Effect of chemical modification of recombinant human acetylcholinesterase by polyethylene glycol on its circulatory longevity

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Post-translational modifications were recently shown to be responsible for the short circulatory mean residence time (MRT) of recombinant human acetylcholinesterase (rHuAChE) [Kronman, Velan, Marcus, Ordentlich, Reuveny and Shafferman (1995) *Biochem. J.* **311**, 959–967; Chitlaru, Kronman, Zeevi, Kam, Harel, Ordentlich, Velan and Shafferman (1998) *Biochem. J.* **336**, 647–658; Chitlaru, Kronman, Velan and Shafferman (2001) *Biochem. J.* **354**, 613–625], which is one of the major obstacles to the fulfilment of its therapeutic potential as a bioscavenger. In the present study we demonstrate that the MRT of rHuAChE can be significantly increased by the controlled attachment of polyethylene glycol (PEG) side chains to lysine residues. Attachment of as many as four PEG molecules to monomeric rHuAChE had minimal effects, if any, on either the catalytic activity ($K_m = 0.09$ mM and $k_{cat} = 3.9 \times 10^5$ min⁻¹) or the reactivity of the modified enzyme towards active-centre

inhibitors, such as edrophonium and di-isopropyl fluorophosphate, or to peripheral-site ligands, such as propidium, BW284C51 and even the bulky snake-venom toxin fasciculin-II. The increase in MRT of the PEG-modified monomeric enzyme is linearly dependent, in the tested range, on the number of attached PEG molecules, as well as on their size. It appears that even low level PEG-conjugation can overcome the deleterious effect of under-sialylation on the pharmacokinetic performance of rHuAChE. At the highest tested ratio of attached PEG-20000/rHuAChE (4:1), an MRT of over 2100 min was attained, a value unmatched by any other known form of recombinant or native serum-derived AChE reported to date.

Key words: clearance, glycosylation, organophosphate bioscavenger, pharmacokinetics.

INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) plays a pivotal role in the cholinergic system, where it functions in the rapid termination of nerve impulse transmission. The high reactivity of the enzyme toward organophosphorus compounds suggests that exogenous cholinesterase can serve as an effective therapeutic agent in the prophylaxis and treatment of organophosphorus poisoning. Indeed the successful exploitation of the scavenging potential of administered cholinesterase has been demonstrated in rodents and in non-human primates [1–5]. The use of human AChE (HuAChE) as an efficient bioscavenger has been advanced by the development of recombinant production systems [6–8], and the introduction of catalytically favourable mutations [9–12]. Yet, the short circulatory residence time of the various recombinant AChE preparations [13–16] represents one of the factors that precludes the development of an efficient AChE-based recombinant bioscavenger, and the formulation of a method for the pharmacokinetic improvement of the enzyme is a major biotechnological challenge.

The circulatory fate of AChE is dictated by a delicate hierarchy of post-translational-related determinants [13,17–19]. Pharmacokinetic studies have shown that the concomitant oligomerization of AChE catalytic units and the sialylation of AChE oligosaccharides exert a synergistic effect upon the circulatory longevity of AChE. Based on these studies, circulatory short-

lived recombinant HuAChE (rHuAChE) or recombinant bovine AChE can be converted into circulatory long-lived species by appropriate genetic engineering of the producer cells [17–19]. The observation that the successful alterations of protein size and surface significantly increased AChE circulatory residence, suggests that other modifications, such as appendage of polymers by chemical modification of the protein surface, may improve circulatory retention.

Several recombinant proteins have been exploited as potent therapeutic agents, following the enhancement of their pharmacological performance by chemical modification of the protein surface by natural or synthetic polymers (for review see [20,21]). Polyethylene glycol (PEG) has mainly been used, but dextrans, heparin, albumin, polyvinylpyrrolidone and other polymers have also been used (for review see [22]). PEG is the most widely used polymer for protein modification due to its lack of toxicity, the relatively simple chemistry required for its covalent binding to proteins, and its commercial availability. Abuchowski et al. [23] were the first to show that extension of circulatory residence of bovine liver catalase can be achieved by PEG conjugation. Since then several studies have demonstrated the therapeutic improvement of recombinant proteins by PEG conjugation (e.g. [24–28]). It is believed that PEG attachment to proteins extends their circulatory residence through interference with protein removal pathways, including kidney glomerular filtration, proteolytic degradation, as well as in active clearance via specific

Abbreviations used: 2AB, 2-aminobenzamide; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BoAChE, bovine acetylcholinesterase; HuAChE, human AChE; Δ C-HuAChE, C-terminal truncated mutant of HuAChE; rHuAChE, recombinant HuAChE; DFP, di-isopropyl phosphorofluoridate; HEK-293 cells, human embryonic kidney 293 cells; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MRT, mean residence time; PEG, polyethylene glycol.

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receptors [29–31]. One of the limitations of PEG conjugation to proteins is that it may lead to loss of biological activity [22].

In view of the fact that AChE is one of the most efficient biocatalysts, with well characterized effects of various post-translational modifications on pharmacokinetic behaviour [13–19], it was interesting to use this promising therapeutic bioscavenger molecule as a target for studies of PEG modifications. In the present study we demonstrate, for the first time, the successful generation of PEG-conjugated rHuAChE, which fully retains its enzymic activity and reactivity towards organophosphates. Most notably, the modification of rHuAChE by PEG improves the pharmacokinetic properties of rHuAChE to the extent that its circulatory longevity surpasses all other known recombinant or native serum-derived forms of AChE.

EXPERIMENTAL

Generation of a C-terminal truncated mutant of HuAChE (Δ C-HuAChE), enzyme production and purification

Truncation of the C-terminus (substitution of the last 40 amino acids with a pentapeptide, ASEAP) of the T-subunit of HuAChE [32], was performed by DNA cassette replacement [33], as described recently [34]. The DNA coding sequence for Δ C-HuAChE was inserted into a tripartite expression vector also expressing the reporter gene *cat* and the selection marker *neo* [6,35]. Generation of stably transfected human embryonic kidney 293 cells (HEK-293 cells) expressing high levels of rHuAChE and purification of the secreted enzyme were performed as described previously [6,35].

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS analysis of glycans

N-glycans of Δ C-HuAChE (approx. 100 μ g of protein) were purified, 2-aminobenzamide (2AB)-labelled and converted into their neutral methylated forms by methyl iodide esterification, as described previously [18]. Mass spectra of 2AB-labelled esterified Δ C-HuAChE glycans were acquired on a Micromass TofSpec 2E reflectron TOF mass spectrometer. Glycan samples (1 μ l) diluted 1:10 in water were mixed with an equal volume of freshly prepared 2,5-dihydroxybenzoic acid [10 mg/ml in 70% (v/v) acetonitrile] and loaded on to the mass spectrometer target. Dried spots were recrystallized by adding 0.5 μ l of ethanol and were allowed to dry again. Glycans were observed as $[M + Na]^+$ ions. A peptide mixture (1 μ l) consisting of renin substrate, adrenocorticotrophic hormone fragment 18–39 and angiotensin (all at a concentration of 10 pmol/ μ l, and all purchased from Sigma), which served as a three-point external calibrant for mass assignment of the ions, was mixed with freshly prepared α -cyano-4-hydroxycinnamic acid (10 mg/ml in 49.5% acetonitrile, 49.5% ethanol and 0.001% TFA), loaded on the mass spectrometer target and allowed to dry. All oligosaccharides were analysed at 20 kV with a single-stage reflectron in the positive-ion mode. Between 100 and 200 scans were averaged for each of the spectra shown.

Sucrose density gradient centrifugation

Analytical sucrose density gradient centrifugation was performed on 5–25% sucrose gradients containing 0.1 M NaCl/50 mM sodium phosphate buffer (pH 8.0). Centrifugation was carried out in an SW41 Ti rotor (Beckman) for 26 h at 160 000 g. Fractions of 0.2 ml were collected and assayed for AChE activity. Alkaline phosphatase, catalase and β -galactosidase were used as sedimentation markers.

PEG conjugation reaction and analysis of the products

Attachment of PEG chains to primary amines in rHuAChE was performed using succinimidyl-propionate-activated methoxy-PEG (Shearwater Polymers, Inc., Huntsville, AL, U.S.A.). Purified Δ C-HuAChE (1–5 μ M) was incubated with PEG-5000 or PEG-20000 in 50 mM phosphate buffer (pH 8.0) for 2 h at room temperature ($23 \pm 2^\circ\text{C}$). There was a significant variance in the extent of the modification and the distribution of the modified products depending on the particular preparation of activated PEG used and the molar ratio of AChE/PEG in the conjugation reaction. A specific set of conjugation conditions and PEG preparation resulting in relatively homogeneous products (no more than three differently PEG-conjugated forms) was chosen for the present study. To generate the PEG-AChE conjugates analysed in this study, PEG was added at a ratio of 5:1 (low ratio) or 25:1 (high ratio) (initial concentration of PEG/initial concentration of AChE primary amines). The modified products were dialysed extensively against PBS. Samples of the proteins were resolved by SDS/PAGE [7.5% (w/v) polyacrylamide], electrotransferred on to nitrocellulose and subjected to Western-blot analysis using mouse polyclonal anti-HuAChE antibodies [33].

Enzyme activity and reaction with inhibitors

AChE activity was measured according to Ellman et al. [36]. Assays were performed in the presence of 50 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM acetylthiocholine, 0.1 mg/ml BSA and 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27°C and monitored by a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). K_m values of HuAChE and PEG-HuAChE for acetylthiocholine were obtained from Lineweaver–Burk plots, and k_{cat} calculations were based on active-site titration [11]. Interactions of HuAChE or PEG-HuAChE with the AChE-specific inhibitors edrophonium, propidium, BW284C51, snake-venom toxin fasciculon-II and the organophosphate compound di-isopropyl phosphorofluoridate (DFP) were analysed as described previously [10].

Pharmacokinetics

Clearance experiments in mice [3–6 CD-1 outbred male mice (Charles River, U.K.) per enzyme sample] and analysis of pharmacokinetic profiles were carried out essentially as described previously [13]. The study was approved by the local ethical committee on animal experiments. Mice were injected with the various rHuAChE preparations (40 μ g/mouse in 0.2 ml of PBS). Residual AChE activity in blood samples was measured and all values were corrected for background hydrolytic activity in the blood (using samples withdrawn 1 h before performing the experiment). AChE activity values in samples removed immediately after injection were assigned a value of 100% and used for calculation of residual activity. Background cholinesterase levels in blood of pre-administered mice were less than 2 units/ml. The clearance patterns of the various enzyme preparations were usually biphasic and could be fitted to a bi-exponential elimination pharmacokinetic model [$C_t = Ae^{-k\alpha t} + Be^{-k\beta t}$, where C_t represents the concentration of the enzyme in the circulation at time (t), $k\alpha$ and $k\beta$ are the first-order constants of the first and second elimination phases respectively, and A and B represent the fractions of the material removed from the circulation in the first fast and second slow elimination phases respectively] as described previously [13]. This model enables determination of the parameters A and B , and $T_{1/2\alpha}$ and $T_{1/2\beta}$, which represent the

circulatory half-life values of the enzyme in the fast and slow phases respectively. The pharmacokinetic parameters, mean residence time (MRT; which reflects the average length of time the administered molecules are retained in the organism) and clearance (which represents the proportionality factor relating the rate of substance elimination to its plasma concentration, and is equal to dose/area under the concentration-time curve), were independently obtained by analysing the clearance data according to a non-compartmental pharmacokinetic model using the WinNonlin computer program [37].

RESULTS AND DISCUSSION

Selection of the HuAChE oligoform for study

AChE is found in multiple molecular forms that are the outcome of alternative splicing events and post-translational organization into multisubunit structures [38]. The recombinant wild-type

enzyme produced in various heterologous systems is comprised of a mixed population of oligomeric forms (monomers, dimers and tetramers) [35]. Recently we have demonstrated that not only glycosylation and sialylation [13,17], but also the oligomerization state of the enzyme [18,19] plays an important role in determining the enzymes circulatory fate. Therefore in order to assess the possible effects of chemical modification of rHuAChE by PEG on the longevity of the resulting modified enzyme it was essential to use homogeneous preparations with respect to post-translational modification of both carbohydrates and subunit assembly. Accordingly we selected a monomeric form of AChE devoid of the C-terminal sequence corresponding to residues 544–583 (i.e. Δ C-HuAChE). This sequence was replaced by the pentapeptide ASEAP, to generate a derivative of the H-subunit of AChE (Figure 1A) [34]. Since the Δ C-HuAChE form does not include the C-terminal cysteine residue (Cys⁵⁸⁰), which forms the interchain disulphide bonds between catalytic subunits [35], this

Figure 1 Generation and characterization of Δ C-HuAChE

(A) Schematic representation of the distribution of lysine residues along the HuAChE sequence and the C-terminal sequences of the wild-type (WT) and Δ C-rHuAChE (Δ C) forms. Lysine residues are marked by arrows. Cys⁵⁸⁰ in the wild-type form, responsible for intermolecular bridging, is marked by an asterisk. Note the clustering of lysine residues at the C-terminus of wild-type HuAChE. (B) Location of lysine residues within the three-dimensional structure of Δ C-HuAChE [34]. (C) Sucrose-gradient sedimentation profiles of Δ C-rHuAChE (●) and rHuAChE Cys⁵⁸⁰ → Ala (○). G1 indicates the sedimentation position of the monomeric form of AChE, and G2 indicates the expected sedimentation position of the dimeric form. Arrows denote the sedimentation positions of the markers included in the samples: alkaline phosphatase (6.1 S); catalase (11.3 S); and β -galactosidase (16 S). (D) MALDI-TOF MS analysis of total N-glycans of Δ C-rHuAChE produced by HEK-293 cells. Purified N-glycans were subjected to 2-AB labelling and iodomethane-mediated esterification prior to MALDI-TOF MS analysis. Molecular masses represent monoisotopic masses of the respective $[M + Na]^+$ ions of the glycan species. Note that an increase of 14.015 Da of all glycan species occurs due to the methylation of the common 2-AB moiety. The abundance of sialylated, partially sialylated and non-sialylated forms was calculated from the relative peak amplitude of each glycan form. □, GlcNAc; ○, Man; ◇, β -Gal; ◇, Fuc; ▼, sialic acid.

Table 1 Comparison of catalytic properties and inhibition constants of various inhibitors for unmodified and PEG-modified Δ C-AChE

Values are means $\pm$ S.D. for at least three independent experiments. Preparation C corresponds to AChE modified by PEG-20000 (low ratio), and preparation D corresponds to AChE modified by PEG-20000 (high ratio).

AChE preparation	Kinetic parameter				Inhibition constants				
	K_m (mM)	k_{cat} ($\times 10^5 \text{ min}^{-1}$)	K_{app} ($\times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$)	K_{ss} (mM)	Edrophonium ($K_i, \mu\text{M}$)	Propidium ($K_i, \mu\text{M}$)	BW284C51 (K_i, nM)	DFP ($K_i, 10^{-4} \text{ M}^{-1} \cdot \text{min}^{-1}$)	Fasciculin (K_i, pM)
Unmodified	0.09 ± 0.01	3.9 ± 0.2	43 ± 2	9 ± 2	0.8 ± 0.2	1.0 ± 0.3	8 ± 1	57 ± 4	0.8 ± 0.1
Preparation C	0.09 ± 0.01	4.0 ± 0.2	44 ± 4	6 ± 2	0.8 ± 0.2	0.6 ± 0.2	10 ± 2	58 ± 3	0.7 ± 0.2
Preparation D	0.09 ± 0.01	$3.9 \pm .01$	43 ± 3	10 ± 3	0.8 ± 0.2	0.7 ± 0.3	6 ± 1	51 ± 2	1.1 ± 0.3

enzyme preparation is monomeric. The use of Δ C-HuAChE for the present study had additional potential advantages. (1) Unlike the monomeric HuAChE Cys⁵⁸⁰ $\rightarrow$ Ala mutant, the truncated Δ C-HuAChE monomer does not carry the tryptophan amphiphilic tail [38,39] involved in the non-covalent association of AChE subunits. This ensures that the Δ C-HuAChE monomeric enzyme preparation will not be contaminated with residual higher oligomeric forms ([35]; and compare HuAChE Cys⁵⁸⁰ $\rightarrow$ Ala with Δ C-HuAChE in Figure 1C). (2) Since masking of a part of the surface of the enzyme by intersubunit interactions is not possible in Δ C-HuAChE we can expect that the entire surface of the enzyme will be accessible to PEG. (3) The three-dimensional structure of Δ C-HuAChE was recently determined by X-ray crystallography [34], permitting precise spatial localization of the potential lysine targets for PEG conjugation (Figure 1B). (4) The truncated Δ C-HuAChE form contains only seven, rather than ten, lysine residues (see Figure 1A). This could be useful in reducing the complexity of the population of PEG-modified enzymes, and may be advantageous for future analysis.

The gene encoding Δ C-HuAChE was introduced into HEK-293 cells, and stably transfected cell clones producing high levels of the truncated enzyme were established as described previously [6,34]. MALDI-TOF MS analysis of the N-glycans attached to the Δ C-HuAChE expressed in HEK-293 cells demonstrated that only 60 % of the N-glycans were sialylated (Figure 1D). Similar levels of partial sialylation were observed for other recombinant AChEs expressed at high levels in HEK-293 cells [17–19]. The biological activity of Δ C-HuAChE is essentially identical to that of the wild-type enzyme as judged by the catalytic characteristics, as well as its reactivity towards non-covalent or covalent active-centre or peripheral-site ligands (Table 1 and [10,33,34]).

Modification of Δ C-HuAChE by PEG

The amino-acid residues to which a PEG chain is attached are determined by the method used for the activation of the PEG precursor [40,41]. For the studies described in the present paper we used succinimidyl-propionate-activated methoxy-PEG. This monovalent version of activated PEG is directed to lysine residues and terminal amines, and forms a stable amide linkage with the conjugated protein. Inspection of the Δ C-HuAChE three-dimensional structure reveals that all of its seven lysine residues are located on the enzyme surface (Figure 1B). Only one of these lysine residues (Lys³⁴⁸) is adjacent to the entrance to the 'catalytic gorge'. To generate modified AChE preparations differing in the number and in the size of attached PEG molecules, we tested various incubation conditions of Δ C-HuAChE with either PEG-5000 or PEG-20000 (see the Experimental section). Modulation of PEG attachment to AChE is possible by altering the reaction conditions (e.g. molar concentration and pH) and the molar

ratio of activated PEG/AChE. Under certain conditions high PEG occupancy levels (as judged by the multiple number of AChE-related bands visible by SDS/PAGE and Western-blot analysis, which can be as high as 8) can be achieved. However, this entails a high level of heterogeneity, which complicates the accurate determination of the impact that the product size has on the pharmacokinetic performance. In the present study, therefore, we chose a set of conjugation conditions which resulted in PEG-modified AChE preparations that were characterized by a relatively high degree of homogeneity with respect to the number of different PEG-AChE products (no more than three differently PEG-conjugated species within one preparation, as judged by SDS/PAGE).

Figure 2 demonstrates the unique migration pattern of discrete bands of the PEG-AChE products generated under the various conditions. At low or high molar ratios of PEG/primary Δ C-HuAChE amines (see the Experimental section) a minimal mixture not exceeding 2–3 discrete polypeptide bands for each reaction mixture was obtained. Incubation of the 66 kDa Δ C-HuAChE molecule with PEG-5000 at the lower ratio of PEG/primary amines resulted in PEG-AChE products with apparent molecular masses of 84 and 98 kDa (Figure 2A; preparation A, bands 1' and 2'), whereas incubation with the same PEG-5000 at a higher ratio of PEG/primary amines resulted in the generation of modified enzymes with apparent molecular masses of 113 and 127 kDa (Figure 2A; preparation B, bands 3' and 4'). These results indicate that increasing the PEG/AChE ratio leads to a higher level of lysine occupancy by PEG. Incubation of Δ C-HuAChE with PEG of higher molecular mass (PEG-20000) resulted in the generation of a similar ladder of larger products of 119, 160, 213 and 250 kDa where again the apparent molecular mass depended on the molar ratio of PEG/ Δ C-HuAChE (Figure 2A; preparations C and D).

The apparent molecular masses of the individual products in each preparation appeared to differ from their nearest neighbours by similar mass units. For example, the increase in molecular mass between bands 1' and 2' was similar to the apparent mass increase between polypeptides 2' and 3' or between 3' and 4' (Figure 2). This is also the case for the products that were generated using PEG-20000 (bands 1''–4'' in Figure 2). This uniform discrete increase in mass between neighbouring bands on a gel is interpreted as the progressive attachment of single PEG subunits (Figure 2B). Accordingly, under these conditions, the number of PEG units conjugated to each AChE molecule can vary between 1 and 4 in correlation with the initial ratio of PEG/AChE in the reaction mixture, independent of the size of the PEG used. We note that PEG conjugation under different conditions can generate less homogeneous AChE preparations with more than four PEG units attached per HuAChE molecule (see the Experimental section). It is likely that under the

Figure 2 Analysis of PEG-AChE products

HuAChE was modified by PEG-5000 or PEG-20000 as indicated, at two PEG/AChE primary amines ratios (preparations A and C, low ratio; preparations B and D, high ratio). **(A)** Western-blot analysis of PEG-HuAChE preparations (A–D) using mouse polyclonal anti-HuAChE antibodies. Individual bands depicted in these preparations are marked 1'–4' for AChE-PEG-5000 and 1''–4'' for AChE-PEG-20000. **(B)** Correlation between the apparent molecular mass of each of the individual PEG-AChE products and the expected number of PEG units attached per AChE molecule (determined from three independent experiments; correlation coefficients > 0.99).

conditions selected for study, the PEG appendage occurs preferentially at some modification-prone sites (see below). Indeed, the phenomenon of incomplete PEG conjugation of all potential targets on proteins, under various conditions, is quite common [25–27,42]. In a recent study by Clark et al. [26] PEG conjugation of growth hormone did not exceed 70% occupancy of the potential attachment sites. While some of the sites of growth hormone are more reactive than others, a well defined correlation between reactivity to PEG and the surface accessibility of the amino acids or their involvement in intramolecular interactions could not be established.

Enzymic characterization of PEG-HuAChE molecules

A major problem associated with PEG modification of proteins is that in many instances it leads to loss of biological activity. For example, loss of 72 and 92% of catalytic activity was documented for PEG-adenosine deaminase and PEG-asparaginase respectively [42,43]. The deleterious effect of PEG modification on activity is believed to be mainly attributed to the attachment of the PEG chains at critical sites on the target molecule, but also to other factors, such as harsh coupling conditions or modification of surface charge at the attachment sites. To assess the effect of PEG modification on Δ C-HuAChE, detailed kinetic studies were performed with enzyme modified with the larger

Figure 3 Pharmacokinetic profiles of non-modified and PEG-modified AChE

(A) Clearance profiles of non-modified AChE (Δ C-rHuAChE), AChE modified by PEG-5000 (preparation B; Prep. B) and AChE modified by PEG-20000 (preparation D; Prep. D). The various exogenous rAChEs were introduced into mice at levels that were at least 30-fold higher than the background level (see the Experimental section). **(B)** Correlation of the MRT and the 'average' molecular mass of the PEG-AChE preparation. $\circ$, unmodified HuAChE; $\square$, HuAChE-PEG-5000 preparations A and B; ∇ , HuAChE-PEG-20000 preparations C and D. The MRT values of the various preparations (Table 2) were plotted against the calculated (see text for details) 'average' apparent molecular masses of each preparation (correlation coefficient was 0.991).

PEG moiety (PEG-20000; preparations C and D; Figure 2A). The K_m and k_{cat} values of the modified enzymes were indistinguishable, within the experimental error, from those of the unmodified enzyme (Table 1). Likewise, the inhibition constants for the classical non-covalent active-site inhibitor edrophonium or the covalent organophosphate DFP were similar to those of the unmodified AChE (Table 1). It was quite interesting to find that even ligands, such as propidium and the bis-quaternary inhibitor BW284C5, that bind to the peripheral anionic site at the entrance to the active-centre gorge [44–48], displayed an affinity to the highly PEG-conjugated HuAChE similar to that of unmodified HuAChE (Table 1). Likewise, the substrate inhibition constant, K_{ss} , representing the dissociation of a second substrate from the peripheral site [44–48], of PEG- Δ C-HuAChE and the unmodified enzyme were similar (Table 1). Remarkably, modification of the lysine residues by PEG conjugation did not affect the affinity of the peptide snake-venom fasciculin-II, which binds tightly to the peripheral site of AChE and occludes the portal of the active-centre gorge (Table 1). Unlike the other peripheral inhibitors (e.g. propidium or

Table 2 Pharmacokinetic parameters of unmodified and PEG-modified AChE

The circulatory clearance profiles of the various AChEs were determined as described in the Experimental section. A , B , $T_{1/2\alpha}$ and $T_{1/2\beta}$ represent the fractions of the material removed from the circulation and half-life values of the first (fast) and second (slow) decay phases respectively. In all cases, the correlation coefficient was > 0.99 . The clearance curves were also analysed by fitting to a non-compartmental pharmacokinetic model for the calculation of MRT and clearance using a Window-based program. Prep., preparation.

AChE preparation	Pharmacokinetic parameters					
	A (% of total)	$T_{1/2\alpha}$ (min)	B (% of total)	$T_{1/2\beta}$ (min)	Clearance (ml/h/kg)	MRT (min)
Unmodified	74 $\pm$ 8	3.6 $\pm$ 0.6	26 $\pm$ 2	44 $\pm$ 3	170.4	42 $\pm$ 3
Prep. A (PEG-5000 low ratio)	56 $\pm$ 9	29 $\pm$ 6	43 $\pm$ 3	390 $\pm$ 50	14.2	510 $\pm$ 70
Prep. B (PEG-5000 high ratio)	46 $\pm$ 3	28 $\pm$ 5	64 $\pm$ 3	540 $\pm$ 70	13.2	740 $\pm$ 80
Prep. C (PEG-20000 low ratio)	35 $\pm$ 3	32 $\pm$ 5	65 $\pm$ 5	750 $\pm$ 130	12	950 $\pm$ 120
Prep. D (PEG-20000 high ratio)	23 $\pm$ 4	35 $\pm$ 15	76 $\pm$ 3	1550 $\pm$ 120	4.3	2100 $\pm$ 200

BW284C5), which are small molecules, fasciculin is a bulky polypeptide of 61-amino-acid residues, and its attachment to HuAChE involves extensive surface contacts, as recently elucidated from X-ray crystallography of the Δ C-rHuAChE–fasciculin-II complex [34]. The fact that binding of fasciculin to AChE is not impaired by PEG implies that the long PEG chains do not wrap around the enzyme, but remain in an extended configuration. It should be noted that one of the seven Δ C-rHuAChE lysine residues, Lys³⁴⁸, is located within the HuAChE surface interaction domain of fasciculin-II [34]. Since PEG conjugation, under the conditions used, did not appear to reduce fasciculin-II binding it is possible that this particular lysine residue does not readily serve as a substrate for PEG modification. It may be interesting to note that Lys³⁴⁸ is located in close proximity to the N-glycosylation site, Asn³⁵⁰. Altogether, it appears that PEG conjugation of Δ C-HuAChE, can be compatible with maintenance of full enzymic activity, with no apparent effect on reactivity toward various ligands or on the scavenging potential of toxic agents, exemplified by the organophosphate DFP.

Pharmacokinetic behaviour of PEG-HuAChE

The pharmacokinetic characteristics of the different PEG-AChE preparations (Figure 3A and Table 2) clearly demonstrate that in all cases circulatory residence is significantly improved by PEG conjugation. The most pronounced effect was observed following modification of Δ C-HuAChE with either PEG-5000 or PEG-20000 at the higher PEG/AChE ratio (preparations B and D respectively; Figure 3A). In the latter case, the MRT was 50 times longer than that of the unmodified enzyme (2100 min and 42 min respectively; Table 2). Such a high MRT value in CD-1 outbred mice, exceeds by far any previously reported value for any type of AChE or butyrylcholinesterase (BChE) molecules from either recombinant, native or serum-derived origin (the maximal MRT values for serum-derived human BChE, horse BChE or the fetal bovine AChE are approx. 1400 min [13,14]).

It appears that the extension of circulatory longevity by PEG conjugation can be attributed both to the number and size of PEG subunits attached to the enzyme. In an attempt to determine the quantitative relationship between the MRT and the molecular mass of the PEG-modified enzyme we computed the 'average' apparent molecular mass for each of the preparations. To this end we determined the relative abundance of PEG-conjugated products in a given preparation by subjecting Western blots profiles to densitometric analysis. For example, preparation A (Figure 2A) is composed of two products with apparent molecular masses of 84 and 98 kDa. The larger product comprises 73 % of

the preparation, and the number of PEG molecules attached to each product according to Figure 2(B) allows us to determine that the 'average' apparent molecular mass of the preparation is 94 kDa, with an average number of 1.7 PEG units attached to each Δ C-HuAChE molecule. In the same way, the 'average' apparent molecular mass and the average number of attached PEG units for preparations B, C and D were determined to be 120 kDa and 3.5 units, 138 kDa and 1.5 units, and 203 kDa and 2.9 units respectively. Using these computed 'average' values we find (Figure 3B) that the MRT is linearly dependent on the overall apparent molecular mass of the PEG-conjugated HuAChE preparation. The finding of the linear relationship in Figure 3(B) suggests that the factor determining the circulatory time of AChE is not the number of the modified sites but the actual increase in molecular size as a consequence of the PEG conjugation. Thus it appears that, in principle, attachment of a single very large PEG unit to HuAChE may be as efficient as PEG conjugation of all potential lysines by smaller PEG subunits.

Several studies with superoxide dismutase addressed the issue of the effect of size and number of PEG modifications on the pharmacokinetics of the enzyme. Whereas some of these studies argued that the number of PEG molecules linked to superoxide dismutase had an important role in its pharmacokinetic performance [49], other studies showed that the predominant factor was the molecular size of the modified enzyme [50]. Another study documented that the circulatory residence time of PEG-modified interleukin-2 correlates with the size of the modified proteins rather than with the number of PEG molecules attached [25]. Our study clearly shows that in the case of rHuAChE, the principal determinant that governs the improvement in the circulatory profile of PEG-HuAChE proteins is their overall size. Although a direct correlation was observed between circulatory residence time and the molecular size of PEG-HuAChE, one should be careful in concluding that this linear relationship will be maintained for PEG-HuAChE molecules with higher levels of PEG occupancy than that tested in the present study.

One of the more striking findings of the present work is that a very substantial extension in circulatory residence can be achieved even with the partially sialylated Δ C-HuAChE and, furthermore, that such an extension can be achieved by the attachment of a relatively limited number of PEG molecules. Recently, we have shown that while oligomerization can increase circulatory MRT of rHuAChE or recombinant bovine AChE, the MRT values depend on a delicate interplay between oligomerization and the extent of glycan variability [18,19]. Thus the completely desialylated tetramers, dimers or monomers of HuAChE had very short MRTs (approx. 3.5 min). The partially sialylated (60 % sialylated N-glycan termini) monomeric Δ C-HuAChE

(66 kDa), dimeric (130 kDa) and tetrameric (approx. 260 kDa) HuAChE display MRT values of 42, 102 and 190 min respectively (Table 2 and [13,19]). On the other hand the fully sialylated tetramers and dimers of HuAChE had MRTs of 700 and 190 min respectively. It was therefore quite unexpected that limited PEG conjugation of the partially sialylated Δ C-HuAChE [resulting in 'average' apparent molecular masses of 94 or 138 kDa (preparations A and C)] exhibited high MRTs of 500–900 min. This is even more striking in view of the fact that these MRTs are in the same range as those determined for the much larger tetramers in their fully sialylated form. These results suggest that PEG conjugation can compensate in a very efficient manner for both oligomerization and, more significantly, for under-sialylation. This observation can be exploited to overcome one of the main hurdles for generating rHuAChE as a therapeutic drug with extended longevity.

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