

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 April 2009 (16.04.2009)

PCT

(10) International Publication Number
WO 2009/047762 A1

(51) International Patent Classification:
C07K 7/08 (2006.01) A61K 38/10 (2006.01)

(21) International Application Number:
PCT/IL2008/001331

(22) International Filing Date: 7 October 2008 (07.10.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/978,605 9 October 2007 (09.10.2007) US

(71) Applicant (for all designated States except US): **YEDA RESEARCH AND DEVELOPMENT CO. LTD** [IL/IL]; at the Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KATCHALSKI-KATZIR, Ephraim** [IL/IL]; 19 Neve Metz, the Weizmann Institute of Science, 76100 Rehovot (IL). **BALASS, Moshe** [IL/IL]; 17/3 Hzaith Street, 71947 Beit Aryeh - Ofarim (IL).

(74) Agent: **BEN-AMI & ASSOCIATES**; P.O. Box 94, 76100 Rehovot (IL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau



WO 2009/047762 A1

(54) Title: COMPOSITIONS AND PEPTIDES FOR TREATMENT OF ENVENOMATION BY PLA₂ CONTAINING VENOMS LIKE BUNGARUS MULTICINCT VENOM

(57) Abstract: The present invention provides pharmaceutical compositions and peptides for treatment of *Bungarus multicinctus* envenomation, more specifically, pharmaceutical compositions comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of phospholipase A₂ (PLA₂), and a peptide capable of specifically binding to α -bungarotoxin (α -BTX) and inhibiting the antagonistic interaction of the α -BTX with muscle nicotinic acetylcholine receptor

COMPOSITIONS AND PEPTIDES FOR TREATMENT OF ENVENOMATION BY PLA2 CONTAINING VENOMS LIKE BUNGARUS MULTICINCT VENOM

FIELD OF THE INVENTION

5 The present invention relates to compositions for treatment of *Bungarus multicinctus* envenomation, more specifically, to compositions comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of phospholipase A₂, and a peptide capable of specifically binding to α -bungarotoxin (α -BTX) and inhibiting the antagonistic interaction of the α -BTX with muscle
10 nicotinic acetylcholine receptor.

BACKGROUND OF THE INVENTION

 The problem of snake envenomation is a major cause of mortality and morbidity in many tropical and subtropical countries. For example, the annually worldwide incidents of snakebites in 1991 were 5,000,000 with approximately
15 40,000 deaths (Galan *et al.*, 2004).

Bungarus is a genus of venomous elapid snakes found in India and South-East Asia, commonly referred to as kraits, including 12 species and 5 subspecies. *Bungarus* species have neurotoxic venom many times more potent than cobra venom. A bite from a krait is very serious and causes respiratory failure in the
20 victim. Before effective antivenom was developed, there was a 75 percent mortality rate among victims. According to some sources, krait bites have a 50% mortality rate even with antivenom, suggesting that the krait is one of the most dangerous snakes in the world.

 The venom of the *Bungarus multicinctus* (Formosan krait) comprises two
25 major proteins, α - and β -bungarotoxin (BTX); both are extremely potent neurotoxins. These two toxins are targeted to the neuromuscular junction of the peripheral muscles and interact with the post- and pre-synaptic membranes, respectively, as schematically shown in **Fig. 1**. In particular, α -BTX is a monomer

that binds with extremely high affinity to the ligand-binding site of the nicotinic acetylcholine receptor (nAChR), a ligand-gated ion channel that is activated upon binding to acetylcholine, and consequently blocks the opening of the receptor ion channel, which is essential for muscles contraction. β -BTX is composed of two
5 polypeptide chains linked by a single disulphide bond: a phospholipase A₂ (PLA₂) subunit and a targeting subunit, which binds certain types of voltage-gated potassium channels at the presynaptic membrane. Binding of β -BTX to the potassium channels on the presynaptic site facilitates membrane degradation by the catalytic activity of the PLA₂ subunit, which finally results in disruption of
10 acetylcholine release to the synapse. The post-synaptic α -BTX and the pre-synaptic β -BTX work in concert to cause peripheral muscle paralysis, which is the ultimate cause of death incurred by the *B. multicinctus* venom.

The inventors of the present invention have previously reported the successful generation of the library-derived synthetic peptide MRYYESSLKSYDP
15 (SEQ ID NO: 13) that binds with moderate affinity (10^{-6} M) to α -BTX (Balass *et al.*, 1997). Based on the NMR structural data (Scherf *et al.*, 1997), the affinity of the library-peptide was improved by disulphide cyclization, and the affinity of the obtained disulphide-constrained cyclic peptide, CRYYYESSLKSYCD (SEQ ID NO:
20 15) to α -BTX, was found to be higher by two orders of magnitude than that of the linear library peptide. The cyclic peptide, herein designated "pep α ", was found to block the antagonistic interaction of α -BTX with the muscle nicotinic acetylcholine receptor, thus to confer full protection against α -BTX lethality in mice, even if administered one hour after toxin injection (Balass *et al.*, 2001).

Antivenom antibodies (antivenin), produced in the serum of horses and
25 sheep, are currently the only available agents for the treatment of envenomated humans and animals. However, the treatment of envenomation with such antivenin elicits an immune response and may cause anaphylactic shock. There is thus a widely recognized need for, and it would be highly advantageous to have, new approaches for treating human envenomation.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a pharmaceutical composition for treatment of *Bungarus multicinctus* envenomation, comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of phospholipase A₂ (PLA₂), and a peptide capable of specifically binding to α -bungarotoxin (α -BTX) and inhibiting the antagonistic interaction of the α -BTX with muscle nicotinic acetylcholine receptor, and a pharmaceutically acceptable carrier.

In another aspect, the present invention relates to a peptide of 12 to 25 amino acid residues, capable of specifically binding to and inhibiting the catalytic activity of PLA₂, selected from:

- (i) a linear peptide comprising a consensus sequence:
Trp-Asp(Glu)-X₁-X₂-X₃-X₄-X₅ [SEQ ID NO: 1]
wherein X₁ is absent or is Met or Lys; X₂ is Leu or Cys; X₃ is Gln, Ala, Tyr or Ser; X₄ is absent or is Gln, Trp or Phe; and X₅ is absent or is Leu, wherein at least one of X₂ and X₅, if present, is Leu;
- (ii) a cyclic derivative of (i);
- (iii) a D-stereomer of (i) or (ii);
- (iv) a dual peptide consisting of two of the same or different peptides (i) to (iii), wherein the peptides are covalently linked to one another directly or through a spacer;
- (v) a multimer comprising a number of the same or different peptides of (i) to (iii);
- (vi) an amide of the C-terminal of a peptide (i) to (iv);
- (vii) an N-acyl derivative of a peptide (i) to (v); or
- (viii) a pharmaceutically acceptable salt thereof.

In a further aspect, the present invention relates to a pharmaceutical composition comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ as defined hereinabove, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

In yet another aspect, the present invention provides a method for treatment of *B. multicinctus* envenomation, comprising administering to an individual or animal following a *B. multicinctus* bite a pharmaceutical composition for treatment of *B. multicinctus* envenomation as defined above, in amounts sufficient to treat the
5 *B. multicinctus* envenomation.

In still another aspect, the present invention provides a method for inhibiting the catalytic activity of the PLA₂ component of a venom of a venomous animal, following envenomation of an individual or animal by said venomous animal, comprising administering to said individual or animal a pharmaceutical composition
10 comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ as defined hereinabove, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, in an amount sufficient to inhibit the catalytic activity of the PLA₂.

BRIEF DESCRIPTION OF THE FIGURES

15 **Fig. 1** shows a schematic representation of the site of action of α - and β -bungarotoxin (BTX) within the cholinergic synapse. α -BTX interacts with the nicotinic acetylcholine receptor (AChR) in the postsynaptic membrane. β -BTX is composed of two subunits, i.e., a PLA₂ subunit and a targeting subunit (Targ.) that interacts with certain types of voltage-gated potassium channels (K⁺-channel) at the
20 presynaptic membrane.

Fig. 2 shows 15% SDS-PAGE gel analysis of the *B. multicinctus* venom and its major neurotoxins without reducing agent. Whole venom (V), α -BTX (α) and β -BTX (β) are indicated by arrows.

25 **Fig. 3** shows the binding of β -BTX and its separated PLA₂ and targeting subunits to pep- β . Binding was assayed on an ELISA plate coated with 1 μ g/ml of pep- β or with unrelated control peptide (CLKWNPDDYGGVKKIC). Aliquots (1 μ g/ml) of each of the biotin-labeled β -BTX, the PLA₂ subunit and the targeting subunit were added to the plate coated with the above peptides, and their binding was monitored by an ELISA reader at 405 nm.

Fig. 4 shows TLC analysis of the inhibition of PLA₂ activity of β-BTX at increasing concentrations of pep-β. The enzymatic reaction was carried out in a total volume of 25 μl, containing 1.6 mg/ml phosphatidylcholine (10 μl), 4 μg/ml β-BTX (5 μl), increasing concentrations of pep-β (5 μl) as indicated, and buffer (5 μl), as described in Materials and Methods. The arrow indicates the IC₅₀ value of the peptide (13 μM). PLA₂ activity was determined by visualization of the iodine stained, residual non-hydrolyzed substrate. S represents the amount of the substrate in the control, wherein no β-BTX was added to the reaction mixture.

Figs. 5A-5D show 3-week old male Balb/c mice 0-60 (**5A**), 60-90 (**5B**), 90-110 (**5C**) and 110-120 (**5D**) minutes following *B.multicinctus* venom (2.5 μg/mouse) injection, indicating four broad phases of visible symptoms, i.e., active, fatigued, paralyzed and dead, respectively.

Fig. 6 shows the protein profile of *Walterinnesia aegyptia*, *V. palaestinae*, *Pseudocerastes persicus fieldi* and *Vipera ammodytes* venoms, as detected by SDS PAGE gel analysis, pointing to the specific proteins having PLA₂ activity.

Fig. 7 shows TLC analysis of the inhibition of the PLA₂ activity of the venom of *P. fieldi* and *V. ammodytes* induced by pep-β, based on both the product (P) accumulation and the substrate (S) disappearance. Venom concentration: 1 μg/ml for *P. fieldi* and *W. aegyptia*, and 5 μg/ml for *V. ammodytes* and *V. palaestinae*. 1=without pep-β; 2=with pep-β (200 μg/ml); 3=with pep-α (200 μg/ml) as a control peptide.

Fig. 8 shows the dose-dependent inhibition of the PLA₂ activity of *P. fieldi*, *V. ammodytes* and *B. multicinctus* venom by pep-β based on both the product (P) accumulation and the substrate (S) disappearance. Venom concentration: 2.5 μg/ml for *B. multicinctus*, *V. ammodytes* and *V. palaestinae*, and 0.5 μg/ml for *P. fieldi*. Incubation time: 1 h at RT, 1 h at 37°C and 6 h at RT. IC₅₀ of 25, 70 and 140 μg/ml was estimated for the venom of *P. fieldi*, *V. ammodytes* and *B. multicinctus*, respectively.

Fig. 9 shows TLC analysis of the inhibition of PLA₂ activity of purified PLA₂ toxins of *P. fieldi* and *V. ammodytes* (0.2 mg/ml) induced by pep-β (1 mg/ml),

based on both the product (P) accumulation and the substrate (S) disappearance. Incubation time: 1 h at RT and 7 h at 37°C. *P. fieldi* PLA₂-1, *P. fieldi* PLA₂-2, *V. Ammodytes* PLA₂-1 and *V. Ammodytes* PLA₂-2 refer to PLA₂-1 and PLA₂-2 isoforms of *P. fieldi* and *V. Ammodytes*, respectively, represented by bands 1 and 2 of these two species in Fig. 6.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new peptides-based method for neutralizing the lethality of the venom of *B. multicinctus*. This method is based on a concurrently neutralization of both α - and β -BTX, the two major neurotoxins in the venom of *B. multicinctus*, thus, antagonizing the lethal effect of the whole venom.

In order to develop a specific peptide inhibitor, exclusively against the lethal effect of β -BTX, a 15-mer phage-peptide library has been screened by β -BTX, and the peptides of SEQ ID NOs: 7-10 were found to bind to β -BTX with low to moderate affinity.

15 Val-Ser-Thr-Trp-Glu-Met-Leu-Gln-Gln-Leu-Asn-Thr-Thr-Arg-Met [SEQ ID NO: 7]
 Gly-Leu-Trp-Arg-Gly-Phe-Trp-Asp-Leu-Ala-Trp-Leu-Pro-Ala-Asp [SEQ ID NO: 8]
 His-Phe-Asp-Tyr-Pro-Ala-Phe-Trp-Tyr-Trp-Glu-Lys-Leu-Tyr [SEQ ID NO: 9]
 Gly-Trp-Ser-Ala-Trp-Asp-Cys-Ser-Phe-Leu-Ser-Cys-Ala-Pro-Ser [SEQ ID NO: 10]

20 The peptide of SEQ ID NO: 7, having the highest affinity to β -BTX among these peptides, was selected for a further process. As known from the scientific literature, linear peptides can assume an indefinite number of different conformations, of which only few may be able to bind a target receptor, whereas constraint of the conformational freedom by a cysteine replacement approach to modify the linear library-selected peptide to a cyclic form leads to a decrease in the entropy of peptides, and may thus result in the generation of higher-affinity derivatives. Such cyclic derivatives may further exhibit an increased stability as compared with their linear counterpart (Giebel *et al.*, 1995; Venkatesh, 2000). Thus, in order to improve the affinity of the peptide of SEQ ID NO: 7 to β -BTX, it was elongated on both sides and constrained into a cyclic structure by oxidation of two

cysteine residues, forming the peptide of SEQ ID NO: 11, herein designated "pep- β ".

Cys-Ala-Glu-Val-Ser-Thr-Trp-Glu-Met-Leu-Gln-Gln-Leu-Asn-
 Thr-Thr-Arg-Met-Pro-Pro-Cys [SEQ ID NO: 11]

5 As shown in Example 1 hereinafter, the affinity of pep- β to β -BTX (4×10^{-9} M) is higher by about three orders of magnitude than that of the linear library-peptide. Furthermore, this peptide binds specifically to the PLA₂ subunit of β -BTX but not to the targeting subunit after reduction of the toxin and separation of the subunits, and neutralizes the PLA₂ catalytic activity of the toxin at micromolar
 10 concentration.

As shown in Example 2, when pep- β is administered to mice it confers full protection from the lethality of β -BTX (2.5 μ g/mouse; subcutaneous injection), even if given up to one hour after the toxin injection. However, separate administration of each of pep- α , which blocks the antagonistic interaction of α -BTX
 15 with the muscle nicotinic acetylcholine receptor and confer full protection against α -BTX lethality, or pep- β , does not rescue mice injected with whole venom of *B. multicinctus* unless it is given concomitantly with the venom. The effect of either α - or β -BTX by itself is probably not sufficient to antagonize the damage incurred by its counterpart, and these results are consistent with those obtained by Crosland
 20 (1991), which used anti-protozoal compounds such as chloroquine and quinacrine to inhibit the PLA₂ activity of the β -BTX in the venom. As described by Crosland (1989a and 1989b), both anti-protozoal compounds were effective in preventing mouse lethality only when injected into mice concomitantly with either whole venom of *B. multicinctus* or β -BTX.

25 The present inventors postulated that a mixture of pep- α and pep- β , each capable of neutralizing one of the two major toxins of the *B. multicinctus* (α - and β -BTX, respectively), would be able to neutralize the *B. multicinctus* venom, and indeed, as shown in Example 3, full protection from the venom's lethality was obtained when a mixture of both peptides (500 μ g each/mouse) was administered
 30 (intraperitoneal, IP, injection) twice, the first dose 10 min and the second 50 min

after venom injection. These results indicate that by administering effective amounts of both pep- α and pep- β , thus concurrently neutralizing both α - and β -BTX, full protection from the venom of *B. multicinctus* is obtained, even when administered after the snakebite. Based on the aforesaid, it is concluded that a mixture of pep- α and pep- β can be used for treatment of human or domesticated animals after being bitten by *B. multicinctus*.

Thus, in one aspect the present invention relates to a pharmaceutical composition for treatment of *B. multicinctus* envenomation, comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂, and a peptide capable of specifically binding to α -BTX and inhibiting the antagonistic interaction of the α -BTX with muscle nicotinic acetylcholine receptor, and a pharmaceutically acceptable carrier.

In view of the sequences of the peptides of SEQ ID NOs: 7-11, the peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂, according to the present invention, may be a peptide of 12 to 25 amino acid residues as defined above.

In one embodiment, the peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ is a linear peptide comprising a consensus sequence of SEQ ID NO:1, wherein X₁ is absent or is Met or Lys; X₂ is Leu or Cys; X₃ is Gln, Ala, Tyr or Ser; X₄ is absent or is Gln, Trp or Phe; and X₅ is absent or is Leu, wherein at least one of X₂ and X₅, if present, is Leu.

In certain preferred embodiment, the linear peptide comprises the consensus sequence of SEQ ID NO: 2, wherein both X₂ and X₅ are Leu, more preferably, the consensus sequence of SEQ ID NO: 3, wherein X₁ is Met, and both X₃ and X₄ are Gln; or the consensus sequence of SEQ ID NO: 4, wherein X₁ is absent, X₃ is Ala and X₄ is Trp.

Trp-Asp(Glu)-X₁-Leu-X₃-X₄-Leu [SEQ ID NO: 2]

Trp-Glu-Met-Leu-Gln-Gln-Leu [SEQ ID NO: 3]

Trp-Asp-Leu-Ala-Trp-Leu [SEQ ID NO: 4]

In other preferred embodiments, the linear peptide comprises the consensus sequence of SEQ ID NO: 5, wherein X₁ is Lys, X₂ is Leu, X₃ is Tyr, and both X₄ and X₅ are absent; or the consensus sequence of SEQ ID NO: 6, wherein X₁ is absent, X₂ is Cys, X₃ is Ser, X₄ is Phe and X₅ is Leu.

- 5 Trp-Glu-Lys-Leu-Tyr [SEQ ID NO:5]
 Trp-Asp-Cys-Ser-Phe-Leu [SEQ ID NO:6]

In more preferred embodiments, the linear peptide is selected from the peptides of SEQ ID NOs: 7, 8, 9 and 10, as defined above, comprising the consensus sequences of SEQ ID NOs: 3, 4, 5 and 6, respectively.

- 10 In another embodiment, the peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ is a cyclic peptide comprising a consensus sequence of SEQ ID NO: 1, preferably the consensus sequence of SEQ ID NOs: 3-6, as defined above, more preferably the consensus sequence of SEQ ID NO: 3.

- 15 In a most preferred embodiment, the peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ is the cyclic peptide of SEQ ID NO: 11, as defined above.

- 20 In a further embodiment, the peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ is a D-stereomer of the linear or the cyclic peptides defined above. The D-stereomer according to the present invention may be any derivative of either the linear or the cyclic peptide defined above, obtained by replacement of one or more natural amino acid residues by the corresponding D-stereomer amino acid residue.

- 25 In still another embodiment, the peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ is a dual peptide consisting of two of the same or different peptides, each comprising the consensus sequence of SEQ ID NO:1 as defined above, a cyclic derivative thereof, or a D-stereomer thereof, wherein said peptides are covalently linked to one another directly or through a spacer. According to the present invention, the spacer may be a small amino acid residue such as a serine or alanine residue, or a C₁-C₄ alkylene.

In yet another embodiment, the peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ is a multimer comprising a plurality of the same or different peptides, each comprising the consensus sequence of SEQ ID NO:1 as defined above, a cyclic derivative thereof, or a D-stereomer thereof.

5 The peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂, as defined hereinabove, may further be amidated in its C-terminus or acylated, e.g., in its N-terminus. Examples of such acyl derivatives correspond to the formula R-X-CO-, wherein R is a substituted or unsubstituted hydrocarbyl, preferably alkyl or aryl, and X is a covalent bond, O, NH or NHCO. Examples of
10 acyl radicals are octanoyl, monomethoxysuccinyl, acetylaminoacetyl, adamantyl-NH-CO-, and more preferably, carbobenzoxy (i.e. benzyl-O-CO-), naphthyl-NH-CO-, and Fmoc (i.e. fluorenylmethyl-O-CO-).

The peptide capable of specifically binding to α -BTX and inhibiting the antagonistic interaction of the α -BTX with muscle nicotinic acetylcholine receptor
15 may be, for example, any one of the peptides previously disclosed by the present inventors (Balass *et al.*, 1997), having such properties, namely the peptides of SEQ ID NOs: 12-19, more preferably the peptide of SEQ ID NO: 13, most preferably the cyclic peptide of SEQ ID NO: 15, herein designated "pep- α ", that was found to confer full protection against α -BTX lethality in mice, even if given one hour after
20 toxin injection.

Pro-Pro-Pro-Ile-Phe-Arg-Tyr-Tyr-Glu-Tyr-Trp-Pro-Thr-Ser-Tyr [SEQ ID NO: 12]

Tyr-Met-Arg-Tyr-Tyr-Glu-Ser-Ser-Leu-Lys-Ser-Tyr-Pro-Asp-Trp [SEQ ID NO: 13]

Glu-Tyr-Met-Arg-Tyr-Tyr-Glu-Ser-Ser-Leu-Asn-Pro-Thr-Arg-Leu [SEQ ID NO: 14]

25 Cys-Arg-Tyr-Tyr-Glu-Ser-Ser-Leu-Lys-Ser-Tyr-Cys-Asp [SEQ ID NO: 15]

Ile-Trp-Arg-Tyr-Tyr-Glu-Asp-Ser-Glu-Leu-Met-Gln-Pro-Tyr-Arg [SEQ ID NO: 16]

Phe-Thr-Tyr-Tyr-Gln-Ser-Ser-Leu-Glu-Pro-Leu-Ser-Pro-Phe-Tyr [SEQ ID NO: 17]

His-Asp-Lys-Leu-Phe-Thr-Phe-Tyr-Gln-Asn-Ser-Pro-Ser-Ser-Tyr [SEQ ID NO: 18]

Thr-Met-Thr-Phe-Pro-Glu-Asn-Tyr-Tyr-Arg-Glu-Arg-Pro-Tyr-His [SEQ ID NO: 19]

In a most preferred embodiment, the pharmaceutical composition for treatment of *B. multicinctus* envenomation comprises the peptides of SEQ ID NOs: 11 and 15.

In a preferred embodiment, the composition for treatment of *B. multicinctus* envenomation is formulated for injection. According to the present invention, such a formulation may comprise the active agents, namely a first peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂, and a second peptide capable of specifically binding to α -BTX and inhibiting the antagonistic interaction of the α -BTX with muscle nicotinic acetylcholine receptor, as defined above, without an adjuvant or, alternatively, emulsified in an adjuvant suitable for human clinical use. Examples of such adjuvants may be, without being limited to, aluminum hydroxide, aluminum hydroxide gel and aluminum hydroxyphosphate.

In view of the high and specific affinity of pep- β to the PLA₂ subunit of β -BTX, and since many snake venoms comprise PLA₂ subunit which in many cases determines the toxicity of these venoms, the capacity of pep- β to effectively inhibit the PLA₂ activity of other snake venoms was evaluated. For that purpose, venoms of *Vipera ammodytes*, *Vipera palaestinae*, *Pseudocerastes persicus fieldi* and *Walterinnesia aegyptia* have been used.

As shown in Example 4 hereinafter, pep- β inhibited the PLA₂ activity of the neurotoxins of *V. ammodytes* and *P. fieldi* in a similar efficacy, but it did not inhibit the PLA₂ activity of the neurotoxins of *V. palaestinae* or *W. aegyptia*. In fact, the inhibition of PLA₂ activity by pep- β in the case of *V. ammodytes* was slightly better than in the case of *B. multicinctus* and about four times higher in the case of *P. fieldi*.

Thus, in another aspect, the present invention relates to a peptide of 12 to 25 amino acid residues, capable of specifically binding to and inhibiting the catalytic activity of PLA₂, as defined above.

In a further aspect, the present invention relates to a pharmaceutical composition comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ as defined above, preferably a peptide comprising the

consensus sequence of SEQ ID NOs. 3, 4, 5 or 6, most preferably the cyclic peptide of SEQ ID NO: 11, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

The aforesaid composition may be used for inhibiting the catalytic activity of PLA₂ component of a venom of a venomous animal following envenomation of a human or an animal, preferably a domesticated animal, by said venomous animal. The venomous animal may be any animal having a venom comprising PLA₂, such as a snake or a scorpion. Examples of such snakes are *B. multicinctus*, *V. ammodytes* and *P. fieldi*.

The pharmaceutical composition provided by the present invention may be prepared by conventional techniques, e.g., as described in Remington: The Science and Practice of Pharmacy, 19th Ed., 1995. The composition may be in any suitable form and may further include pharmaceutically acceptable fillers, carriers or diluents, and other inert ingredients and excipients. The composition can be administered by any suitable route, e.g., intravenously, intraperitoneally, subcutaneously, or transdermally.

In a preferred embodiment, the pharmaceutically acceptable carrier is a liquid and the pharmaceutical composition is an injectable solution.

The use of peptides for treatment of snakebites offers a method superior to that of the standard antibodies (antivenin) produced in horses or sheep, particularly in view of the risk of anaphylactic shock and the high production costs. Contrary to the effect of antibodies in the human body, peptide drugs do not elicit any immune response, thus excluding the risk of anaphylactic shock. Moreover, the stability of cyclic peptides enables their transportability and immediate use at the site of the event. Since peptide synthesis has become more simple and cost effective, the compositions and the vaccines of the invention provide a powerful method for treating snake envenomations, provided the peptides included bind with a high affinity to the major toxic components of the venom to be neutralized, and effectively inhibit its activity.

Thus, in yet another aspect, the present invention provides a method for treatment of *B. multicinctus* envenomation, comprising administering to an individual or animal following a *B. multicinctus* bite a pharmaceutical composition for treatment of *B. multicinctus* envenomation as defined above, in amounts
5 sufficient to treat the *B. multicinctus* envenomation.

In a preferred embodiment, said method comprises the administration to said individual or animal a pharmaceutical composition comprising the peptides of SEQ ID NOs: 11 and 15, most preferably, repeatedly by intraperitoneal (IP) injection, about 10 minutes and about 50 minutes following a *B. multicinctus* bite.

10 In still another aspect, the present invention provides a method for inhibiting the catalytic activity of the PLA₂ component of a venom of a venomous animal, following envenomation of an individual or animal by said venomous animal, comprising administering to said individual or animal a pharmaceutical composition comprising a peptide capable of specifically binding to and inhibiting the catalytic
15 activity of PLA₂ as defined above, preferably the peptide of SEQ ID NO:11, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, in an amount sufficient to inhibit the catalytic activity of the PLA₂.

The invention will now be illustrated by the following non-limiting Examples.

20

EXAMPLES

Materials and Methods

*(i) Isolation of α - and β -BTX from *B. multicinctus* venom by HPLC*

α - and β -bungarotoxin (BTX) of *B. multicinctus* venom (Sigma T3019) were isolated from the venom by a C₁₈ hydrophobic column [LiChrosorb RP-18 (7 μ m)
25 250-10, Merck, Germany], employing a binary gradient of 0.1% trifluoroacetic acid (TFA) in water (solution A) and 0.1% TFA in acetonitrile (solution B). The venom fractions were separated using a gradient of 20-70% acetonitrile over 60 min at 1.5 ml/min and monitored at 220 nm. α - and β -BTX were detected by 15% SDS PAGE,

as shown in Fig. 2. The α - and β -BTX peaks were eluted from the column at 22% and 42% acetonitrile, respectively.

α - and β -BTX fractions were further purified to homogeneity on a C₈ column [LiChrospher RP-8 (5 μ m) 250-4 Merck, Germany], employing the above binary gradient. β -BTX was separated between 25-45% acetonitrile over 60 min at 1 ml/min, and the purified β -BTX was eluted as five tandem peaks (isoforms) between 33-38% acetonitrile. α -BTX was separated between 20-45% acetonitrile over 60 min at 1 ml/min, and the purified α -BTX was eluted as two peaks (isoforms), the first at 28% and the second at 30% acetonitrile.

10 (ii) *Biotinylation of α - and β -bungarotoxin*

For biotinylation, 100 μ g of the purified α - and β -BTX in 100 μ l of 0.1 M NaHCO₃, pH 8.6, were incubated for 1 hour at room temperature with 5 μ g of biotin amido caproate N-hydroxysuccinimide ester (Sigma, B2643) taken from a stock solution of 2 mg/ml in dimethylformamide. The reaction mixture was dialyzed at 15 4°C against phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M phosphate buffer, pH 7.4).

(iii) *Isolation of phage-clones that react with α - and β -BTX from a 15-mer phage-peptide library*

α - and β -BTX were applied for screening a 15-mer phage-peptide library 20 (kindly provided by George P. Smith, University of Missouri), according to Scott and Smith (1990). Phage-clones reacting with either toxin were selected from a library sample containing about 10¹⁰ infectious phage particles using three cycles of bio-panning (phage selection and amplification). In each cycle, the reaction mixtures were incubated for 30 min on a streptavidin-coated 30 mm polystyrene 25 petri dish. Unbound phages were removed by extensive washing (10 times with PBS containing 0.5% Tween-20) and the remaining phages were eluted with 0.2 N HCl, pH 2.2. The eluate was neutralized with 2 M Tris and used to infect *E.coli* cells (strain K91). After the third cycle of biopanning, individual amplified phage-clones were tested for their ability to bind to α - or β -BTX.

(iv) Synthetic peptides: preparation and cyclization

Synthetic peptides were prepared on a large scale (0.5-1 g) in the Chemical Services Unit of the Hebrew University of Jerusalem, using the solid-phase automated method described by Merrifield (1963).

5 The cysteine-containing pep- α , CRYYESLKS α YCD (SEQ ID NO: 15), and pep- β , CAEVSTWEM β LQQLNTTRMPPPC (SEQ ID NO: 11), were solubilized in buffer (0.1 mg/ml peptide in 5 mM Tris-HCl, pH 8.0) and left for complete oxidation by stirring overnight at room temperature in open air. The extent of cysteine oxidation was assayed by using the Ellman reagent (Ellman, 1959). The oxidized cyclic forms of pep- α and pep- β were tested for binding with α - and β -BTX, respectively. The oxidized synthetic preparations of these peptides were purified by HPLC using the C₁₈ column and employing a binary gradient of 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B). Separations of the oxidized from the non-oxidized forms of pep- α and pep- β were obtained using
10 gradients of 18-23% acetonitrile over 25 min and 30-60% over 45 min, respectively. The flow rate was 1.5 ml/min, and the peptide peaks were monitored at 230 nm. Pep- α was eluted at 22% acetonitrile and pep- β at 41%. Purified peptides were analyzed by MALDI mass-spectrometry (VG Fison, Altvincham, UK), which validated that both peptides possess a cyclic structure with molecular weights of
15 1,614 and 2,533 dalton for pep- α and pep- β , respectively. The calculated molecular weights of the non-oxidized forms are 1,616.6 and 2,535 dalton, respectively. No fragments were detected when a sample of the cyclic peptide was further examined by MALDI-TOF mass spectrometry on a scale of 1,000-10,000 dalton, indicating that only one form of the cyclic peptide was generated.

25 The binding affinity of both purified and crude preparations of pep- α and pep- β to α -BTX and β -BTX, respectively, was inhibited to the same extent. For the sake of clarity we denote the crude oxidation product of pep- α and pep- β as oxidized pep- α and pep- β , whereas HPLC-purified active material is referred to as a cyclic peptide. The following experiments were thus carried out with the oxidized
30 crude peptide preparation.

(v) HPLC purification of chains A and B of β -BTX following reduction with β -mercaptoethanol

Purified β -BTX fraction (200 μ g) was incubated at 37°C for 1 h with 100 mM β -mercaptoethanol in a buffer containing 40 mM Na-acetate (pH 5) and 0.6 M NaCl. Following reduction, the toxin was loaded on a C₈ column [LiChrospher RP-8 (5 μ m) 250-4 Merck, Germany], employing a binary gradient of 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B). Separation of the subunits was obtained by running a gradient of 20-60% acetonitrile over 60 min at 1ml/min. Three peaks containing chain B (the targeting subunit), the non-split toxin, and chain A (the PLA₂ subunit) were collected at 23%, 31% and 35%, respectively.

(vi) Determining the PLA₂ enzymatic activity of β -BTX

HPLC-purified β -BTX and its separated isoforms were assayed for phospholipase A₂ (PLA₂) activity as follows. The substrate L- α -phosphatidylcholine (L- α -lecithin) type XVI-E from fresh egg yolk (Sigma P-3556) was dissolved in chloroform: methanol, (2:1 v/v). Aliquots of 2 mg were evaporated to dryness in a Speed Vac (Savant, USA) and kept at -20°C until used. The PLA₂ reaction was carried out in a total volume of 25 μ l containing 10 μ l (8 mg/ml) substrate, 5 μ l buffer (50 mM glycylglycine pH 8, 20 mM CaCl₂, 0.5% Triton (v/v)), 5 μ l peptide (or 5 μ l water) and 5 μ l PLA₂. The reaction was shaken (250 rpm) overnight at 37°C. Product and substrate left in the enzyme reaction mixture were assayed by TLC. Samples (5 μ l) taken from the enzyme reaction mixture were loaded for TLC and run using a mixture of chloroform, methanol, water and acetic acid (190, 50, 4, 4 v/v, respectively) as a solvent system. In order to visualize spots of the substrate (phosphatidylcholine) and the products (free fatty acid and lysophospholipid), the chromatograms were stained with iodine vapor. Under these experimental conditions the hydrolytic product moves considerably faster than the substrate on the chromatograph.

(vii) Injection of *B. multicinctus* whole venom into mice

In order to induce lethality in mice about 2 hour after the venom injection, Balb/c mice (3-4 week old, 13-18 g) were subcutaneously (SC) injected with 2.5 μ g

B. multicinctus whole venom (Sigma V6625) in 0.5 ml PBS. A dose response curve for the venom was obtained in order to determine the LD₅₀ (50% lethality). Mice treated with the venom alone became paralyzed about 1.5 hours after the venom injection and died after an additional 0.5 hour.

5 **Example 1. Design and preparation of pep- α and pep- β that bind to α - and β -BTX, respectively**

α - and β -bungarotoxin (BTX), the two major neurotoxins in the *B. multicinctus* venom, were subjected to a peptide library in order to select a specific peptide inhibitor for each toxin.

10 In a previous study (Balass *et al.*, 1997), applying α -BTX to a phage peptide library, we obtained the peptides of SEQ ID NOs: 12-19, as listed in **Table 1** hereinafter. The library-peptide MRYYESSLKSYPD (SEQ ID NO: 13) was tested to inhibit the interaction between *Torpedo* nicotinic acetylcholine receptor (AChR) and its antagonist α -BTX, and was found to inhibit this interaction with an IC₅₀
15 value of 10⁻⁶ M. However, this peptide does not protect mice from α -BTX lethality when injected concomitantly with the toxin. In order to increase the peptide binding affinity to α -BTX, we modified the library peptide and prepared the cyclic peptide CRYYESLKSYPD (SEQ ID NO: 15, herein designated “pep- α ”) as described in Materials and Methods. Since pep- α inhibited the binding of α -BTX to AChR at an
20 IC₅₀ value of 10⁻⁸ M, being a more potent inhibitor than the library-peptide, it has been further used for *in vivo* experiments (Balass *et al.*, 2001).

Our next step was to apply β -BTX to screen a phage-peptide library and we consequently detected four different peptides with the consensus sequence **WD(E)xLxxL** (SEQ ID NO: 2), as shown in **Table 2** hereinafter. The peptide
25 **VSTWEMLQQLNTTRM** (SEQ ID NO: 7) that showed the highest binding affinity to β -BTX was used as a lead peptide for a further study. In order to improve the binding affinity of said peptide to β -BTX, several amino acids, including two cysteines, were added to its termini (**CAEVSTWEMLQQLNTTRMPPPC**), and a disulphide-constrained cyclic form of the extended peptide (SEQ ID NO: 11, herein

designated pep- β) was prepared as described in Materials and Methods. As found, pep- β binds to β -BTX with an affinity about three orders of magnitude higher ($4 \times 10^{-9} \text{M}$) than that of the lead peptide. Furthermore, as shown in **Fig. 3**, pep- β binds specifically to the separated phospholipase A₂ (PLA₂) subunit of β -BTX, but not to the targeting subunit of the toxin after their separation, and it is able to inhibit the catalytic activity of the PLA₂ subunit at micromolar concentration, as shown in **Fig. 4**. For the following *in vivo* experiments, we administered a mixture of cyclic pep- α and pep- β to protect mice from the lethal effect of *B. multicinctus* venom.

Table 1: Peptides selected from a phage peptide library using α -BTX as a selector

Sequence of peptide insert	SEQ ID NO.	No. of phage clones identified ^a	Binding affinity (M) ^b
PPPIFRYYEYWPTSY	12	1	10^{-7}
YMRYYESLKSYPDW	13	16	10^{-8}
EYMRYYESLNPTL	14	3	10^{-8}
IWRYYEDELMPYR	16	10	10^{-9}
FTYYQSSLEPLSPFY	17	3	10^{-8}
HDKLFTFYQNSPSSY	18	1	10^{-7}
TMTFPENYYRERPYH	19	2	10^{-7}
RYYESSL (consensus)			

10 ^a Number of phage clones out of a group of 36 clones arbitrarily chosen from the third biopanning cycle α -BTX positive binders.

^b Binding affinity (half maximal binding) of α -BTX to the sepecified phage clone immobilized on an ELISA plate.

Table 2: Peptides selected from a phage peptide library using β -BTX as a selector

Sequence of peptide insert	SEQ ID NO.	No. of phage clones identified ^a	Binding affinity (M) ^b
VSTWEMLQQLNTRM	7	10	10^{-8}
GLWRGFWD—LAWLPAD	8	21	10^{-7}
HFDYPAFWYWDKLY	9	9	10^{-7}
GWSAWD---CSFLSCAPS	10	3	10^{-7}
(consensus) WDXLXXL			

15 ^a Out of a group of 43 clones arbitrarily chosen from the third biopanning cycle β -BTX positive binders.

^b Half maximal binding) of β -BTX to the sepecified phage clone immobilized on an ELISA plate.

Example 2. Concomitant injection of *B. multicinctus* venom and a mixture of pep- α and pep- β into mice confers protection from venom lethality

Following injection of *B. multicinctus* venom to mice, paralysis occurs and soon afterwards death, mainly due to respiratory failure. During the 2 hour lapse
5 from venom injection to death, four broad phases of visual symptoms can be observed, as shown in **Figs. 5A-5D**.

For *in vivo* experiments, we used 3-4 week old mice (Balb/c) which were injected (SC) with *B. multicinctus* whole venom (2.5 $\mu\text{g}/\text{mouse}$) and either pep- α , pep- β or a mixture thereof. As shown in **Table 3** hereinafter, a significant delay of
10 lethality, but not protection, was observed when either pep- α or pep- β was given (50 μg each) concomitantly with *B. multicinctus* whole venom. Full protection from venom lethality was attained by each peptide only when an increased amount of either pep- α or pep- β (0.5 mg each) was administered to mice concomitantly with the venom (data not shown).

15 In order to determine the neutralizing potency of a pep- α and pep- β mixture, 3-4 week old Balb/c mice were concomitantly injected (SC) with *B. multicinctus* whole venom (2.5 $\mu\text{g}/\text{mouse}$) and the peptide mixture. As shown in **Table 3**, full protection from venom lethality was observed even when a mixture containing low dose of the peptides (25 μg each) was injected, concomitantly with the venom;
20 however, when the dose of each peptide in the mixture was reduced to 5 $\mu\text{g}/\text{mouse}$, it only delayed lethality. Based on these results, it was decided to administer, in the following *in vivo* experiment, a mixture containing an increased amount of both peptides (500 μg each/mouse).

Example 3. Administering a mixture of pep- α and pep- β after injection of *B. multicinctus* venom protects mice from venom lethality

The therapeutic value of administering peptide drugs after envenomation is of crucial importance for the treatment of human and animal victims of snakebites. In order to evaluate the ability of a mixture of pep- α and pep- β to protect mice from the *B. multicinctus* venom lethality after the venom injection, and based on the
30 findings described in Example 2 hereinabove, a series of *in vivo* experiments was

carried out, in which a mixture containing 500 µg of each of pep-α and pep-β was injected to mice following SC injection of the venom. Several parameters were considered related to the time lapse from the venom injection and the mode of injection of the peptide mixture (pep-α and pep-β, 1:1).

5 As shown in **Table 4** hereinafter, intraperitoneal (IP) injection of the peptide mixture 10 min after the SC injection of the venom (2.5 µg/mouse) delayed lethality by 1-2 hours as compared to SC injection of the mixture. No detectable change in the lethality was observed when the mixture was administered by intravenous (IV) route rather than IP injection. However, full protection from venom lethality was
10 observed when a mixture containing 500 µg of each peptide was given twice, by IP administrations, 10 min and then 50 min following the venom injection.

Table 3: Concomitant injection of the venom with or without peptide(s)

No. of mice	Venom (2.5 µg/mouse)	Peptide(s) (µg/mouse)	Response*
4	+	-	All D (2 hours)
4	-	Pep-α (500)	All A (>2 days)
4	-	Pep-β (500)	All A (>2 days)
4	+	Pep-α (50)	2 D (9-20 hours) 2 A (>40 hours)
4	+	Pep-β (50)	All D (2-5 hours)
4	+	Pep-α+Pep-β (5 each)	All D (5-30 hours)
4	+	Pep-α+Pep-β (25 each)	All A (>2 days)

*Related to the time lapse from the venom injection (A-active D-dead)

15 **Table 4:** Administering pep-α (500 µg), pep-β (500 µg) or a mixture of both (500 µg each) following SC injection of the venom (2.5 µg/mouse)

No. of mice	Treatment (µg/mouse)	Response*
4	PBS (IP)	All D (2 hours)
4	Pep-α after 10 min (IP)	All D (2-3 hours)
4	Pep-β after 10 min (IP)	All D (2-3 hours)
4	Pep-α+Pep-β after 10 min (SC)	All D (2-3 hours)
4	Pep-α+Pep-β after 10 min (IP)	All D (4 hours)
4	Pep-α+Pep-β after 10 min (IV)	All D (4 hours)
4	Pep-α+Pep-β after 10 and 20 min (IP)	All D (3-6 hours)
3	Pep-α+Pep-β after 10 and 50 min (IP)	All A (>3 days)

*Related to the time lapse from the venom injection (A-active D-dead)

Example 4. Pep- β inhibits the PLA₂ activity of *V. ammodytes* and *P. fieldi*

Most of the snake venoms comprise neurotoxins composed of phospholipase A₂ (PLA₂) subunit, which in many cases determine the toxicity of these venoms. Therefore, in order to evaluate if the pep- β is capable to inhibit the PLA₂ activity of other snake venoms, we have used venoms of *Walterinnesia aegyptia*, *Vipera palaestinae*, *Pseudocerastes persicus fieldi* and *Vipera ammodytes*.

The protein profile of each one of these venoms, as detected by 12% SDS-PAGE gel analysis, is shown in **Fig. 6**, pointing to the specific protein bands having PLA₂ activity.

Fig. 7 shows TLC analysis of PLA₂ activity within the venom of the snakes *P. fieldi*, *V. ammodytes*, *V. palaestinae* and *W. aegyptia*, with (200 μ g/ml) or without pep- β , as well as with a control peptide (200 μ g/ml pep- α). As shown, based on both the product accumulation and substrate disappearance, pep- β inhibited the PLA₂ activity in the neurotoxins of *V. ammodytes* and *P. fieldi*, with a similar efficacy, but it did not inhibit the PLA₂ activity of the neurotoxins of *V. palaestinae* or *W. aegyptia*.

Evaluation of increasing concentrations of pep- β (at 25, 50, 100 and 200 μ g/ml) to inhibit the PLA₂ activity of the various examined venoms (2.5 μ g/ml for *B. multicinctus*, *V. ammodytes* and *V. palaestinae*, and 0.5 μ g/ml for *P. fieldi*) showed that the inhibition efficacy in the case of *V. ammodytes* was slightly better (based on the product accumulation) than in the case of *B. multicinctus* (**Fig. 8**). It was further found that the inhibition efficacy of pep- β in the case of *P. fieldi*, as expressed by IC₅₀ values, was ~4 times higher, as shown in **Fig. 8** and IC₅₀ of 25, 70 and 140 μ g/ml was estimated for the venom of *P. fieldi*, *V. ammodytes* and *B. multicinctus*, respectively, as summarized in **Table 5** hereinafter.

Fig. 9 shows the inhibitory effect of increasing concentrations of pep- β (25, 50, 100 and 200 μ g/ml) on PLA₂ activity of purified fractions of PLA₂ isotoxins of *P. fieldi* and *V. ammodytes*. As shown, PLA₂-1 and PLA₂-2 isoforms of *P. fieldi* as well as PLA₂-1 and PLA₂-2 isoforms of *V. ammodytes* (represented by bands 1 and

2 of *P. fieldi* and *V. ammodytes* in Fig. 6, respectively), were inhibited by pep- β at the IC₅₀ values of 1.3X10⁻³M, 8X10⁻³M, 2.6X10⁻³M and 1.7X10⁻³M, respectively.

Table 5: IC₅₀ values of pep- β for the inhibition of PLA₂ activity in the venom of *B. multicinctus*, *V. ammodytes* and *P. fieldi* venom

Snake venom	IC ₅₀ (M)*
<i>B. multicinctus</i>	6.3 X 10 ⁻³
<i>V. ammodytes</i>	4.7 X 10 ⁻³
<i>P. fieldi</i>	1.7 X 10 ⁻³

5

*Obtained by an ELISA test

REFERENCES

- Balass M., Kalef E., Fuchs S., Katchalski-Katzir E., A cyclic peptide with high affinity to α -bungarotoxin protects mice from the lethal effect of the toxin, *Toxicon*, **2001**, 39, 1045-1051
- 5 Balass M., Katchalski-Katzir E., Fuchs S., The α -bungarotoxin binding site on the nicotinic acetylcholine receptor: Analysis using a phage-epitope library, *PNAS*, **1997**, 94, 6054-6058
- Crosland R.D., Effect of drugs on the lethality in mice of the venoms and neurotoxines from sundryl snakes, *Toxicon*, **1991**, 29, 613-631
- 10 Crosland R.D., Effect of chloroquine, promazine and quinacrine on the lethality in mice of the venoms and neurotoxines from several snakes, *Toxicon*, **1989a**, 27, 655-663
- Crosland R.D., Effect of chlorpromazine on toxicity in mice of the venom and neurotoxines from the snake *Bungarus mulicinctus*, *J. Pharmacol. Exp. Ther.*,
15 **1989b**, 246, 992-995
- Ellman G.L., Tissue sulfhydryl groups, *Arch. Biochem. Biophys.*, **1959**, 82, 70-77
- Giebel L.B., Cass R., Milligan D.L., Young D., Arze R., Johnson C., Screening of cyclic peptide phage libraries identifies ligands that bind strepavidin
20 with high affinities, *Biochemistry*, **1995**, 34, 15430-15435
- Galan J.A., Sanchez E.E., Rodriguez-Acosta A., Perez J.C., Neutralization of venoms from two Southern Pacific Rattlesnakes (*Crotalus helleri*) with commercial antivenoms and endothermic animal sera, *Toxicon*, **2004**, 43, 791-799
- Merrifield R.B., Solid phase peptide synthesis. I. The synthesis of a
25 tetrapeptide, *J. Am. Chem. Soc.*, **1963**, 85, 2149-2154
- Scherf T., Balass M., Fuchs S., Katchalski-Katzir E., Anglister J., Three-dimensional solution structure of the complex of α -bungarotoxin with a library-derived peptide, *PNAS*, **1997**, 94, 6059-6064
- Scott J.K., Smith G.P., Searching for peptide ligands with an epitope library,
30 *Science*, **1990**, 240, 386-390

Venkatesh N., Prevention of passively transferred experimental autoimmune myasthenia gravis by a phage library-derived cyclic peptide, *PNAS*, **2000**, 97, 761-766

CLAIMS

1. A pharmaceutical composition for treatment of *Bungarus multicinctus* envenomation, comprising a peptide capable of specifically binding to and inhibiting the catalytic activity of phospholipase A₂ (PLA₂), and a peptide capable of specifically binding to α-bungarotoxin (α-BTX) and inhibiting the antagonistic interaction of the α-BTX with muscle nicotinic acetylcholine receptor, and a pharmaceutically acceptable carrier.
- 5
2. The pharmaceutical composition of claim 1, wherein said peptide capable of specifically binding to and inhibiting the catalytic activity of PLA₂ is a peptide of 12 to 25 amino acid residues, selected from:
- 10
- (i) a linear peptide comprising a consensus sequence:
Trp-Asp(Glu)-X₁-X₂-X₃-X₄-X₅ [SEQ ID NO: 1]
wherein X₁ is absent or is Met or Lys; X₂ is Leu or Cys; X₃ is Gln, Ala, Tyr or Ser; X₄ is absent or is Gln, Trp or Phe; and X₅ is absent or is Leu, wherein at least one of X₂ and X₅, if present, is Leu;
 - (ii) a cyclic derivative of (i);
 - (iii) a D-stereomer of (i) or (ii);
 - (iv) a dual peptide consisting of two of the same or different peptides (i) to (iii), wherein the peptides are covalently linked to one another directly or through a spacer;
 - (v) a multimer comprising a number of the same or different peptides of (i) to (iii);
 - (vi) an amide of the C-terminal of a peptide (i) to (iv);
 - (vii) an N-acyl derivative of a peptide (i) to (v); or
 - (viii) a pharmaceutically acceptable salt thereof.
- 15
- 20
- 25
3. The pharmaceutical composition of claim 2(i), wherein said linear peptide comprises the consensus sequence:

Trp-Asp(Glu)-X₁-Leu-X₃-X₄-Leu [SEQ ID NO: 2]

wherein X₁ is absent or is Met or Lys; X₃ is Gln, Ala, Tyr or Ser; and X₄ is absent or is Gln, Trp or Phe.

4. The pharmaceutical composition of claim 3, wherein said linear peptide comprises the consensus sequence:

5 Trp-Glu-Met-Leu-Gln-Gln-Leu [SEQ ID NO: 3] or
Trp-Asp-Leu-Ala-Trp-Leu [SEQ ID NO: 4]

5. The pharmaceutical composition of claim 2(i), wherein said linear peptide comprises the consensus sequence:

10 Trp-Glu-Lys-Leu-Tyr [SEQ ID NO: 5] or
Trp-Asp-Cys-Ser-Phe-Leu [SEQ ID NO: 6]

6. The pharmaceutical composition of claim 4, wherein said linear peptide is Val-Ser-Thr-Trp-Glu-Met-Leu-Gln-Gln-Leu-Asn-Thr-Thr-Arg-Met

[SEQ ID NO: 7] or
15 Gly-Leu-Trp-Arg-Gly-Phe-Trp-Asp-Leu-Ala-Trp-Leu-Pro-Ala-Asp
[SEQ ID NO: 8].

7. The pharmaceutical composition of claim 5, wherein said linear peptide is His-Phe-Asp-Tyr-Pro-Ala-Phe-Trp-Tyr-Trp-Glu-Lys-Leu-Tyr

[SEQ ID NO: 9] or
20 Gly-Trp-Ser-Ala-Trp-Asp-Cys-Ser-Phe-Leu-Ser-Cys-Ala-Pro-Ser
[SEQ ID NO: 10].

8. The pharmaceutical composition of claim 2(ii), wherein said cyclic peptide comprises the consensus sequence of SEQ ID NO: 1.

9. The pharmaceutical composition of claim 8, wherein said cyclic peptide comprises the consensus sequence of SEQ ID NOs: 3-6, preferably the consensus
25 sequence of SEQ ID NO: 3.

10. The pharmaceutical composition of claim 9, wherein said cyclic peptide is

Cys-Ala-Glu-Val-Ser-Thr-Trp-Glu-Met-Leu-Gln-Gln-Leu-Asn-Thr-Thr-Arg-
 Met-Pro-Pro-Cys [SEQ ID NO: 11]

11. The pharmaceutical composition of any one of claims 1 to 10, wherein said peptide capable of specifically binding to α -BTX and inhibiting the antagonistic interaction of the α -BTX with muscle nicotinic acetylcholine receptor is selected from the peptides of SEQ ID NOs: 12 to 19:

Pro-Pro-Pro-Ile-Phe-Arg-Tyr-Tyr-Glu-Tyr-Trp-Pro-Thr-Ser-Tyr
 [SEQ ID NO: 12]

10 Tyr-Met-Arg-Tyr-Tyr-Glu-Ser-Ser-Leu-Lys-Ser-Tyr-Pro-Asp-Trp
 [SEQ ID NO: 13],

Glu-Tyr-Met-Arg-Tyr-Tyr-Glu-Ser-Ser-Leu-Asn-Pro-Thr-Arg-Leu
 [SEQ ID NO: 14]

15 Cys-Arg-Tyr-Tyr-Glu-Ser-Ser-Leu-Lys-Ser-Tyr-Cys-Asp [SEQ ID NO: 15],
 Ile-Trp-Arg-Tyr-Tyr-Glu-Asp-Ser-Glu-Leu-Met-Gln-Pro-Tyr-Arg
 [SEQ ID NO: 16]

Phe-Thr-Tyr-Tyr-Gln-Ser-Ser-Leu-Glu-Pro-Leu-Ser-Pro-Phe-Tyr
 [SEQ ID NO: 17],

His-Asp-Lys-Leu-Phe-Thr-Phe-Tyr-Gln-Asn-Ser-Pro-Ser-Ser-Tyr
 [SEQ ID NO: 18]

20 Thr-Met-Thr-Phe-Pro-Glu-Asn-Tyr-Tyr-Arg-Glu-Arg-Pro-Tyr-His
 [SEQ ID NO: 19]

12. The pharmaceutical composition of claim 11, comprising the peptides of SEQ ID NOs: 11 and 15.

13. The pharmaceutical composition of claim 1, formulated for injection.

- 25 14. The pharmaceutical composition of claim 13, wherein said peptides are emulsified in an adjuvant suitable for human clinical use.

15. The pharmaceutical composition of claim 14, wherein said adjuvant is selected from aluminum hydroxide, aluminum hydroxide gel, or aluminum hydroxyphosphate.

16. A peptide of 12 to 25 amino acid residues, capable of specifically binding to and inhibiting the catalytic activity of PLA₂, selected from:

(i) a linear peptide comprising a consensus sequence:

Trp-Asp(Glu)-X₁-X₂-X₃-X₄-X₅ [SEQ ID NO:1]

wherein X₁ is absent or is Met or Lys; X₂ is Leu or Cys; X₃ is Gln, Ala, Tyr or Ser; X₄ is absent or is Gln, Trp or Phe; and X₅ is absent or is Leu, wherein at least one of X₂ and X₅, if present, is Leu;

(ii) a cyclic derivative of (i);

(iii) a D-stereomer of (i) or (ii);

(iv) a dual peptide consisting of two of the same or different peptides (i) to (iii), wherein the peptides are covalently linked to one another directly or through a spacer;

(v) a multimer comprising a number of the same or different peptides of (i) to (iii);

(vi) an amide of the C-terminal of a peptide (i) to (iv);

(vii) an N-acyl derivative of a peptide (i) to (v); and

(viii) a pharmaceutically acceptable salt thereof.

17. The peptide of claim 16(i), wherein said linear peptide comprises the consensus sequence of SEQ ID NO: 2.

18. The peptide of claim 17, wherein said linear peptide comprises the consensus sequence of SEQ ID NO: 3 or 4.

19. The peptide of claim 16(i), wherein said linear peptide comprises the consensus sequence of SEQ ID NO: 5 or 6.

20. The peptide of claim 18, wherein said linear peptide is the peptide of SEQ ID NO: 7 or 8.
21. The peptide of claim 19 wherein said linear peptide is the peptide of SEQ ID NO: 9 or 10.
- 5 22. The peptide of claim 16(ii), wherein said cyclic peptide comprises the consensus sequence of SEQ ID NO: 1.
23. The peptide of claim 22, wherein said cyclic peptide comprises the consensus sequence of SEQ ID NO: 3.
24. The peptide of claim 23, wherein said cyclic peptide is the peptide of SEQ ID
10 NO: 11.
25. A pharmaceutical composition comprising a peptide according to any one of claims 16 to 24 or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.
26. The pharmaceutical composition of claim 25, comprising the cyclic peptide
15 of SEQ ID NO: 11.
27. The pharmaceutical composition of claim 25, for inhibiting the catalytic activity of the phospholipase A₂ (PLA₂) component of a venom of a venomous animal following envenomation by said venomous animal.
28. The pharmaceutical composition of claim 27, wherein said venomous animal
20 is a snake selected from *Bungarus multicinctus*, *Vipera ammodytes* or *Pseudocerastes persicus fieldi*.
29. The pharmaceutical composition of claim 28, wherein said venomous animal is *Bungarus multicinctus*.
30. A method for treatment of *Bungarus multicinctus* envenomation, comprising
25 administering to an individual or animal following a *B. multicinctus* bite a

pharmaceutical composition of claim 1, in amounts sufficient to treat the *B. multiauratus* envenomation.

31. The method of claim 30, wherein said pharmaceutical composition comprises the peptides of SEQ ID Nos: 11 and 15.

5 32. The method of claim 31, wherein said pharmaceutical composition is administered repeatedly by intraperitoneal (IP) injection, about 10 minutes and about 50 minutes following said *B. multiauratus* bite.

33. A method for inhibiting the catalytic activity of the phospholipase A₂ (PLA₂) component of a venom of a venomous animal, following envenomation of an individual or animal by said venomous animal, comprising administering to said individual or animal a pharmaceutical composition of claim 25 in an amount sufficient to inhibit the catalytic activity of the PLA₂.

10 34. The method of claim 33, wherein said pharmaceutical composition comprises the peptide of SEQ ID No: 11.

15

20

1/5

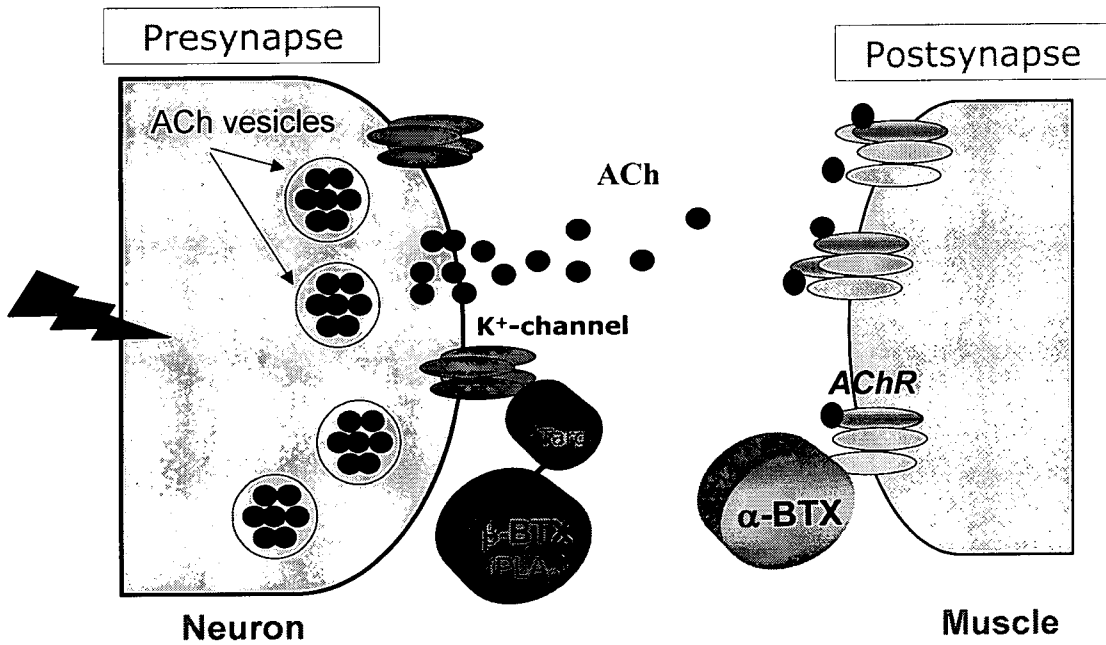


Fig. 1

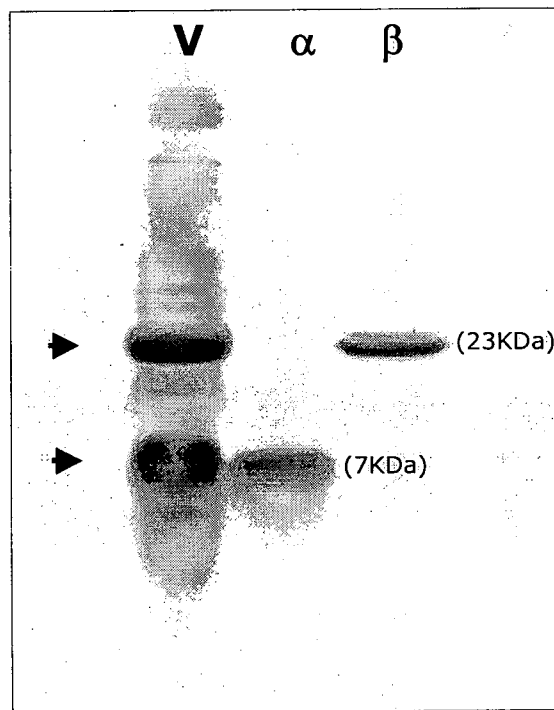


Fig. 2

2/5

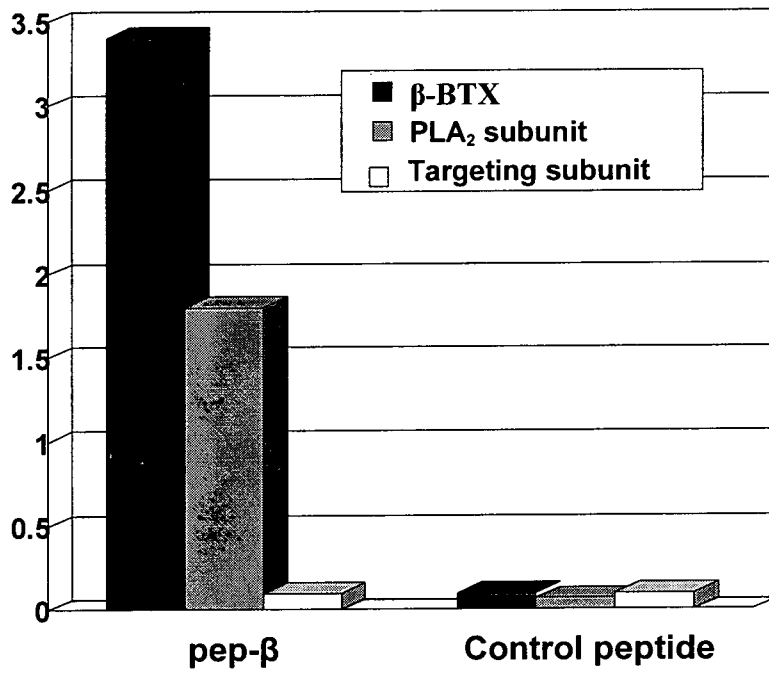


Fig. 3

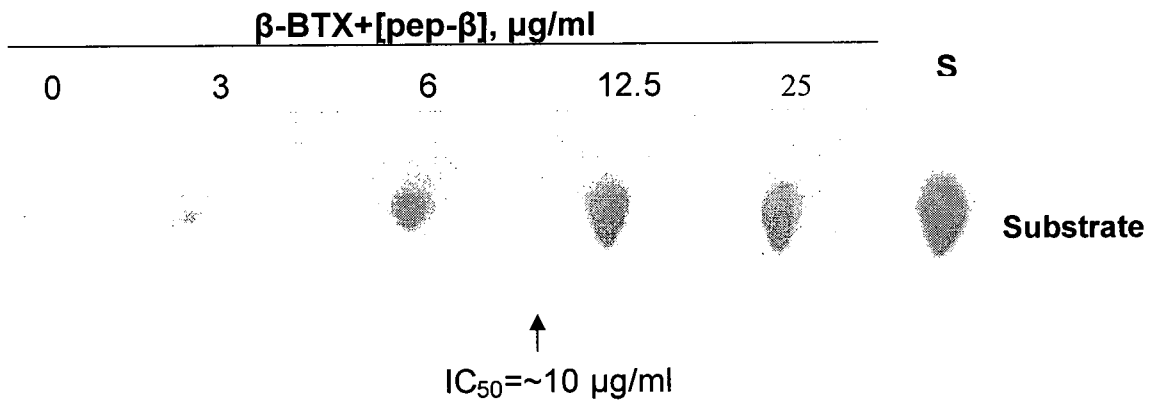


Fig. 4

3/5

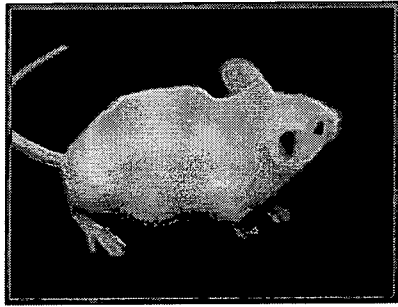


Fig. 5A

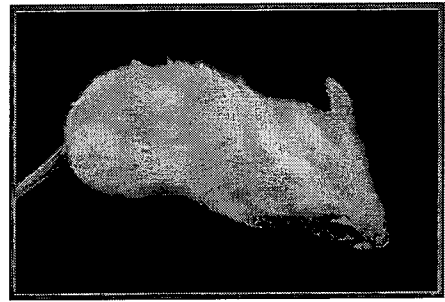


Fig. 5B



Fig. 5C

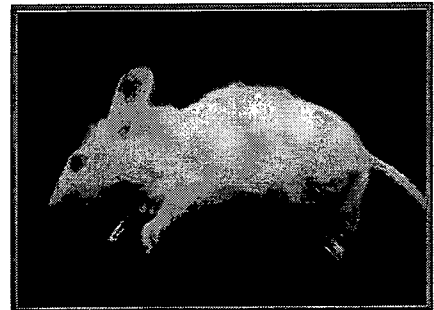


Fig. 5D

4/5

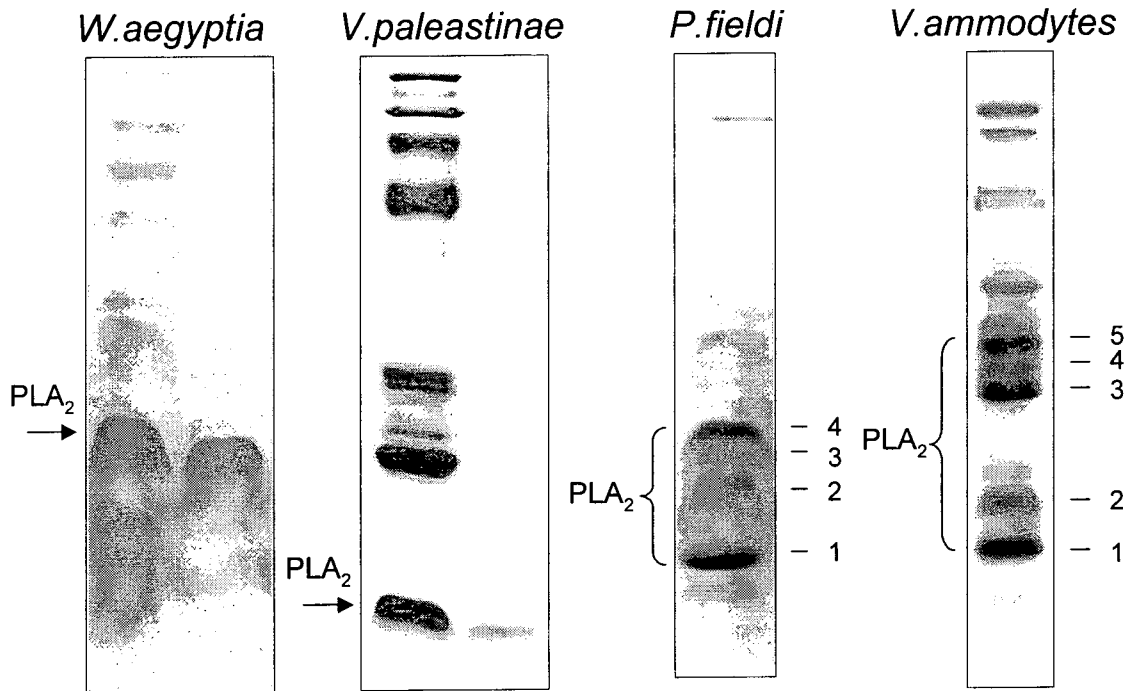


Fig. 6

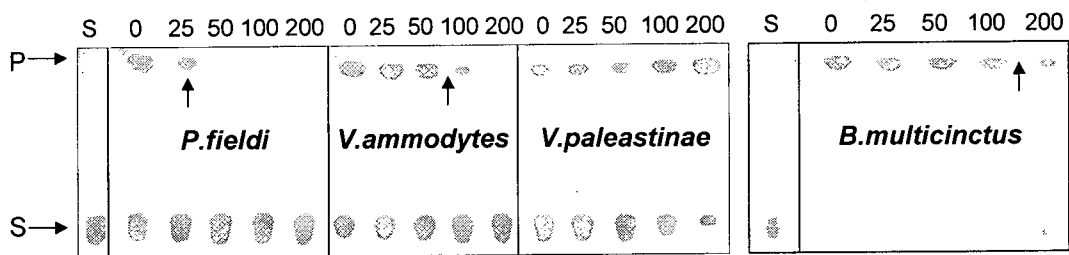


Fig. 7

5/5

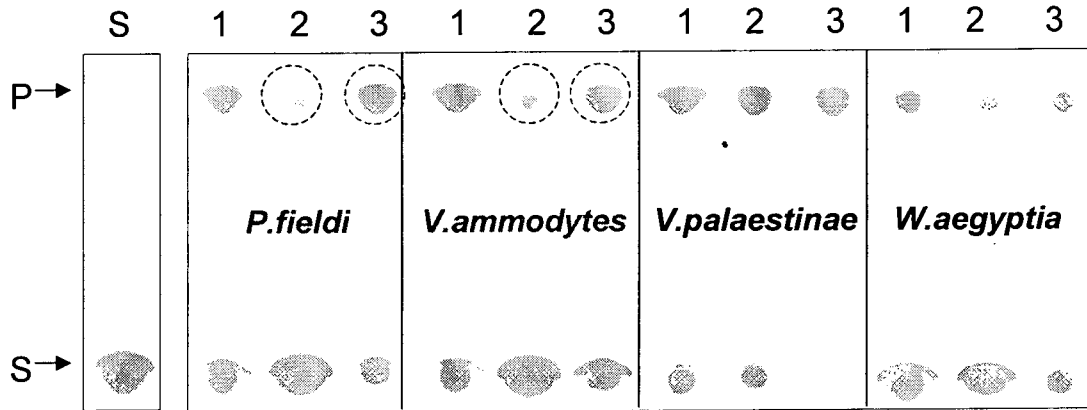


Fig. 8

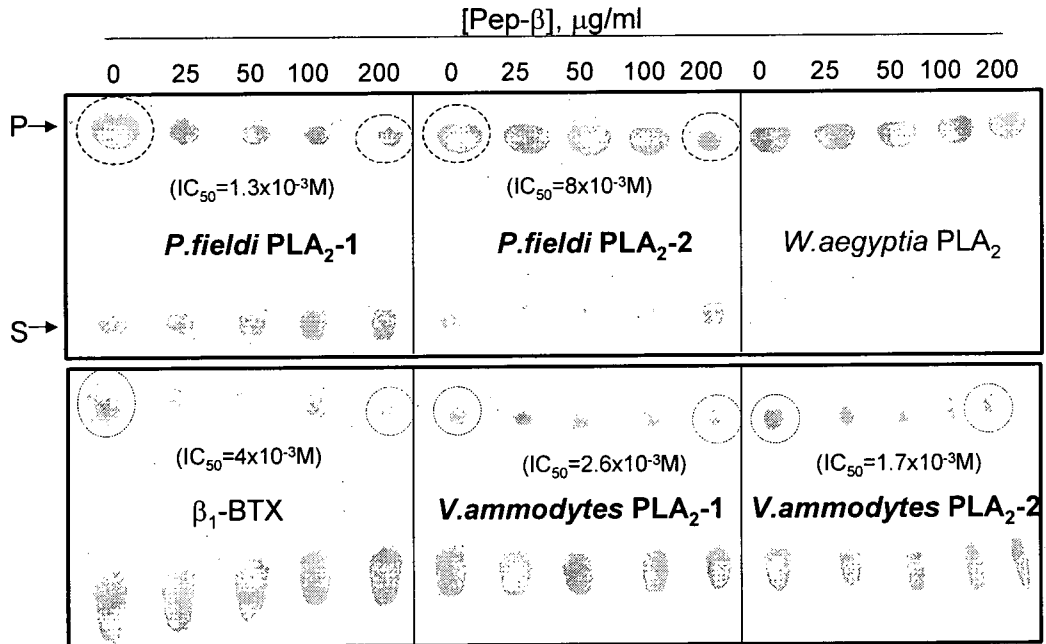


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2008/001331

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K7/08 A61K38/10		
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) C07K A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BALASS M ET AL: "Mode of inhibition of phospholipase A2 activity of beta-bungarotoxin by a characteristic library-derived peptide" 12TH ANNUAL MEETING OF THE ISRAEL SOCIETY FOR NEUROSCIENCE; EILAT, ISRAEL; DECEMBER 14-16, 2003, NEURAL PLASTICITY, vol. 10, no. 3, 16 December 2003 (2003-12-16), pages 181-182, XP008101597	16-29, 33, 34
Y	abstract	1-15, 30-32
X	US 2003/224390 A1 (FOWLKES DANA M [US] ET AL) 4 December 2003 (2003-12-04) Tab.7, peptide 17PT	16, 17
----- -/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> 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 document 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 9 February 2009		Date of mailing of the international search report 18/02/2009
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Groenendijk, Matti

INTERNATIONAL SEARCH REPORT

International application No

PCT/IL2008/001331

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/018778 A1 (CAPLAN MICHAEL J [US]) 14 February 2002 (2002-02-14) Tab.4, peptides 3-5; claims 1-67 -----	16,25
Y	BALASS M ET AL: "The alpha-bungarotoxin binding site on the nicotinic acetylcholine receptor: analysis using a phage epitope library" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC.; US, vol. 94, 1 June 1997 (1997-06-01), pages 6054-6058, XP002228100 ISSN: 0027-8424 cited in the application the whole document -----	1-15, 30-32
Y	BALASS M ET AL: "A cyclic peptide with high affinity to alpha-bungarotoxin protects mice from the lethal effect of the toxin" TOXICON, ELMSFORD, NY, US, vol. 39, 1 July 2001 (2001-07-01), pages 1045-1051, XP002228102 ISSN: 0041-0101 cited in the application the whole document -----	1-15; 30-32
A	CROSLAND R D: "EFFECT OF DRUGS ON THE LETHALITY IN MICE OF THE VENOMS AND NEUROTOXINS FROM SUNDRY SNAKES" TOXICON, vol. 29, no. 6, 1991, pages 613-632, XP002513635 ISSN: 0041-0101 the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No PCT/IL2008/001331
--

Patent document cited in search report	Publication date	Publication date	Patent family member(s)	Publication date
US 2003224390	A1	04-12-2003	AU 7890101 A	21-01-2002
			CA 2414572 A1	17-01-2002
			EP 1360501 A2	12-11-2003
			JP 2004523726 T	05-08-2004
			WO 0204956 A2	17-01-2002
US 2002018778	A1	14-02-2002	AU 1951201 A	12-06-2001
			AU 2065801 A	12-06-2001
			WO 0140264 A2	07-06-2001
			WO 0139799 A2	07-06-2001