

## 90. RECOMBINANT CHOLINESTERASES: DEVELOPING AN IDEAL BIOSCAVENGER FOR PROTECTION AGAINST ORGANOPHOSPHATE NERVE AGENTS

Ashima Saxena<sup>1</sup>, Yacov Ashani<sup>2</sup>, Palmer Taylor<sup>3</sup>, Donald M. Maxwell<sup>4</sup>, and B.P. Doctor<sup>1</sup>

<sup>1</sup>Walter Reed Army Institute of Research, Silver Spring, MD; <sup>2</sup>Israel Institute for Biological Research, Ness-Ziona, Israel; <sup>3</sup>University of California at San Diego, La Jolla, CA; and <sup>4</sup>U. S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD.

### INTRODUCTION

Cholinesterases (ChEs) were shown to be effective prophylactic agents in preventing organophosphate (OP) toxicity in mice and rhesus monkeys (1,2). Although the use of enzyme as a single pretreatment drug for OP toxicity was sufficient to provide complete protection, a relatively large (stoichiometric) amount of enzyme was required to neutralize OP *in vivo*. Significant improvements in OP/enzyme stoichiometry were achieved *in vitro* as well as *in vivo* by combining enzyme pretreatment with oxime reactivation, so that the aging of OP-inhibited FBS AChE was minimized and the catalytic activity of AChE was rapidly and continuously restored (3).

Several recent studies have demonstrated that mutations of the key residues in the active-site gorge of ChEs can have a dramatic effect on the catalytic properties of the enzyme. Using this technique it is possible to obtain mutant enzymes which possess an increased affinity for OPs (4), or are more easily reactivated by oximes (5), and/or possess a reduced rate of aging (6,7). In an effort to improve the bioscavenging performance of ChEs by site-directed mutagenesis, we developed mutants of mouse (Mo) AChE, F295L, F297I, and E202Q AChE. The effect of these mutations on the rate of inhibition, reactivation by HI-6, and aging of Mo AChE inhibited by the two potent stereoisomers of soman were determined. The *in vitro* detoxification of soman and sarin by wild-type, F295L, F297I, and E202Q AChE in the presence of 2 mM HI-6, were also studied.

ChEs are highly glycosylated proteins, with up to 24% of their molecular weight consisting of carbohydrates, which are present primarily as asparagine-linked side chains (8). The successful use of plasma-derived ChEs as OP bioscavengers stems from their relatively long residence time in circulation. To evaluate the possible use of recombinant (r) ChEs as bioscavengers *in vivo*, we compared the mean residence time (MRT) in circulation of five tissue-derived and two rChEs *i.v.* injected in mice with their oligosaccharide profiles. The results detail the carbohydrate composition and oligosaccharide profiles of recombinant ChEs. The comparative pharmacokinetic study allowed us to examine the possible relationship between protein size, monosaccharide composition, fraction of acidic oligosaccharides and circulatory stability of ChEs from diverse sources.

### MATERIALS AND METHODS

Wild-type and mutants of Mo AChE were expressed, purified and characterized with respect to catalytic parameters as described (9). Soman and sarin were obtained from the Chemical Research, Development and Engineering Center (Aberdeen Proving Ground, MD). The two P (-)-epimers of soman were obtained as described (10). Concentrations of soman solutions were determined by titration of the solution with a known amount of fetal bovine serum (FBS) AChE and measurement of residual activity (1 nmol of FBS AChE is equivalent to 400 units). The oxime, HI-6, was obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Silver Spring, MD). The monomeric (m) and tetrameric (t) forms of FBS AChE were resolved by gel permeation chromatography of purified FBS AChE on Biogel A 1.5 m column (1.5 x 170 cm) equilibrated with 50 mM sodium phosphate, pH 8.0. Dr. Patrick Masson and Dr. Israel Silman provided purified BChE from human serum (HuS) and AChE from *Torpedo californica*. Dr. Oksana Lockridge and Dr. Palmer Taylor provided purified recombinant human (rHu) BChE and recombinant mouse (rMo) AChE, respectively.

The inhibition, reactivation, aging, and detoxification studies with rMo AChEs were performed as described (11). The glycan analysis and pharmacokinetic studies were conducted as reported previously (12).

### RESULTS AND DISCUSSION

Single site substitutions of glutamate E202 (199) located next to the active-site serine S203 (200) with glutamine, or phenylalanines at positions 295 (288) and 297 (290) located in the acyl pocket of Mo AChE with leucine and isoleucine, respectively, generated mutant enzymes with altered inhibition and reactivation properties. To examine the effect of these mutations on the stereoselectivity of AChE, we compared the bimolecular rate constants for the inhibition of wild-type, F295L, F297I, and E202Q AChE by the two P(S)-diastereomers of soman (Table 1). Racemic soman is a mixture of four stereoisomers of which only the two P(S)-diastereomers are potent inhibitors of AChE (13,14). Although the reactivity of F295L AChE toward the two diastereomers of soman was similar to that

of wild-type AChE, mutations of phenylalanine at position 297 and glutamic acid at position 202 caused a 5- to 10-fold decrease in the bimolecular rate constants for inhibition by these diastereomers. A 2-fold difference in the bimolecular rate constant of wild-type AChE for the two P(S)-diastereomers was observed compared to the 3- and 4.5-fold differences observed for F295L and E202Q AChE, respectively. Previous inhibition studies with stereoisomers of soman also showed that the P(S)C(S)-soman was a slightly more potent inhibitor of ChEs compared to P(S)C(R)-soman (14,15). However, mutations of the two residues in the acyl pocket did not affect the reactivity of mutant enzymes toward sarin.

The influence of these mutations on 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyaminopyridinium)-dimethyl ether hydrochloride (HI-6) induced reactivation of wild-type and mutant AChEs inhibited by the two P(S)-diastereomers of soman were also studied. For AChE inhibited with either of the two P(S)-diastereomers of soman, the second order reactivation rate constant for F297I AChE was essentially the same as that for wild-type AChE (Table 1). However, a 4- to 6-fold increase and an 8- to 10-fold decrease in the rate of reactivation were observed for F295L and E202Q AChE, respectively. For all enzymes, P(S)C(R)-inhibited-AChE reactivated at a 7- to 10-fold slower rate as compared to P(S)C(S)-inhibited-AChE. Similar effects were demonstrated for the reactivation of racemic 7-(methylethoxyphosphinyloxy)-methyquinolinium iodide (MEPQ)-inhibited E202Q and F295L Mo AChE by 2-PAM and HI-6 (5). For the F295L Mo AChE, the enhancement was observed with half of the racemic MEPQ-inhibited enzyme (5), and the second half appeared to reactivate at an extremely slow rate. For E202Q Mo AChE, the mutation affects the rate of reactivation as well as the extent of reactivation especially with P(S)C(R)-soman-AChE. These results are consistent with molecular modeling studies which showed that steric hindrance between the methyl group at the chiral carbon of P(S)C(R)-soman and H440 can reduce the efficiency of nucleophilic reactivation of P(S)C(R)-soman-AChE compared to P(S)C(S)-soman-AChE (16). The results are also consistent with *in vitro* studies with human, electric eel, and Plaice AChE, which demonstrated that reactivation of P(S)C(S)-soman-AChE by HI-6 was more effective than the reactivation of P(S)C(R)-soman-AChE (14,15).

A reaction that counteracts reactivation of soman-inhibited AChE is aging. We examined the rate of aging of wild-type and mutant AChEs inhibited by the two P(-)-diastereomers of soman by their incubation at pH 8.0, and subsequent measurement of the extent of reactivation by 2 mM HI-6. No differences in the rate constants for the aging of wild-type AChE inhibited with P(S)C(S)- and P(S)C(R)-soman were observed. These results are consistent with previous observations made with human, eel, and bovine erythrocyte AChE, which showed that the rate of aging was practically independent of the configuration at the  $\alpha$ -carbon atom of the pinacolyl moiety of soman (14,17). Replacement of phenylalanines in the acyl pocket did not have any significant effect on the rate of aging at pH 8.0. However, for E202Q AChE, at pH 8.0 and above, the rate of aging was so slow that it could not be measured under present experimental conditions (Table 1).

Aging has proven to be the major barrier to achieving oxime reactivation of AChE inhibited by the more potent OPs (3). Recombinant enzymes without this liability would confer a superior characteristic in the development of catalytic scavengers of OPs. To test this possibility, we carried out *in vitro* detoxification of soman and sarin by wild-type F295L, F297I, and E202Q AChE in the presence of 2 mM HI-6. The results of this experiment shown in Figure 1, demonstrate that despite superior reactivation properties, F295L and F297I AChEs were not an improvement over wild-type AChE in detoxifying soman and sarin. On the other hand, E202Q AChE was 2-3 times more effective in detoxifying soman and sarin compared to wild-type AChE. These results suggest that the improved detoxification properties of E202Q AChE may be due to its reduced rate of aging compared to wild-type AChE. The combination of mutagenized enzymes with oxime-induced OP turnover suggests a promising future for the detoxification of OP compounds by enzyme bioscavengers.

The major requirements for an enzyme to be an effective bioscavenger for OP toxicity are: (a) relatively long half-life in circulation, (b) relatively high turnover number, (c) immunocompatibility, and (d) availability in sufficient quantities for use as a pretreatment drug. Therefore, in addition to the catalytic properties, the circulatory stability of these enzymes is imperative for their use as bioscavengers *in vivo*. Since glycan structures play a significant role in the circulatory stability of enzymes, we compared the glycan structures and pharmacokinetics of recombinant DNA-derived ChEs with plasma-derived ChEs.

HuS BChE contained the greatest amount of carbohydrate (31% by weight of protein) compared to other plasma-derived ChEs such as tFBS AChE and Eq BChE which contained 9% and 23% carbohydrate by weight, respectively. *Torpedo* AChE, mFBS AChE, rMo AChE, and rHu BChE contained 9%, 9%, 10%, and 13% carbohydrate by weight of protein, respectively (Table 2). The relatively high content of mannose suggested the presence of N-linked oligosaccharides, and the presence of N-acetylgalactosamine indicated the presence of O-linked oligosaccharides in all enzymes. The presence of galactose indicated that the majority of glycans in all ChEs except *Torpedo* AChE were of the complex or hybrid type rather than the high-mannose type. In addition,

substantial amounts of the oligosaccharides in all ChEs except Eq BChE were fucosylated. The total number of complex carbohydrate chains/subunit calculated from their mannose content for various ChEs are listed in Table 2.

Charge-based separation of the 2-aminobenzamide labeled oligosaccharides showed that they consist of neutral as well as acidic components (2). In all cases, no acidic oligosaccharides were detectable after neuraminidase treatment suggesting that the acidic substituent on the oligosaccharide chain was a covalently linked terminal outer-arm sialic acid residue.

Fractionation of the total pool of desialylated oligosaccharides by high resolution gel permeation chromatography, provided a basis for identifying the N-linked units present in various ChEs. One major (11.2 glucose units (gu)) and several minor distinct structural components for HuS BChE, Eq BChE, and tFBS AChE, were identified. The structures of these glycans were determined to be of the complex biantennary type (18,19). In contrast, size-based fractionation of the desialylated oligosaccharide pools yielded three-to-four major oligosaccharides for *Torpedo* AChE, mFBS AChE, rMo AChE, and rHu BChE. The glycans eluting at 11.2 gu and 12.2 gu are most likely of the complex biantennary type (19) and the other peaks probably correspond to high mannose, hybrid, tri- and tetra-antennary structures.

The molecular forms of various ChEs used in this study were determined by sucrose density gradient centrifugation analysis. HuS BChE, Eq BChE, and tFBS AChE, the three ChEs derived from plasma sources are tetrameric (t) in form (20,21), *Torpedo* AChE is dimeric in form (22) and mFBS AChE migrates as a monomer (m) (23). Of the two recombinant ChEs tested, rHu BChE is a mixture of monomers, dimers and tetramers, whereas rMo AChE exists in monomeric form only.

Following i.v. injection, the plasma activity of all ChEs declined in two phases, and curve fitting was carried out as described (12). In order to permit a meaningful correlation between the pharmacokinetic characteristics and the structural features of ChEs from various sources, we compared their circulatory properties by determining volume of distribution ( $V_{ss}$ ), MRT, total body clearance (CL), and elimination rate constant ( $k_{el}$ ), using a non-compartmental analysis (12). The results are summarized in Table 3.

$V_{ss}$  was compared with the initial volume of distribution that is approximated by the plasma volume ( $V_p$ ). A 1.1 to 1.4-fold increase in  $V_{ss}$  over  $V_p$  was observed for plasma-derived tetrameric forms of Eq BChE, HuS BChE, and FBS AChE. The less stable enzymes, mFBS AChE, rMo AChE and rHu BChE, clearly equilibrated with a slightly larger volume ( $V_{ss}/V_p \sim 2$ ).

Similarly the enzymes could be separated into two major groups according to their total body residence time. As indicated above for  $V_{ss}$ , one group contains the tetrameric forms of plasma-derived Eq BChE, HuS BChE, and FBS AChE, which are characterized by extended durations in the body (MRTs ~ 1902-3206 min). The second group contains mFBS AChE, rMo AChE, and rHu BChE, with MRT values of 205-304 min, which are 6- to 15-fold shorter than the tetrameric enzymes. MRT and  $V_{ss}$  were reasonably correlated with the molecular weight of the proteins that reflect the assembly of the ChE subunits, and with the fraction of sialylated oligosaccharides.

The molar ratio of sialic acid-to-galactose residues on HuS BChE, rMo AChE, and rHu BChE was ~1.0, suggesting that all the terminal galactose residues were capped with sialic acid (Table 2). However the MRT of HuS BChE was 9- and 14-fold greater than that of rMo AChE and rHu BChE, suggesting that the capping of galactose with sialic acid by itself is not sufficient to confer circulatory stability to ChEs. For tFBS AChE (MRT, 1902 min), Eq BChE (MRT, 3206 min), *Torpedo* AChE (MRT, 44 min) and mFBS AChE (MRT, 304 min), this ratio was ~0.5 (Table 2), suggesting that only half of the terminal galactose residues were capped with sialic acid, yet these enzymes greatly differed in their circulatory stability. These observations suggest that although the presence of sialic acid appears to be essential for maintaining ChEs in circulation, the location rather than the number of the non-sialylated galactose residues may be affecting circulatory stability.

## CONCLUSIONS

The results presented here demonstrate that it is indeed possible to improve the detoxification properties of ChEs by site-directed mutagenesis. The combination of mutant enzymes and oxime reactivation, have very useful applications in many areas. An effective formulation can be developed for use in medical, surgical or skin decontamination of exposed subjects. Such formulations can also be used for decontamination of equipment, transportation vehicles and other environmental objects. This approach can be employed to develop effective methods for the safe disposal of stored nerve agents. The advantages of this approach will be relative ease of handling, cost-effectiveness, and relative safe disposal of detoxification products. In addition, the presence of residual AChE in the detoxifying media itself serves as an end point for completeness of the decontamination process.

To evaluate the possible use of rChEs as bioscavengers *in vivo*, we compared the mean residence time (MRT) in circulation of five tissue-derived and two rChEs i.v. injected in mice with their oligosaccharide profiles. The results

presented here reveal differences in the oligosaccharides of native and recombinant ChEs with regard to the total carbohydrate content and charge- and size-based oligosaccharide profiles. However, neither the carbohydrate composition nor the oligosaccharide profile could be completely correlated with the pharmacokinetic parameters of these enzymes. While the correlation between glycan characteristics and pharmacokinetic parameters is not fully understood, it is noteworthy that the glycans of recombinant ChEs and mFBS AChE displayed a remarkable heterogeneity in size and consist of hybrid and complex bi-, tri- and tetra-antennary structures. *Torpedo* AChE also contains high-mannose structures. The three plasma ChEs, on the other hand, contain mature glycans, which are predominantly of the complex biantennary type, confirming that these structures are responsible for the extended MRTs of the enzymes. The molar ratio of sialic acid-to-galactose residues on ChEs suggest that, location rather than the number of the non-sialylated galactose residues may be affecting circulatory stability. Site-specific analysis of glycan structures may elucidate the structures responsible for the rapid clearance of non-plasma ChEs and suggest suitable manipulations for improving the circulatory stability of rChEs.

## REFERENCES

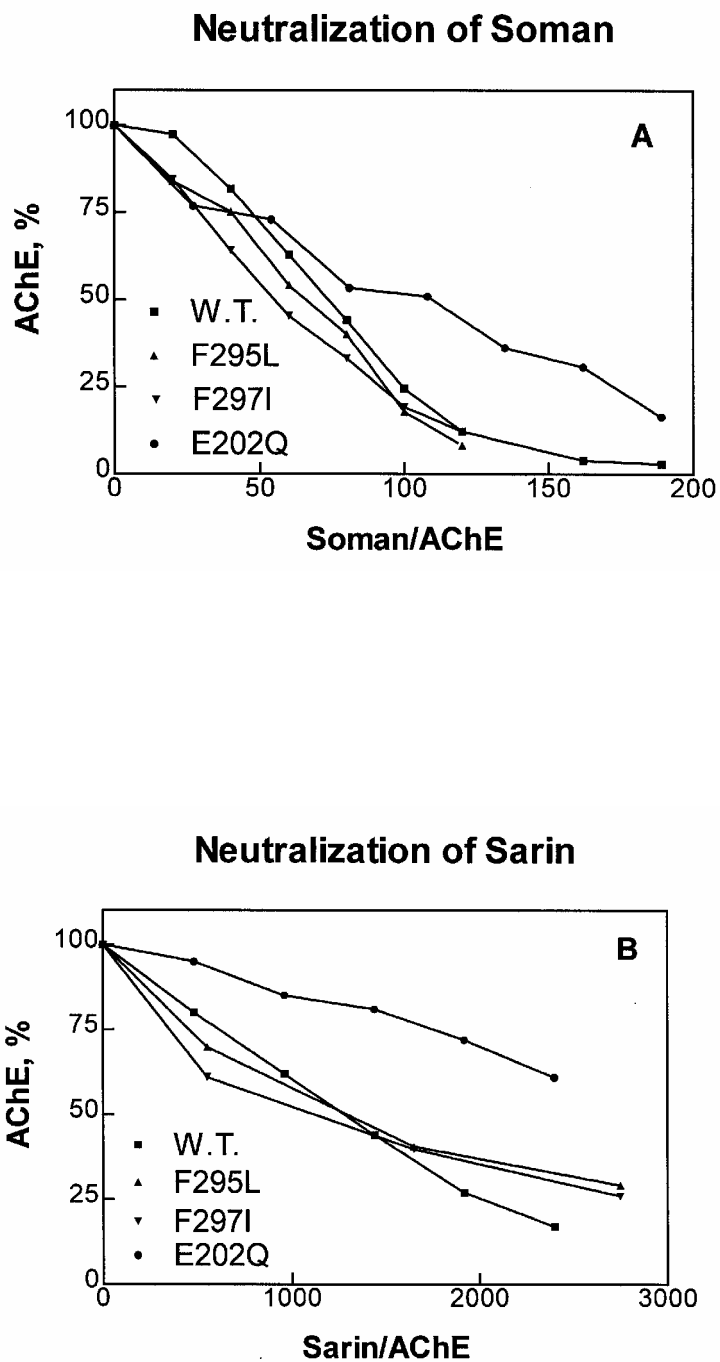
1. Ashani, Y., Shapira, S., Levy, D., Wolfe, A. D., Doctor, B. P., and Raveh, L. (1991) *Biochem. Pharmacol.*, 41, 37-41.
2. Wolfe, A. D., Blick, D. W., Murphy, M. R., Miller, S. A., Gentry, M. K., Hartgraves, S. L., and Doctor, B. P. (1992) *Toxicol. Appl. Pharmacol.*, 117, 189-193.
3. Caranto, G. R., Waibel, K. H., Asher, J. M., Larrison, R. W., Brecht, K. M., Shutz, M. B., Raveh, L., Ashani, Y., Wolfe, A. D., Maxwell, D. M., and Doctor, B. P. (1994) *Biochem. Pharmacol.*, 47, 347-357.
4. Ordentlich, A., Barak, D., Kronman, Ariel, N., Segall, Y., Velan, B., and Shafferman, A. (1996) *J. Biol. Chem.*, 271, 11953-11962.
5. Ashani, Y., Radic, Z., Tsigelny, I., Vellom, D. C., Pickering, N. A., Quinn, D. M., Doctor, B. P., and Taylor, P. (1995) *J. Biol. Chem.*, 270, 6370-6380.
6. Saxena, A., Doctor, B. P., Maxwell, D. M., Lenz, D. E., Radic, Z., and Taylor, P. (1993) *Biochem. Biophys. Res. Commun.*, 197, 343-349.
7. Ordentlich, A., Kronman, C., Barak, D., Stein, D., Ariel, N., Marcus, D., Velan, B., and Shafferman, A. (1993) *FEBS Lett.*, 334, 215-220.
8. Haupt, H., Heide, K., Zwisler, O., and Schwick, H.G. (1966) *Blut* 14, 65-75.
9. Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S., and Taylor, P. (1993) *Biochem.*, 32, 12074-12084.
10. Benschop, H. P. and De Jong, L. P. A. (1988) *Acc. Chem. Res.*, 21, 368-374.
11. Saxena, A., Maxwell, D. M., Quinn, D. M., Radic, Z., Taylor, P., and Doctor, B. P. (1997) *Biochem. Pharmacol.*, 54, 269-274.
12. Saxena, A., Ashani, Y., Raveh, L., Stevenson, D., Patel, T., and Doctor, B. P. (1998) *Mol. Pharmacol.*, 53, 112-122.
13. Benschop, H. P., Konings, C. A. G., and De Jong, L. P. A. (1981) *J. Am. Chem. Soc.*, 103, 4260-4262.
14. Benschop, H. P., Konings, C. A. G., van Genderen, J., and De Jong, L. P. A. (1984) *Toxicol. Appl. Pharmacol.* 72, 61-74.
15. De Jong, L. P. A., and Wolring, G. Z. (1984) *Biochem. Pharmacol.*, 33, 1119-1125.
16. Qian, N., and Kovach, I. M. (1993) *FEBS Lett.*, 336, 263-266.
17. Bucht, G., and Puu, G. (1984) *Biochem. Pharmacol.*, 33, 3573-3577.
18. Ohkura, T., Hada, T., Higashino, K., Ohue, T., Kochibe, N., Yamashita, K. (1994) *Cancer Res.*, 54, 55-61.
19. Saxena, A., Raveh, L., Ashani, Y., Doctor, B. P. (1997) *Biochem.*, 36, 7481-7489.
20. Lockridge, O., Eckersen, H. W., La Du, B. N. (1979) *J. Biol. Chem.*, 254, 8324-8330.
21. Ralston, J. S., Rush, R. S., Doctor, B. P., Wolfe, A. D. (1985) *J. Biol. Chem.*, 260, 4312-4318.
22. Silman, I., Futerman, A. H. (1987) *Eur. J. Biochem.*, 170, 11-22.
23. Saxena, A., Ashani, Y., Brady, D. R., Gentry, M. K., Hur, R. S., Hively, H., Larrison, R., Caranto, G., Doctor, B. P. (1991) in *Proceedings of the 1991 Medical Defense Bioscience Review*, pp. 499-502.

## KEYWORDS

Bioscavenger, cholinesterases, site-directed mutagenesis, glycans, pharmacokinetics

## FIGURES AND TABLES

Figure 1.



*In vitro* Detoxification of sarin or soman by Mouse Wild-Type and Mutant AChEs in the presence of HI-6. The reactivation of mouse wild-type (■), F295L (>), F297I (?) and E202Q (•) AChE (0.011 nmol) was carried out in the presence of 50 mM sodium phosphate, pH 8.0 containing 0.05 % BSA and 2 mM HI-6 after repeated additions of soman (1.8 nmol, Panel A) at 24-hr intervals, or sarin (0.275 nmol, Panel B) at 6-hr intervals. Residual enzyme activity was plotted against the cumulative amount of soman or sarin present in the reaction mixture. Data shown is representative of three experiments.

**Table 1.** Inhibition, reactivation, and aging Constants for recombinant mouse acetylcholinesterases

Inhibitor	Bimolecular rate constant $k \times 10^{-8} (\text{M}^{-1} \text{min}^{-1})$			
	Wild-Type	E202Q	F295L	F297I
P(S)C(S)-Soman	1.20±0.27	0.200±0.017	0.99±0.01	0.10±0.007
P(S)C(R)-Soman	0.69±0.09	0.044±0.004	0.33±0.01	0.12±0.01
Sarin	0.08±0.03		0.11±0.02	0.07±0.01

**Second Order Reactivation rate constant for HI-6**

	$k (\text{mM}^{-1} \text{min}^{-1})$			
	Wild-Type	E202Q	F295L	F297I
P(S)C(S)-Soman	0.50±0.07	0.05±0.01	2.10±0.46	0.60±0.15
P(S)C(R)-Soman	0.05±0.01	0.005±0.001	0.33±0.03	0.06±0.01
Sarin	0.51±0.06		0.66±0.04	2.60±0.32

**Table 2.** Number of glycans and the content of acidic oligosaccharides in cholinesterases

Enzyme	Mannose	N-acetyl glucosamine	Galactose	Sialic acid	Non-sialylated galactose <sup>a</sup>	Number of glycans <sup>b</sup>	Fraction of acidic oligosaccharides <sup>c</sup>
				nmol/nmol subunit			
Eq BChE	0	0	0	0	9	11	0
HuS BChE	0	51	0	0	2	12	0
tFBS AChE	9.5	0	0	3.5	0	3	0
<i>Torpedo</i> AChE	0	0	0	0	0	4	0
mFBS AChE	8.5	0	0	0	0	3	0
rMo AChE	0	0	0	0	0	3	0
rHu BChE	0	0	0	0	0	5	0

<sup>a</sup> Difference between galactose and sialic acid content<sup>b</sup> Calculated from mannose content (three residues of this sugar per complex oligosaccharide)<sup>c</sup> Calculated from area under the peaks (12)

**Table 3.** Noncompartmental analysis of the time course of various recombinant and tissue-derived cholinesterases in plasma of mice<sup>a</sup>

Enzyme	MRT (min)	CL (mL/hr.kg)	Elimination half-life (min)	V <sub>p</sub> (mL/kg) <sup>b,c</sup>	V <sub>ss</sub> (mL/kg) <sup>b</sup>
Eq BChE	3206±317	1.2	2379±230	41.6±1.9	57.7±2.5
HuS BChE <sup>d</sup>	2791	1.3	1683	51	58.3
tFBS AChE	1902±129	1.3	1210±84	30.8±2.4	40.9±2.8
Torpedo AChE	44±2	72	40±7	41.2±0.9	51.8±1.4
mFBS AChE	304±99	16.2	215±14	39.5±4.8	81.5±8.2
rMo AChE	301±40	21.6	230±31	49.8±4.2	107.8±10.9
rHu BChE	205±62	33	144±36	54.6±4.0	114±20

<sup>a</sup>Values are mean ± SD, (n=3-6).

<sup>b</sup>Normalized to body weight.

<sup>c</sup>Calculated by dividing the administered dose by ChE plasma concentration at t=0, estimated by log linearized extrapolation of the initial time course data.