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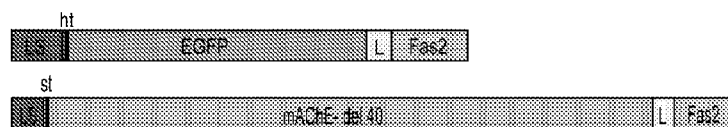


FIG. 1

(57) Abstract: Described herein are chimeric proteins, pharmaceutical compositions and methods for treating organophosphate exposure and poisoning (e.g., nerve agent poisoning, pesticide poisoning) in a subject (e.g., a human subject) and preventing organophosphate poisoning in a subject at risk of exposure to an organophosphate. The chimeric AChE-Fas2 protein used in the experiments described herein was demonstrated to bind specifically to the neuromuscular junction in vivo where it restored enzyme activity to the synapse after irreversible inactivation.



CHIMERIC PROTEINS, COMPOSITIONS AND METHODS FOR RESTORING CHOLINESTERASE FUNCTION AT NEUROMUSCULAR SYNAPSES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of Provisional Application Serial No. 61/713,293, filed October 12, 2012, which is hereby incorporated by reference in its entirety, for all purposes, herein.

FIELD OF THE INVENTION

[0002] The present invention is directed generally to the fields of cell biology and medicine. More particularly, the present invention provides novel chimeric proteins, pharmaceutical compositions and methods for treating organophosphate poisoning in an individual by administering novel chimeric proteins that restore cholinesterase (e.g., acetylcholinesterase (AChE)) enzymatic activity.

BACKGROUND

[0003] Organophosphate pesticides and nerve agents act by irreversibly inhibiting the enzyme AChE at the neuromuscular junction (NMJ) and other cholinergic synapses in the central and peripheral nervous systems. More than 70,000 fatalities occur each year from exposure to organophosphate pesticides according to United Nations figures (United Nations, 2005), many of these from self administration, with more than one million related injuries (United Nations, 2004). In addition, the widespread availability of organophosphate nerve agents such as sarin and soman, and the ease with which these compounds can be synthesized and delivered, makes them potentially useful by nefarious groups such as the release of sarin in the Tokyo subway in 1995 that killed more than a dozen people and injured thousands. Although treatments for exposure to organophosphates have been developed, including the use of reactivators of cholinesterases like 2-pyridine aldoxime methiodide (2-PAM), the rapid aging of modern organophosphates precludes their use under most circumstances. Some organophosphates such as tabun and soman, for example, become irreversibly bound to AChE, a process called aging, within minutes of administration after which the enzyme can no longer be reactivated. The consequence of AChE

inactivation is the prolongation of cholinergic transmission at cholinergic synapses, such as the NMJs of diaphragm muscles, leading to respiratory failure and death. There is thus a pressing need for improved treatments of organophosphate poisoning, including nerve agent exposure.

SUMMARY

[0004] Described herein are novel chimeric proteins, pharmaceutical compositions and methods for the treatment of victims (e.g., mammals such as humans) exposed to organophosphate compounds based on replacement of inactive enzyme molecules such as cholinesterases (e.g., AChE) present on accessible surfaces of target tissues including nerves, muscles and many glands. None of the currently available treatments, or even those under development, will restore function to cholinergic synapses whose AChE has been inactivated by rapidly aging organophosphate nerve agents. A novel means of targeting catalytically active AChE molecules to the neuromuscular synapse for replacing the damaged AChE molecules with new ones to restore esteratic function was developed. Fasciculin-2 (Fas2) is a 61 amino acid snake neurotoxin that binds with extremely high affinity to the peripheral anionic site of AChE. Expressible plasmids encoding chimeric proteins including murine AChE fused with Fas2 at its carboxyl terminus were developed and tested. These chimeric AChE proteins were shown to bind to endogenous mammalian AChE, and attach to the sites of nerve-muscle contact in vivo. They can thus be used to replace inactivated enzyme at neuromuscular synapses in animals (including humans) exposed to organophosphates. Although the experiments described herein involve AChE, any esterase that hydrolyses acetylcholine, such as butyrylcholinesterase (BuChE), can be included in the chimeric proteins described herein. Similarly, in addition to Fas2, any other Fas-like molecule that can bind to the peripheral anionic site of AChE or other catalytically active esterase-like protein that can hydrolyze acetylcholine into choline and acetic acid (e.g., BuChE) with high affinity can be used.

[0005] Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0006] As used herein, “protein” and “polypeptide” are used synonymously to mean any peptide-linked chain of amino acids, regardless of length or post-translational modification, e.g., glycosylation or phosphorylation.

[0007] By the term “gene” is meant a nucleic acid molecule that codes for a particular protein, or in certain cases, a functional or structural RNA molecule. A complementary DNA

(cDNA) is a single-stranded DNA that is complementary to messenger RNA or DNA that has been synthesized from messenger RNA by reverse transcriptase, and can be used for expression of a particular protein.

[0008] As used herein, the terms “nucleotide sequence,” “nucleic acid,” and “nucleic acid molecule” mean a chain of two or more nucleotides such as RNA (ribonucleic acid) and DNA (deoxyribonucleic acid).

[0009] The terms "patient," "subject" and "individual" are used interchangeably herein, and mean a mammalian (e.g., human, rodent, non-human primates, canine, bovine, ovine, equine, feline, etc.) subject to be treated and/or to obtain a biological sample from.

[0010] As used herein, "bind," "binds," or "interacts with" means that one molecule recognizes and adheres to a particular second molecule in a sample or organism, but does not substantially recognize or adhere to other structurally unrelated molecules in the sample. Generally, a first molecule that "specifically binds" a second molecule has a binding affinity greater than about 10^8 to 10^{12} moles/liter for that second molecule and involves precise “hand-in-a-glove” docking interactions that can be covalent and noncovalent (hydrogen bonding, hydrophobic, ionic, and van der Waals).

[0011] The term "organophosphates" refers to compounds capable of inactivating cholinesterases by phosphorylating the serine hydroxyl group located on the active site of the enzyme. Phosphorylation inactivates the enzyme when a covalent bond forms between the organophosphate molecule and the enzyme molecule. Exemplary organophosphates include insecticides such as malathion, parathion, diazinon, fenthion, dichlorvos, and chlorpyrifos and nerve gases such as soman, sarin, tabun, and VX. Another example of an organophosphate is the more generally available diisopropylfluorophosphate (DFP), used in many laboratories as a surrogate nerve agent.

[0012] The novel chimeric proteins described herein are capable of replacing endogenous AChE. "Replacing" refers to functionally replacing inactivated or otherwise damaged AChE molecules with new AChE molecules by attaching an active chimeric AChE molecule to the inactivated endogenous one to restore function.

[0013] The term "labeled," with regard to a probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody.

[0014] When referring to a nucleic acid molecule or protein, the term "native" refers to a naturally-occurring (e.g., a wild-type (WT)) nucleic acid or protein.

[0015] The phrases "isolated" and "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

[0016] The term "sample" is used herein in its broadest sense. A sample including polynucleotides, peptides, antibodies and the like may include a bodily fluid, a soluble fraction of a cell preparation or media in which cells were grown, genomic DNA, RNA or cDNA, a cell, a tissue, skin, hair and the like. Examples of samples include saliva, serum, urine, blood and plasma.

[0017] As used herein, the term "treatment" is defined as the application or administration of a therapeutic agent to a patient or a test animal, or application or administration of the therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease, or the predisposition toward disease.

[0018] As used herein, the phrase "safe and effective amount" refers to the quantity of a component which is sufficient to yield a desired 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 invention. By "therapeutically effective amount" is meant an amount of a composition of the present invention effective to yield the desired therapeutic response, for example, an amount effective to prevent or ameliorate organophosphate poisoning in an individual. The specific safe and effective amount or therapeutically effective amount will vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal or animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed.

[0019] As used herein, the terms "therapeutic," and "therapeutic agent" are used interchangeably, and are meant to encompass any molecule, chemical entity, composition, drug, therapeutic agent, or biological agent capable of preventing, ameliorating, or treating a disease or other medical condition.

[0020] Although chimeric proteins, compositions, kits, and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable chimeric proteins, compositions, kits, and methods are described below. All publications, patent applications, and patents mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. The particular embodiments discussed below are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1 is a schematic of the design of secretable green fluorescent protein (GFP) and AChE Fas2 chimeric proteins: The chimeric proteins were constructed by inserting a leader sequence (LS) in front of the GFP (a) in pEGFP-C1 followed by a 15 amino acid linker peptide (L) and the humanized Fas2 coding sequence (hereinafter referred to as “GFP-Fas2”). For the AChE-Fas2 (b) the GFP was removed and replaced with the mutant AChE that does not bind Fas2 nor contains the 40 amino acid C-terminal t-peptide.

[0022] FIG. 2 is a series of micrographs showing binding of AChE-Fas2 to the NMJ in vivo 30 min after 5 μ g intravenous (iv) injection.

[0023] FIG. 3A is a graph showing that injection of 5 μ g of AChE-Fas2 protein rescues mice following exposure to 2x LD₅₀ DFP.

[0024] FIG. 3B is a set of photomicrographs showing that AChE activity is restored to the NMJs of mice surviving 2x LD₅₀ DFP after AChE-Fas2 injections.

DETAILED DESCRIPTION

[0025] Described herein are compositions (e.g., chimeric proteins) and methods for treating organophosphate poisoning (e.g., nerve agent poisoning, pesticide poisoning) in a subject (e.g., a human subject). Two functional Fas2 chimeric proteins that bind to native wild type AChE were produced. The marker fusion protein, GFP-Fas2, when injected into mice, homed in to the neuromuscular synapse where it bound to the endogenous enzyme and co-localized with the nicotinic acetylcholine receptor (AChR). Also produced is a functional AChE-Fas2 chimeric protein that binds to the mammalian neuromuscular synapse and functionally replaces the endogenous enzyme on frozen sections of skeletal muscle and in vivo. Chimeric AChE-Fas2

protein rescued mice exposed to a lethal dose of organophosphate cholinesterase inhibitor when they were injected with 2 x LD₅₀ DFP (2.9 µg/g BW) in saline followed 5 minutes later by an injection of 0.2 µg/g BW chimeric AChE protein or saline alone. At this dose of DFP, 75% of the saline-injected mice usually die within 30 minutes, whereas only 1 mouse out of 12 died following treatment with the chimeric protein (it is possible that this mouse was not successfully injected). These results show the development of a novel therapy for exposure to organophosphate nerve agents consisting of, in one embodiment, a targetable chimeric AChE molecule coupled to an AChE-specific snake neurotoxin, Fas2, that targets the protein to the neuromuscular synapse, thereby replacing the inactivated AChE molecules with new functional ones.

Biological Methods

[0026] Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as *Molecular Cloning: A Laboratory Manual*, 3rd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; and *Current Protocols in Molecular Biology*, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates).

Compositions and Methods for Treating Organophosphate Poisoning In an Individual

[0027] Described herein are compositions (e.g., chimeric proteins) and methods for treating organophosphate poisoning in an individual (e.g., a human subject). Examples of organophosphates include nerve gases such as sarin, soman, VX and tabun, and pesticides such as malathion, parathion, diazinon, fenthion, dichlorvos, chlorpyrifos, etc. The therapeutic compositions and methods of the invention (which include prophylactic treatment) in general include administration of a therapeutically effective amount of the compositions described herein to a subject in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for organophosphate poisoning or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider.

[0028] A typical chimeric protein for treating organophosphate poisoning as described herein includes a catalytically active AChE protein and a neuromuscular synapse targeting domain. However, any catalytically active esterase-like protein that can hydrolyze acetylcholine into choline and acetic acid, e.g., butyrylcholinesterase, can be used. Any suitable neuromuscular synapse targeting domain (e.g., Fas2 domain) can be used. By “neuromuscular synapse targeting domain” is meant any peptide or protein that is capable of binding to AChE or other catalytically active esterase-like protein that can hydrolyze acetylcholine into choline and acetic acid (e.g., BuChE) at the NMJ. In the experiments described herein, the 61 amino acid snake toxin Fas2 was used (i.e., a Fas2 encoded by a nucleotide sequence optimized for mammalian expression) as a neuromuscular synapse targeting domain. However, Fasciculin-1 (Fas1), or any other snake toxin, or derivative thereof, that binds to any catalytically active esterase-like protein that can hydrolyze acetylcholine into choline and acetic acid such as AChE or BuChE can be used. Typically, the catalytically active AChE protein in a chimeric AChE-Fas2 protein as described herein lacks an oligomerization domain to make it monomeric and includes at least one mutation (e.g., a deletion, insertion, amino acid substitution) which prevents dimerization and binding of the catalytically active AChE protein to the Fas2. Examples of other neuromuscular synapse targeting domains include portions of the collagenic tail, a related toxin called Fasciculin 1 (Fas-1), from the same snake that differs by only one or two amino acids, and active peptides derived from Fas-2 or Fas-1.

[0029] Any suitable chimeric protein, e.g., any chimeric AChE-Fas2 protein or BuChE-Fas2 protein that is able to bind to a neuromuscular synapse and restore AChE activity to the neuromuscular synapse in vivo after inactivation of endogenous AChE at the neuromuscular synapse can be used for treating and preventing organophosphate poisoning in an individual. For treating a human, typically a human AChE or human BuChE protein is used. Human AChE and BuChE amino acid sequences are known. For example, an amino acid sequence for a human AChE protein is accession number NM_000665 and an amino acid sequence for a human BuChE is accession number NM_000055.2 (e.g., SEQ ID NO:6). The amino acid sequence for a human AChE variant (variant E4-E6) is as follows:

MRPPQCLLHTPSLASPLLLLLLWLLGGGVGAEGREDAELLVTVRGGRLRGIRLKTTPGGP
VSAFLGIPFAEPPMGPRRFLPPEPKQPWSGVVDATTFQSVCYQYVDTLYPGFEGTEMWN
PNRELSDECLYLNWVTPYPRPTSPTPVLVWIYGGGFYSGASSLDVYDGRFLVQAERTVL

VSMNYRVGAFGFLALPGSREAPGNVGLLDQRLALQWVQENVAAFGGDPTSVTLFGESA
GAASVGMHLLSPPSRGLFHRAVLQSGAPNGPWATVGMGEARRRATQLAHLVGCPPGG
TGGNDTELVACLRTRPAQVLVNHEWHVLPQESVFRFSFVPVVDGDFLSDTPEALINAGD
FHGLQVLVG VVKDEGSYFLVYGAPGFSKDNESLISRAEFLAGVRVGVPQVSDLA AEAV
VLHYTDWLHPEDPARLREALSDVVG DHDNVVCPVAQLAGRLAAQG ARVYAYVFEHRAS
TLWPLWMGVPHGYEIEFIFGIPLDPSRNYTAEKIFAQRLMRYWANFARTGDPNEPRD
PKAPQWPPYTAGAQQYVSLDLRPLEVRRGLRAQACAFWNRFLPKLLSATDTLDEAERQ
WKA EFHRWSSY MVHWKNQFDHYSKQDRCSDL (SEQ ID NO:1).

[0030] Any suitable Fasciculin sequence can be used (the amino acid sequence of Fas2 is described in Bourne et al., Cell vol. 83:503-512, 1995) in the chimeric polypeptides described herein. However, when generating chimeric proteins as described herein for use in humans, a nucleotide sequence encoding Fasciculin that has been optimized for mammalian expression (e.g., human expression) is preferred. An example of a Fas2 amino acid sequence (encoded by a nucleotide sequence optimized for mammalian expression) is TMCYSHTTTSRAILTNCGENSCYRKSRRHPPKMVLGRGCGCPPGDDNLEVKCCTSPDKC NY (SEQ ID NO:2). A typical amino acid sequence for a chimeric AChE-Fas2 protein having a human AChE and a Fas2 sequence (encoded by a nucleotide sequence optimized for mammalian expression) includes a leader sequence followed by human AChE followed by the 15 amino acid linker sequence followed by the Fas2 sequence. However, any variants, mutants, fragments, analogs or derivatives thereof that bind to a neuromuscular synapse and restore AChE activity to the neuromuscular synapse can be used. In one example of an amino acid sequence for a chimeric AChE-Fas2 protein, a human AChE variant (variant E4-E6) including a linker sequence, a Fas2 sequence and a leader sequences is as follows:

[0031] MRPPQCLLHTPSLASPLLLLLLWLLGGGVGAEGREDAELLVTVRGGRLRGIR
LKTPGGPVSAFLGIPFAEPPMGPRRFLPPEPKQPWSGVVDATTFQSVCYQYVDTLYPGFE
GTEMWNP NRELSEDCLYLN VWTPYPRPTSPTPVLVWIYGGGFYSGASSLDVYDGRFLV
QAERTVLVSMNYRVGAFGFLALPGSREAPGNVGLLDQRLALQWVQENVAAFGGDPTS
VTLFGESAGAASVGMHLLSPPSRGLFHRAVLQSGAPNGPWATVGMGEARRRATQLAH
LVGCPPGGTGGNDTELVACLRTRPAQVLVNHEWHVLPQESVFRFSFVPVVDGDFLSDTP
EALINAGDFHGLQVLVG VVKDEGSYFLVYGAPGFSKDNESLISRAEFLAGVRVGVPQVS
DLAAEAVVLHYTDWLHPEDPARLREALSDVVG DHDNVVCPVAQLAGRLAAQG ARVYA

YVFEHRASTLSWPLWMGVPHGYEIEFIFGIPLDPSRNYTAEKIFAQRLMRYWANFART
 GDPNEPRDPKAPQWPPYTAGAQQYVSLDLRPLEVRRGLRAQACAFWNRFLPKLLSATD
 TLDEAERQWKAEFHRWSSYMVHWKNQFDHYSKQDRCSDLSGGGGSGGGGSGGGGSE
 FTMCYSHTTTSRAILTNCGENSCYRKSRRHPPKMVLGRGCGCPPGDDNLEVKCCTSPDK
 CNY(SEQ ID NO:3).

[0032] A nucleotide sequence encoding a chimeric AChE-Fas2 protein can be determined using the protocols and cloning details described herein. Nucleic acid molecules encoding chimeric proteins as described herein include variants, such as those that encode fragments, analogs and derivatives of a chimeric AChE-Fas2 protein or chimeric BuChE-containing protein. The nucleotide sequence of such variants can feature a deletion, addition, or substitution of one or more nucleotides. In other embodiments, variant chimeric AChE-Fas2 proteins or chimeric BuChE-containing proteins displaying substantial changes in structure can be generated by making nucleotide substitutions that cause less than conservative changes in the encoded polypeptide. Examples of such nucleotide substitutions are those that cause changes in (a) the structure of the polypeptide backbone; (b) the charge or hydrophobicity of the polypeptide; or (c) the bulk of an amino acid side chain. Those skilled in the art can create nucleic acid molecules that have minor variations in their nucleotide sequence, by, for example, standard nucleic acid mutagenesis techniques or by chemical synthesis.

[0033] A chimeric protein may also include a linker peptide interposed between the catalytically active AChE protein, for example, and the neuromuscular synapse targeting domain to facilitate folding of each protein independently, and/or a sequence tag (e.g., StrepTag®, His tag, HA tag, etc.) at the amino terminus for purification of the chimeric protein.

[0034] A pharmaceutical composition for treating or preventing organophosphate poisoning in an individual is also described herein. Such a pharmaceutical composition typically includes a chimeric protein as described herein, and a pharmaceutically acceptable carrier, the chimeric protein present in an amount effective for restoring AChE activity to many or most neuromuscular synapses in the individual after inactivation of endogenous AChE at neuromuscular synapses due to exposure to an organophosphate (e.g., organophosphate poisoning).

[0035] The chimeric proteins and pharmaceutical compositions described herein may be packaged as a kit. A typical kit for treating or preventing organophosphate poisoning in an

individual includes a therapeutically effective amount of a chimeric protein, a pharmaceutically acceptable excipient or carrier, instructions for use, appropriate packaging, and optionally, a means of administering. If desired, the kit also contains an effective amount of a therapeutic agent for ameliorating central nervous system (CNS) symptoms of organophosphate exposure (e.g., pyridostigmine, atropine and/or oximes such as 2-PAM). In some embodiments, the kit includes a sterile container which contains a therapeutic or prophylactic composition; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

[0036] Also described herein are methods of treating an individual exposed to an organophosphate (e.g., nerve gases such as sarin, soman, VX and tabun, or pesticides such as malathion, parathion, diazinon, fenthion, dichlorvos, chlorpyrifos, etc.). A typical method includes administering to the individual a chimeric protein or pharmaceutical composition as described herein in an amount effective to restore AChE activity to neuromuscular synapses in vivo after inactivation of endogenous AChE at neuromuscular synapses due to organophosphate exposure (poisoning). The individual is typically a human, and may be suffering from respiratory failure due to exposure to the organophosphate. Administration of the chimeric proteins and pharmaceutical composition restores functional neuromuscular transmission in the individual, due to the chimeric protein localizing to neuromuscular synapses in the individual. Typically, the chimeric protein is at a concentration of about 20 $\mu\text{g/kg}$ body weight to about 200 $\mu\text{g/kg}$ (e.g., 19 $\mu\text{g/kg}$, 20 $\mu\text{g/kg}$, 25 $\mu\text{g/kg}$, 50 $\mu\text{g/kg}$, 100 $\mu\text{g/kg}$, 150 $\mu\text{g/kg}$, 200 $\mu\text{g/kg}$, 205 $\mu\text{g/kg}$) in the pharmaceutical composition. The pharmaceutical composition may be administered via any suitable means. For example, the chimeric protein can be administered as soon as possible after exposure to organophosphates, usually as a single intravenous dose. The method can further include administering to the individual a therapeutic agent for ameliorating CNS symptoms of organophosphate exposure concomitant with or subsequent to administration of the chimeric proteins or pharmaceutical compositions described herein. Examples of such therapeutic agents/therapies include pyridostigmine, atropine and/or oximes such as 2-PAM (e.g., a combination of two or more of pyridostigmine, atropine and oxime). The chimeric proteins and pharmaceutical compositions described herein may be administered to a subject in need thereof in combination with one or more additional treatments as necessary.

Preventing Organophosphate Poisoning In a Subject

[0037] Although the chimeric proteins described above (e.g., chimeric AChE proteins, chimeric BuChE proteins, etc.) are generally for use in treating a subject who has been exposed to and/or is suffering from organophosphate poisoning, these or modified versions of these chimeric proteins could be used for preventing organophosphate poisoning in an individual, e.g., administering such modified proteins to an individual at risk of being exposed to organophosphate poisoning (before the individual is exposed to organophosphate poisoning). In one example of a chimeric AChE protein or BuChE protein for use in preventing organophosphate poisoning in an individual, the AChE protein or BuChE protein of the chimeric protein can be mutated such that it is resistant to organophosphates and pesticides. In another example, the AChE protein or BuChE protein of the chimeric protein can be mutated such that it is more easily reactivated. Any chimeric proteins as described herein used for prophylaxis would be resistant to the organophosphates (e.g., nerve agents) they are designed to protect an individual from.

Additional Chimeric Proteins

[0038] In addition to targeting AChE protein to NMJs, the linker and Fasciculin (e.g., Fas2) portions of the chimeric proteins described herein can be used to target other proteins to cholinergic synapses in the peripheral nervous system (PNS). For example, a growth factor or a trophic factor (e.g., a neurotrophin) or another molecule such as Agrin or a portion of a receptor such as LRP4 could be delivered to the NMJ. In general, any protein that when delivered to a NMJ can enhance or promote AChE protein expression, activity, NMJ formation/signaling, etc., can be delivered to NMJs using the compositions and methods described herein.

Administration of Compositions

[0039] The chimeric proteins provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and carriers. The formulations of the invention are useful for parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, or intraperitoneal administration. In some embodiments, a chimeric protein as

described herein is administered orally. In accordance with the invention, the preferred routes of administration are intravenous, intramuscular, and subcutaneous. The compositions can be formulated for administration to humans or other animals (e.g., invertebrates, vertebrates such as nonhuman primates, canines, felines, bovines, etc.) in therapeutically effective amounts (e.g., amounts which eliminate or reduce the patient's or subject's pathological condition) to provide therapy for the organophosphate poisoning described above. Exemplary formulations include aqueous solutions or lyophilized powders for dilution. The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical arts, for example, as described in Remington: The Science and Practice of Pharmacy (Remington: The Science & Practice of Pharmacy), Pharmaceutical Press; 21 edition (October 7, 2011). Formulations for parenteral administration may contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, and the like.

[0040] As indicated above, the chimeric proteins and pharmaceutical compositions described herein may be in a form suitable for sterile injection. To prepare such a composition, the suitable active therapeutic(s) are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution, and isotonic sodium chloride solution and dextrose solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl, ethyl or n-propyl p-hydroxybenzoate).

Effective Doses

[0041] The chimeric proteins and pharmaceutical compositions described herein are preferably administered to a subject or patient (e.g., rodent, human) in an effective amount, that is, an amount capable of producing a desirable result in a treated subject or patient (e.g., restoring AChE function and treating or preventing organophosphate poisoning in the subject or patient). Toxicity and therapeutic efficacy of the compositions utilized in methods of the invention can be determined by standard pharmaceutical procedures. As is well known in the medical and veterinary arts, dosage for any one animal depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, time and route of administration, general health, and other drugs being administered concurrently. The amount of

the therapeutic agent to be administered varies depending upon the manner of administration, the age and body weight of the subject, and with the clinical symptoms. Typical dose ranges would be from about 20 µg/kg to about 200 µg/kg of body weight per day. The chimeric proteins and pharmaceutical compositions described herein are useful for administration to humans suffering from organophosphate poisoning characterized by, for example, inactivated AChE as described above. When administered to a mammal (e.g., human, bovine, ovine, canine, feline, equine, rodent, etc.), these novel proteins effectively neutralize organophosphate poisoning by, for example, replacing inactive AChE molecules with catalytically active AChE molecules.

EXAMPLES

[0042] The present invention is further illustrated by the following specific examples. The examples are provided for illustration only and should not be construed as limiting the scope of the invention in any way.

Example 1 – Construction of the test GFP-Fas2 and the AChE-Fas2 chimeric proteins

[0043] In the experiments described herein, a mouse chimeric AChE-Fas2 protein was tested. The amino acid sequence of the murine AChE-Fas2 chimeric protein (the processed protein without the leader sequence) is:

EDWRHPQFGGPQLLVRVRGGQLRGIRLKAPGGPVSAFLGIPFAEPPVGSRRFMPPEPKRP
 WSGVLDATTFQNVQYQYVDTLYPGFEGTEMWNPNNRELSDCLYLNWVTPYPRPASPTP
 VLIWIYGGGFYSGAASLDVYDGRFLAQVEGAVLVSMNYRVGTFGFLALPGSREAPGNV
 GLLDQRLALQWVQENIAAFGGDPMSVTLFGESAGAASVGMHILSLPSRSLFHRAVLQSG
 TPNGPWATVSAGEARRRATLLARLVGCPPGGAGGNDTELIACLRTRPAQDLVDHEWHV
 LPQESIFRFSFVPVVDGDFLSDTPEALINTGDFQDLQVLVGCVKDEGSYFLVYGVPGFSK
 DNESLISRAQFLAGVRIGVPQASDLAAEAVVLHYTDWLHPEDPTHLRDAMSAVVGDNH
 VVCPVAQLAGRLAAQGARGVYAYIFEHRASLTWPLWMGVPHGYEIEFIFGLPLDPSLNY
 TTEERIFAQRLMKYWTNFARTGDPNDPRDSKSPQWPPYTTAAQQYVSLNLKPLEVRRG
 LRAQTCAFWNRFLPKLLSATCTSGGGGSGGGGSGGGGSEFTMCYSHTTTSTRAILTNCGE
 NSCYRKSRRHPPKMVLGRGCGCPPGDDNLEVKCCTSPDKCNY (SEQ ID NO:4). In this chimeric protein, the murine AChE has a deletion of the last 40 amino acids that form the dimerization domain, resulting in a monomeric enzyme of about 65 kDa. This AChE was also

modified by mutating three critical amino acids in the Fas2 binding site to produce a monomeric AChE enzyme that does not bind to Fas2 (i.e., especially its own Fas2 domain of the chimeric protein). This chimeric protein was demonstrated to bind specifically to the neuromuscular junction where it restored active AChE enzyme activity to the synapse after irreversible inactivation. A marker fusion protein, GFP-Fas2, was also generated and tested as described in more detail in Example 2.

Example 2 - Replacement of inactive acetylcholinesterase molecules at the neuromuscular synapse in vivo

[0044] A novel approach was developed for restoring functional neuromuscular transmission following irreversible AChE inhibition using a chimeric AChE protein molecule that can home in to sites of nerve muscle contact in vivo (the NMJ) and replace the inactivated enzyme molecules. This approach is capable of rescuing mice exposed to lethal doses of organophosphates when administered shortly after exposure. This novel approach has the potential to protect humans from pesticide and nerve agent exposure as well as rescue them once exposed.

[0045] A means of targeting catalytically active AChE, or other protein molecules, specifically to the NMJ was developed. In these experiments, Fas2 was used. To determine the feasibility of this approach, we first constructed a chimeric fluorescent reporter protein consisting of GFP linked via a 15 amino acid linker peptide to the 61 amino acid snake toxin Fas2 (Fig. 1). A leader sequence was included to specify secretion, and a 6xHis tag was added at the amino terminus to facilitate purification. The construct was transfected into HEK293 cells and a stable cell line established by selection and cloning. The chimeric protein was subsequently purified from defined medium or cell extracts from these cells using a nickel column and confirmed by SDS-PAGE and Western blotting. Initial tests showed that the chimeric GFP-Fas2 protein bound to the neuromuscular junctions on frozen sections of mouse skeletal muscle where it co-localized with the nicotinic acetylcholine receptor.

[0046] To determine whether the GFP-Fas2 protein could bind to the neuromuscular junction in vivo, mice were injected with either 2-3 μ g Alexa-488 conjugated Fas2 or the chimeric GFP-Fas2 protein and sacrificed 30 minutes later. The EDL and gastrocnemius muscles were dissected from the hind limbs and labeled with Alexa-555 α Btx to visualize the nicotinic

receptors and the fibers teased apart and mounted on microscope slides. Both the Alexa 488-Fas2 and the 27 kDa monomeric GFP-Fas2 protein localized to the neuromuscular synapse following intravenous injection where they bound to the existing AChE molecules associated with the synaptic basal lamina. These results indicate the feasibility of using the snake toxin to target proteins to the neuromuscular synapse.

[0047] The extracellular mammalian AChE normally exists as higher order oligomeric forms such as membrane-bound tetramers at CNS synapses or the neuromuscular junction form consisting of three tetramers covalently linked to a three stranded collagen-like tail. These forms are too large to diffuse into the synaptic space at the neuromuscular junction. On the other hand, antibodies at around 150 kDa are still capable of diffusing into the neuromuscular synapse as this is the basis for several autoimmune diseases such as myasthenia gravis and Lambert-Eaton Myasthenic Syndrome. To construct a mouse AChE chimera that could still penetrate the synaptic space, we first truncated the enzyme by deleting the last 40 amino acids that form the dimerization domain as described by Bourne et al (Cell vol. 83:503-512, 1995). This results in a monomeric enzyme of about 70 kDa. We then mutated the three critical amino acids in the Fas2 binding site as described by Bourne et al. (Cell vol. 83:503-512, 1995) to produce a monomeric enzyme that does not bind to fasciculin. The addition of a 15 amino acid linker (SGGGGSGGGGSGGGG (SEQ ID NO: 5)) followed by Fas2 at the carboxyl terminus, and the 8 amino acid streptavidin binding sequence at the amino terminus following the cleavable leader sequence completed the construct. The advantage of inserting the StrepTag® sequence at the amino terminus is that the tag can be used for both purifying the chimeric protein by affinity chromatography as well as localizing the molecule using fluorescent StreptTactin, a genetically engineered form of streptavidin that has a higher affinity for the 8 amino acid StrepTag® sequence (IBA Biotagnology, Gottingen, Germany). The construct was confirmed by sequencing and used to establish stable HEK293 cell lines secreting the chimeric protein for purification from conditioned medium.

[0048] To test the chimeric mAChE-Fas2 protein for its ability to localize to the NMJ in vitro, frozen sections of mouse skeletal muscle mounted on microscope slides were pre-treated with Phosphate-buffered saline (PBS), or PBS plus 10^{-4} M DFP to irreversibly inhibit AChE at the NMJ, followed by incubation with either buffer alone containing bovine serum albumin (BSA) or buffer with the recombinant chimeric AChE-Fas2 protein. The chimeric AChE-Fas2

protein bound specifically to the NMJ where it restored active enzyme activity to the synapse after irreversible inactivation. To determine whether the chimeric enzyme would localize to the neuromuscular synapse in vivo, mice were injected with either saline alone or containing 2 μ g AChE-Fas2. The mice were sacrificed 60 minutes later, the EDL and gastrocnemius muscles dissected, labeled with Alexa-555 α Btx to visualize the nicotinic receptors and Oyster-556 StrepTactin to visualize the chimeric AChE. The AChE-Fas2 protein was able to diffuse and attach to existing AChE molecules at the neuromuscular synapse in vivo.

[0049] Ultimately, the purpose of developing the targetable AChE molecule is to restore function to neuromuscular synapses inactivated by irreversible organophosphate inhibitors. To test this, we used the commercially available organophosphate AChE inhibitor DFP as a surrogate for nerve agents and pesticides. Injection of mice with 2 x LD₅₀ DFP followed by an intravenous injection of saline alone 5 minutes later resulted in rapid death. However injection of 5 μ g of the chimeric AChE-Fas2 protein 5 minutes after injection of DFP rescued the majority of the animals. To confirm that the chimeric protein was restoring function specifically to the NMJ, the muscles of the injected mice were removed and labeled with Alexa 488- α Btx to visualize the nicotinic receptors together with the histochemical visualization of catalytically active AChE using the Karnovsky and Roots protocol. Injection of the chimeric AChE-Fas2 protein completely restored active enzyme at the neuromuscular synapse in those mice that survived.

[0050] The present studies demonstrate the feasibility of using a chimeric AChE-Fas2 protein to restore function to an otherwise lethally damaged neuromuscular synapse. Since the proximal cause of death following exposure to organophosphates is respiratory failure, treatment of victims with the chimeric AChE protein would be sufficient to allow recovery from the initial phase of poisoning and follow up with the more conventional treatments to ameliorate the CNS symptoms of organophosphate exposure. Although fasciculin itself is an inhibitor of AChE, the endogenous enzyme molecules to which it is attaching would already be inactivated under its conditions of use, hence that would not be a concern. The effective dose of the chimeric protein is generally less than 200 μ g/kg; this dose is sufficient to rescue the majority of the animals. Moreover, the amount of this protein needed to restore function to the neuromuscular synapse is much less than the amount injected because a large fraction will be bound by the cell surface AChE molecules on erythrocytes.

Other Embodiments

[0051] Any improvement may be made in part or all of the compositions, kits, and method steps. All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended to illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. Any statement herein as to the nature or benefits of the invention or of the preferred embodiments is not intended to be limiting, and the appended claims should not be deemed to be limited by such statements. More generally, no language in the specification should be construed as indicating any non-claimed element as being essential to the practice of the invention. This invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contraindicated by context.

What is claimed is:

1. A chimeric protein comprising: i) a catalytically active acetylcholinesterase (AChE) protein or a catalytically active butyrylcholinesterase (BuChE) protein, and ii) a neuromuscular synapse targeting domain, wherein the chimeric protein is able to bind to a neuromuscular synapse and restore cholinesterase activity to the neuromuscular synapse in vivo after inactivation of endogenous AChE at the neuromuscular synapse.
2. The chimeric protein of claim 1, wherein the chimeric protein comprises the amino acid sequence set forth in SEQ ID NO:3.
3. The chimeric protein of claim 1, wherein the neuromuscular synapse targeting domain is Fasciculin-2 (Fas2).
4. The chimeric protein of claim 3, wherein the Fas2 comprises a deletion of the last 40 amino acids of wild-type Fas2 and has the sequence set forth in SEQ ID NO:2.
5. The chimeric protein of claim 1, wherein the catalytically active AChE protein lacks a dimerization domain and comprises at least one mutation, deletion, or insertion which prevents binding of the catalytically active AChE protein to Fas2.
6. The chimeric protein of claim 5, wherein the catalytically active AChE enzyme has the amino acid sequence set forth in SEQ ID NO:1.
7. The chimeric protein of claim 1, further comprising a linker peptide interposed between the catalytically active AChE protein and the neuromuscular synapse targeting domain.
8. The chimeric protein of claim 1, further comprising a sequence tag for purification.
9. A pharmaceutical composition for treating organophosphate poisoning in an individual, the composition comprising the chimeric protein of claim 1 and a pharmaceutically acceptable carrier, the chimeric protein in an amount effective for restoring cholinesterase

- activity to a neuromuscular synapse in the individual after inactivation of endogenous AChE at the neuromuscular synapse from organophosphate poisoning.
10. The pharmaceutical composition of claim 9, wherein the chimeric protein comprises the amino acid sequence set forth in SEQ ID NO:3.
 11. The pharmaceutical composition of claim 9, wherein the neuromuscular synapse targeting domain is Fas2.
 12. The pharmaceutical composition of claim 9, wherein the catalytically active AChE protein lacks a dimerization domain and comprises at least one or more mutations, insertions or deletions which prevent binding of the catalytically active AChE protein to Fas2.
 13. The pharmaceutical composition of claim 12, wherein the catalytically active AChE protein has the amino acid sequence set forth in SEQ ID NO:1
 14. The pharmaceutical composition of claim 9, wherein the chimeric protein further comprises a linker peptide interposed between the catalytically active AChE protein and the neuromuscular synapse targeting domain.
 15. The pharmaceutical composition of claim 9, wherein the chimeric protein further comprises a sequence tag for purification.
 16. A method of treating an individual exposed to an organophosphate, the method comprising administering to the individual the pharmaceutical composition of claim 9 in an amount effective to restore cholinesterase activity to the neuromuscular synapse in vivo after inactivation of endogenous AChE at the neuromuscular synapse.
 17. The method of claim 16, wherein the individual is a human suffering from respiratory failure due to exposure to the organophosphate.

18. The method of claim 16, wherein administration of the pharmaceutical composition restores functional neuromuscular transmission in the individual.
19. The method of claim 16, wherein the organophosphate is one selected from the group consisting of: sarin, soman, VX and tabun.
20. The method of claim 16, wherein the chimeric protein localizes to neuromuscular synapses after administration of the pharmaceutical composition to the individual.
21. The method of claim 16, wherein the chimeric protein is at a concentration of about 20 μ g to about 200 μ g per kilogram body weight.
22. The method of claim 21, wherein the pharmaceutical composition is administered intravenously once after exposure with additional treatments as necessary.
23. The method of claim 21, further comprising administering to the individual a therapeutic agent for ameliorating central nervous system symptoms of organophosphate exposure concomitant with or subsequent to administration of the pharmaceutical composition, wherein the therapeutic agent is atropine, pralidoxime chloride (2-PAM), or a combination thereof.
24. The method of claim 16, wherein the organophosphate is a pesticide selected from the group consisting of: malathion, parathion, diazinon, fenthion, dichlorvos, and chlorpyrifos.
25. A method of preventing organophosphate poisoning in an individual at risk of being exposed to an organophosphate pesticide or nerve gas, the method comprising:
administering to the individual a pharmaceutical composition comprising a chimeric protein consisting essentially of: a catalytically active AChE protein or a catalytically active BuChE protein; and a neuromuscular synapse targeting domain, wherein the AChE

protein or BuChE protein is mutated such that it is resistant to organophosphates and pesticides or such that it can be reactivated, and wherein the chimeric protein is able to bind to a neuromuscular synapse and restore cholinesterase activity to the neuromuscular synapse in vivo after inactivation of endogenous AChE by organophosphates.

26. The method of claim 25, wherein administration of the pharmaceutical composition prevents inactivation of AChE at neuromuscular synapses in the individual during or subsequent to exposure of the individual to an organophosphate pesticide or nerve gas.
27. The method of claim 25, wherein the organophosphate is one selected from the group consisting of: sarin, soman, VX and tabun.
28. The method of claim 25, wherein the chimeric protein localizes to neuromuscular synapses after administration of the pharmaceutical composition to the individual.
29. The method of claim 25, wherein the chimeric protein is at a concentration of about 20 μ g to about 200 μ g per kilogram body weight.
30. The method of claim 29, wherein the pharmaceutical composition is administered once after exposure with additional treatments as necessary.
31. The method of claim 25, further comprising administering to the individual a therapeutic agent for ameliorating central nervous system symptoms of organophosphate exposure concomitant with or subsequent to administration of the pharmaceutical composition.
32. The method of claim 25, wherein the organophosphate is a pesticide selected from the group consisting of: malathion, parathion, diazinon, fenthion, dichlorvos, and chlorpyrifos.
33. A kit for treating or preventing organophosphate poisoning in an individual comprising a therapeutically effective amount of a chimeric protein according to claim 1, a

pharmaceutically acceptable excipient or carrier, instructions for use, and packaging.

1/1

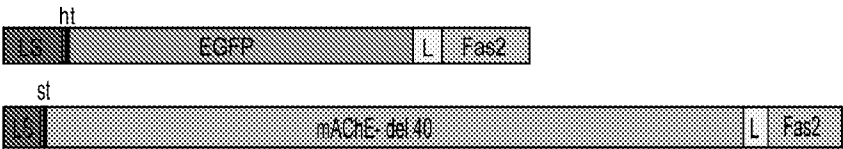


FIG. 1

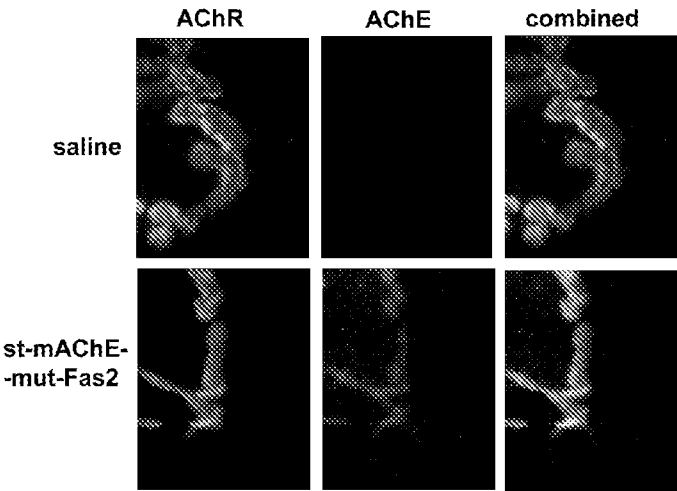


FIG. 2

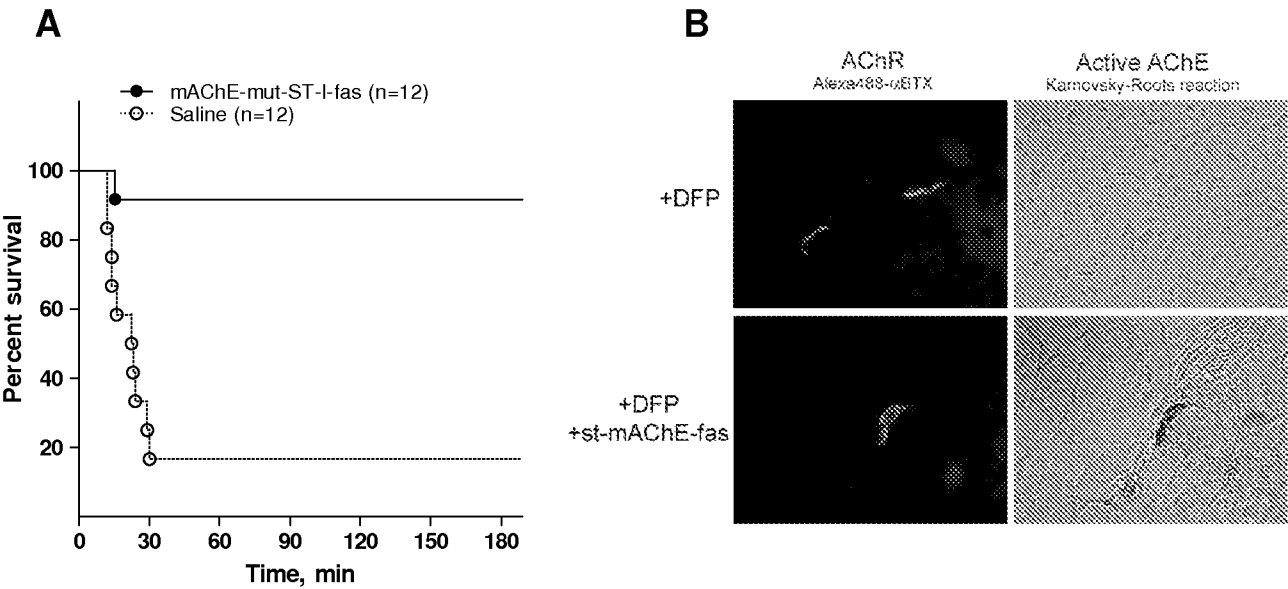


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/064793

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61P 21/00 (2014.01)

USPC - 435/20

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 31/15, 31/46, 38/16, 38/17; A61P 39/02, 25/00, 25/14; C07K 14/00, 14/705, 19/00; C12N 9/16, 9/64, 15/09, 15/11, 15/12, 15/62, 15/63 (2014.01)

USPC - 435/6.16, 6.18, 7.1, 7.92, 20, 375; 514/1.1, 1.5, 18.1, 21.3; 530/324, 387.3; 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, 2039/505; C07K 2319/00; C12Q 1/46, 1/6883; G01N 33/564 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/076024 A2 (ROTUNDO et al) 20 July 2006 (20.07.2006) entire document	1, 3, 8, 9, 11, 15-24, 33
Y		4-7, 12-14, 25-32
Y	VILJOEN et al. "Snake Venom Toxins: THE PURIFICATION AND AMINO ACID SEQUENCE OF TOXIN FvII FROM DENDROASPIS ANGUSTICEPS VENOM." The Journal of Biological Chemistry. Vol. 218, No. 14. Pages 4915-4919. 25 July 1973 (25.07.1973) entire document	4
Y	RADIC et al. "Site of Fasciculin Interaction with Acetylcholinesterase" The Journal of Biological Chemistry Vol. 269, No. 15. Pages 11233-11239. 15 April 1994 (15.04.1994) entire document	5, 6, 12, 13, 25-32
Y	WO 1995/23158 A1 (SOREQ'et al) 31 August 1995 (31.08.1995) entire document	6, 13
Y	US 2011/0223188 A1 (LANGERMANN) 15 September 2011 (28.09.2011) entire document	7, 14

☐ Further documents are listed in the continuation of Box C.

* 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

13 January 2014

Date of mailing of the international search report

29 JAN 2014

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/064793

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 filed or furnished:

a. (means)



on paper



in electronic form

b. (time)



in the international application as filed



together with the international application in electronic form



subsequently to this Authority for the purposes of search

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 in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Specifically, SEQ ID NOs: 1-3 were searched.