Chemico-Biological Interactions 203 (2013) 172–176

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Effect of polyethylene glycol conjugation on the circulatory stability of plasma-derived human butyrylcholinesterase in mice ☆

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ARTICLE INFO

Article history:
Available online 7 December 2012

Keywords:
Butyrylcholinesterase
Bioscavenger
Polyethylene glycol
Organophosphorus compounds
Circulatory stability
Mice

ABSTRACT

Exogenously administered human serum butyrylcholinesterase (Hu BChE) was demonstrated to function as a bioscavenger of highly toxic organophosphorus (OP) compounds in several animal species. Since the enzyme is isolated from human serum, it is currently the most suitable pretreatment for human use. A dose of 200–300 mg/70 kg human adult is projected to provide protection from 2 X LD_{50} of soman. Due to the limited supply of Hu BChE, strategies aimed at reducing the dose of enzyme are being explored. In this study, we investigated the effect of modification with polyethylene glycol (PEG) on the *in vivo* stability of Hu BChE. Mice were given two injections of either Hu BChE or Hu BChE modified with PEG-5K or PEG-20K, six weeks apart. Pharmacokinetic parameters, such as mean residence time (MRT), maximal concentration (C_{max}), elimination half-life ($T_{1/2}$), and area under the plasma concentration time curve extrapolated to infinity (AUC), were determined. For the first injection, values for MRT, $T_{1/2}$, C_{max}, and AUC for PEG-5K-Hu BChE and PEG-20K-Hu BChE were similar to those for Hu BChE. These values for the second injection of Hu BChE as well as PEG-Hu BChEs were lower as compared to those for the first injections, likely due to antibody-mediated clearance.

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1. Introduction

In humans, two types of cholinesterases catalyze the hydrolysis of acetylcholine: acetylcholinesterase (Hu AChE, 3.1.1.7) and butyrylcholinesterase (Hu BChE, EC 3.1.1.8). The primary role of AChE predominantly present in the muscle and nervous system is to terminate impulse transmission at cholinergic synapses by hydrolyzing acetylcholine [1]. Although human butyrylcholinesterase (Hu BChE) is present in nearly every tissue, it is primarily synthesized in the liver and secreted into plasma, where it circulates as a soluble, globular tetrameric form consisting of four identical subunits, each containing 574 amino acids [2]. The molecular weight of each subunit is 85 kDa, of which 65 kDa is for the protein and 20 kDa for the carbohydrate. It is thought that the physiological role of BChE is to protect against man-made and naturally occurring toxic compounds. It rapidly sequesters organophosphorus (OP) compounds by stoichiometric binding and hydrolyzes cocaine and the muscle relaxants succinylcholine and mivacurium, which make it suitable for many prophylactic and therapeutic applications [3]. Exogenously administered purified Hu BChE was successfully shown to protect animals from toxicity of OP pesticides and nerve agents and for treating the toxic effects of cocaine [4–6]. Similarly, partially purified enzyme was shown to alleviate succinylcholine-induced apnea in humans [7].

To advance the use of Hu BChE as a bioscavenger in humans, methods were developed for the purification of gram quantities of enzyme from Cohn Fraction IV-4 paste [8]. The enzyme had a specific activity of ~700 U/mg, migrated as a major band of 85 kDa on SDS–PAGE, and displayed a long shelf-life [9]. It also was highly stable in the circulation of mice, guinea pigs, monkeys, and minipigs, exhibited no systemic or behavioral toxicity in these animals, and protected them from the toxic effects of several OP nerve agents [9–18]. Hu BChE was registered as an Investigational New Drug by the FDA in 2006 and completed a phase I clinical trial in 2009. It was projected that 200–300 mg of Hu BChE would provide protection to an adult human against 2 × LD_{50} of soman [19]. However, production of native Hu BChE in such quantities is a challenge because of the high cost of purification and inadequate supply of outdated human plasma.

The prophylactic use of Hu BChE as a bioscavenger is because of its high reactivity with OP nerve agents and the observed half-life of 8–11 days in human circulation [20,21]. One strategy for reducing the dose of enzyme is by extending its circulatory stability. Previous studies have reported that conjugation of recombi-

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0009-2797/$ - see front matter Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.cbi.2012.11.021

nant (r) Hu BChE with polyethylene glycol (PEG) prolonged its circulatory stability while retaining its bioscavenging properties [22–24]. Since multiple administrations will be necessary for maintaining therapeutic levels of Hu BChE in circulation, we investigated the pharmacokinetics of unmodified and PEG-modified Hu BChE following repeated administrations. Mice were given two injections of either Hu BChE or Hu BChE modified with PEG-5K or PEG-20K, six weeks apart. Pharmacokinetic parameters, such as mean residence time (MRT), maximal concentration (C_{max}), elimination half-life ($T_{1/2}$), time to reach maximal concentration (T_{max}), and area under the plasma concentration time curve extrapolated to infinity (AUC), were calculated. For the first injection, values for MRT, $T_{1/2}$, C_{max}, and AUC for PEG-5K-Hu BChE and PEG-20K-Hu BChE were similar to those for Hu BChE. These values for the second injection of Hu BChE as well as PEG-Hu BChEs were lower as compared to those for the first injections.

2. Materials and methods

2.1. Modification of Hu BChE with PEG

Hu BChE isolated from Cohn fraction IV-4 paste [8] was used for these studies. Succinimidyl propionate-activated methoxy PEG-5K (SPA-PEG-5K) or SPA-PEG-20K- from Nektar Inc.(Birmingham, AL) was used for the chemical modification of Hu BChE. PEG in 50 mM sodium phosphate buffer, pH 8.0, was added to purified Hu BChE (0.5 mg/ml) at a ratio of 25:1 (w:w) and the mixture was gently rotated at room temperature for 2 h [22]. Unconjugated PEG was removed by extensive dialysis of samples against 10 mM sodium phosphate buffer, pH 8.0. Samples were freeze-dried and stored at −20 °C until use. The PEGylation of Hu BChE was confirmed by non-denaturing polyacrylamide gel electrophoresis (PAGE) using 4–15% gradient gels. Kinetic constants (K_m and K_{ss}) for unmodified and PEG-modified Hu BChE were determined as described [22].

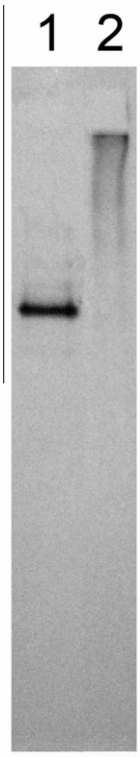

Fig. 1. Analysis of PEG-modified Hu BChE by non-denaturing polyacrylamide gel electrophoresis. Hu BChE was conjugated with PEG-20K polymer as described in Section 2 and analyzed by PAGE under non-reducing/non-denaturing conditions, using 4–15% gradient gels as described [8]. Samples are: lane 1, Hu BChE (0.006 U) and lane 2, PEG-20K-Hu BChE (0.005 U).

2.2. Determination of pharmacokinetic parameters

All animal studies were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations stated in the Guide for the Care and Use of Laboratory Animals (NRC Publication, 1996 edition). All procedures with animals received prior approval from the WRAIR/NMRC Institutional Animal Care and Use Committee and were performed in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Freeze-dried samples of Hu BChE and PEG-5K-Hu BChE and PEG-20K-Hu BChE stored at −20 °C were reconstituted in sterile saline (1500–2000 U/ml). Four groups of Balb/c mice (male, 8 weeks old, $n = 6$) received saline or one of the following enzymes: (1) Hu BChE, (2) PEG-5 K-Hu BChE, and (3) PEG-20K-Hu BChE. Each group was delivered saline or 400 U (570 μg) of enzyme by i.p. injection on day 1 (first injection) and on day 42 (second injection). Five microlitre of blood was drawn from the tail vein at various time intervals for the determination of circulating BChE activity. The following pharmacokinetic parameters were determined from the time course curve of blood BChE concentration: MRT, C_{max}, T_{max}, $T_{1/2}$, and AUC, using PK Solutions 2.0 (Summit Research Services, Montrose, CO). Group means and standard deviations were calculated for all pharmacokinetic parameters and statistical evaluations were performed using the two-way ANOVA test.

2.3. Assay for BChE activity

Samples were assayed for BChE activity using 1 mM butyrylthiocholine and 0.5 mM 5,5′-dithiobis 2-nitrobenzoic acid in 50 mM sodium phosphate buffer, pH 8.0, at 22 °C. The formation of 5-thio-2-nitrobenzoic acid was followed by monitoring the increase in absorbance at 412 nm using a molar extinction coefficient of 13,600 M^{-1} [25]. Activity was reported as U/ml, where 1 U represents 1 μmole of butyrylthiocholine hydrolyzed per min. Mouse blood samples were diluted 20-fold in water, prior to assay for BChE activity.

3. Results and discussion

Several studies over the last two decades have demonstrated that rodents, minipigs, and non-human primates pretreated with Hu BChE survived exposure to multiple LD_{50}'s of OP nerve agents, and were devoid of any symptoms observed in untreated animals. But, the dose required to confer protection is large, which prompted efforts to express rHu BChE in mammalian cells [22,26,27], in the milk of transgenic goats [24] and in plants [28]. However, rHu BChE was much less stable in the circulation of animals as compared to Hu BChE limiting its use for the prophylaxis of OP nerve agents. PEGylation of rHu BChE resulted in a dramatic improvement in its MRT, but the bioavailability was 70–80% that of Hu BChE, which meant that a higher dose of enzyme would be needed for protection. On the other hand, if the MRT and bioavailability of Hu BChE in circulation could be enhanced by PEGylation, it would reduce the dose required and make its use more feasible. Therefore, in this study, we investigated the pharmacokinetics of unmodified and PEG-modified Hu BChEs in mice. Hu BChE was modified with PEG-5K or PEG-20K using the same procedure that was used for the PEGylation of rHu BChE [22]. Fig. 1 shows the electrophoretic mobility of Hu BChE (lane 1) and PEG-20K-Hu