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In vitro characterization of cationic copolymer-complexed recombinant human butyrylcholinesterase

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ABSTRACT

Effective use of exogenous human BChE as a bioscavenger for organophosphorus toxicants (OPs) is hindered by its limited availability and rapid clearance. Complexes made from recombinant human BChE (rhBChE) and copolymers may be useful in addressing these problems. We used *in vitro* approaches to compare enzyme activity, sensitivity to inhibition, stability and bioscavenging capacity of free enzyme and copolymer-rhBChE complexes (C-BCs) based on one of nine different copolymers, from combinations of three molecular weights (MW) of poly-L-lysine (PLL; high MW, 30–70 kDa; medium MW, 15–30 kDa; low MW, 4–15 kDa) and three grafting ratios of poly(ethylene glycol) (PEG; 2:1, 10:1, 20:1). Retarded protein migration into acrylamide gels stained for BChE activity was noted with all copolymers as the copolymer-to-protein ratio was increased. BChE activity of C-BCs was lower relative to free enzyme, with the 2:1 grafting ratio showing generally greater reduction. Free enzyme and C-BCs showed relatively similar *in vitro* sensitivity to inhibition by paraoxon, but use of the 20:1 grafting ratio led to lower potencies. Through these screening assays we selected three C-BCs (high, medium and low MW; 10:1 grafting) for further characterizations. BChE activity was higher in C-BCs made with the medium and low compared to high MW-based copolymer. C-BCs generally showed higher stability than free enzyme when maintained for long periods at 37 °C or following incubation with chymotrypsin. Free enzyme and C-BCs were similarly effective at inactivating paraoxon *in vitro*. While these results are promising for further development, additional studies are needed to evaluate *in vivo* performance.

1. Introduction

Organophosphorus anticholinesterases (OPs) are among the most toxic of synthetic chemicals [1]. Their pronounced acute toxicity derives from potent inhibitory effects on the enzyme acetylcholinesterase (AChE). OPs bind covalently to the active site serine residue (Ser203) of AChE, blocking the catalytic hydrolysis of the neurotransmitter acetylcholine [2]. Extensive AChE inhibition leads to accumulation of acetylcholine at cholinergic synapses and resulting prolonged stimulation of cholinergic receptors in a variety of tissues. This mode of action led to widespread use of OPs as insecticides and, more notoriously, their misuse as weapons of chemical warfare and terrorism [3].

Systemic administration of exogenous bioscavenger proteins (*i.e.*, circulating proteins that can bind to toxicants before they distribute to target tissues) was first shown to block OP toxicity over two decades ago [4,5]. Bioscavengers can prevent tissue AChE inhibition and block the expression of some of the most debilitating consequences of OP intoxication, *i.e.*, respiratory depression, seizures, and neuropathology [6,7]. Several types of OP bioscavengers have been evaluated, with human butyrylcholinesterase (hBChE) and paraoxonase (PON1) being the leading stoichiometric and catalytic bioscavengers, respectively [8,9].

Butyrylcholinesterase is the "sister" enzyme of AChE, distributed throughout the body but in highest concentrations in the liver and plasma [10]. Purified hBChE has been shown to be a stable, effective, and safe bioscavenger against the most toxic OP nerve agents in animal models [11]. Intravenous administration of BChE has been used in humans since the 1950s to treat OP intoxications, succinylcholine toxicity, and more recently in Phase I clinical trials (reviewed in Ref. [10]). A major limitation in its application

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however is the need for a large amount of purified protein, due in part to its relatively rapid clearance from the circulation. Parenterally administered hBChE has a mean retention time of about 50–100 h in multiple species [8,11–15] although it may have longer residence in humans [16]. Production of sufficient amounts of purified hBChE is also challenging due to the limited supply of human plasma, the relatively limited yield of enzyme from plasma, and significant expense, all of which have hindered the application of hBChE as prophylactic agent against OP intoxications [10].

Recombinant hBChE (rhBChE), because it can be expressed in high levels using cells, animals and even plants, remains a promising alternative for the hBChE. The human enzyme exists primarily in tetrameric form, while recombinant enzymes are often monomers or dimers. Some recombinant BChE enzymes have a very short circulatory residence time, *e.g.*, human BChE expressed in CHO cells (predominantly monomers and dimers) has a half-life of only about two minutes. In contrast, these same investigators reported that the recombinant tetramer (produced by inclusion of polyproline) led to much longer circulation (about 16 h [17]). Chilikuri et al. [18] reported that a rhBChE tetramer produced in HEK 293 cells had a mean circulatory residence time in mice of about 18 h. Thus, even the tetramer recombinant enzymes circulate with reduced time compared to the human serum enzyme. The shorter circulation time, instability, and potential immunogenicity compared to the serum enzyme pose obstacles for use of recombinant BChE as a bioscavenger [10].

One approach that may address the problem of clearance is the use of polymers to protect the recombinant enzyme and thereby extend its circulation time [19–24]. Recombinant BChE complexed with poly(ethylene glycol) (PEG) increased mean retention time in mice from 18 h to 36 h and the PEG-enzyme complex was more resistant than the unmodified enzyme to inactivation *in vitro* by the protease chymotrypsin [18]. Increased circulation residence for catalytic bioscavengers has also been shown with PEG-modified enzyme [25]. A grafted copolymer of poly-L-lysine (PLL) and PEG (PLL-g-PEO) was used by Gaydess et al. [26] to form a copolymer-BChE complex (C-BC) using native human serum BChE or horse serum BChE. This C-BC was shown to enter the brain within 2 h after parenteral administration, while no accumulation of BChE in brain was noted with administration of the free enzyme. These findings suggested that polymer or copolymer complexes with BChE can lead to different pharmacokinetic properties compared to the free enzyme, and that a C-BC might be useful as a bioscavenger.

We report the relative *in vitro* enzyme activity, sensitivity to the OP inhibitor paraoxon, stability (to prolonged incubation at 37 °C and inactivation by chymotrypsin), and bioscavenging potency against paraoxon in free and copolymer-complexed rhBChE. The *in vitro* findings described herein provide information on the design and characteristics of copolymer-based complexes containing rhBChE that may be useful in the further development of a bioscavenger.

2. Materials and methods

2.1. Chemicals and materials

Methoxypoly(ethylene glycol), functionalized with *n*-hydroxysuccinimide ester was purchased from Creative PEGworks (Chapel Hill, NC). Glutaraldehyde (50%) was purchased from Fisher Scientific (Pittsburg, PA). Zeba Spin Desalting Columns (7 kDa cut-off) were purchased from Pierce Chemicals (Dallas, TX). Pre-cast nondenaturing polyacrylamide gradient gels (4–20%) were purchased from Bio-Rad (Hercules, CA). Paraoxon (*O,O′*-diethyl-*p*-nitrophenyl phosphate, >98% purity) was purchased from Chem-Service (West Chester, PA). Tritium-labeled acetylcholine iodide, specific activity 80.1 mCi/mmol, was purchased from PerkinElmer (Boston, MA). Poly-L-lysine, pig liver carboxylesterase, α-chymotrypsin from bovine pancreas (Type II) and all other reagents/chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Recombinant human butyrylcholinesterase (rhBChE) purification

A total of twenty C57BL/6 mice were injected through the tail vein with 10^{13} particles of AAV8-VIP viral vector encoding human BChE. After four weeks (to reach maximum levels of expressed transgene), the mice were euthanized by overdose with pentobarbital and blood was collected by cardiac puncture. After centrifugation (10 min, 1000 × *g*) serum was collected and dialyzed overnight at 4 °C against 20 mM sodium acetate, 1 mM EDTA. The dialyzed serum was centrifuged at 10,000 × *g* for 10 min and the supernatants were incubated overnight at 4 °C with Q Sepharose equilibrated with 20 mM sodium acetate, 1 mM EDTA pH 4.0. The Sepharose beads were then washed with the same buffer until the OD_{280} of the solution dropped below 0.04. Protein was then eluted with 500 mM NaCl in 20 mM sodium acetate, 1 mM EDTA, pH 4.0. The two eluents with the highest BChE activity were combined and desalted by spin desalting columns. The eluent was adjusted to pH 7–8 with 1 M Tris–Cl pH 8.5 and incubated at 4 °C with procainamide Sepharose 4B (generously supplied by Dr. Oksana Lockridge, University of Nebraska), which had been equilibrated overnight with 20 mM potassium phosphate, 1 mM EDTA pH 7.0. The beads were then loaded onto a glass column and washed with 20 mM potassium phosphate, 1 mM EDTA, pH 7.0, followed by 0.2 M NaCl in 20 mM potassium phosphate, 1 mM EDTA, pH 7.0. BChE protein was eluted with 1 M NaCl in the same buffer. The two eluents with the highest BChE activity were combined, then desalted and concentrated over Amicon Ultra Centrifugal Filters (Ultracel-50K). Purity assessed by SDS PAGE gel electrophoresis (reducing conditions) was greater than 90%, with a prominent band at approximately 85 kDa. The purified enzyme was stable during storage at 4 °C in 20 mM potassium phosphate, 1 mM EDTA, pH 7.0 containing 0.02% NaN_3.

2.3. Copolymer synthesis

A library of nine copolymers was prepared using three different sizes of poly-L-lysine (PLL) and three different grafting ratios of poly(ethylene glycol) (PEG). Methoxypoly(ethylene glycol, mPEG) with an amine-reactive, *n*-hydroxysuccinimide ester (NHS) on the distal end was reacted with PLL to produce a PLL-grafted-PEG (PLL-g-PEG) copolymer. A 50/50 mixture of 2 and 5 kDa PEG was used along with each of the three PLLs (high molecular weight (MW), 30–70 kDa; medium MW, 15–30 kDa; low MW, 4–15 kDa). The ratio of PEG to PLL was varied to produce three grafting ratios (2:1, 10:1 and 20:1). The mPEG-NHS and PLL were dissolved in PBS and allowed to react at room temperature for 2 h. The resulting copolymer was purified using a centrifugal concentrator with a 10 kDa cutoff filter, rinsed with ultrapure water, then lyophilized and stored at −20 °C. Grafting ratios were determined by ^{1}H NMR spectroscopy using a Bruker Avance INOVA 400 MHz spectrometer.

2.4. Copolymer-BChE complex preparation

The method for synthesis of copolymer-BChE complexes (C-BCs) was similar to that reported by Gaydess et al. [26]. As BChE and PLL carry opposing net charges at physiological pH, mixing BChE and the copolymer at neutral pH leads to spontaneous complex formation. Each copolymer was dissolved in PBS immediately prior to adding to the enzyme. A constant amount of rhBChE (25 μl, 0.266 mg/ml) was first aliquoted into a 1.5 ml Eppendorf tube, and then 7.5 μl of either PBS (to produce the free