Chemical polysialylation of human recombinant butyrylcholinesterase delivers a long-acting bioscavenger for nerve agents in vivo

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The creation of effective bioscavengers as a pretreatment for exposure to nerve agents is a challenging medical objective. We report a recombinant method using chemical polysialylation to generate bioscavengers stable in the bloodstream. Development of a CHO-based expression system using genes encoding human butyrylcholinesterase and a proline-rich peptide under elongation factor promoter control resulted in self-assembling, active enzyme multimers. Polysialylation gives bioscavengers with enhanced pharmacokinetics which protect mice against 4.2 LD_{50} of *S*-(2-(diethylamino)ethyl) *O*-isobutyl methanephosphonothioate without perturbation of long-term behavior.

organophosphate | N-acetylneuraminic acid | Russian VX | pesticide | mass spectrometry

According to the World Health Organization, 200,000 people die annually in developing countries as a result of poisoning by organophosphorus (OP) pesticides. The most dangerous OPs are chemical warfare agents. They were classified as weapons of mass destruction by the United Nations in April 1991 and their production, stockpiling, and use is banned under the Chemical Weapons Convention of 1993. Nonetheless, there have been several episodes of the use of nerve agents and OP pesticides as terror weapons. Further, the destruction of huge chemical warfare agents stocks may result in accidental poisoning to civilians and military personnel. In consequence, there is a clear demand for efficient, therapeutic anti-OP agent to overcome the adverse effects of drugs used for prophylaxis, emergency, and postexposure treatments (1–3).

Recently, DNA-encoded bioscavengers have been reported, including both enzymes and abzymes, thereby launching a new era of bioscavengers. Several biocatalysts were found to serve as potential natural protection against OP poisoning, such as plasma butyrylcholinesterase (BChE) (4), liver carboxylesterases (5), acetylcholinesterase (6, 7), paraoxonase 1 (8), catalytic antibodies (9–11), prolidase (12), PAF-acetylesterase (13), and even albumin (14, 15).

BChE was chosen as the most suitable template for development of stoichiometric bioscavengers for four reasons: (*i*) it is a well-studied enzyme, (*ii*) it is constitutive in humans, (*iii*) it can specifically and rapidly bind OP molecules, and (*iv*) human plasma BChE has investigational drug status from the US Food and Drug Administration. Human plasma BChE undoubtedly has become "gold standard" in treating and prophylaxis of OP poisoning. Unfortunately, use of the human plasma BChE is limited by current manufacturer protocol, which requires large amounts of outdated human plasma for generation of a single dose of bioscavenger, thus making the resulting drug cost too high for wide application. Recent publication by the Mor and colleagues (16) vividly describes that "with current technology, creating a small stockpile of plasma-derived BChE (1 kg ≈ 5,000 doses) would require dedicating the entire annual US supply of outdated plasma to a purification effort at a considerable cost." The other hidden problem of using outdated plasma is possible contamination with viruses. Recombinant technology provides controlled contamination-free environment, fixed price, and yield, thus making possible the generation of a drug stockpile on demand. Numerous attempts to achieve transgenic expression of BChE have been developed in the past 10 y, including transgenic plants (16), mice, and goats (17). However, none of these has yet been economically effective or received approval for administration to humans. Although the CHO cell expression system is very widely used for different US Food and Drug Administration–approved protein drugs (18–22), the recently reported CHO-based expression of BChE has delivered only modest production and thus cannot be adopted for medical application (23).

Pharmacokinetic properties of the recombinant BChE are affected by both glycosylation and size of the recombinant protein (24, 25). Oligosaccharide profiles of recombinant human BChEs showed significant difference compared with native plasma enzyme (24, 26). However, the most important characteristic for the bioscavenger, i.e., half-elimination time (27), is mainly dependent on the size of the protein. Thus, recent studies on stoichiometric bioscavengers display focus shifted to increase of the mass of recombinant (r) BChE to prolong its stability in bloodstream. To perform this, two strategies have been suggested: (*i*) chemical modification of the protein and (*ii*) oligomerization. To overcome difficulties in expression of the tetrameric form of rBChE, a natural tetramerization peptide (28, 29) can be

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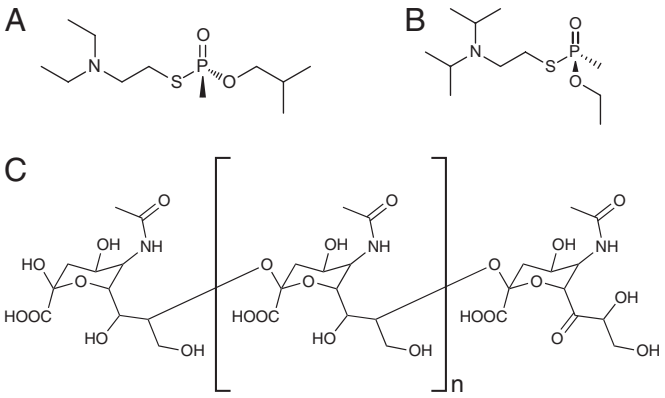

Fig. 1. Chemicals mentioned in this work. (*A*) VR, *S*-(2-(diethylamino)ethyl) *O*-isobutyl methanephosphonothioate A racemic mixture of VR was used in this study. (*B*) VX, *S*-(2-(diisopropylamino)ethyl) *O*-ethyl methanephosphonothioate. (*C*) Colominic (polysialic) acid, average $n = 25$.

introduced into medium (30) or to the expression system in vivo (31). Alternatively or simultaneously, PEGylation can be used to improve pharmacokinetic stability of native and recombinant BChE (16, 25, 32). In additional to PEG, polysialic acids can be used to increase the molecular size of rBChE. These molecules were shown to improve pharmacokinetics of test drugs (33), leaving the drug active after modification (34, 35). Unlike other hydrophilic polymers, such as PEG or dextran, polysialic acids are biodegradable, less likely to cause immunogenic response, and their catabolic product (i.e., NeuNAc) is not known to be toxic (36). Thus, the chemical polysialylation of cholinesterase and BChE particularly has been considered a promising route to the creation of the next generation of bioscavengers.

In this study, we designed a CHO expression system to force in vivo subunit reassembly of recombinant BChE. To protect this complex from degradation in the bloodstream, we propose chemical polysialylation of this stoichiometric bioscavenger without any loss of functional activity. This polysialylated bioscavenger has delivered protection to rodents against one of the most potent nerve agents, *S*-(2-(diethylamino)ethyl) *O*-isobutyl methanephosphonothioate (VR, Russian VX) (Fig. 1). This result may lead to the elaboration of a long-acting pretreatment providing an effective counter measure for human OP poisoning.

Results and Discussion

To select an efficient expression system, three expression vectors (pGS/CMV/BCHE, pcDNA/CMV/BCHE, and pBudCE/EF/BCHE) were transiently transfected into CHO-K1 cells (Fig. 2*A* and Fig. S1). Transfectomas growing media were screened after 3 d for BChE activity using Ellman's method. Results showed that CHO-K1 cells transfected with pBudCE/EF/BCHE produced up to 3 mg·L^{-1}, whereas cells transfected with pGS/CMV/BCHE and pcDNA/CMV/BCHE produced only 0.5–0.6 mg·L^{-1}.

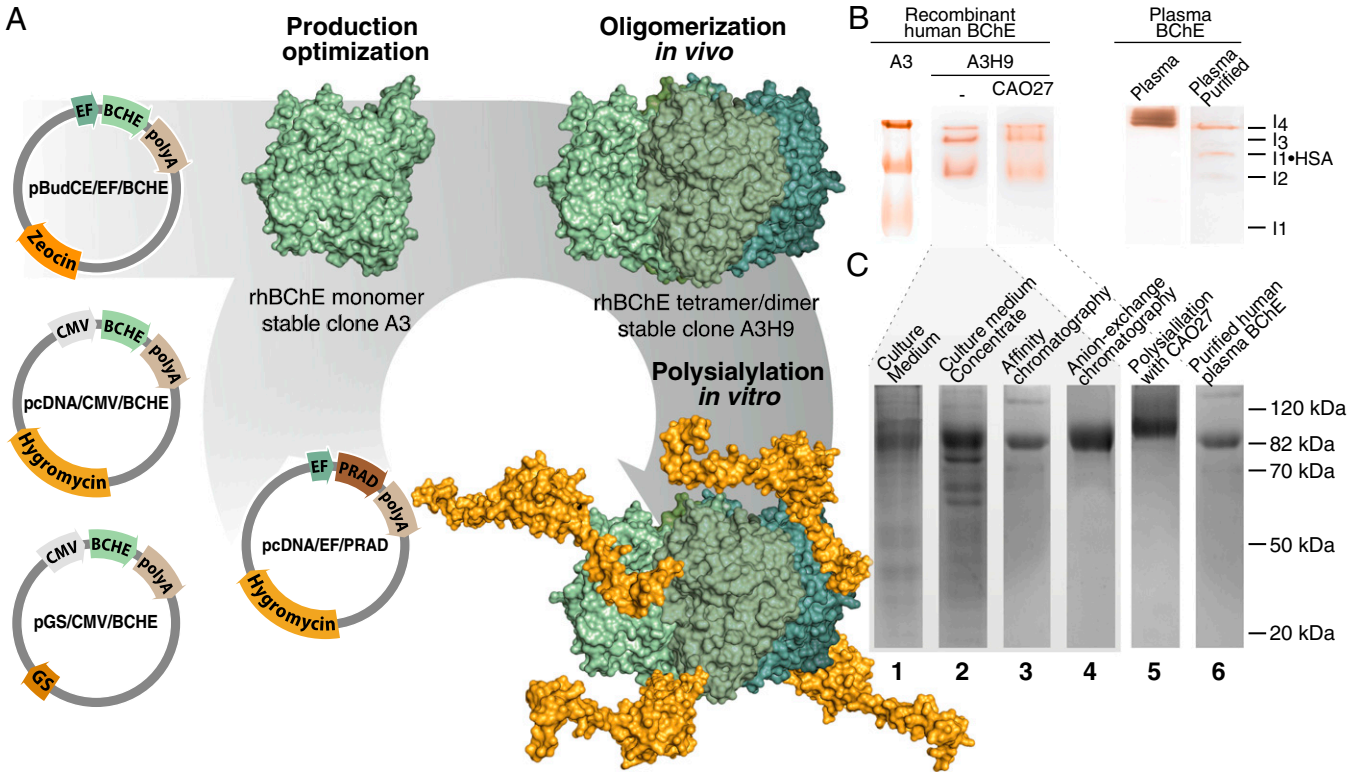

Fig. 2. CHO-based expression system using genes encoding butyrylcholinesterase and proline-rich peptide (PRAD) under control of elongation factor (EF) promoter result in self-assembled active enzyme multimers, revealing enhanced pharmacokinetics on polysialylation. (*A*) Outline strategy of current method. Two eukaryotic expression vectors pBudCE/EF/BChE and pcDNA/CMV/BCHE were designed using a BChE gene from pGS/CMV/BCHE plasmid. Plasmid pcDNA/EF/PRAD was cotransfected to clone A3 to coexpress PRAD peptide with rhBChE to create pharmacologically more stable oligomeric enzyme. rhBChE produced by A3H9 clone was used for chemical polysialylation in vivo experiments. (*B*) Analysis of hBChE oligomers (I_1–I_4): samples loaded onto native 4–30% PAGE and stained according to Karnovsky and Roots (*SI Materials and Methods*). rhBChE produced by A3H9 clone is expressed predominantly as dimer (I_2) and tetramer (I_4). I_1•HSA represents heterodimer of hBChE with human serum albumin persisting in human plasma. (*C*) rhBChE purification and modification analysis: A3H9 culture medium (lane 1), ultraconcentrated (lane 2), purified by two-step chromatography (lanes 3 and 4), and finally chemically polysialylated (lane 5). Plasma hBChE control (lane 6).

Table 1. Chemical polysialylation has no effect on enzymatic properties and enhances pharmacokinetics of recombinant bioscavenger

	Kinetic					Pharmacokinetics		
	With BTC				VR			
Enzyme	K_M, μM	k_{cat}, s^{-1}	K_{ss}, μM	b	k_i, M^{-1} s^{-1}	MRT, min	$t^{1/2}{}_{dist}$, min	$t^{1/2}{}_{el}$, min
rhBChE	25 ± 1	820 ± 10	250 ± 30	2.4 ± 0.2	(8.5 ± 0.8)·10^5	220 ± 50	4 ± 1	180 ± 30
rhBChE-CAO27	28 ± 2	830 ± 10	230 ± 20	2.2 ± 0.2	(10 ± 1)·10^5	1,400 ± 200	21 ± 13	1,000 ± 140
hBChE	23 ± 2 (37)	665 ± 30 (37)	140 ± 20 (37)	2.5 ± 0.1 (37)	(7.2 ± 0.7)·10^5	2,791 (24)	—	1,683 (24)

Characteristics of nonmodified and chemically polysialylated rhBChE-CAO27 compared with hBChE. "±" indicates SD, enzyme kinetic constants (n = 3), inhibition by VR (n = 12).

pBudCE/EF/BCHE transfectomas were selected under pressure of 300 μg·mL^{-1} Zeocin (Invitrogen) antibiotic, resulting in production of monoclone A3. After optimization of expression conditions, we finally achieved a 35 mg·L^{-1} production level of the recombinant product (Fig. S2). Analysis of rhBChE isoforms produced by clone A3 showed that enzyme was expressed primarily as monomer (Fig. 2*B*). Natural tetramerization peptide was introduced to obtain the tetrameric form of rhBChE, which has shown better stability in the bloodstream than the monomeric enzyme, suggesting that tetramerization plays an even more important role than accurate glycosylation (23) (Fig. 2*A*).

BChE tetramers are known to have better stability in the bloodstream compared with monomers, suggesting that tetramerization plays a more important role than accurate glycosylation (24, 37). To achieve this, a pcDNA/EF/PRAD vector, containing PRAD tetramerization peptide, was cotransfected in A3 clone (Fig. 2*A*). After selection under pressure of 300 μg·mL^{-1} Zeocin (Invitrogen) and 6 mg·mL^{-1} G418 (Invitrogen) antibiotics, an A3H9 clone was produced. Analysis of rhBChE isoforms produced by this newly designed clone showed that dimers and tetramers were expressed primarily and no monomer was detected in the growth medium (Fig. 2*B*). A3H9 demonstrated stable productivity for 30 generations, providing a good source of the rhBChE. It is thus feasible to use this clone for bulk production in modern bioreactors, which generally provide a dramatic increase in productivity of up to several grams per liter of growth medium.

We developed the purification protocol to achieve 85% recovery (Fig. S3) of ≥95% pure rhBChE (Fig. 2*C*). The protocol was optimized to avoid rhBChE proteolysis during purification because C′-end proteolysis leads to degradation of dimers and tetramers. Both human plasma BChE and purified rhBChE were loaded onto 8% (mass/vol) SDS/PAGE gel in the presence and absence of β-mercaptoethanol (Fig. S3). A band at 160 kDa in the absence of β-mercaptoethanol represents disulfide-bonded dimer with an intact C′-end demonstrating minimal degradation of BChE during purification,

The rhBChE was shown to hydrolyze butyrylthiocholine iodide (BTC) with K_M 25 ± 1 μM and k_{cat} 820 ± 13 s^{-1}, substrate activation typical for this enzyme (37) (Table 1). Thus, rhBChE produced by clone A3H9 does not show any remarkable significant difference in catalytic properties compared with the naturally occurring human plasma enzyme.

Oligosaccharide profiles are critical for enzyme stability in the bloodstream. These profiles of recombinant human BChEs have been found to show differences compared with native plasma enzyme (16, 25, 32). PEGylation has been proposed (16) for long-acting DNA-encoded ChE-based bioscavengers. Recently, polysialic acids or colominic acids [polymers of *N*-acetylneuraminic acid with α(2→8) ketosidic linkages] have been tested as alternatives to nonbiodegradable PEG. Linear homo- and heteropolymers of *N*-acetylneuraminic acid are not known to have any receptors in the human body, and their catabolic products are nontoxic (24). These benefits make polysialylation more suitable for creation of long-acting bioscavengers, bearing potential

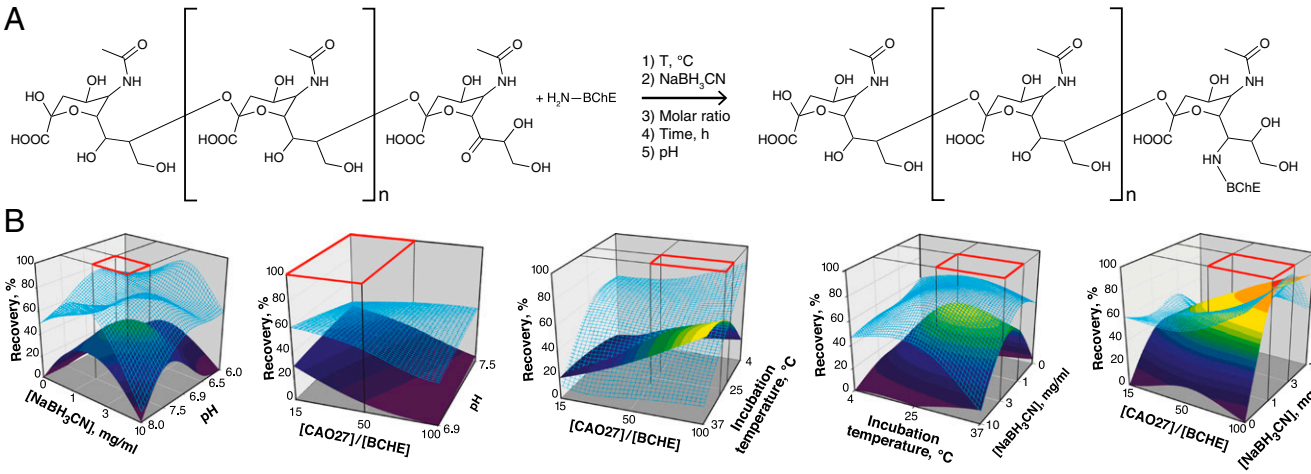

Fig. 3. Combinatorial optimization of chemical polysialylation for production of recombinant bioscavenger that is long-lived in the bloodstream. (*A*) General scheme of the chemical polysialylation BChE by colominic oxidized acids and most crucial reaction parameters (molar ration, temperature, concentration of reduction agent, pH, reaction time). Reaction of 7′-keto group of CAO27 with protein NH_2 groups leads to formation of Schiff-bases, then reduced with $NaBH_3CN$. Step-by-step reaction optimization (*B*) revealed optimal conditions when affordable activity loss (cyan framework) is accompanied with high recovery of polysialylated BChE (colored surface).

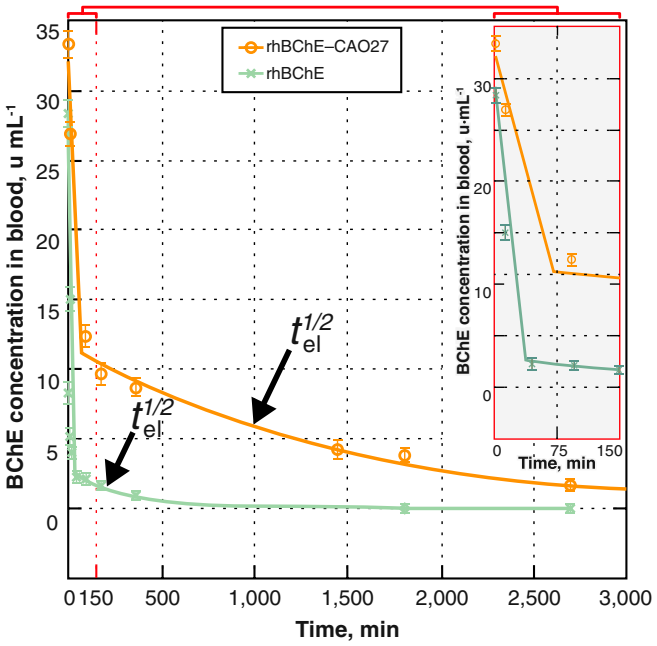

Fig. 4. The polysialylated bioscavenger displayed sixfold enhanced pharmacokinetic properties. rhBChE and polysialylated rhBChE (rhBChE-CAO27) administered i.v. to BALB/c mice ($n = 10$) in dose of 100 U per mouse (5 mg·kg^{-1}). According to a two-compartment pharmacokinetic model, the half-elimination time of rhBChE and rhBChE-CAO27 (arrows) is 180 ± 20 and 1,000 ± 140 min, respectively. (*Inset*) Swift-flowing modified and nonmodified rhBChE distribution period between two compartments (error bars, SD, $n = 10$).

for injection into humans in significant amounts for pretreatment against OPs with remarkable reduction of side effects, including immune response (34, 38, 39).

Oxidized colominic acids of average mass of 27 kDa (CAO27) were prepared to modify the primary amino groups of rhBChE. This modification alters the N′-end amino group of the protein and NH_2 groups of lysine side chains. Reaction of the 7′-keto group of CAO27 with an NH_2 group of the protein leads to a Schiff-base that is stabilized by reduction with $NaBH_3CN$ (Fig. 3*A*). We applied combinatorial screening of reaction conditions to optimize the chemical polysialylation process (Fig. 3*B*); this achieved at least 80% yield of modified protein with less than 15% loss of activity. The protein-to-colominic acid ratio was 1:6 on average by resorcinol assay. MS analysis identified six peptides that carried modified residues (Fig. S4). To control the modification process, rhBChE-CAO27 conjugate samples were loaded onto 8% SDS/PAGE. Both rhBChE bandshift and increased shear were observed for the modified protein (Fig. 2*C*, line 5). Modified rhBChE was shown to hydrolyze BTC with K_M 28 ± 2 μM and k_{cat} 830 ± 10 s^{-1} (Table 1). Analysis of rhBChE molecular forms after this modification showed no change compared with unmodified rhBChE (Fig. 2*C*). Having adequate quantities of chemically derivatized DNA-encoded human BChE-based bioscavenger on hand, we moved to in vivo studies.

Appropriate glycosylation is one of the most important BChE posttranslational modifications. The oligosaccharide coat represents 25% of the molecular weight of human plasma BChE. In a remarkable work, Saxena has demonstrated the importance of accurate glycosylation and its impact on the pharmacokinetics and stability of BChE in vivo (24). CHO cells were found to produce BChE lacking the oligosaccharides, which resulted in low values of mean residence time (MRT) (205 ± 62 min) and relatively short half-elimination time (144 ± 36 min) when administered to mice. These data are relevant to our results. Pharmacokinetics was determined with BALB/c mice for two BChE preparations: (*i*) nonmodified rhBChE and (*ii*) polysialylated rhBChE-CAO27 (Fig. 4). The activity time course was fitted using a two-compartment pharmacokinetic model (Fig. S5). BChE produced by A3H9 clone showed 220 ± 50 min MRT and 180 ± 20 min half-elimination time, whereas rhBChE-CAO27 showed a sixfold increase of stability (1,400 ± 200 min MRT) and 1,000 ± 120 min half-elimination time in comparison with unmodified enzyme (Table 1). These observed parameters are relevant to the "gold standard" human plasma BChE (24, 27, 40–42). With a half-elimination time approaching 17 h, the rhBChE-CAO27 conjugate shows very good potential for prophylactic or pretreatment as a biopharmaceutical against OP poisoning.

Nerve agent VR (Fig. 1) has a slightly higher toxicity than agent VX and works primarily on AChE at neuromuscular junctions and central cholinergic synapses, causing lethal neurotransmission failure (43). Our data show that rhBChE and rhBChE-CAO27 enzymes are inhibited by VR with k_I $(10 \pm 1)\cdot 10^5$ $M^{-1}\cdot s^{-1}$ and $(8.5 \pm 0.8)\cdot 10^5$ $M^{-1}\cdot s^{-1}$, respectively (Table 1). These in vitro data show that polysialylated rhBChE has a very high binding rate with VR and can therefore serve as a potential bioscavenger.

To investigate the efficacy of the polysialylated rhBChE, mice were preadministrated with enzyme before OP treatment. The human plasma BChE was taken as a reference enzyme. Doses of OP ranged from nonlethal to totally lethal (LD_{100}) to determine the exact protection efficacy. A 21 mg·kg^{-1} dose of rhBChE-CAO27 (equivalent to 400 U) gave an efficacy index (EI) score of 4.2, meaning that it protects mice against 4.2 LD_{50} of VR exposure administered intramuscularly. The EI of an equivalent 400 U dose for human plasma BChE was 4.7 (Table 2 and Table S1). We suggest that the small difference of rhBChE and hBChE efficacy indexes is due to the difference in half-elimination time of these proteins (Table 1), so that result can be interpreted as *equal* efficacy of hBChE and rhBChE toward intramuscular injection of VR saline.

To study the behavior and physical endurance of mice after OP treatment, animals were subjected to a series of open-field and treadmill tests at premedication, preexposure, and postexposure

Table 2. Polysialylated bioscavenger reveals enhanced protection of mice against 4.2 LD_{50} of agent VR

	Median lethal dose of VR ($LD_{50} \times 10^3$, mg·kg^{-1})			
		95% confidence limits		
Test group	Estimate	Lower bound	Upper bound	Efficacy index, $LD_{50}^{(Pretreated)}/LD_{50}^{(Control)}$
rhBChE-CAO27 ($n = 20$)	79	69	90	4.2
hBChE ($n = 24$)	89	80	100	4.7
Control ($n = 61$)	19	18	20	—

The protective efficacy of polysialylated rhBChE was assessed in male SPF mice i.v. injected with 21 mg·kg^{-1} of enzyme followed by VR exposure at 30 min after injection. The number of mice in rhBChE-CAO27, hBChE, and control groups were 20, 24, and 61, respectively. Calculations were performed according to Finney's method using SPSS software (IBM).

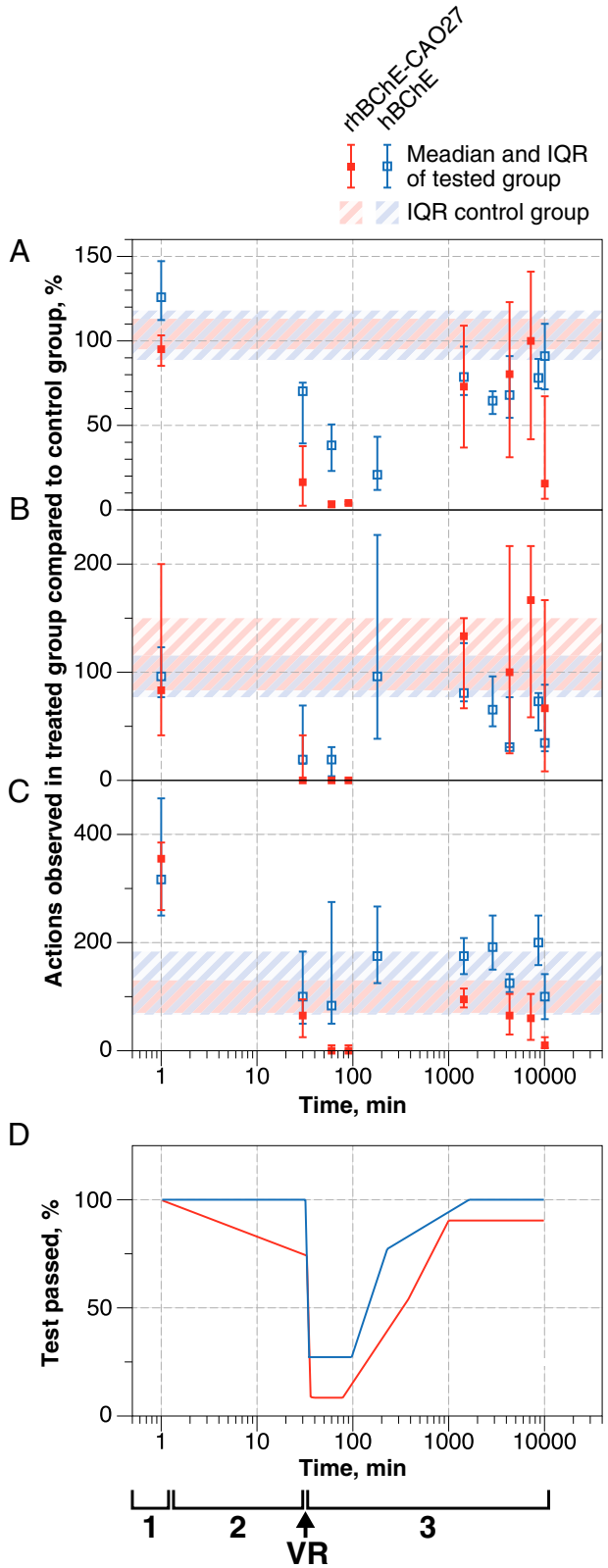

Fig. 5. Polysialylated bioscavenger rhBChE-CAO27 protects mice against nerve gas VR exposure without long-term behavioral perturbation. Two groups of mice were administrated with 21 mg·kg^{-1} dose of hBChE (n = 10) or rhBChE-CAO27 (n = 8) followed by intramuscular injection of VR, shown in blue and red, respectively. To control nonconspicuous results in behavior and physical endurance of mice after intoxication, surviving mice were subjected to a series of open-field tests (*A*–*C*) and treadmill testing (*D*) at premedication, preexposure, and postexposure times (brackets 1, 2, and 3, respectively). Animals from both groups displayed a similar drop in all activities studied during the first hours after VR injection. Nonetheless, they showed full recovery 24 h after VR administration.

times. The two groups of mice injected with either human plasma BChE or rhBChE-CAO27 conjugate showed the same profile in both open-field and treadmill tests (Fig. 5). Full recovery of normal behavior in each group was observed within 24 h after OP exposure.

Conclusions

We succeeded in developing a CHO-based recombinant human BChE expression system with delivering a high production level of rhBChE. Protein produced by clone A3H9 primarily consisted of tetramers and dimers. We successfully used polysialic acids for capping modification to enhance rhBChE stability in the bloodstream. Combinatorial optimization of chemical polysialylation has given a high yield of rhBChE-CAO27 conjugate with low loss of activity. Animal studies show that this polysialylated rhBChE is sixfold more stable than unmodified enzyme when administered to BALB/c mice. The rhBChE modified in this way is an effective stoichiometric bioscavenger and has no long-term side effects, thereby protecting mice against 4.2 LD_{50} of the highly toxic nerve agent VR.

Materials and Methods

Chemicals. VR (Fig. 1*A*) was synthetized in the State Research Institute of Organic Chemistry and Technology, Moscow.

NMR of VR was performed on an AVANCE 300 spectrograph (Brucker) in $CDCl_3$ solution using standard procedures (Figs. S6 and S7).

Chemical Modification with Polysialic Acid and Conjugate Purification. Colominic oxidized acid with average molecular mass of 27 kDa (CAO27, Fig. 1C) was produced at the Lipoxen facilities. Optimal conditions were selected using a combinatorial approach. The following reaction conditions were varied: pH (6.0–8.0), rhBChE:CAO molar ratio (1:15–1:100), incubation temperature (4–37 °C), and $NaBH_3CN$ concentration (0–10 mg·mL^{-1}). Each reaction was performed in a 30-μL final volume. Residual rhBChE activity was analyzed using Ellman's method, and sialylation efficiency was analyzed by PAGE. Gels were digitized using VersaDoc Imaging System (Bio-Rad), and calculations were performed with Quantity One software. Preparative conjugation was performed in optimal conditions: 0.1 M potassium phosphate buffer, pH 6.9, molar ratio protein:CAO = 1:50, final concentration of $NaBH_3CN$ of 3 mg·mL^{-1} during 48 h at 25 °C. The rhBChE-CAO27 conjugate was purified by affinity chromatography with procainamide-Sepharose sorbent.

In Vivo Experiments. In vivo experiments were approved by the Institutional Animal Care and Use Committees of Pushchino Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Pushchino, Russia. BALB/c male mice weighing 25–30 g (n = 10) were administered with rhBChE and rhBChE-CAO27 conjugate at a dose of 100 u per mice by fast i.v. injection. Following enzyme administration, 10 μL of blood was drawn from the eye sinus at various time intervals over 5 d. Data were reviewed for compliance with the criteria of the two-compartment pharmacokinetic model [*SI Text* and ref. (44)]. Blood samples were diluted in PBS for determination of total blood BChE activity. MRT, elimination and distribution half-life time, and C_{max} and AUC parameters were calculated from the time course curve of blood BChE concentration. All calculations were performed on KaleidaGraph software (Synergy Software).

Determination of rhBChE-CAO27 Efficacy Against VR Exposure. The protective efficacy of polysialylated rhBChE was assessed in male SPF mice injected with 21 mg·kg^{-1} (400 u) enzyme i.v., followed 30 min later by exposure to VR. Doses of OP were varied from nonlethal to totally lethal (LD_{100}) to determine the precise protection efficacy. VR was injected intramuscular (gluteus maximus muscle) in water solution in 50–100 μL per animal. Animals were observed for 5 d after exposure and survival was scored at 30 min after exposure. Data (Table S1) were analyzed with probit analysis in SPSS software (IBM) according to Finney's method (45).

Animal Behavior and Physical Endurance Research. Behavior and physical endurance tests were performed in the State Research Institute of Organic

Chemistry and Technology, Moscow, Russia. The open field contained a 50- × 30-cm sized chamber with black acrylate floor marked into 40 squares. The floor also contained 28 2-cm diameter lookup holes. Animals were placed in the center of the open field and the following events were counted for 3 min: (*i*) number of line crosses representing horizontal activity, (*ii*) number of stand-ups representing vertical activity, and (*iii*) number of hole inspections representing exploratory activity. All these parameters were monitored during pretreatment, posttreatment, and posttreatment times. The physical endurance test consisted of a 30-min run on treadmill. Descriptive statistics on behavior data were calculated using Statistica software (StatSoft Inc.).

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