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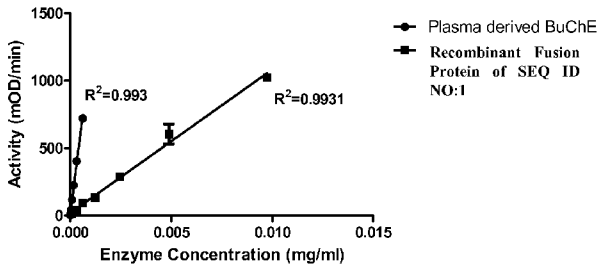
[Continued on next page]

(54) Title: ALBUMIN-BCHE IN DETOXIFICATION OF NEUROTOXINS

FIGURE 1



Baxter BuChE								
mg/mL	0.00061	0.000305	0.000152	7.62E-05	3.81E-05	1.91E-05	9.53E-06	4.76E-06
nM	7.2	3.6	1.8	0.9	0.45	0.225	0.1125	0.05625
	724.1143	397.6286	219.1857	115.0286	44.3	18.28571	9.114286	4.371429
	718.1857	408.1714	232.2143	120.5571	42.45714	21.94286	6.6	1.3
Average	721.15	402.9	225.7	117.7929	43.37857	20.11429	7.857143	2.835715
St Dev	4.192133	7.454926	9.212592	3.909291	1.303097	2.585991	1.777869	2.171828

Recombinant Fusion Protein of SEQ ID NO:1								
mg/mL	0.009756	0.004878	0.002439	0.00122	0.00061	0.000305	0.000152	7.62E-05
nM	0.077	0.0385	0.01925	0.009625	0.004813	0.002406	0.001203	0.000602
	1042.775	678.6833	299.6583	137.6833	104.9667	43.83333	35.38333	16.53333
	1004.692	530.0833	278.1833	132.5083	77.69167	35.025	20.18333	11.78333
Average	1023.733	604.3833	288.9208	135.0958	91.32917	39.42917	27.78333	14.15833
St Dev	26.92898	105.0761	15.18512	3.659278	19.28634	6.228432	10.74802	3.358757

(57) Abstract: The present invention provides the recombinant fusion protein of SEQ ID No :1. and a method of treating a mammal for exposure to a neurotoxin or for preventing or ameliorating the Neurotoxic Symptoms of exposure to neurotoxin which comprises administering an effective amount of the recombinant fusion protein of SEQ ID No :1. SEQ ID No:1 is a fully recombinant fusion polypeptide composed of the mature form of human serum albumin (HSA) fused at its amino terminus to the carboxy- terminus of a truncated and mutated human BChE.

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ALBUMIN-BCHE IN DETOXIFICATION OF NEUROTOXINS

This application claims the priority of U.S. Provisional Application No. 61/986,227, filed April 30, 2014, the contents of which are hereby incorporated by reference.

Throughout this application, various publications are referenced by author and publication date. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Background of the Invention

Nerve agents (NA) are highly lethal chemicals, of which many belong to the organophosphorus (OP) compound group; they are among the most toxic substances identified. OP compounds were originally developed for use as insecticides, but due to their extreme toxicity were adopted as weapons of chemical warfare. Nerve agents act by irreversibly inhibiting the enzyme acetylcholinesterase (AChE) which is responsible for the breakdown of acetylcholine in the synapse. Current antidotes to OP exposure include pretreatment with pyridostigmine coupled with therapeutic administration (via auto-injector) of an anti-convulsant, an oxime and atropine. All current treatments carry significant side-effect risks. The pretreatment of subjects exposed to NAs with a human biologic is an attractive alternative, as protection lasting 1-2 weeks could be afforded to those in potentially contaminated environments; the same approach could be used by emergency medical workers responding to a contamination event.

In the last 2 decades both human butyrylcholinesterase (hBChE) purified from either human plasma or from transgenic goat milk, and acetylcholinesterase (AChE) from various sources were studied extensively as stoichiometric antidotes (single turnover) against intoxications with multiple LD50 doses of

OP-based nerve agents and/or pesticides. Studies of combined treatment by cholinesterases and oxime reactivators suggest that these mixtures may constitute a pseudo-catalytic antidotal concept with turnovers >1. Two sources of hAChE were examined as potential bioscavengers: a. recombinant hAChE expressed in a human cell line, and b. hAChE expressed in transgenic plant cells. Despite the fact that hAChE reacts faster than hBChE with some OPs, the latter (obtained from human plasma) has the advantage of being already recorded as a safe protein administered in numerous cases in humans with no apparent toxic side effects and with long circulatory half-life of the "naked" administered enzyme. In several cases biological half-life of exogenously administered plasma derived hBChE in humans were reported at 10-12 days (3). Recombinant enzyme products of either hBChE or hAChE are required to be encapsulated to comply with requirements of extended plasma stability.

In order for hBChE (or any bioscavenger) to qualify as drug candidate against a variety of OPs (i.e., a broad-spectrum antidote), it must fulfill several requirements: It should react rapidly with all types of OP threat nerve agents (e.g., sarin, soman, cyclosarin, tabun, VX, Russian VX) in the circulation so as to prevent them from reaching AChE in physiologically important targets. The ideal prophylaxis (or post-exposure treatment)would be to reduce the concentration of the toxic dose of OPs to below 1/10 of its LD50 value within 0.5-1 min (approximately 1 blood circulation time); Bioscavengers should protect by a tolerable protein dose at least against 2xLD50 s of OPs, preferably (but not necessarily) without the need of post-exposure additional standard therapy (e.g., atropine, oxime reactivators, anti-convulscants); The Bioscavenger should be effective not only as a prophylactic drug but also as a post-exposure treatment with or without standard therapeutic regimen, whenever such therapeutic window exists (e.g., skin contamination, long-term

low-level exposure) and should have long circulatory half-life so that a single dose is sufficient to protect against OP intoxication for 2-3 days, preferably 1 week. Additional requirements for such a bioscavenger is that it should be produced from a reliable and safe source, obtainable by a large-scale production process that will guarantee continuous supply at low cost and that it has shelf-life stability of at least 3 years when stored at room temperature (RT) as a lyophilized powder and 5 years at 4°C.

To date, the need for an efficious, safe and commercially available, long acting and diverse bioscavenger for the treatment of exposure to several types of Neurotoxins still remains.

Summary of the Invention

The present invention provides a method of treating a mammal for exposure to a neurotoxin or for preventing or ameliorating the Neurotoxic Symptoms of exposure to a neurotoxin which comprises administering an effective amount of a recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1. SEQ ID No:1 is a fully recombinant fusion polypeptide composed of the mature form of human serum albumin (HSA) fused at its amino terminus to the carboxy-terminus of a truncated and mutated human BChE.

Brief Description of the Figures

Figure 1. Hydrolytic activity of huBuChE (Baxter) vs. the recombinant fusion protein of SEQ ID NO:1 against BtCh substrate

Both the recombinant fusion protein of SEQ ID NO:1 and huBuChE display linear concentration/activity relationships for the hydrolysis of BtCh under the conditions used. When compared to huBuChE, the recombinant fusion protein of SEQ ID NO:1 possesses approximately nine times less hydrolytic activity against BtCh on a per milligram basis. The recombinant fusion protein of SEQ ID NO:1 (126,000 g/mol) is approximately 1.5 times the molecular weight of huBuChE (85,000 g/mol). Thus, the difference in hydrolytic activity between huBuChE and the recombinant fusion protein of SEQ ID NO:1 is actually closer to six-fold, which is similar to the value that has been previously reported (Yang W, Zhan C-G et al, 2010); this modest difference in activity may be due to the use of a buffer at a slightly lower pH (7.0 used here, 7.5 used in the previous study).

Figure 2. Non-linear regression fit of the recombinant fusion protein of SEQ ID NO:1 hydrolysis of BtCh substrate as determined by isothermal titration calorimetry

2A. A single injection experiment was performed (MicroCal Manual) and a substrate inhibition model was used to analyze the data and generate the required K_M and K_i (substrate inhibition constant) of BtCh with the recombinant fusion protein of SEQ ID NO:1. BtCh was present at a final concentration of 250 μM in the reaction well as this would allow for maximal velocity without causing substantial substrate inhibition. The concentration of enzyme that generated a linear change in absorbance at 412nm over a 5 minute measurement period was determined before each iteration of the experiment; generally, 150 to 200 ng of enzyme per reaction well was sufficient to meet this criterion.

2B. Agent (GA, GB, GD, GF, VX, VR, or VM at varying concentrations from 1 pM to 1 mM) and substrate (at a fixed 250 µM final concentration) were simultaneously added to the recombinant fusion protein of SEQ ID NO:1 samples, and the absorbance was measured continuously for five minutes at 412nm. The resulting curves were fit using a nonlinear regression analysis to determine the rate of inhibition at each agent concentration, which was then plotted as described by Hart and O'Brien (Hart GJO et al, 1973) to determine the inhibition rate constants for each agent tested. Inhibition rate constants of the recombinant fusion protein of SEQ ID NO:1 with several OP nerve agents, determined using the continuous method. Error bars indicate standard deviation of three independent replicates. Inset shows only the values for the recombinant fusion protein of SEQ ID NO:1 with VX and VM. In all cases, the numerical values for each OP are shown above the corresponding bar.

Figure 3. In vivo PK (by residual activation of the recombinant fusion protein of SEQ ID NO:1) ex vivo in guinea pigs

Pharmacodynamic time-course of the recombinant fusion protein of ID NO:1 in guinea pigs (n = 3 per dose) after i.v. administration of A) 25 mg/kg or B) 50 mg/kg. Error bars show the standard deviation in the activity level at each time point. The concentration of the recombinant fusion protein of SEQ ID NO:1 was determined by comparison to a standard curve generated using purified enzyme.

Figure 4. Summary of Experimental Results

Detailed Description of the Invention

SEQ ID NO: 1 has the following amino acid sequence:

EDDIIIATKNGKVRGMNLTVFGGTVTAFLGIPYAQPPLGRLRFKKPQSLTKWSDIWNATKYA
NSCCQNIDQSFPGFHGSEMWNPNTDLSEDCLYLNVWIPAPKPKNATVLIWIYGGGFQTGTSS
LHVYDGKFLARVERVIVVSMNYRVGALGFLALPGNPEAPGNMGLFDQQLALQWVQKNIAAFG
GNPKSVTLFGESSGAASVSLHLLSPGSHSLFTRAILQSGSFNAPWAVTSLYEARNRTLNLAK
LTGCSRENETEIIKCLRNKDPQEILLNEAFVVPYGTPLGVNFGPTVDGDFLTDMPDILLELG
QFKKTQILVGVNKDEGTWFLVGGAPGFSKDNNSIITRKEFQEGLKIFFPGVSEFGKESILFH
YTDWVDDQRPENYREALGDVVGDYNFICPALEFTKKFSEWGNNAFFYYFEHRSSKLPWPEWM
GVMHGYEIEFVFGLPLERRDNYTKAEEILSRSIVKRWANFAKYGNPNETQNNSTSWPVFKST
EQKYLTLNTESTRIMTKLRAQQCRFWTSFFPKVDAHKSEVAHRFKDLGEENFKALVLIAFAQ
YLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCC
AKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPE
LLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWA
VARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKE
CCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHP
DYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEY
KFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKK
QTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL
(SEQ ID NO:1)

As used herein, and unless stated otherwise, each of the following terms shall have the definition set forth below.

As used herein, "effective," as in an amount effective to achieve an end, means the quantity of a component that is sufficient to yield an indicated therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this disclosure. For example, an amount effective to treat a mammal or a human patient exposed to a neurotoxin. The specific effective amount will vary with such factors as the age and gender of the mammal,

the particular condition being treated, the physical condition of the mammal, the type of neurotoxin or nerve agent to which the mammal was exposed to and the nature of concurrent therapy (if any), including Oximes therapy, and the specific formulations employed.

As used herein, "ameliorating" a neurotoxic symptom or neurotoxic poisoning means slowing, stopping, inhibiting or reversing the progression of the neurotoxic symptom or neurotoxic poisoning, and/or lessening, alleviating or removing the neurotoxic symptom or the neurotoxic poisoning.

A "Neurotoxin" or "Neutoxins" also referred to as "nerve agents" are substances, which are generally prepared by chemical synthesis or extracted from natural sources (natural or artificial toxic substances). Neurotoxins may cause deleterious or undesirable effects to a mammal if inhaled, absorbed, ingested, or otherwise encountered because of their high reactivity with and inhibition of cholinesterases. Exposure to "Neutoxins" or "nerve agents" could be through inhalation, ingestion and/or dermal exposure.

Examples of Neurotoxins include but are not limited to "organophosphorus compounds" (OPC) or Organophosphates (OP), such as diisopropylfiuorophosphate (DFP), CA (tabun), GB(sam), GD (soman), GE (cyclosarin), CV, yE, VG(amiton), VM, VR (RVX or Russian VX), VS, VX, and combinations thereof.

"Organophosphorate pesticides" include but are not limited to acephate, azinphos-methyl, bensulide, cadusafos, chlorethoxyfos, chlorpyrifos, chlorpyrifos methyl, chlorthiophos, coumaphos, dialifior, diazinon, diehlorvos(DDVP), dierotophos, dimethoate, dioxathion, disulfoton, ethion, ethoprop, ethyl parathion, fenamiphos, fenitrothion, fenthion, fonofos, isazophos methyl, isofenphos, malathion, methamidophos, methidathion, methyl parathion, mevinphos, monocrotophos, naled, oxydemeton methyl, phorate,

phosalone, phosmet, phosphamidon, phostebupirim, pirimiphos methyl, profenofos, propetamphos, sulfotepp, sulprofos, temephos, terbufos, tetraehlorvinphos, tribufos (JDEF), trichlorfon.

"Nerve agent poisoning" or "neurotoxic poisoning" relate to deleterious or undesirable effects to a mammal that are the result of exposure to a Neurotoxin or a nerve agent.

Neurotoxic symptoms can be "mild", "moderate" or "severe" in nature. "Mild" symptoms include but are not limited to bronchoconstriction, chest tightness, dim or blurred vision, conjunctival injection, fasciculations at site of exposure, increased sweating at site of exposure, mild increase in bronchial secretions, miosis (pupillary constriction) with eye pain or headache or rhinorrhea and combinations thereof. "Moderate" symptoms include coughing, wheezing, fasciculations, generalized weakness, nausea, vomiting, diarrhea, generalized weakness, shortness of breath, dyspnea and combinations thereof. "Severe" symptoms include coma, seizures, flaccid paralysis, apnea, severe bronchorrhea and bronchospasm, generalized fasciculations, generalized secretions and death.

"Acetylcholinesterase inhibitors" are chemicals whose primary toxic effect is to block the normal breakdown of the neurotransmitter acetylcholine. Acetylcholinesterase inhibitors occupy and block the site where the neurotransmitter, acetylcholine, attaches to the enzyme acetylcholinesterase. With toxic doses, the result is that excessive levels of the acetylcholine build up in the synapses and neuromuscular junctions and glands.

"Oximes" restore AChE activity by hydrolytically cleaving the "OPC"s from the active site of inhibited AChE. AChE reactivators are mono- or bis-quaternary pyridinium salts bearing in their molecule a functional oxime group able to

split the bond between the OPC inhibitor and the enzyme, releasing free functional enzymes, which are therefore able to be once again physiologically active in the organism. Examples of oxime include but are not limited to: pralidoxime chloride (2-PAM), mesylate (P2S),H16, toxogonin, MMB-4, HS-6 and TMB4 (A. BARELLI).

A "bioscavenger molecule" is any molecule derived from a biological source such as recombinant DNA technology and/or being expressed from a biological organism, such as mammalian or bacterial cell culture (for example not being artificially synthesized like a peptide or small molecule), which can scavenge or bind/block/remove another molecule from the immediate environment which otherwise would have posed a threat to the host organism. Unlike receptor-based scavengers found on the surface of cells or small molecule scavengers, a bio-scavenger is a large molecular entity derived from a biological source which can scavenge (remove and deactivate) toxic substances that otherwise would pose a threat to the immediate tissue environment

List of Abbreviations

As used herein, the following abbreviations have the meaning set out below throughout this disclosure:

PD	Pharmacodynamic
PK	Pharmacokinetic
TBU	Teva Biopharmaceuticals USA
US	United States
WFI	Water for Injection
NA	Nerve agents
OP	organophosphorus
AChE	acetylcholinesterase
hBChE	human butyrylcholinesterase
SC	Subcutaneously
IV	Intravenously
IM	intramuscularly

The present invention provides a method of treating a mammal for exposure to a neurotoxin or for preventing or ameliorating

the Neurotoxic Symptoms of exposure to neurotoxin which comprises administering an effective amount of the recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1. SEQ ID No:1 is a fully recombinant fusion polypeptide composed of the mature form of human serum albumin (HSA) fused at its amino terminus to the carboxy-terminus of a truncated and mutated human BChE.

In an embodiment, the invention is a method for preventing or ameliorating the neurotoxic symptoms of exposure to neurotoxin in a mammal which comprises administering an effective amount of the recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1.

In a further embodiment, the invention is a method for preventing or ameliorating neurotoxic poisoning in a mammal which comprises administering an effective amount of the recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1.

In an embodiment, the invention is a method for preventing or ameliorating the neurotoxic symptoms of exposure to neurotoxin or for preventing or ameliorating neurotoxic poisoning in a mammal wherein the neurotoxic poisoning or neurotoxic symptoms are expressed in the central, the peripheral, or the autonomic nervous systems, or in skeletal muscles.

The neurotoxic poisoning or neurotoxic symptoms could be associated with mild, moderate or severe neurotoxic symptoms or combinations thereof. The mild symptoms could be selected but are not limited to: bronchoconstriction, chest tightness, dim or blurred vision, conjunctival injection, fasciculations at site of exposure, increased sweating at site of exposure, mild increase in bronchial secretions, miosis (pupillary constriction) with eye pain or headache or rhinorrhea and combinations thereof.

The moderate symptoms could be selected but are not limited to: symptoms selected from: coughing, wheezing, fasciculations, generalized weakness, nausea, vomiting, diarrhea, generalized weakness, shortness of breath, dyspnea and combinations thereof.

The severe symptoms could be selected but are not limited to: symptoms selected from: coma, seizures, flaccid paralysis, apnea, severe bronchorrhea and bronchospasm, generalized fasciculations, generalized secretions, death and combinations thereof.

In yet another embodiment, the recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1 is administrated prior to exposure to said neurotoxin.

In one embodiment of the invention the fusion protein whose amino acid sequence is set forth as SEQ ID No:1 is administrated at least 12 hours prior to exposure to said neurotoxin or at least 24 hours prior to exposure to said neurotoxin or at least 48 hours prior to exposure to said neurotoxin or at least 72 hours prior to exposure to said neurotoxin

In one embodiment of the invention the fusion protein whose amino acid sequence is set forth as SEQ ID No:1 is administrated immediately after exposure to said neurotoxin.

In an embodiment, the invention is a method for preventing or ameliorating the neurotoxic symptoms of exposure to neurotoxin or for preventing or ameliorating neurotoxic poisoning in a mammal wherein the neurotoxin is a natural or artificial toxic substance. In an embodiment, the neurotoxin is an organophosphorate pesticide or an organophosphorous compound. In an embodiment, the organophosphorous compound is sarin (O-isopropyl-methylphosphonofluoridate, GB), VX (ethyl-S-2-diisopropylaminoethyl-phosphano-thiolate), MEPQ (7-

(methylethoxyphosphinyloxy)-1-methylquinolinium iodide), soman (pinacolylmethyl-phosphonofluoridate, GD), DFP (diisopylfluorophosphate paraoxon), malathion, parathion, tabun (GA), and cyclosarin (GF), Russian VX (VR), paraoxon, Chlorpyrifos-oxon, VM or a combination thereof.

In one embodiment of the invention the exposure to the neurotoxin is through inhalation, ingestion, dermal exposure or a combination thereof.

In one embodiment of the invention the fusion protein whose amino acid sequence is set forth as SEQ ID No:1 is administered subcutaneously (SC), intravenously (IV) or intramuscularly (IM).

In an embodiment, the invention is a method for preventing or ameliorating the neurotoxic symptoms of exposure to neurotoxin or for preventing or ameliorating neurotoxic poisoning in a mammal which comprises co-administering an effective amount of the recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1 with an oxime, wherein said oxime reactivates said bioscavenger molecule. In an embodiment, the oxime is selected from the group consisting of 2-PAM, H16, toxogonin, MMB-4, HS-6 TMB4, and combinations thereof.

This invention will be better understood by reference to the Examples which follow, which are set forth to aid in an understanding of the subject matter but are not intended to, and should not be construed to, limit in any way the claims which follow thereafter.

Examples

Study Product

SEQ ID No.1 is a fully recombinant fusion polypeptide with a molecular mass of approximately 140 kDa. It is composed of the mature form of human serum albumin (HSA) fused at its amino terminus to the carboxy-terminus of a truncated and mutated human BChE. Four mutations were made in the BChE amino acid sequence in order to increase cocaine hydrolytic activity. In addition, the last 45 amino acids of native human BChE molecule are responsible for tetramerization of the native molecule and these amino acids were removed from the recombinant enzyme molecule to facilitate expression of the enzyme in monomer form.

Example 1: In Vivo Experiments

1.1. Hydrolytic Activity against Butyrylthiocholine (Figure 1)

The catalytic activity of the recombinant fusion protein of SEQ ID No:1 against butyrylthiocholine needed to be established under the conditions utilized at the United States Army Medical Research Institute of Chemical Defense (USAMRICD). These data were used to determine appropriate enzyme and substrate concentrations for use in subsequent assays.

The recombinant fusion protein of SEQ ID No:1 was resuspended according to manufacturer instructions. The seal of the vial containing recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1 was flipped off and a needle was placed on a 3ml syringe. 1.0 ml of WFI was withdrawn into the syringe while ensuring that no air was trapped within the syringe when measuring the desired volume. The needle was inserted through the septum at an approximately

a 90° angle while holding the plunger. Once the needle penetrated the septum, it was directed towards the side of the vial and the plunger was depressed slowly. Once all the WFI was dispensed into the vial the needle was slowly removed from the septum. The vial gently rolled between the palms of the hand for at least 6 minutes (fully dissolved) or was put on a mini-rotator (Glas-Col) with 20rpm for 20±4 minutes after it was initially rolled by hand for approximately 1 minute. The vial was not vigorously shaken and each vial was gently tapped for several seconds upon completion of reconstitution.

The resuspended recombinant fusion protein of SEQ ID No:1 was diluted to concentrations ranging from 10 nM to 35 nM (assuming a molecular weight of 126,000 g/mol) using 0.1 M potassium phosphate buffer at pH 7.0. Two microliters of each enzyme solution was added to 100µL of 0.1 M potassium phosphate buffer at pH 7.0 in the wells of polystyrene 96-well flat bottom plates (Fisher Scientific). After mixing, 100µL of a solution containing 0.5 mM butyrylthiocholine (BtCh) and 1 mM dithionitrobenzoic acid (DTNB) (Ellman et al, 1961) in 0.1 M potassium phosphate buffer at pH 7.0 was added to each well and the change in absorbance at 412 nm was measured using a Spectromax plate reader (Molecular Devices) over a period of five minutes; only the linear portion of each assay curve was used for data analysis. A similar experiment was performed in parallel using human plasma-derived butyrylcholinesterase (huBuChE; Baxter BioSciences).

1.2. Inhibition Rate Constants Determined Using a Continuous Measurement Method (Figure 2A, 2B, Table 1)

A continuously measured competition assay was used to determine the inhibition rate constants for both the recombinant fusion protein of SEQ ID NO:1 and huBuChE for several OP nerve agents as an alternative, more reproducible method.

In order to quantitatively compare the efficiency with which the recombinant fusion protein of SEQ ID NO:1 sequesters nerve agents as compared to ability of huBuChE to bind the same compounds, a continuous measurement method was used. This method utilized the principle of competitive inhibition and the fact that all nerve agent inhibition of BuChE is an inactivating covalent modification of the active site (Hart GJO et al, 1973). For this approach to be used, the KM of the enzyme with BtCh must be determined. Isothermal titration calorimetry was used to determine the KM value of the recombinant fusion protein of SEQ ID NO:1 with BtCh due to the fact that the KM was too low to measure using the standard 96-well plate assays described in earlier experiments. In brief, while this continuous method is not a direct measurement of the bimolecular association of OP nerve agents with an enzyme, this assay more closely mimics the conditions under which OP nerve agent would compete with a native substrate in vivo to achieve inhibition of either acetylcholinesterase or butyrylcholinesterase. Interestingly, while the absolute values of the inhibition rate constants determined by the continuous method are not identical to the bimolecular association rate constants determined using the discontinuous method, the relative inhibitory capacity of each OP for the recombinant fusion protein of SEQ ID NO:1 remains nearly the same. Finally, in experiments conducted as part of another study, values determined from the continuous method using acetylcholinesterase with the same panel of OPs closely approximate previously reported values for the same parameters (Hart GJO et al, 1973).

TABLE 1. Recombinant Fusion Protein of SEQ ID NO:1 Ki rate constants versus Baxter human plasma derived BuChE

	Recombinant Fusion Protein of SEQ ID NO:1		Human Plasma Derived BuChE	
Agent	ki (1/M*min)	St Dev	ki (1/M*min)	St Dev
GA	6.12E+05	8.26E+04	4.93E+06	1.14E+05
GB	1.03E+06	7.73E+04	8.62E+06	3.85E+05
GD	1.09E+07	4.87E+05	8.52E+07	1.33E+06
GF	2.03E+07	5.24E+05	1.61E+08	3.23E+06
VX	3.98E+04	4.60E+03	6.92E+06	1.02E+05
VM	3.39E+04	2.87E+03	2.62E+06	4.77E+04
VR	1.78E+06	4.30E+04	1.08E+08	6.25E+06

1.3. Reactivation Capacity of Various Oximes with the recombinant fusion protein of SEQ ID NO:1 (Table 2)

Small molecules such as oximes have the potential to regenerate active cholinesterase enzyme after exposure to OP nerve agents (an otherwise irreversible covalent inhibitor). The time required to reactivate fifty percent of an OP-inhibited enzyme population (t1/2) is an indication of the efficiency with which a specific reactivator acts on an enzyme inhibited by a specific OP nerve agent. The t1/2 for several commonly tested reactivators was determined for the recombinant fusion protein of SEQ ID NO:1 after inhibition by a panel of OP nerve agents.

Equal volumes of the recombinant fusion protein of SEQ ID NO:1 and dilute OP nerve agent in the buffers described above in previous experiments were incubated at room temperature for 10 minutes to achieve complete inhibition of

butyrylcholinesterase activity. Excess nerve agent was removed by passing the samples through a small volume G25/150 size exclusion column (Centrisep, Princeton Separations, Inc., Adelphia, NJ). Inhibited enzyme samples and positive control, uninhibited enzyme samples were diluted and incubated in the presence of 1 mM Oxime or buffer control, as appropriate. Periodically over the course of 2 hours, the capacity of the incubated samples and controls to hydrolyze BtCh was measured using the general method described in previous experiments. The activity of the uninhibited enzyme over the 2 hour time course was fixed as the maximum possible activity, and the percentage of reactivation for the inhibited sample was determined as the fraction of the maximum possible at each time point. The percent reactivation was plotted versus time, and was then fit linearly to determine the half-time for reactivation (t1/2).

TABLE 2. Half-times of reactivation (t1/2) for Recombinant Fusion Protein of SEQ ID NO:1 in the presence of nerve agents and oxime reactivators

OP Agent	2PAM $t_{1/2}$ (min)	MMB4 $t_{1/2}$ (min)	HI6 $t_{1/2}$ (min)
GA	-	-	ND
GB	-	-	108 ± 0.7
GD	ND	ND	ND
GF	28 ± 2	13 ± 1	5.3 ± 0.9
VX	> 500	110 ± 2	177 ± 4
VR	-	-	7.4 ± 1

Note that 'ND' = None Detected and "-" = Not Tested

Example 2: In Vivo Experiments

2.1. Pharmacodynamic Determination of the recombinant fusion protein of SEQ ID NO:1 Circulatory Stability (Figure 3)

The circulatory stability of any bioscavenger candidate is an important factor for determining the usefulness of the

candidate for the purposes of protecting against nerve agent intoxication, and indeed may define the concept(s) of operation (ConOps) under which the enzyme can be useful. The experiments described here were performed in order to determine the time frame after administration of the recombinant fusion protein of SEQ ID NO:1 that enzyme activity could be detected in the plasma of injected guinea pigs. This information was required to design appropriate protective efficacy experiments (see the following experimental section).

Male Hartley guinea pigs (300 - 350 grams, Charles River Laboratories) were injected intramuscularly (i.m.) in the rear thigh or intravascularly (i.v.) via a commercially implanted carotid catheter with either 25 mg/kg or 50 mg/kg of recombinant fusion protein of SEQ ID NO:1 (n = 3 animals per dose and route). Blood samples (~50 ul each) were collected via toe nail clip into lithium-heparin lined collection microcuvettes (Sarstedt, Germany), processed immediately to plasma and stored at -80 °C. Timepoints collected for i.m. administered drug were 0, 1, 3, 16, 24, 48, 72, and 190 hours. Timepoints for i.v. administered drug were 0, 0.5, 1, 4, 20, 28, 44, 72, and 190 hours. Plasma samples were examined for the capacity to hydrolyze the substrate butyrylthiocholine using the assay conditions described above. The total amount of the recombinant fusion protein of SEQ ID NO:1 was determined in each sample by extrapolation using an in vitro generated standard curve of the recombinant fusion protein of SEQ ID NO:1 with the same substrate. The BtCh hydrolysis values derived from the 0 hour samples of guinea pig plasma were subtracted as background from each sample to account for the presence of endogenous BuChE in the guinea pig plasma.

The recombinant fusion protein of SEQ ID NO:1 activity appears to reach a maximum at or before 30 minutes after i.v. administration. Butyrylcholinesterase activity returns to baseline levels by 28 hours (25 mg/kg dose) or 72 hours (50

mg/kg dose) after administration, indicating that the protein is cleared quickly from the system. Preliminary analyses indicate an elimination half-time for the recombinant fusion protein of SEQ ID NO:1 from guinea pig plasma of ~60 minutes. In results not shown, plasma samples collected from guinea pigs injected i.m. with either 25 or 50 mg/kg of the recombinant fusion protein of SEQ ID NO:1 were found to have no enhanced butyrylcholinesterase activity above baseline levels at any of the time points sampled, suggesting that little or no of the recombinant fusion protein of SEQ ID NO:1 enters the blood of guinea pigs after i.m. administration of a dose as high as 50 mg/kg.

2.2. Protective Efficacy Testing in Guinea Pigs (Table 3)

The experiment conducted is an initial proof-of-concept in vivo test to determine if any protection against OP nerve agent intoxication can be provided via prophylactic administration of the recombinant fusion protein of SEQ ID NO:1.

Male Hartley guinea pigs (300-350 grams, purchased with implanted carotid catheters from Charles River Laboratories) were injected with the recombinant fusion protein of SEQ ID NO:1 at 50 mg/kg i.v. Animals were exposed subcutaneously (s.c.) to 2 x LD50 of the OP nerve agents GA (tabun), GB (sarin), GD (soman), GF (cyclosarin), VX, or VR at 30 minutes after enzyme injection (n = 3 animals/agent, with the exception of experiments with VX, as detailed below). OP compounds were diluted in saline such that injection volumes were 50 to 200 µl per animal, and s.c. injections were made into the left side of the animal below the rib cage. In an additional experiment, 3 guinea pigs were administered 250 mg/kg of the recombinant fusion protein of SEQ ID NO:1 and subsequently exposed to 2 x LD50s of VX; in this experiment,

two additional animals were tested with VX after injection with 50 mg/kg. All exposed animals were scored for survival at 24 hours post-OP exposure.

TABLE 3. Survival rates for guinea pigs exposed to nerve agents for 30 mins after adminstration of the recombinant fusion protein of SEQ ID NO:1

OP Agent	Dose of OP (µg/kg)	24 hour Survival
GA	240	1 / 3
GB	87	3 / 3
GD	56	3 / 3
GF	108	3 / 3
VX	18	2 / 5
VR	22.6	3 / 3
VX*	18	3 / 3

*These animals received an enzyme dose approximately 5-fold higher than that administered to the other animals, followed by exposure to 2 x LD_{50} dose of VX.

Discussion

The results indicate that the modified human butyrylcholinesterase moiety present in of the recombinant fusion protein of SEQ ID NO:1 is capable of hydrolyzing the substrate butyrylthiocholine, albeit at a ~6-fold slower rate than the wild-type human enzyme. This enzymatic activity is inhibited by a broad spectrum of OP nerve agents (GA, GB, GD, GF, VX, VR, and VM), and this inhibition occurs with different bimolecular rates and inhibition rates, depending on the OP in question. Both the discontinuous and continuous methods of assessing OP interaction with the recombinant fusion protein of SEQ ID NO:1 indicated that the relative capacity of these OPs to inhibit the recombinant fusion protein of SEQ ID NO:1 can be rank ordered GF > GD > VR > GB > GA > VX ≥ VM.

The capacity of HI6 and other oximes to reactivate the recombinant fusion protein of SEQ ID NO:1's butyrylcholinesterase activity after inhibition by a panel of nerve agents indicates that none of the oximes tested are expected to afford broad spectrum reactivation, but that HI6 does reactivate GF- and VR-inhibited recombinant fusion protein of SEQ ID NO:1 at a rate sufficiently fast to potentially allow pseudo-catalytic hydrolysis of these OP compounds in vivo. The reactivation rates for the recombinant fusion protein of SEQ ID NO:1 inhibited by other OPs are much longer, and as such do not afford much promise for in vivo reactivation even in non-acute situations (when pseudo-catalysis is not required, and one is only attempting to regenerate active enzyme to provide protection against a subsequent exposure), because the circulatory half-life of the oximes is much shorter than the half-time for reactivation. Thus, the oximes would be expected to degrade or otherwise be cleared from circulation before causing significant reactivation in vivo of the recombinant fusion protein of SEQ ID NO:1.

The pharmacodynamic profile of the recombinant fusion protein of SEQ ID NO:1 in guinea pig plasma after i.v. administration indicates that this protein is extremely short lived in circulation, with a half-life of roughly one hour. This short half-life is most likely the reason that no increase in butyrylcholinesterase activity was detected after i.m. administration, as this route of protein administration generally results in lower Cmax values and reduced bioavailability as compared with i.v. administration. The recombinant fusion protein of SEQ ID NO:1 appears to be suited for use as an immediate post-exposure intervention for OP poisoning; this ConOp requires both rapid bioavailability of an enzyme in circulation after administration, and sufficient circulatory stability of the enzyme to outlast the toxicokinetics of the OP.

Finally, the results from protective efficacy tests of the recombinant fusion protein of SEQ ID NO:1 administered as a prophylactic against a broad spectrum of OP agents indicates that if present in the blood at sufficient quantities at the time of exposure, of the recombinant fusion protein of SEQ ID NO:1 can afford symptom-free protection against 2 x LD50s of GB, GD, GF, VX, and VR. The amount of enzyme necessary to provide protection against VX (and presumably GA) is higher than that provided by a 50 mg/kg dose of the recombinant fusion protein of SEQ ID NO:1. In the case of GA, this may be a consequence of the fact that the LD50 of GA is low compared to that of other OP nerve agents (240 µg/kg, or ~1500 nmoles/kg, for 2 x LD50s); at 50 mg/kg of the recombinant fusion protein of SEQ ID NO:1, the animals receive only ~400 nmoles of enzyme per kg. The results with VX, however, suggest that the situation is more complex than a simple measure of the stoichiometry of OP and enzyme. For VX, the dose of OP that constitutes 2 x LD50s is 67 nmoles/kg, so a dose of 50 mg/kg represents a nearly seven-fold molar excess of enzyme over agent. The failure of the recombinant fusion

protein of SEQ ID NO:1 to provide symptom-free protection against VX is most likely a consequence of the low binding affinity of VX for the recombinant fusion protein of SEQ ID NO:1 (reflected in the bimolecular rates and inhibition rates shown in Figures 2A and 2B). Interestingly, this explanation may apply for GA as well, as the inhibition rates for GA with the recombinant fusion protein of SEQ ID NO:1 are also low as compared to values for other OPs. By analogy, it is expected that the recombinant fusion protein of SEQ ID NO:1 at 50 mg/kg would also not provide complete protection against VM, although this compound was not tested in vivo. Regardless of the reason for the poor ability of the recombinant fusion protein of SEQ ID NO:1 to protect against 2 x LD50 of VX at 50 mg/kg, the results also indicate that administration of five-fold more protein does provide protection against the same dose of VX.

Claims

Claim 1. A method for preventing or ameliorating a neurotoxic symptom of exposure to a neurotoxin in a mammal which comprises administering to the mammal an effective amount of a recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1.

Claim 2. The method of claim 1 wherein the neurotoxic symptom is expressed in the central nervous system, the peripheral nervous system, the autonomic nervous systems, skeletal muscle, or a combination thereof.

Claim 3. The method of claim 1 or claim 2 wherein the neurotoxic symptom is a mild, a moderate or a severe neurotoxic symptom or a combination thereof.

Claim 4. A method for preventing or ameliorating neurotoxic poisoning in a mammal which comprises administering to the mammal an effective amount of a recombinant fusion protein whose amino acid sequence is set forth as SEQ ID No:1.

Claim 5. The method of claim 4 wherein the neurotoxic poisoning is of the central nervous system, the peripheral nervous system, the autonomic nervous systems, skeletal muscle, or a combination thereof.

Claim 6. The method of claim 4 or claim 5 wherein the administration of the recombinant fusion protein prevents or ameliorates a mild, a moderate, or a severe neurotoxic symptom of the neurotoxic poisoning, or a combination thereof.

Claim 7. The method of claim 3 or claim 6 wherein the mild neurotoxic symptom is bronchoconstriction, chest tightness, dim or blurred vision, conjunctival injection, fasciculations at the site of exposure, increased sweating at the site of exposure, mild increase in bronchial secretions, miosis

(pupillary constriction) with eye pain or headache or rhinorrhea, or a combination thereof.

Claim 8. The method of any one of claims 3, 6, or 7, wherein the moderate neurotoxic symptom is coughing, wheezing, fasciculations, generalized weakness, nausea, vomiting, diarrhea, generalized weakness, shortness of breath, dyspnea, or a combination thereof.

Claim 9. The method of any one of claims 3 or 6-8 wherein the severe neurotoxic symptom is coma, seizures, flaccid paralysis, apnea, severe bronchorrhea and bronchospasm, generalized fasciculations, generalized secretions, death, or a combination thereof.

Claim 10. The method of any one of claims 1-9 wherein the recombinant fusion protein is administrated prior to exposure to said neurotoxin.

Claim 11. The method of claim 10 wherein the recombinant fusion protein is administrated at least 12 hours prior to exposure to said neurotoxin.

Claim 12. The method of claim 10 wherein the recombinant fusion protein is administrated at least 24 hours prior to exposure to said neurotoxin.

Claim 13. The method of claim 10 wherein the recombinant fusion protein is administrated at least 48 hours prior to exposure to said neurotoxin.

Claim 14. The method of claim 10 wherein the recombinant fusion protein is administrated at least 72 hours prior to exposure to said neurotoxin.

Claim 15. The method of any one of claims 1-14 wherein the recombinant fusion protein is administrated after exposure to said neurotoxin.

Claim 16. The method of claim 15 wherein the recombinant fusion protein is administered within six hours of exposure to said neurotoxin.

Claim 17. The method of claim 15 wherein the recombinant fusion protein is administered within one hour of exposure to said neurotoxin.

Claim 18. The method of claim 15 wherein the recombinant fusion protein is administered within 30 minutes of exposure to said neurotoxin.

Claim 19. The method of claim 15 wherein the recombinant fusion protein is administered within 10 minutes of exposure to said neurotoxin.

Claim 20. The method of claim 15 wherein the recombinant fusion protein is administered within 5 minutes of exposure to said neurotoxin.

Claim 21. The method of any one of claims 1-20 wherein the neurotoxin is a natural or an artificial toxic substance.

Claim 22. The method of claim 21 wherein the neurotoxin is an organophosphorous compound.

Claim 23. The method of claim 22 wherein the organophosphorous compound is selected from the group consisting of sarin (O-isopropyl-methylphosphonofluoridate, GB), VX (ethyl-S-2-diisopropylaminoethyl-phosphano-thiolate), MEPQ (7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide), soman (pinacolylmethyl-phosphonofluoridate, GD), DFP (diisopylfluorophosphate paraoxon), malathion, parathion, tabun (GA), and cyclosarin (GF), Russian VX (VR), paraoxon, Chlorpyrifos-oxon, VM and combinations thereof.

Claim 24. The method of claim 22 wherein the neurotoxin is an organophosphorate pesticide.

Claim 25. The method of any one of claims 1-24 wherein the exposure to the neurotoxin is through inhalation, ingestion, dermal exposure or a combination thereof.

Claim 26. The method of any one of claims 1-25, wherein the recombinant fusion protein is administered subcutaneously.

Claim 27. The method of any one of claims 1-25, wherein the recombinant fusion protein is administered intravenously (IV).

Claim 28. The method of any one of claims 1-25, wherein the recombinant fusion protein is administered intramuscularly (IM).

Claim 29. The method of any one of claims 1-28, which further comprises co-administration of an oxime, wherein said oxime reactivates said recombinant fusion protein.

Claim 30. The method of claim 29 wherein said oxime is selected from the group consisting of 2-PAM, H16, toxogonin, MMB-4, HS-6, TMB4, and combinations thereof.

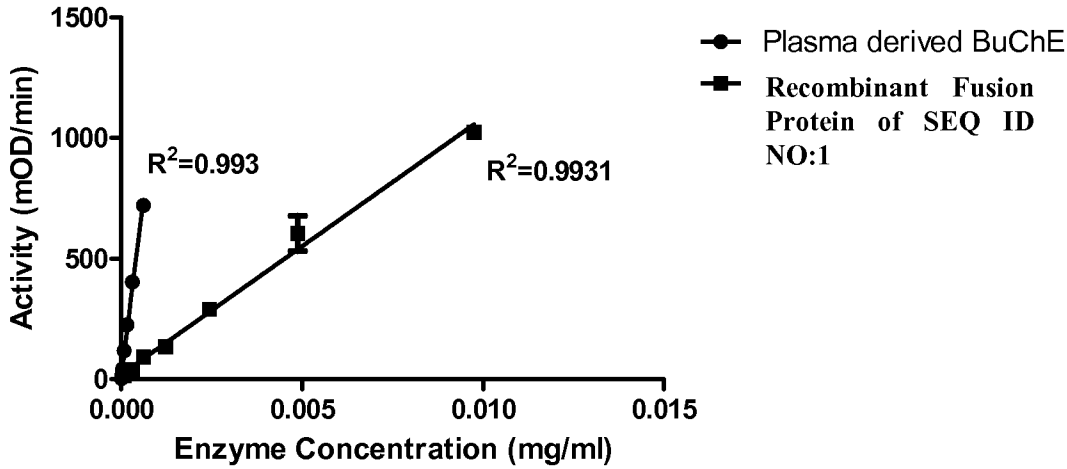
Claim 31. The method of any one of claims 29-30, wherein the amount of the recombinant fusion protein and the amount of the oxime when taken together is more effective to treat the mammal than when each agent is administered alone.

Claim 32. The method of any one of claims 29-31, wherein the amount of the recombinant fusion protein in combination with the amount of oxime is less than is clinically effective when administered alone.

Claim 33. The method of any one of claims 29-32, wherein the amount of the oxime in combination with the amount of the recombinant fusion protein is less than is clinically effective when administered alone.

Claim 35. The method of any one of claims 1-34, wherein the effective amount of the recombinant fusion protein is 5 mg/kg to 250 mg/kg.

FIGURE 1



Baxter BuChE								
mg/mL	0.00061	0.000305	0.000152	7.62E-05	3.81E-05	1.91E-05	9.53E-06	4.76E-06
nM	7.2	3.6	1.8	0.9	0.45	0.225	0.1125	0.05625
	724.1143	397.6286	219.1857	115.0286	44.3	18.28571	9.114286	4.371429
	718.1857	408.1714	232.2143	120.5571	42.45714	21.94286	6.6	1.3
Average	721.15	402.9	225.7	117.7929	43.37857	20.11429	7.857143	2.835715
St Dev	4.192133	7.454926	9.212592	3.909291	1.303097	2.585991	1.777869	2.171828
Recombinant Fusion Protein of SEQ ID NO:1								
mg/mL	0.009756	0.004878	0.002439	0.00122	0.00061	0.000305	0.000152	7.62E-05
nM	0.077	0.0385	0.01925	0.009625	0.004813	0.002406	0.001203	0.000602
	1042.775	678.6833	299.6583	137.6833	104.9667	43.83333	35.38333	16.53333
	1004.692	530.0833	278.1833	132.5083	77.69167	35.025	20.18333	11.78333
Average	1023.733	604.3833	288.9208	135.0958	91.32917	39.42917	27.78333	14.15833
St Dev	26.92898	105.0761	15.18512	3.659278	19.28634	6.228432	10.74802	3.358757

FIGURE 2A

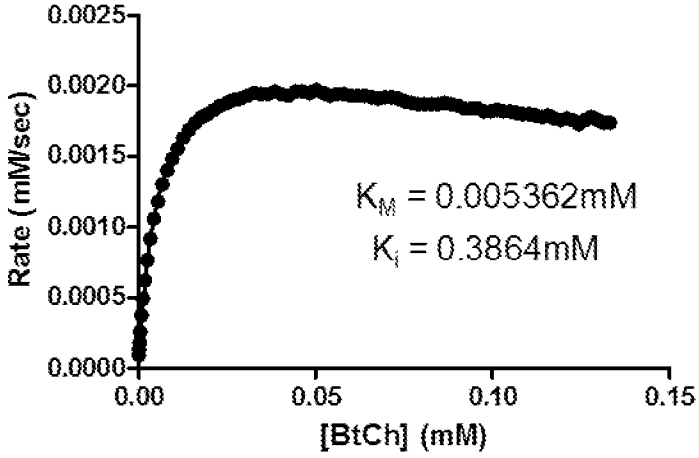
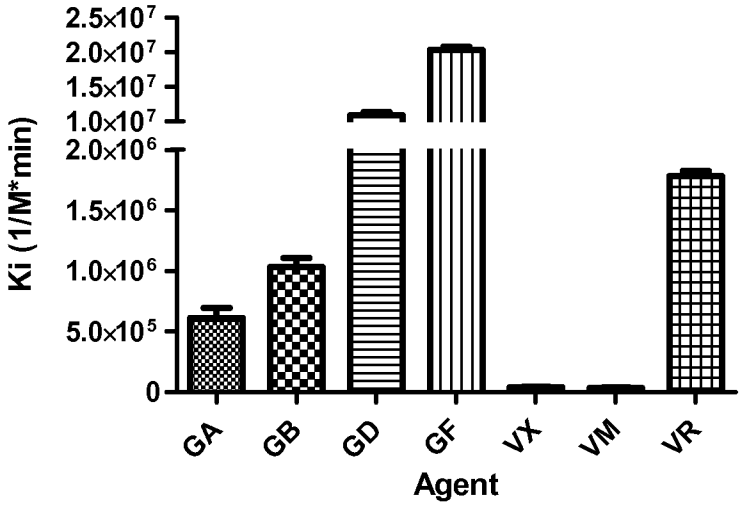


FIGURE 2B



	Recombinant Fusion Protein of SEQ ID NO:1	
Agent	ki (1/M*min)	St Dev
GA	6.12E+05	8.26E+04
GB	1.03E+06	7.73E+04
GD	1.09E+07	4.87E+05
GF	2.03E+07	5.24E+05
VX	3.98E+04	4.60E+03
VM	3.39E+04	2.87E+03
VR	1.78E+06	4.30E+04

FIGURE 3

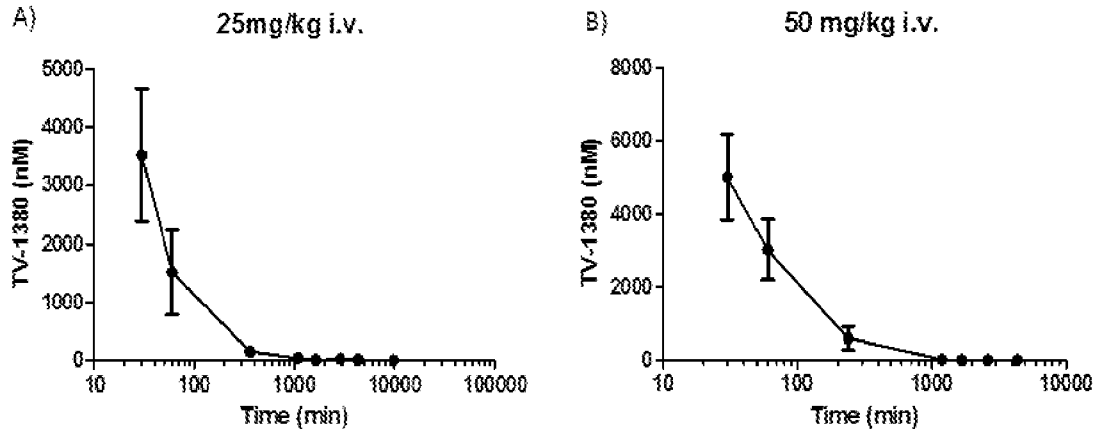


FIGURE 4

All GPs; recombinant fusion protein of SEQ ID NO:1 RoA = IC artery; Give recombinant fusion protein of SEQ ID NO:1 then NAs(SC) 30-mins later; PK data separate

[SEQ ID NO: 1] DOSE (N/#GROUP)	Nerve Agent (SC 2*LD50)	24 hr Nerve Agent Poisoning?	24hr-Survival Post Exposure?	Half-life PK ($t_{1/2}$) hr Mean (ELISA)
[50 mg/kg] (3)	GD (Soman)	No	Yes; 100%	-
[50 mg/kg] (3)	GB (Sarin)	No	Yes; 100%	-
[50 mg/kg] (2)	GF (Cyclosarin)	No	Yes; 100%	-
[50 mg/kg] (3)	VR (VX-Russian)	No	Yes; 100%	-
[50 mg/kg] (3)	GA (Tabun)	Yes; major/minor	Partial; 50%	-
[50 mg/kg] (3)	VX	Yes; major	No; 0%	-
[50 mg/kg] (2)	VX	Yes; major	Yes; 100%	-
[266-267 mg/kg] (3)	VX	No	Yes; 100%	-
[25 mg/kg] (3)	-	-	-	IM (15±0.7) IV (29±6.9)
[50 mg/kg] (3)	-	-	-	IM (18±0.7) IV (12.4±5.1)

Biomolecular Rates (M/min), and Oxime Reactivation ($t_{1/2}$ mins; x<2 min threshold), N=1

BuChe	GA	GB	GD	GF	VX	VR
WT Baxter	3.56E8	4.47E8	4.02E8	3.35E8	6.03E9	4.57E8
SEQ ID NO: 1	3.35E7	6.04E7	1.59E8	3.00E8	1.33E7	6.93E7
SEQ ID NO: 1 Oxime Reactivation (mins) (y<2 req)	ND	HI6 (108±7)	ND	2PAM (28±2); MMB4 (13±1) HI6 (5.3±0.3)	2PAM (X>500) MMB4 (110±2) HI6 (177±4)	HI6 (7.4±1)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/028291

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 9/18 (2015.01)
CPC - C12N 9/18 (2015.07)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 38/00, 38/38; C12N 9/18 (2015.01)
USPC - 424/94.6; 435/197; 530/362; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 38/00, 38/38, 38/465; C07K 14/76, 14/765, 14/4702, 2319/00, 2319/31; C12N 9/00, 9/16, 9/18 (2015.07) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Pub Med

Search terms used: butyrylcholinesterase BchE albumin fusion chimera neurotoxin neurotoxic neurotoxicity

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2009/0029914 A1 (ROSEN et al) 29 January 2009 (29.01.2009) entire document	1, 2, 4, 5 ---------- 3, 6
Y	US 2011/0312900 A1 (SKLAIR-TAVRON et al) 22 December 2011 (22.12.2011) entire document	3, 6
A	US 2011/0002888 A1 (ROSEN et al) 06 January 2011 (06.01.2011) entire document	1-6
A	US 2008/0194481 A1 (ROSEN et al) 14 August 2008 (14.08.2008) entire document	1-6
A	CARMONA et al. "Butyrylcholinesterase accelerates cocaine metabolism: in vitro and in vivo effects in nonhuman primates and humans," Drug Metab Dispos. March 2000, Vol. 28, Pgs. 367-371. entire document	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 August 2015	01 SEP 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/028291

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☒ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☒ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NO: 1 was searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/028291

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-33, 35
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.