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*Research Article*

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# Nano-Intercalated Organophosphorus-Hydrolyzing Enzymes in Organophosphorus Antagonism

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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 (PO) and diisopropylfluorophosphate (DFP) were used as model organophosphorus compounds. Changes in plasma cholinesterase activity were monitored. The cholinesterase activity was proportional to the concentrations of DFP or PO. Plasma cholinesterase activity was higher in animals receiving enzyme and oxime before the organophosphates than in the oxime-only pretreated 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.

**KEY WORDS:** dendritic polymer; diisopropylfluorophosphate (DFP); organophosphorus acid anhydrolase (OPAA); paraoxon; 2-PAM.

## 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 systems. 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, a 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 (1). 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 (2) which demonstrates substrate specificity for the P–F bond of sarin, soman, and diisopropylfluorophosphate (DFP) (3), while OPH has a broader substrate specificity and is capable of hydrolyzing P–O [paraoxon (PO)], 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

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cells prepared by hypotonic dialysis as a carrier platform (4,5). Subsequently, carrier red blood cells (CRBC) were evaluated as enzyme carriers, originally in cyanide antagonism (6–9), and more recently in OP antagonism (10,11). In addition to the CRBC, these enzymes have been deployed with other enzyme delivery platforms, including sterically stabilized liposomes (SL) (12–15). Encapsulation of OPAA and OPH within sterically stabilized liposomes (SL-OPAA and SL-OPH, respectively) and the successful prophylactic/therapeutic application in OP antagonism have been reported (16–19).

Dendritic polymers (DP) have become a focus of research interest in the area of drug delivery systems (20–26). Their general chemical structure is $–(CH_2CH_2NR)–$, where R is H, methyl, ethyl, or other alkyl groups. Recently, a novel hyperbranched polymer (HBP) with a $CH_3–(CH_2)_{17}$ surface-modified hyperbranched poly(2-ethyloxazoline) polymer was synthesized (27). 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. The grafted hydrophobic $C_{18}$ chains act as a "smart arm" for enzyme encapsulation, trapping the enzymes internally yet allowing substrate and product to diffuse freely. 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 which possess an interior void space, 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 (24).

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 PO, 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, DFP, atropine sulfate, and 2-PAM were purchased from Sigma-Aldrich (St. Louis, MO). When necessary, paraoxon was further purified by aqueous sodium bicarbonate extraction (10). 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°C in 1 μM DTT (3,28). 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 (29). AChE was purchased from Sigma-Aldrich (St. Louis, MO; 930 units/ml).

### Animals

Male BALB/C mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing between 18 and 20 g were housed in room temperature- and light-controlled rooms, (21 ± 2°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 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. The name of the approval committee for Animal Subjects at Sam Houston State University (Huntsville, TX) is Institutional Animal Care and Use Committee.

### 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) (30). 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 $MgCl_2 \cdot 6H_2O$, 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 (31) using Bio-Rad protein assay reagents (Bio-Rad, Richmond, CA). One unit of OPAA is defined as the amount of enzyme which hydrolyzed 1 μmol of DFP to fluoride and isopropyl phosphate per minute.

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 1 μmol of paraoxon per minute. Protein assays were performed by the Bradford method (31) 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.* (27). The hyperbranched 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 milliliter. 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. When preparing DP-OPAA, 20 mg of lyophilized OPAA was added to 1 ml of water containing 200 mg of DP. When preparing DP-OPH, 10 mg of lyophilized OPH was added to 1 ml water containing 200 mg of DFP. Enzyme activities were determined by the abovementioned methods. 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 and 20 units of OPH) for each test animal. These enzyme amounts were determined based on earlier studies with OPH/paraoxon and OPAA/DFP, according to the substrate specificity of these enzymes (24). In lyophilized form, the encapsulated OPAA or OPH are stable for months (32).

### *In Vitro* AChE Inhibition by OPs

AChE was resuspended to 1 mg/ml in 100 mM phosphate buffer, pH 7.4 and allowed to hydrate for 24 h. Inhibition levels were established for each OP by incubating 0.1 mg/ml AChE for 24 h with a series of OP concentrations. After incubation, the enzyme was further diluted to 0.033 mg/ml. Acetylthiocholine (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 2 min after OP injection and centrifuged to separate the plasma. Cholinesterase activity was determined by the method of Ellman *et al.* (33) 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 $s^{-1}$ $\mu l^{-1}$ DP-OPAA) or DP-OPH (0.159 mM *p*-NP $s^{-1}$ $\mu l^{-1}$ 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 1 min 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 that 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 nM paraoxon 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). Figure 3a shows the *in vivo* plasma cholinesterase activity in mice after administering sublethal 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

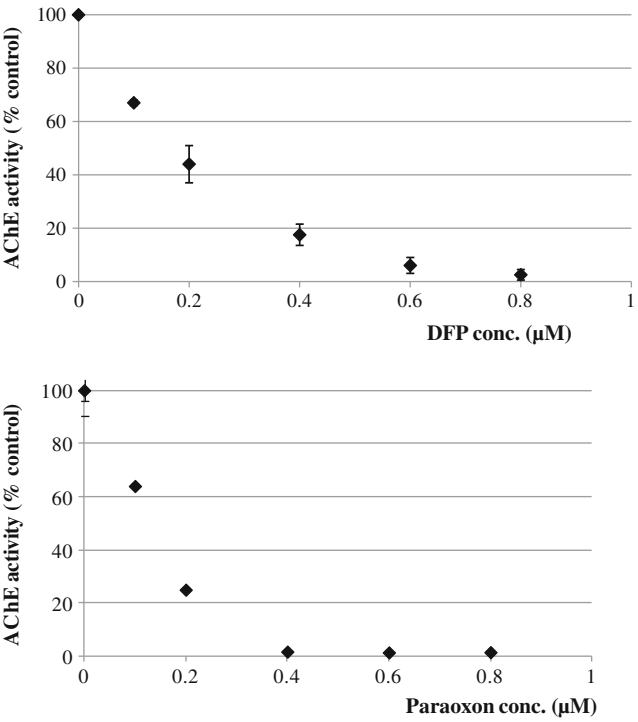


**Fig. 1.** AChE inhibition as a function of increasing amounts of **a** paraoxon and **b** DFP. The inhibition assays contained 0.5 mM acetylthiolcholine iodide, 33 nM DTNB, and 0.12 units of AChE in a final volume of 3 ml. Data are expressed as the mean±SD of three experiments

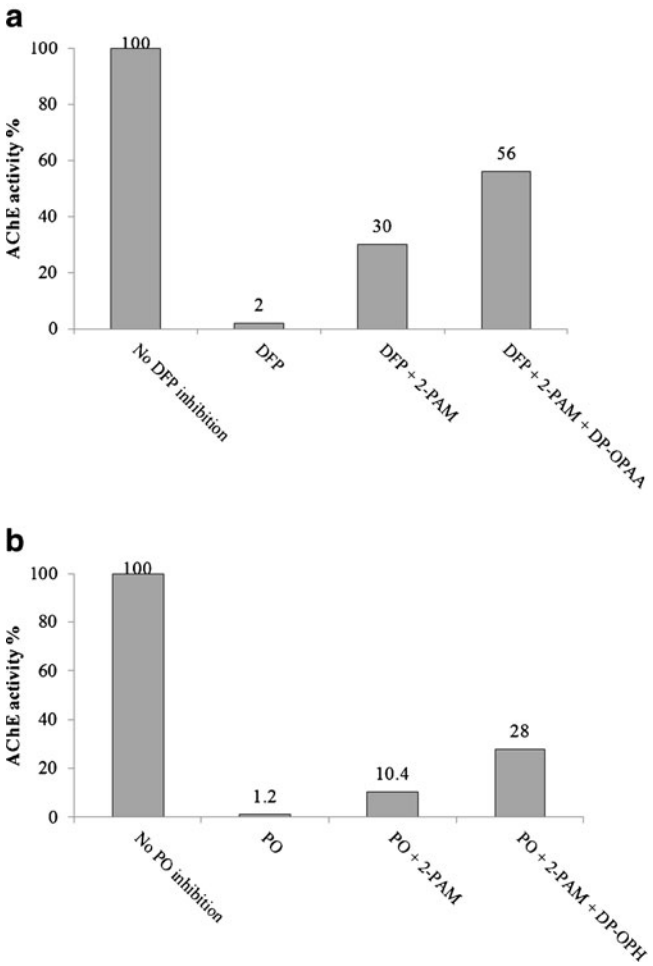


**Fig. 2.** *In vitro* AChE activity in the presence of treatment combinations. The assay mixture contained 0.5 mM acetylcholine iodide, 33 mM DTNB, 0.12 U AChE, and 1.3 mM 2-PAM. Reactions represented in **a** all contained 18 nM DFP in all reactions, except the control, and 5 units of DP-OPAA as indicated. The reactions represented in **b** all contained 0.7 nM POparaoxon and 20 units of DP-OPH where indicated. In all cases, the control is uninhibited cholinesterase activity. Each data point is the average of triplicate assays; in all cases, the experimental error was less than 10%

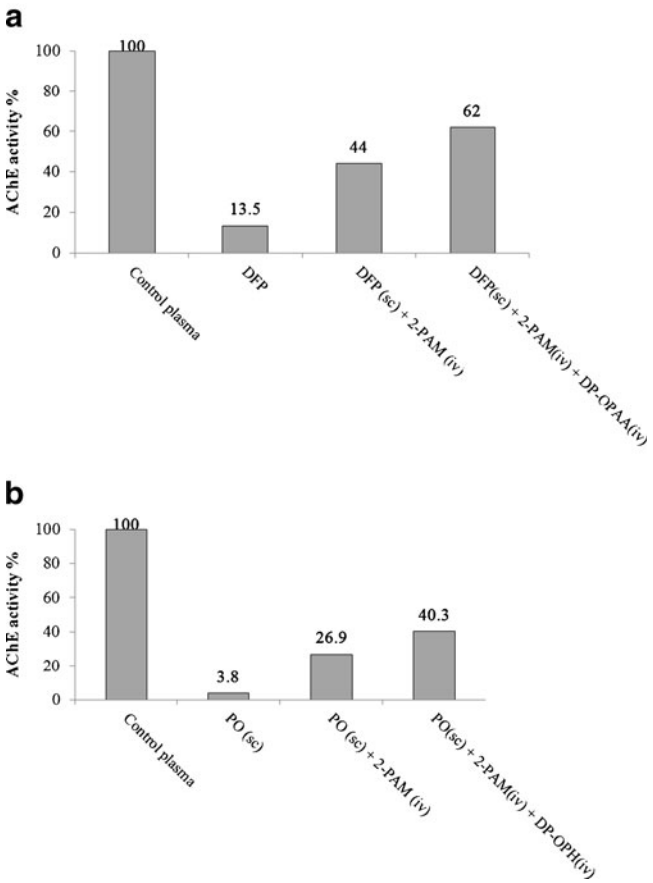


**Fig. 3.** *In vivo* plasma cholinesterase activity in mice. Mice were pretreated with 2-PAM (45 mg/kg, iv) or with 2-PAM+DP enzyme combination. Blood samples were taken 2 min after subcutaneous injection of either 3.5 mg/kg DFP or 0.65 mg/kg paraoxon. **a** DFP exposure. DP-OPA was administered as 100 U iv. **b** Paraoxon exposure. One to 2 units of DP-OPH was administered as indicated. Each data point is the average of triplicate assays; in all cases, the experimental error was less than 10%

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 that can react with the thiol reagent 5,5′-dithio-bis-2-nitrobenzoic acid of the Ellman's method (34–36). 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, then 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 USA 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 are found to be greatly increased if enzymes are entrapped into drug delivery biocarriers such as sterically stabilized liposomes (16–18) or carrier red blood cells (6–11). Employing tree-like (dendritic) polymer-based DP offers a number of advantages, including better water solubility, nontoxic 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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## REFERENCES

1. Tong HS, Way JL. Protection against 1-alkyl-phosphate intoxication by intercerebral injection of 1-methyl-2-formylpyridinium iodide oxime (2-PAM). J Pharmacol Exp Ther. 1962;138:218–23.
2. Aldridge WN, Hoskin FCG, Reiner E, Walker CH. Suggestions for a nomenclature classification of enzymes hydrolyzing organophosphorus compounds. In: Reiner E, Aldridge WN, Hoskin FCG, editors. Enzymes hydrolyzing organophosphorus compounds. Chichester: Ellis Horwood Ltd.; 1989. p. 246–53.
3. Cheng TC, Harvey SP, Stroup AN. Purification and properties of a highly active organopsosphorus acid anhydrolase from *Alteromonas undina*. Appl Environ Microbiol. 1993;59:3138–40.
4. DeLoach JR, Harris RL, Ihler GM. An erythrocyte encapsulation dialyzer used in preparing large quantities of erythrocyte ghosts and encapsulation of pesticide in erythrocyte ghosts. Anal Biochem. 1980;102:220–7.
5. Ihler GM, Glew RH, Schnure FW. Enzyme loading of erythrocytes. Proc Natl Acad Sci U S A. 1973;70:2663–6.
6. Cannon EP, Zitzer AH, McGuinn WD, Petrikovics I, Leung P, Way JL. Antagonism of lethal effects of cyanide with rhodanese containing murine carrier erythrocytes. Proc West Pharmacol Soc. 1992;35:187–90.
7. Leung P, Ray LE, Sander C, Way JL. Encapsulation of thiosulfate: cyanide sulfurtransferase by mouse erythrocytes. Toxicol Appl Pharmacol. 1986;83:101–10.
8. Petrikovics I, Cannon EP, McGuinn WD, Way JL. Cyanide antagonism with organic thiosulfonates and carrier red blood cells containing rhodanese. Fundam Appl Toxicol. 1995;24:1–8.
9. Way JL, Leung P, Ray L, Saunder C. Erythrocyte encapsulated thiosulfate sulfurtransferase. Bibl Haematol. 1985;51:75–81.
10. Pei L, Omburo G, McGuinn WD, Petrikovics I, Dave K, Raushel JL, Wild JR, DeLoach JL, Way JL. Encapsulation of phosphothiesterase within murine erythrocytes. Toxicol Appl Pharmacol. 1994;124:296–301.
11. Pei L, Petrikovics I, Way JL. Antagonism of the lethal effect of paraoxon by carrier erythrocytes containing organophosphorous acid anhydrase. Fundam Appl Toxicol. 1995;28:209–14.
12. Allen TM. Long circulating (sterically stabilized) liposomes for targeted drug delivery. Adv Drug Del Rev. 1994;13:285–309.
13. Lasic DD, Papahadjopoulos D. Medical applications of liposomes. Amsterdam: Elsevier; 1998.
14. Szoka F, Papahadjopoulos D. Comparative properties and methods of preparation of lipid vesicles (liposomes). Ann Rev Biophys Bioenerg. 1980;9:467–508.
15. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta. 1992;1113:171–99.
16. Petrikovics I, Hong K, Papahadjopoulos D, Pei L, Hu Q, McGuinn WD, *et al*. *In vitro* properties of sterically stabilized liposomes encapsulating recombinant phosphotriesterase. Toxicol Appl Pharmacol. 1999;156:56–63.
17. Petrikovics I, Yin R, Cheng TC, DeFrank JJ, McGuinn WD, Sylvester D, *et al*. *In vitro* studies on sterically stabilized liposomes (SL) as enzyme carriers in organophosphorous antagonism. Drug Deliver. 2000;7:1–7.
18. Petrikovics I, Cheng TC, Papahadjopoulos D, Hong K, DeFrank JJ, Jang J, *et al*. Long circulating liposomes encapsulating organophosphorus acid anhydrolase in diisopropylfluorophosphate antagonism. Toxicol Sci. 2000;57:16–21.
19. Petrikovics I, Papahadjopoulos D, Hong K, Cheng TC, Baskin SI, Jiang J, *et al*. Comparing therapeutic and prophylactic protection against the lethal effect of paraoxon. Toxicol Sci. 2004;77:258–62.
20. Dagani R. Chemists explore potential of dendritic macromolecules as functional materials. Chem Eng News. 1996;74:30–8.
21. Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, *et al*. A new class of polymers: starburst-dendritic macromolecules. Polym J. 1985;17:117–32.
22. Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, *et al*. Dendritic macromolecules: synthesis of starburst dendrimers. Macromolecules. 1986;19:2466–8.
23. Tomalia DA, Esfand R. Dendrons, dendrimers and dendrigrafts. Chem Ind. 1997;11:416–20.
24. Petrikovics I, Wales ME, Jaszberenyi JC, Budai M, Baskin SI, Szilasi M, *et al*. Enzyme-based intravascular defense against organophosphorus neurotoxins: synergism of dentritic–enzyme complexes with 2-PAM and atropine. Nanotoxicology. 2007;1:85–92.
25. Petrikovics I, Baskin SI, Beigel KM, Schapiro BJ, Rockwood GA, Manage ABW, *et al*. Nano-intercalated rhodanese in cyanide antagonism. Nanotoxicology. 2010;4:247–54.
26. Yin R, Zhu Y, Tomalia DA. Architectural copolymers: rod-shaped, cylindrical dendrimers. J Amer Chem Soc. 1998;120:2678–9.
27. Yin R, Qin D, Tomalia DA, Kukowsky-Latallo J, Baker JR. From dendrimers to dendrigrafts. Synthesis, characterization and applications. Polym Mater Sci Eng. 1997;77:206–7.
28. DeFrank JJ, Cheng TC. Purification and properties of an organophosphorus acid anhydrase from halophilic bacterial isolate. J Bacter. 1991;173:1938–43.
29. Lai K, Dave KI, Wild JR. Bimetallic binding motifs in organophosphorus hydrolase are important for catalysis and structural organization. J Biol Chem. 1994;269:16579–89.
30. Hoskin FCG, Roush AH. Hydrolysis of nerve gas by squid-type diisopropyl phosphorofluoridate hydrolyzing enzyme on agarose resin. Science. 1982;215:1255–7.
31. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
32. Cheng TC, Harvey SP, DeFrank JJ, Petrikovics I, Rastogi VP. Bacterial enzymes—potential applications for personnel/casualty decontamination against G, V, and HD chemical agents. In: Flora SJS, Romano JA, Baskin SI, Sekhar K, editors. Pharmacological perspectives of toxic chemicals and

their antidotes. New Delhi: Narosa Publishing House; 2004. p. 87–95.
33. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acethylcholinesterase activity. Biochem Pharmacol. 1961;7:88–95.
34. Guarisco JA, Donnell JCO, Skovira JW, McDough JH, Shih TM. *In vivo* oxime administration does not influence Ellman acetylcholinesterase assay results. Toxicol Mech Meth. 2009; 19:379–85.
35. Kern RJ, Wales ME, Tiffany-Castiglioni E, Wild JR. Protection of acetylcholinesterase from organophosphates: kinetics insights into bioscavengers. In: Gupta RC, editor. Handbook of toxicology of chemical warfare agents. New York: Academic Press, San Diego, CA; 2009. p. 1041–53.
36. Sinko G, Calic M, Bosak A, Kovarik Z. Limitation of the Ellman method: cholinesterase activity measurements in the presence of oximes. Anal Biochem. 2007;370:223–7.