic polymers (DP) have become a focus of research interest in the area of drug delivery systems (Dagani, 1996; Tomalia et al., 1985, 1986, 1997; Petrikovics et al., 2007, 2010; Yin et al., 1998). Their general chemical structure is -(CH_2CH_2NR)-, where R is H, methyl, ethyl, or other alkyl groups. Recently, a novel hyper-branched polymer (HBP) with a CH_3-$(CH_2)_{17}$ surface-modified hyperbranched poly(2-ethyloxazoline) polymer was synthesized (Yin et al., 1997). With the internal tertiary amide functional group on repeating ethyloxazoline units and external C_{18} chains, this HBP possesses a hydrophilic core and a hydrophobic shell. Dendritic polymers, including dendrimers, dendrigraft, and hyperbranched polymers, exhibit very different properties than the traditional linear polymers. DPs are three dimensional, tree-like, spherical macromolecules whose size and shape resemble traditional micelles. However, unlike traditional lipid-based micelles, which tend to exist in equilibrium between self-assembled and randomly distributed lipids, the dendritic polyoxazoline polymeric micelles can withstand a variety of environmental conditions, including solvent, temperature and pH extremes. These DPs have been used to encapsulate OPH (DP-OPH) and OPAA (DP-OPAA) in the presence of a surface decontaminant (EcoTru, Enviro Systems, Inc., San Jose, CA) and tested as potential catalyst-based dual-use CWA decontamination formulation (Chen et al., 2004; Petrikovics et al., 2007).

This study describes the *in vitro* efficacy of OPAA and OPH, when encapsulated within a dendritic poly(2-alkyloxazoline) polymer based nanocapsules, in decreasing AChE inhibition by DFP and paraoxon, respectively. Since OP compounds are cholinesterase inhibitors,

monitoring the AChE level in biologic fluids is utilized as an indicator of the efficacy of the different OP antidotal systems.

Material and Methods

Chemicals

Paraoxon and DFP were purchased either from Sigma-Aldrich (St. Louis, MO). When necessary, paraoxon was further purified by aqueous sodium bicarbonate extraction (Pei et al., 1994). Atropine sulfate and 2-PAM solutions were prepared fresh daily. OPAA was a generous gift from the laboratory of T.C. Cheng (US Army Edgewood Chemical & Biological Center, Aberdeen Proving Ground, MD 21010-5423, USA) and stored at –70 ºC in 1 μM DTT (Cheng et al., 1993; DeFrank et al., 1991). Recombinant OPH (E.C. 3.1.8.1) was purified from *Escherichia coli* DH5α containing the plasmid expression vector pOP419, and purified as previously reported (Lai et al., 1994). AChE was purchased from Sigma-Aldrich (930 Units/ml).

Animals

Male Balb/C mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing between 18-20 g were housed in room temperature and light controlled rooms, (21 ± 2 ^{o}C, 12-h light/dark cycle) and were furnished with water and 4% fat Rodent Chow (Teklad HSD, Inc., WI) *ad libitum*. All animal procedures were conducted in accordance with the guidelines in *The Guide for the Care and Use of Laboratory Animals* (National Academic Press, 1996), credited by *AAALAC* (American Association for the Assessment and

Accreditation of Laboratory Animal Care, International). At the termination of the experiments, surviving animals were euthanized with "Aerrane" (Isoflurane) from Fort Dodge Animal Heath, (Forth Dodge, IA) in accordance with the 1986 report of the AVMA Panel of Euthanasia.

Determination of enzymatic activity (OPAA and OPH)

OPAA activity was measured by monitoring the production of fluoride from DFP with a fluoride ion sensitive electrode (Orion Research Inc., Boston, MA) (Hoskin & Roush 1982). The assay solution for the enzyme fractions contained 70 mM Tris, pH 7.2, with 70 mM NaCl, 280 mM KCl, and 3.44 mM DFP. The solution for determining the activity of DP encapsulated enzyme was isotonic (290 mOsm), and composed of 10 mM phosphate buffer, 144 mM NaCl, 2.0 mM $MgCl_2{*}6H_2O$ and 5 mM dextrose. The total volume of the reaction mixture was 5 ml. The electrode potential was recorded as a function of time and potential values were converted to concentration using the Nernst equation. Protein assays were accomplished by the Bradford method (Bradford, 1976) using BioRad protein assay reagents (BioRad, Richmond, CA). One unit of OPAA is defined as the amount of enzyme which hydrolyzed one µmol of DFP to fluoride and isopropyl phosphate per min.

OPH activity was measured at room temperature spectrophotometrically, following the rate of formation of *p*-nitrophenol (*p*-NP) from paraoxon. The standard solution used to determine OPH activity was isotonic (290 mOsm) containing 15 mM phosphate buffer (pH 7.8), 216 mM NaCl, 0.08 mM $ZnCl_2$, 3.0 mM $MgCl_2$, and varying amounts of DP-OPH in a final volume of 2.0 ml. The reaction was initiated with the addition of DP-OPH, and

monitored at 400 nm. One unit of OPH is defined as that amount of enzyme which hydrolyzes one μmol of paraoxon per min. Protein assays were performed by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA).

Preparation of nano-intercalated OPAA and OPH

The synthetic work was performed as reported by Yin et al. (1997). The hyper-branched poly(2-ethyloxazoline) with $CH_5(CH_2)_{17}$ chain modified surface was synthesized by a convergent method and is referred to as C_{18}-$HBP_{20/100}$, where the subscript 20/100 represents the average number of repeat units for interior branches and the center core molecule (*i.e.*, 20 and 100 repeat units, respectively).

The DP was dissolved in Bis-Tris-Propane buffer (BTP) (50 mM, pH 8.5) and then combined with the BTP solution of OPAA or OPH to make up the final solution of 20 mg dendrimer-enzyme mixture per ml. Various ratios of polymer to enzyme (w/w) were evaluated, and the ratios of 10:1 and 20:1 worked optimally for the OPAA and OPH enzyme encapsulation, respectively. The DP carrier systems encapsulating the OPAA or OPH were then lyophilized, giving a powder of DP-OPAA or DP-OPH with the enzyme activity of 1-5 units/mg. For *in vivo* studies they were dissolved in sterile water to provide the required enzyme units (100 units of OPAA, 20 units of OPH) for each test animal. In lyophilized form, the encapsulated OPAA or OPH are stable for months (Cheng et al., 2004).

In vitro AChE inhibition by OP's

AChE was re-suspended to 1 mg/ml in 100 mM phosphate buffer, pH 7.4 and allowed to

hydrate for 24 hours. Inhibition levels were established for each OP by incubating 0.1 mg/ml AChE for 24 hours with a series of OP concentrations. After incubation the enzyme was further diluted to 0.033 mg/ml. Acetylthiocholine (ASCh, 1 mM) was used as the substrate and the reaction was monitored spectrophotometrically at 405 nm for the reaction product using 1 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The amount of OP used in the protection studies corresponded to the level that inhibited the enzyme to >95%; the absolute concentration varied by lot of AChE.

In vivo plasma cholinesterase studies

Male mice received 100 units of DP-OPAA or 20 units of DP-OPH and 2-PAM (45 mg/kg) in a maximum volume of 10 μl/mg mouse, intravenously (dorsal tail vein injection) Sublethal doses of the OPs, DFP (3.5 mg/kg) or paraoxon (0.65 mg/kg), were injected subcutaneously. Blood was taken two min after OP injection, and centrifuged to separate the plasma. Cholinesterase activity was determined by the method of Ellman et al. (1961) as previously described for the standard assay, with the following modifications: 50 μl of plasma was diluted into 6.0 ml phosphate buffer (100 mM, pH 8.0). Two and a half milliliters of this plasma/buffer solution was substituted for the phosphate buffer in the standard assay. The reaction was blanked against 2.5 ml plasma/buffer and the absorbance recorded at 412 nm for 6 min.

Results

Formation of fluoride ions was directly proportional to the amount of DP-OPAA and the

formation of *p*-nitrophenol was directly proportional to the amount of DP-OPH, indicating that, under the conditions defined in this study, increases in amount of DP-OPAA (0.224 mM F^- ion sec^{-1} μl^{-1} DP-OPAA) or DP-OPH (0.159 mM *p*-NP $sec^{-1}\mu l^{-1}$ DP-OPH) are equivalent to increases in amounts of enzyme present in the reaction mixture. The hydrolysis rates for both the DFP and paraoxon were linear over one minute for the concentration ranges of enzyme and substrate employed in these studies. The DP without enzyme did not hydrolyze DFP or paraoxon. It is important to note that in these studies the amount of encapsulated enzymes used was standardized, but not optimized.

AChE inhibition was proportional to the concentration of DFP and paraoxon in the system (Fig. 1.), which indicates these compounds can effectively simulate an OP nerve agent. In the presence of 18 mM DFP, cholinesterase was inhibited greater than 95%. When 2-PAM was employed with DFP, the cholinesterase activity was still inhibited up to 70%. The application of DP-OPAA further reduced the DFP-mediated inhibition of cholinesterase to approximately 44% (Fig. 2.). Similar experiments with paraoxon showed that 0.7 nMparaoxon inhibited 0.12 U of AChE greater than 98%. The application of 2-PAM alone limited the inhibition to 90%, while the 2-PAM + DP-OPH combination decreased the inhibition to approximately 70% (Fig. 2.). Fig. 3A. shows the *in vivo* plasma cholinesterase activity in mice after administering sub-lethal doses of DFP alone, and in conjunction with 2-PAM and 2-PAM + DP-OPAA/DP-OPH. Blood samples taken 2 min after DFP administration indicated that DFP inhibited the cholinesterase activity by approximately 90%. This inhibition was significantly less when 2-PAM was employed alone, or in a combination with DP-OPAA. Similar effects on cholinesterase activity were observed when

paraoxon was employed with and without (DP-OPH) and/or 2-PAM (Fig. 3B.). Comparatively DP-OPH appeared to provide a better protection of cholinesterase activity than DP-OPAA.

Discussion

This study demonstrates the use of synthetic polymeric nanocapsules (DP) as an enzyme delivery system to antagonize the lethal effects of OP anticholinesterases. This relatively new concept combines recombinant enzyme biotechnology with recent developments in polymer chemistry to produce synthetic enzyme carriers. The present study was focused on the *in vitro* indication of DFP and paraoxon effects alone, in the presence of a reactivator, 2-PAM, and/or a DP-encapsulated OP-metabolizing enzyme, DP-OPAA or DP-OPH, by measuring the cholinesterase activity. Monitoring the cholinesterase activity in the biological system (Fig. 3.) was also used to identify OP exposure, as well as detoxification by the OP antidotes. (While acetylcholinesterase (3.1.1.7) can be determined from whole blood, when plasma is analyzed, we measure butyrylcholinesterase: (3.1.1.8) activity with acetylthiocholine as a substrate). It has to be noted that oximes, such as 2-PAM, can react with acetylthiocholine (oximolysis), producing thiocholine what can react with the thiol reagent 5,5'-dithio-bis-2-nitrobenzoic acid of the Ellman's method (Guarisco et al., 2009; Kern et al., 2009; Sinko et al., 2007). This suggests that the Ellman method has to be employed critically in the presence of oximes: if the reaction of oximolysis is faster than the Ellman reaction under the experimental conditions, than the observed 5-thio-2-nitrobenzoic acid concentration can originate from both the cholinesterase reaction and the oximolysis of acetylthiocholine.

The antidotal regimen currently in use in the United States for the treatment of OP toxicity is the combination of 2-PAM and atropine. While this treatment can relieve the symptoms of OP toxicity, it does not remove the nerve agents from the body. Therefore, nerve agents may persist long after exposure, thus necessitating multiple applications of the 2-PAM + atropine regime. Presently, there are no antidotes approved for use that can eliminate OP agents from the body, although both OPAA and OPH have been shown to effectively decontaminate most of the OP agents and pesticides. This study demonstrates that the direct injection of appropriate amounts of enzyme can provide greater protection against the lethal effects of OP agents than the current treatment regimen of (2-PAM + Atropine). The magnitude and duration of this protection is found to be greatly increased if enzymes are entrapped into drug delivery biocarriers such as sterically stabilized liposomes (Petrikovics et al., 1999, 2000a, 2000b), or carrier red blood cells (Cannon et al., 1992; Leung et al., 1986; Pei et al., 1994, 1995; Petrikovics et al., 1995; Way et al., 1985). Employing tree-like (dendritic) polymer based DP offers a number of advantages, including better water solubility, non-toxic nature, and most importantly, ease of encapsulation for delivery. The preparation of core shell highly branched polymers usually requires a multi-step synthesis. This poly(2-ethyloxazoline) based core shell DP was prepared by a one-pot polymerization process starting from commercially available monomers. The use of external enzymes as drug antidotes is a relative new concept in the history of drug antidotal therapy, and the present and previously reported results suggest that this approach has a great potential. These studies represent one of the biomedical applications of nanotechnology, the development of nano-structure delivery systems as therapeutic agents for drug antidotal therapy.

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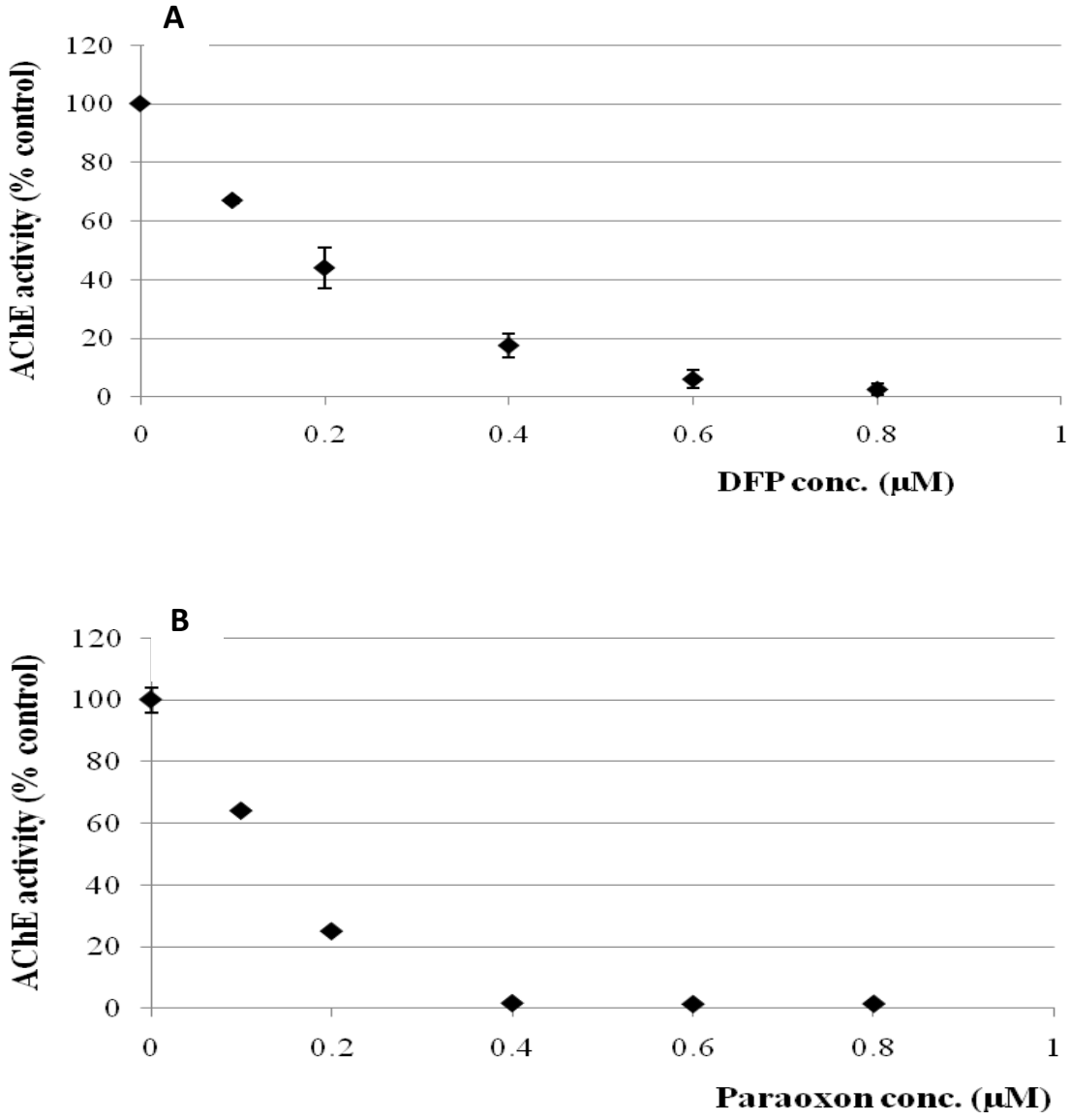
Figure legends

Figure 1. AChE inhibition as a function of increasing amounts of (A) paraoxon and (B) DFP. The inhibition assays contained 0.5 mM acetylthiolcholine iodide, 33 nM DTNB, and 0.12 units of AChE in a final volume of 3 ml.

Figure 2. *In vitro* AChE activity in the presence of treatment combinations. The assay mixture contained 0.5 mM acetylcholine iodide, 33 mM DTNB, 0.12 U AChE, 1.3 mM 2-PAM. Reactions represented in (A) all contained 18 nM DFP in all reactions, except the control, and 5 units of DP-OPAA as indicated. The reactions represented in (B) all contained 0.7 nM paraoxon and 20 units of DP-OPH where indicated. In all cases, the control is uninhibited cholinesterase activity.

Figure 3. *In vivo* plasma cholinesterase activity in mice. Mice were pre-treated with 2-PAM (45 mg/kg, iv.), or with 2-PAM + DP-enzyme combination. Blood samples were taken 2 min after subcutaneous injection of either 3.5 mg/kg DFP or 0.65 mg/kg paraoxon. A) DFP exposure. DP-OPA was administered as 100 U i.v. B) Paraoxon exposure. One to two units of DP-OPH was administered as indicated.

Figure 1.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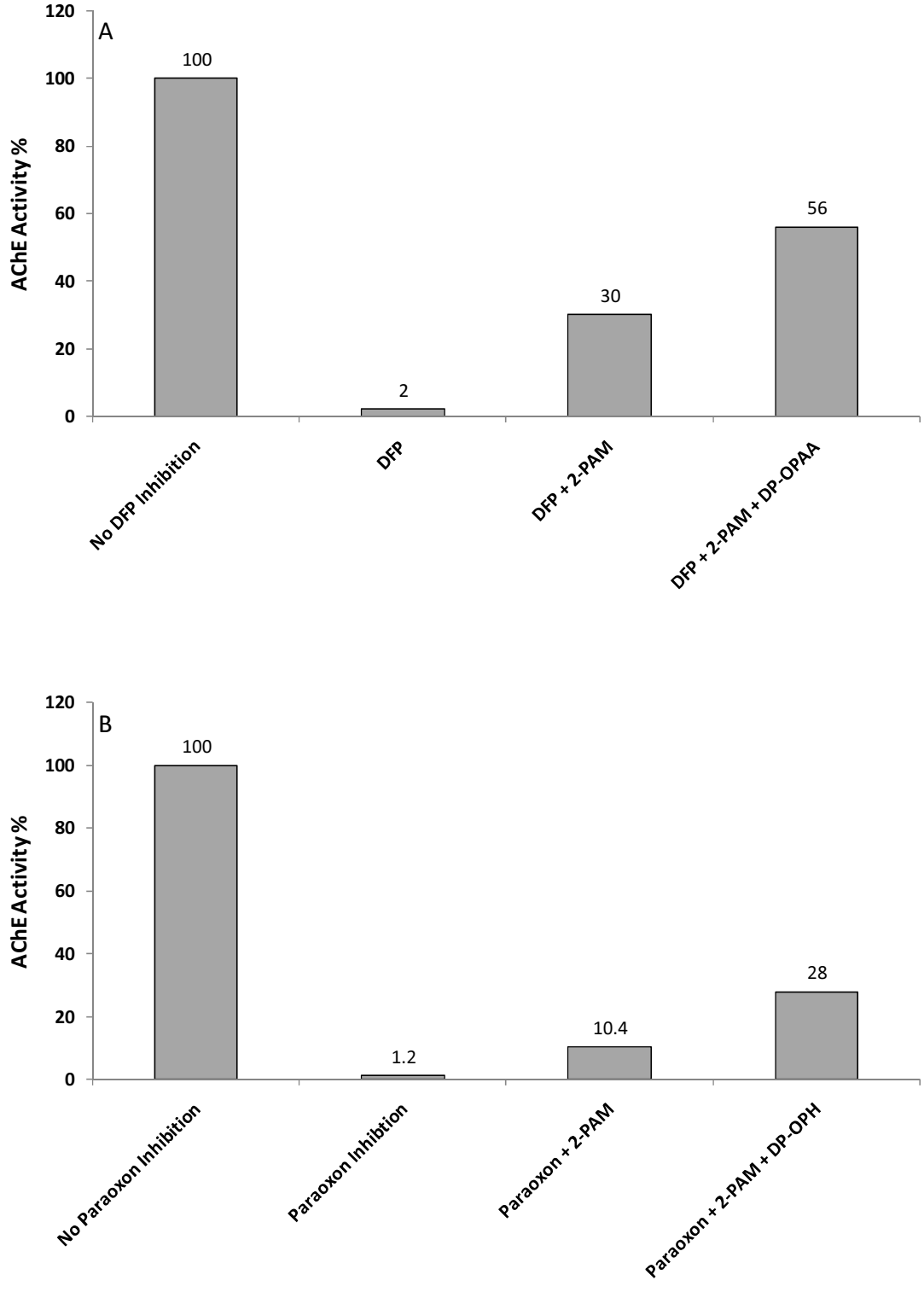
AChE activity (% control)
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
DFP conc. (µM)
B
AChE activity (% control)
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
Paraoxon conc. (µM)

Figure 2.


A
AChE Activity %
120
100
80
60
40
20
0
100
2
30
56
No DFP Inhibition
DFP
DFP + 2-PAM
DFP + 2-PAM + DP-OPAA
B
AChE Activity %
120
100
80
60
40
20
0
100
1.2
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Paraoxon Inhibtion
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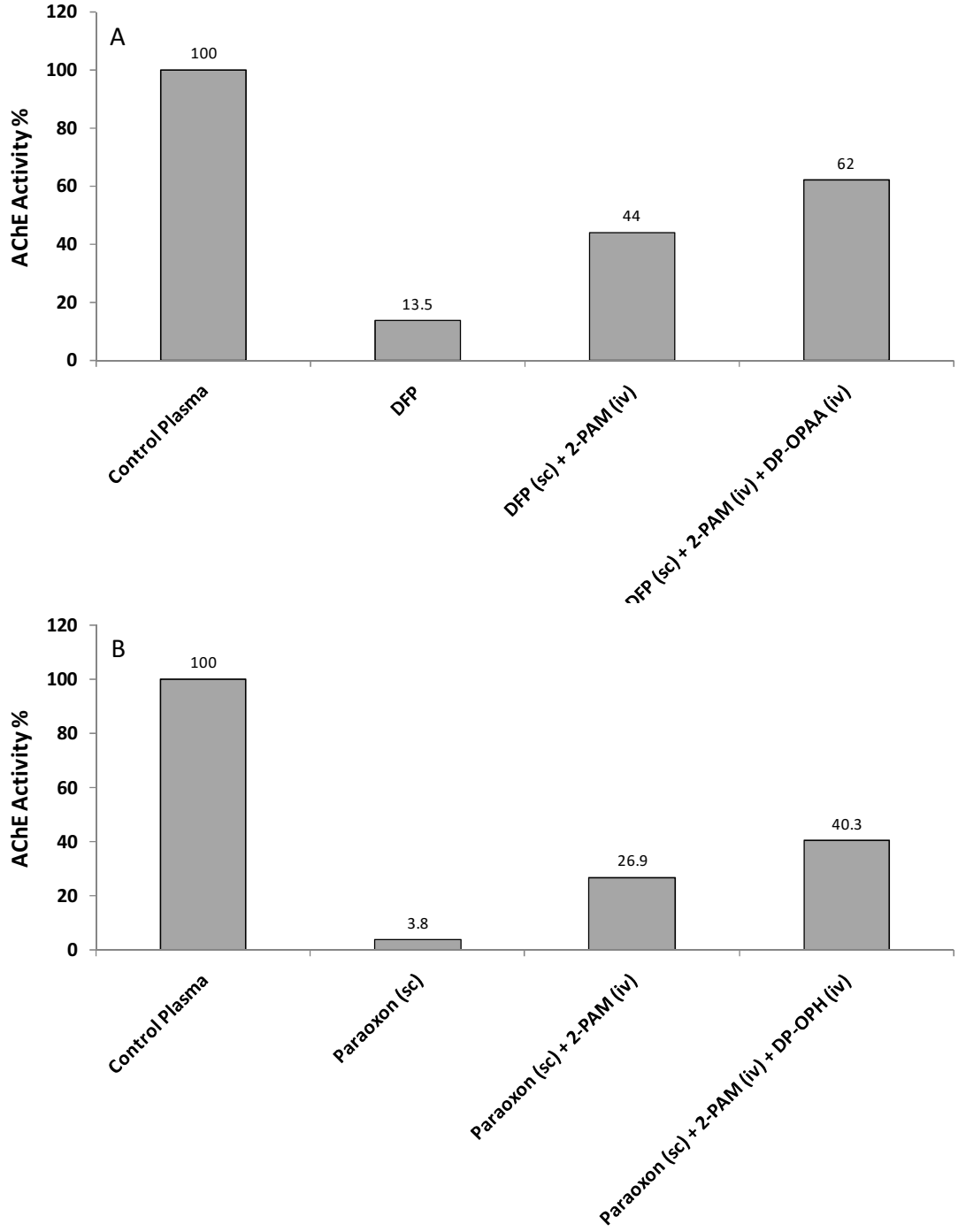
Paraoxon + 2-PAM + DP-OPH

Figure 3.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AChE Activity %
120
100
80
60
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20
0
100
13.5
44
62
Control Plasma
DFP
DFP (sc) + 2-PAM (iv)
DFP (sc) + 2-PAM (iv) + DP-OPAA (iv)
B
AChE Activity %
120
100
80
60
40
20
0
100
3.8
26.9
40.3
Control Plasma
Paraoxon (sc)
Paraoxon (sc) + 2-PAM (iv)
Paraoxon (sc) + 2-PAM (iv) + DP-OPH (iv)