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Design and synthesis of peptides that bind α-bungarotoxin with high affinity and mimic the three-dimensional structure of the binding-site of acetylcholine receptor☆

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Abstract

α-Bungarotoxin (α-BTX) is a highly toxic snake neurotoxin that binds to acetylcholine receptor (AChR) at the neuromuscular junction, and is a potent inhibitor of this receptor. In the following we review multi-phase research of the design, synthesis and structure analysis of peptides that bind α-BTX and inhibit its binding to AChR. Structure-based design concomitant with biological information of the α-BTX/AChR system yielded 13-mer peptides that bind to α-BTX with high affinity and are potent inhibitors of α-BTX binding to AChR (IC_{50} of 2 nM). X-Ray and NMR spectroscopy reveal that the high-affinity peptides fold into an anti-parallel β-hairpin structure when bound to α-BTX. The structures of the bound peptides and the homologous loop of acetylcholine binding protein, a soluble analog of AChR, are remarkably similar. Their superposition indicates that the toxin wraps around the binding-site loop, and in addition, binds tightly at the interface of two of the receptor subunits and blocks access of acetylcholine to its binding site. The procedure described in this article may serve as a paradigm for obtaining high-affinity peptides in biochemical systems that contain a ligand and a receptor molecule.

Keywords: α-Bungarotoxin; Acetylcholine receptor; Binding site; High-affinity peptide; Systematic residue replacement.

Abbreviations: AChR, acetylcholine receptor; α-BTX, α-bungarotoxin; Hap, high-affinity peptide; AChBP, acetylcholine-binding protein

☆ The sad news about John Edsall's passing reached us soon after his death. We are sorry that he was not able to celebrate his 100th birthday with us, but hope that he was able to enjoy the manuscripts that were sent to him directly, and that this dedicatory volume will be a fitting tribute.

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1. Introduction

In the present article, we summarize our joint efforts to design and synthesize a peptide that binds tightly and specifically to α-bungarotoxin (α-BTX), the highly toxic component of the venom of the snake *Bungarus multicinctus*. α-BTX binds to nicotinic acetylcholine receptor (AChR) at the post-synaptic membrane of the neuromuscular junction, and is a potent antagonist of this receptor. The synthesized high-affinity peptide (Hap) was found to protect mice against α-BTX lethality. Furthermore, determination of the three-dimensional structure of the complex between α-BTX and the high-affinity peptide enabled us to elucidate the mode by which α-BTX blocks access of acetylcholine to its AChR binding site.

The nicotinic acetylcholine receptor is a ligand-gated ion channel that is activated upon binding of the ligand acetylcholine [1,2]. α-Neurotoxins, such as α-BTX, bind specifically to AChR and exhibit high toxicity due to inhibition of AChR function at the neuromuscular junction [3]. It was therefore our expectation that synthetic peptides that bind α-BTX tightly might yield inhibitors that prevent the binding of the toxin to AChR, and hence result in potential lead compounds for effective antidotes against α-BTX poisoning. Moreover, since the α-neurotoxins bind AChR with very high affinity and specificity, understanding the nature of this binding is obviously of considerable importance in the study of the ligand-binding site of AChR.

The acetylcholine binding-site of AChR is located mainly at the α-subunit, in the vicinity of Cys192 and Cys193 [4–6], and partially overlaps the binding domain for α-BTX (*Torpedo* AChR residues α185–196, [7]). The tertiary structure of AChR has not yet been determined; its hydrophobic character hampers its crystallization for solving its X-ray structure, whereas its large size prevents NMR structure determination. There have been some unsuccessful attempts to crystallize the extracellular domain of the AChR α-subunit. Most recently, the X-ray structure of an acetylcholine binding protein (AChBP), a water-soluble homologue of the extracellular domain of the nicotinic AChR isolated from snail, has been solved [8].

2. Identification of a lead peptide, selected from a random combinatorial peptide library, that binds α-BTX specifically

In attempts to obtain peptides that bind α-BTX, we have employed a combinatorial phage-display peptide library and identified a library lead-peptide (MRYYESSLKSYPD) that specifically binds α-BTX and inhibits its binding to AChR with a concentration giving 50% inhibition (IC_{50} value) in the low-micromolar range [9]. This peptide contains the motif YYXSS, which is homologous to the AChR consensus motif YYXCC, located at the putative ligand binding-site. This peptide was thus chosen as a lead compound in our search for peptides that bind α-BTX with high affinity.

3. The structure of the complex between α-BTX and the library lead peptide as determined by two-dimensional ^{1}H-NMR

The structure of the complex between α-BTX and the library lead peptide was determined using two-dimensional ^{1}H-NMR spectroscopy [10] (Fig. 1). The bound peptide was found to adopt a globular conformation around a hydrophobic core created by a side chain of Tyr11 of the peptide, whereas the free peptide in solution was characterized by a rather random conformation.

The amino acid residues that bind tightly with α-BTX, or for which the side chains interact internally with other residues in the peptide, were identified. These are Arg2, Tyr3, Tyr4, Glu5, Ser7, Leu8 and Tyr11 [10].

Additional information as to amino acid residues of the AChR that may be important for binding to α-BTX came from a previous comparative sequence analysis of the AChR ligand-binding site from animal species that are sensitive (mouse, *Torpedo*, chick) or resistant (snake, mongoose) to α-BTX [11–13]. Such an analysis demonstrated major amino-acid differences between sensitive and resistant animal species in four residue positions, 187, 189, 194 and 197 of the AChR α-subunit [12]. A more recent study [14], employing point mutations in the AChR α-subunit of the mongoose (resistant to α-BTX) and comparing them to the mouse α-subunit (sensitive to α-BTX),

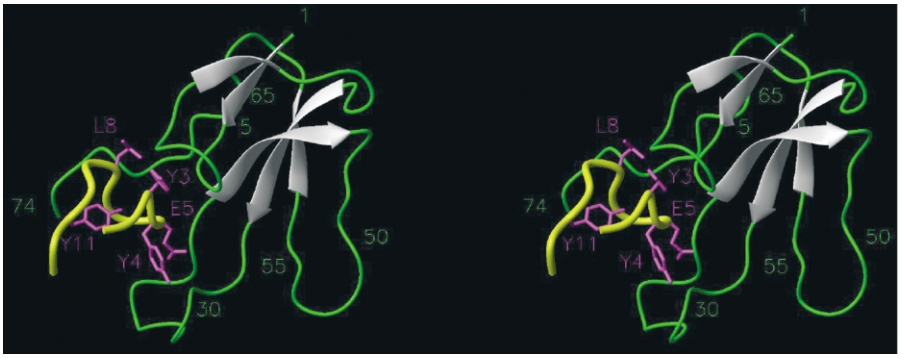

Fig. 1. Stereoscopic view of the complex of α-BTX with the library-lead peptide as determined by NMR spectroscopy. α-BTX residues are shown in gray (β-sheet regions) and green, and the peptide backbone is shown in yellow. Side chains of peptide residues interacting with the toxin (Y3, Y4, E5 and L8), and forming the hydrophobic core of the peptide (Y11), are shown in pink. The figure was generated using the program MOLMOL [26], and is reprinted from Proc. Natl. Acad. Sci. USA 94, T. Scherf, M. Balass, S. Fuchs, E. Katchalski-Katzir, J. Anglister, Three-dimensional solution structure of the complex of α-bungarotoxin with a library-derived peptide, 6059–6064, Copyright (1997), with permission from the National Academy of Sciences, USA.

led to the definition of two subsites at the α-BTX binding-domain of AChR that are important for binding of the toxin: the proline subsite, which is critical for binding and includes Pro194 and Pro197; and the aromatic subsite, which includes residues at positions 187 and 189.

4. Design and synthesis of peptides that bind α-BTX with high affinity

In view of the information gathered thus far, we decided to design and prepare peptides that bind α-BTX with high affinity and inhibit its interaction with AChR, by taking into consideration the structural data available (NMR information) and the functional biological information on the system studied. In fact, it was expected that the novel information to be acquired during this procedure would shed new light on the nature of the AChR residues that are responsible for binding of α-BTX and for determining the biological specificity of this interaction.

In an attempt to design peptides with increased affinity to α-BTX, we designed and synthesized a new peptide library based on systematic single-amino-acid replacement of some of the residues of the library lead peptide [15]. Taking into consideration NMR information from the structural analysis of the complex of α-BTX with the library lead peptide, replacements were introduced only at positions that do not contribute to α-BTX binding, nor to intra-bound-peptide interactions. Thus, in this new library of peptides, replacements were carried out at positions 1, 6, 9, 10, 12 and 13 (see Table 1).

With the object of reducing the number of prepared peptides, the 20 natural amino acids were categorized according to the chemical nature of their side chains, as well as their classification as proposed elsewhere [16]. Six groups of amino acids were suggested ([15]; see Fig. 2) as follows: amino acids with hydrophobic side-chains (Ile, Val, Leu); amino acids with aromatic side chains (Phe, Trp, Tyr); amino acids with positively charged side chains (Lys, Arg, His); and amino acids with negatively charged side-chains, or their corresponding amides capable of hydrogen bonding (Asp, Glu, Asn, Gln). The fifth group consisted of amino acids with the smallest side chain (Gly and Ala), and the sixth group was represented by proline alone, since it affects the backbone conformation in a unique way. Cysteine has an unusual structural role in proteins, in which a disulfide bond with a remote cysteine residue is created. It was therefore excluded from the six categories because we preferred not to involve cyclic peptides in the design. Serine, threonine and methionine were also excluded in most cases.

Table 1
Inhibition of the binding of α-BTX to *Torpedo* AChR by the library-lead peptide and by peptides 1–38, designed by systematic residue replacement of the library-lead peptide

Peptide	Position													IC_{50} (M)
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Library	M	R	Y	Y	E	S	S	L	K	S	Y	P	D	3.3×10^{-7}
1	**L**	R	Y	Y	E	S	S	L	K	S	Y	P	D	2.9×10^{-7}
2	**Y**	R	Y	Y	E	S	S	L	K	S	Y	P	D	1.0×10^{-6}
3	**K**	R	Y	Y	E	S	S	L	K	S	Y	P	D	5.0×10^{-6}
4	**D**	R	Y	Y	E	S	S	L	K	S	Y	P	D	5.0×10^{-6}
5	**G**	R	Y	Y	E	S	S	L	K	S	Y	P	D	5.0×10^{-6}
6	**P**	R	Y	Y	E	S	S	L	K	S	Y	P	D	1.5×10^{-7}
7	M	R	Y	Y	E	**L**	S	L	K	S	Y	P	D	5.0×10^{-6}
8	M	R	Y	Y	E	**Y**	S	L	K	S	Y	P	D	1.1×10^{-7}
9	M	R	Y	Y	E	**K**	S	L	K	S	Y	P	D	5.0×10^{-6}
10	M	R	Y	Y	E	**D**	S	L	K	S	Y	P	D	No inhibition
11	M	R	Y	Y	E	**T**	S	L	K	S	Y	P	D	5.0×10^{-6}
12	M	R	Y	Y	E	**G**	S	L	K	S	Y	P	D	5.0×10^{-6}
13	M	R	Y	Y	E	**P**	S	L	K	S	Y	P	D	No inhibition
14	M	R	Y	Y	E	S	**T**	L	K	S	Y	P	D	1.0×10^{-5}
15	M	R	Y	Y	E	S	**Y**	L	K	S	Y	P	D	No inhibition
16	M	R	Y	Y	E	S	**N**	L	K	S	Y	P	D	1.0×10^{-5}
17	M	R	Y	Y	E	S	S	L	**L**	S	Y	P	D	3.3×10^{-7}
18	M	R	Y	Y	E	S	S	L	**F**	S	Y	P	D	5.0×10^{-6}
19	M	R	Y	Y	E	S	S	L	**R**	S	Y	P	D	1.0×10^{-6}
20	M	R	Y	Y	E	S	S	L	**D**	S	Y	P	D	1.5×10^{-7}
21	M	R	Y	Y	E	S	S	L	**S**	S	Y	P	D	5.0×10^{-6}
22	M	R	Y	Y	E	S	S	L	**G**	S	Y	P	D	5.0×10^{-6}
23	M	R	Y	Y	E	S	S	L	**P**	S	Y	P	D	No inhibition
24	M	R	Y	Y	E	S	S	L	K	**L**	Y	P	D	1.0×10^{-5}
25	M	R	Y	Y	E	S	S	L	K	**Y**	Y	P	D	5.0×10^{-6}
26	M	R	Y	Y	E	S	S	L	K	**K**	Y	P	D	1.0×10^{-5}
27	M	R	Y	Y	E	S	S	L	K	**D**	Y	P	D	5.0×10^{-6}
28	M	R	Y	Y	E	S	S	L	K	**G**	Y	P	D	2.1×10^{-7}
29	M	R	Y	Y	E	S	S	L	K	**P**	Y	P	D	3.2×10^{-8}
30	M	R	Y	Y	E	S	S	L	K	S	Y	**L**	D	5.0×10^{-6}
31	M	R	Y	Y	E	S	S	L	K	S	Y	**Y**	D	5.0×10^{-6}
32	M	R	Y	Y	E	S	S	L	K	S	Y	**K**	D	5.0×10^{-6}
33	M	R	Y	Y	E	S	S	L	K	S	Y	**D**	D	5.0×10^{-6}
34	M	R	Y	Y	E	S	S	L	K	S	Y	**G**	D	5.0×10^{-6}
35	M	R	Y	Y	E	S	S	L	K	S	Y	P	**F**	5.0×10^{-6}
36	M	R	Y	Y	E	S	S	L	K	S	Y	P	**K**	5.0×10^{-5}
37	M	R	Y	Y	E	S	S	L	K	S	Y	P	**E**	1.4×10^{-7}
38	M	R	Y	Y	E	S	S	L	K	S	Y	P	**G**	5.0×10^{-6}

Amino acid residues that were replaced in the library-lead peptide are in bold. IC_{50} data are from [15].

Based on these considerations, a series of 38 peptides was prepared by systematic residue replacement at positions 1, 6, 9, 10, 12 and 13 of the library lead peptide, one at a time, with one representative residue of each of the six categories (see Table 1; [15]). Three replacements were also introduced at position Ser7, despite its participation in intra-peptide interaction. The inhibition of the binding of α-BTX to *Torpedo* AChR by the newly synthesized peptides was measured, and the IC_{50} values were compared with that of the original library lead peptide (3.3×10^{-7} M). Interestingly, large differences in inhibitory potency of some of the peptides were observed (Table 1), although all

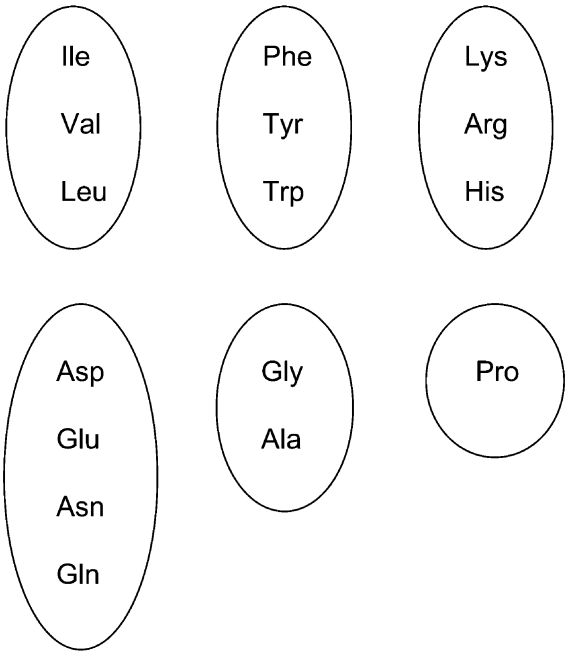

Fig. 2. Suggested categorization of natural amino acids according to the chemical nature of their side chains. The figure is reprinted from Chemistry and Biology 8, R. Kasher, M. Balass, T. Scherf, M. Fridkin, S. Fuchs, E. Katchalski-Katzir, Design and synthesis of peptides that bind α-bungarotoxin with high affinity, 147–155, Copyright (2001), with permission from Elsevier Science.

of the peptides within this series differ from each other by only one residue. As shown in Table 1, one peptide (number 29) exhibited one order of magnitude increase in the inhibitory activity ($IC_{50}=3.2\times10^{-8}$ M) as compared with that of the library lead peptide. Interestingly, this peptide represents a replacement of Ser10 by Pro, which is the residue present at this corresponding position in the neuronal α_7 AChR (see later).

Additional series of peptides were synthesized based on data available from a comparative analysis of amino acid sequences in the AChR binding site of animal species that are sensitive or resistant to α-BTX. We assumed that incorporation into the library lead peptide of residues uniquely present in the sensitive animal species might yield peptides with increased affinity for α-BTX. As reported earlier [11–13] and mentioned above, Trp187 of AChR is important for α-BTX binding, since it is present in the α-subunits of AChRs that bind α-BTX (mouse, chick, *Torpedo*) and not in AChRs of non-sensitive species (hedgehog, cobra, mongoose). In view of the importance of Trp187 for α-BTX binding, we prepared a peptide in which Met1 is replaced by Trp (peptide 39, Table 2) to mimic the aromatic subsite (positions 187 and 189) of AChR. A second replacement based on the biological data was of Ser10 by proline, to mimic the proline subsite of neuronal AChR. Peptides 39 and 29 indeed showed IC_{50} values of

Table 2
Inhibition of binding of α-BTX to *Torpedo* AChR by peptides 39–47 designed on the basis of biological information

Peptide	Position														IC_{50} (M)
	1	2	3	4	5	6	7	8	9	10	11	12	13		
Library	M	R	Y	Y	E	S	S	L	K	S	Y	P	D		3.3×10^{-7}
39	**W**	R	Y	Y	E	S	S	L	K	S	Y	P	D		3.5×10^{-8}
40	M	**V**	Y	Y	E	S	S	L	K	S	Y	P	D		5.0×10^{-6}
41	M	R	**F**	Y	E	S	S	L	K	S	Y	P	D		5.0×10^{-6}
42	M	R	**W**	Y	E	S	S	L	K	S	Y	P	D		No inhibition
43	M	R	Y	Y	E	S	S	**P**	K	S	Y	P	D		5.0×10^{-5}
44	M	R	Y	Y	E	S	S	**E**	K	S	Y	P	D		5.0×10^{-5}
45	M	R	Y	Y	E	S	S	**K**	K	S	Y	P	D		No inhibition
29	M	R	Y	Y	E	S	S	L	K	**P**	Y	P	D		3.2×10^{-8}
46	M	R	Y	Y	E	S	S	**P**	**T**	**T**	**P**	**Y**	**L**	**D**	No inhibition
47	**W**	**V**	**F**	**Y**	**S**	**C**	**C**	L	K	S	Y	P	D		No inhibition
Torpedo	W	V	Y	Y	T	C	C	P	D	T	P	Y	L	D	

Amino acid residues that were replaced in the library-lead peptide are in bold. IC_{50} data are from [15].

Table 3
Inhibition of binding of α-BTX to AChR by peptides 48–56 obtained by two or more amino-acid residue substitutions of the library-lead peptide, and by peptides 57–58 derived from the α-subunit of muscle AChR (α1) and neuronal AChR (α7)

Peptide	Position														IC_{50} (M)
	1	2	3	4	5	6	7	8	9	10	11	12	13		
Library	M	R	Y	Y	E	S	S	L	K	S	Y	P	D		3.3×10^{-7}
48	**W**	R	Y	Y	E	S	S	L	K	**P**	Y	P	D		1.0×10^{-8}
49	**W**	R	Y	Y	E	S	S	L	**D**	**P**	Y	P	D		3.8×10^{-9}
50 (Hap1)	**W**	R	Y	Y	E	S	S	L	**E**	**P**	Y	P	D		2.0×10^{-9}
51	**W**	R	Y	Y	E	S	S	**K**	**E**	**P**	Y	P	D		5.8×10^{-8}
52	**W**	R	Y	Y	E	**Y**	S	L	**D**	**P**	Y	P	D		1.6×10^{-9}
53	**W**	R	Y	Y	E	S	S	L	**D**	**P**	Y	P	E		4.8×10^{-9}
54 (Hap2)	**W**	R	Y	Y	E	S	S	L	**L**	**P**	Y	P	D		1.9×10^{-9}
55	M	R	Y	Y	E	**C**	**C**	L	K	S	Y	P	D		3.3×10^{-8}
56	**W**	R	Y	Y	E	**C**	**C**	L	**D**	**P**	Y	P	D		1.9×10^{-9}
57, α1 (muscle)	W	V	Y	Y	T	C	C	P	D	T	P	Y	L	D	2.6×10^{-8}
58, α7 (neuronal)	E	K	F	Y	E	C	C	K	E	P	Y	P	D		1.8×10^{-5}

Amino acid residues that were replaced in the library-lead peptide are in bold. IC_{50} data are from [15].

3.5×10^{-8} and 3.2×10^{-8} M, respectively, i.e. one order of magnitude better than that of the original library lead peptide (3.3×10^{-7} M; Table 2).

In an attempt to increase further the inhibitory potency of the peptides, we designed a new series of peptides having two or more replacements. Peptides 29 and 39, encompassing single replacements of Ser10Pro and Met1Trp, were the most potent inhibitors of α-BTX binding to AChR thus far. Accordingly, we prepared a peptide in which the corresponding two amino-acid replacements were carried out concomitantly. The resulting peptide (peptide 48, Table 3) with the double replacement has $IC_{50}=1\times10^{-8}$ M, which represents a 30-fold increase in the inhibitory potency relative to the library lead peptide ($IC_{50}=3.3\times10^{-7}$ M), and a three-fold affinity increase compared to the peptides with single replacements (peptides 29 and 39). It thus indicates that the affinity of the peptide for α-BTX could possibly be further improved by concomitantly introducing numerous replacements into the library lead peptide. We therefore prepared more peptides derived from peptide 48, with additional replacements (see Table 3). This study [15] resulted in peptides that bind α-BTX with high affinity and inhibit its binding to AChR with $IC_{50}=2\times10^{-9}$ M. As shown (Table 3), the inhibitory potency of the high-affinity peptides is stronger by at least two orders of magnitude than inhibition obtained by the original phage library-lead peptide or by a 13-mer peptide derived from the binding site domain of AChR (α187–199) [9]. Peptides 49, 50, 52, 53 and 56, in which Lys9 in the lead peptide was replaced by Asp or Glu (which are the residues present at the corresponding position in neuronal α7 AChR), exhibited IC_{50} values of $1.6–3.8\times10^{-9}$ M. Interestingly, replacing Lys9 by Leu (Table 3, peptide 54) also resulted in increased affinity ($IC_{50}=1.9\times10^{-9}$ M).

The synthetic peptides depicted in Table 3 with IC_{50} values in the nanomolar range represent the best inhibitors of α-BTX binding to AChR. These peptides are significantly better inhibitors than the analogous peptides derived from natural amino-acid sequences (residues 187–199) of either muscle (α_1) or neuronal (α_7) AChR ([9] and Table 3, peptide 58). It should be noted that even a 14-mer peptide corresponding to residues 187–200 of the α-subunit of muscle AChR that includes Asp200 ($IC_{50}=2.6\times10^{-8}$ M, M. Balass, unpublished results; and peptide 57, Table 3 [15]) has an IC_{50} value that represents a one-order-of-magnitude weaker inhibitory potency than that of peptides 49, 50, 52, 53, 54 and 56 (nanomolar range; Table 3). Asp200 of muscle receptors aligns with Asp199 in the neuronal AChR and Asp13 in the lead peptide. Thus, the high-affinity peptides exhibit

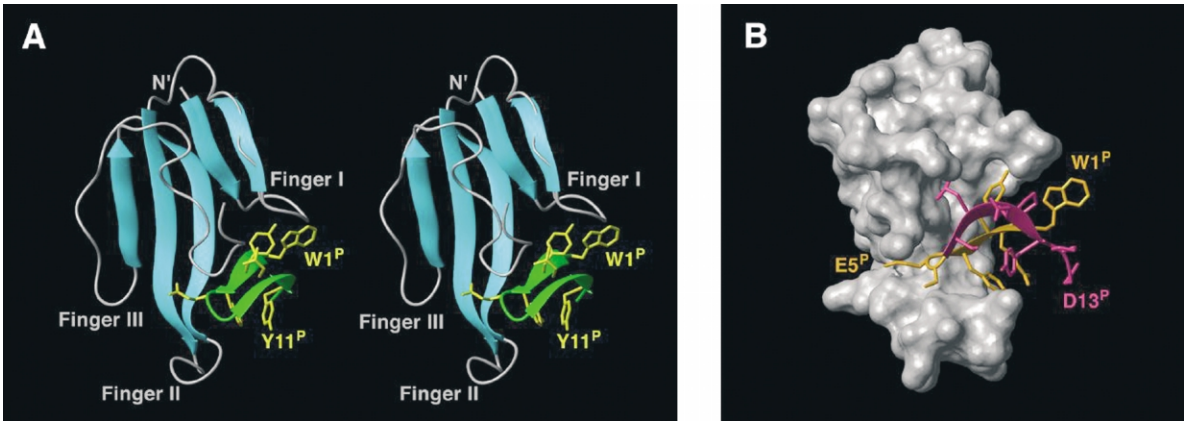

Fig. 3. Solution structure of the Hap1/α-BTX complex. (a) Stereoscopic view of the complex: α-BTX backbone is shown in cyan (β-sheet regions) and in gray. Peptide backbone is shown in green; side chains of peptide residues W1, Y3, Y4, E5, L8 and Y11 are shown in yellow. (b) Surface view of the complex. α-BTX is shown in gray, peptide is shown in orange (residues 1–6) and pink. The figure is reprinted from Proc. Natl. Acad. Sci. USA 98, T. Scherf, R. Kasher, M. Balass, M. Fridkin, S. Fuchs, E. Katchalski-Katzir, A β-hairpin structure in a 13-mer peptide that binds α-bungarotoxin with high affinity and neutralizes its toxicity, 6629–6634, Copyright (2001), with permission from the National Academy of Sciences, USA.

inhibitory potency that is better than any known peptide derived from the binding site of AChR, and is equal to that of the whole α-subunit of AChR [17]. Our results thus show that the approach of systematic residue replacement yielded peptides with high affinity that could not have been achieved by the preparation of peptides corresponding to the amino acid sequence of the α-BTX binding site of AChR.

For further studies, Hap1 was chosen as the representative high-affinity α-BTX-binding peptide (peptide 50, Table 3). In a set of in vivo preliminary studies, we showed that when Hap1 is injected into mice under appropriate experimental conditions, it is capable of neutralizing the toxic effect of α-BTX [15].

5. A β-hairpin structure of Hap1 when complexed with α-BTX

The structure of the complex between α-BTX and the 13-mer peptide Hap1 (Table 3), which binds the toxin with high affinity, has been solved by ^{1}H-NMR spectroscopy [18].

A schematic ribbon diagram of the NMR-derived structure of the bound toxin in its complex with Hap1 is shown in Fig. 3. The bound peptide folds into a β-hairpin structure created by two antiparallel β-strands (Fig. 4), which combine with the existing triple-stranded β-sheet of the toxin to form a five-stranded, intermolecular, antiparallel β-sheet. Peptide residues Y3, E5 and L8 have the highest intermolecular contact area, indicating their importance in the binding of α-BTX. W1, R2 and Y4 also contribute significantly to the binding. A large number of characteristic hydrogen bonds, as well as electrostatic and hydrophobic interactions, are observed in the complex.

The α-BTX structure illustrates the characteristic element of the three-finger toxin family: three long fingers and a C-terminal tail protrude from the tangle-like region of the molecule (Fig. 3). Fingers II and III combine to form a triple-stranded anti-parallel β-sheet element. It should be indicated that, in the present structure, the two β-strands that form the second finger of α-BTX stretch further than in the lead peptide/α-BTX complex [10].

The β-hairpin structure of the bound peptide is formed by two antiparallel β-strands, consisting of residues 2–4 and 7–11 of the peptide (Fig. 4). The two strands are connected by an elongated loop characterized by several short-range distances (Y4/S6, Y4/S7 and E5/S7), as revealed by NMR.

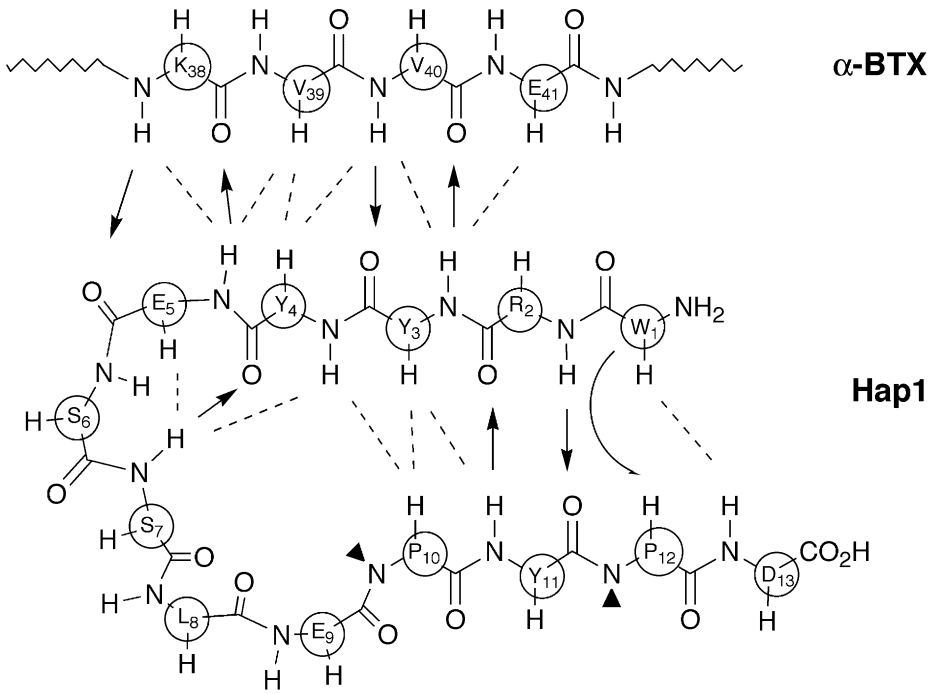

Fig. 4. Schematic representation of intra- and inter-molecular β-sheet interactions between Hap1 and residues 38–41 of α-BTX as found by NMR. One-letter code of each residue is circled at the ᵅC position. Dashed lines indicate inter-strand NOEs, arrows show hydrogen bonds and the curved arrow represents interactions in which side-chain atoms are involved. The figure is reprinted from Proc. Natl. Acad. Sci. USA 98, T. Scherf, R. Kasher, M. Balass, M. Fridkin, S. Fuchs, E. Katchalski-Katzir, A β-hairpin structure in a 13-mer peptide that binds α-bungarotoxin with high affinity and neutralizes its toxicity, 6629–6634, Copyright (2001), with permission from the National Academy of Sciences, USA.

The fact that a short peptide (Hap1) in its bound form has such a well-organized and a well-defined structural element, which exists both intra- and inter-molecularly, seems to be a unique phenomenon. Frequently the conformation of a peptide bound to a protein consists of one or more β-turns [19,20]. In other cases, the bound peptide adopts a β-hairpin-type conformation [21–23]. However, the formation of a short stretch of anti-parallel β-sheet not only within the bound peptide, but also between the peptide and the protein, represents a novel structural motif, as previously reported [24]. It was thus assumed that this rather unique structure accounts for most of the increased binding affinity of Hap1 to α-BTX.

A careful analysis of the amino acid sequence of Hap1, in comparison with muscle as well as neuronal AChR sequences, reveals similarities in all residues, except for positions 6, 7 and 8 of the peptide (Table 3). W1 that corresponds to position 187 of muscle AChR (Table 3) contributes, through its side chain, to the formation of both intermolecular contacts (hydrophobic interactions and hydrogen bonding with A7, T8 and S9 of α-BTX), as well as intra-peptide interactions. The presence of Pro10 and Pro12 seems to represent the proline subsite of muscle AChR that was shown to be of critical importance in establishing the affinity of the receptor to the toxin [14]. Hydrophobic interactions of Trp1 and Pro10 provide further stabilization of the bound peptide by holding together the two peptide ends. Finally, it is pertinent to note that Hap1 has a leucine residue at position 8, which was shown by NMR to play a major role in the binding and to interact directly with several toxin residues. L8 protrudes from the

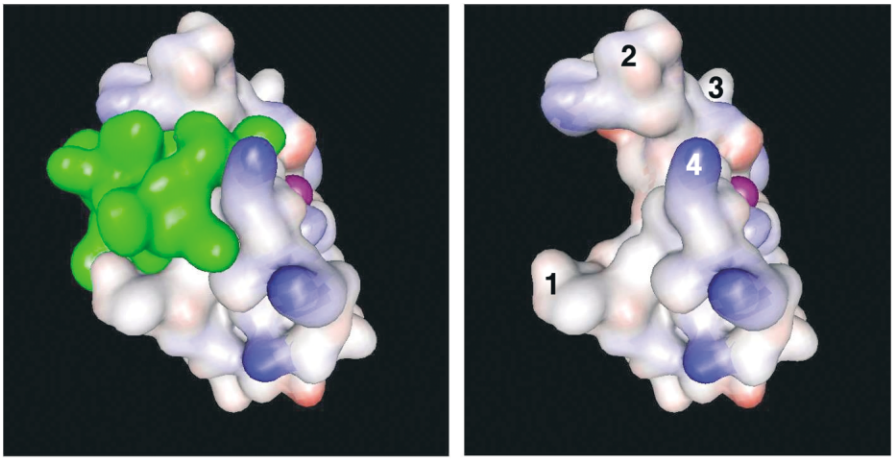

Fig. 5. Three-dimensional surface drawing of the structure of α-BTX/Hap2 complex, determined by X-ray diffraction. Color of the α-BTX corresponds to the electrostatic charge, with blue positive and red negative. (a) α-BTX/Hap2 complex; Hap2 is shown in green. (b) α-BTX with Hap2 removed. α-BTX fingers are labeled 1–4. The figure is reprinted from Neuron 32, M. Harel, R. Kasher, A. Nicolas, J.M. Guss, M. Balass, M. Fridkin, A.B. Smit, K. Brejc, T.K. Sixma, E. Katchalski-Katzir, J.L. Sussman, S. Fuchs, The binding site of acetylcholine receptor as visualized in the X-ray structure of a complex between α-bungarotoxin and a mimotope peptide, 265–275, Copyright (2001), with permission from Elsevier Science.

β-hairpin tongue (Fig. 3), raising the possibility that it may mimic a leucine residue of the receptor [1] outside its known binding region (α184–200), and thus forming a conformational epitope.

6. The structure of the binding site of acetylcholine receptor as visualized in the X-ray structure of Hap2/α-BTX complex

In parallel to the studies of the Hap1/α-BTX complex in solution, an attempt was made to obtain well-diffracting crystals of the complex and to elucidate its 3D structure by X-ray crystallography. We were successful in obtaining such crystals of the complex of α-BTX with Hap2 (WRYYESSLLPYPD, Table 3), and have determined the crystal structure at 1.8-Å resolution [25]. The structure obtained (Fig. 5) agrees well with the NMR findings described in Section 5. This high-resolution structure permitted us to observe in detail the snug fit of the peptide to the toxin. It adopts, as was observed in the NMR studies, a β-hairpin conformation. Of particular interest was the finding that the structures of the bound peptide and the homologous loop of acetylcholine-binding protein (AChBP) (an analogue of the soluble extracellular domain of acetylcholine receptor [8]) are remarkably similar. Based on the superposition of the bound peptide and the corresponding loop of AChBP, it was possible to build a molecular model of the interaction of α-BTX with the AChBP and, by analogy, to visualize the interaction of α-BTX with AChR. In this detailed model complex (Fig. 6), α-BTX wraps around the receptor-binding site loop and, in addition, binds tightly at the interface of two of the receptor subunits, where it inserts a finger into the ligand-binding site, thus blocking access to the acetylcholine binding site and explaining its strong antagonistic activity.

The detailed conformational analysis presented in Fig. 7 shows that Tyr3 of Hap2 forms a snug fit into a loop region of α-BTX. The formation of two H-bonds from its hydroxyl to residues Thr8 and Ile11 of α-BTX makes the tyrosine at that position an ideal candidate for forming binding interactions with α-BTX. Indeed, this tyrosine is known to play a crucial role in α-BTX binding.

Muscle (α_1) or neuronal (α_7) AChRs that bind α-BTX all have a Tyr, or sometimes Phe, at

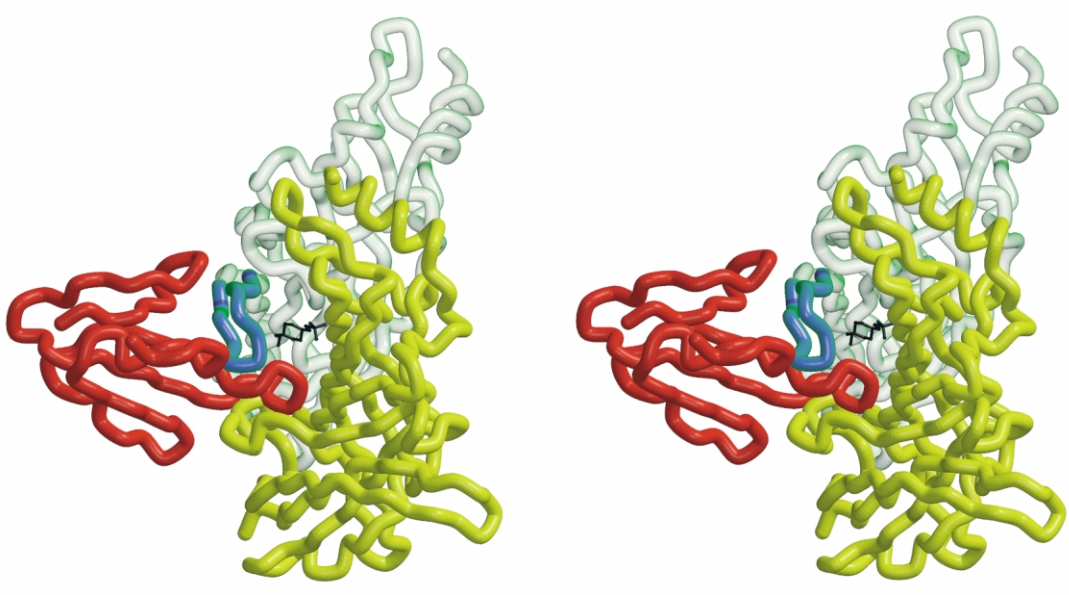

Fig. 6. A stereo view of the combined model of α-BTX/Hap2 (*red and blue*) [25] and AChBP structure [8] with subunit A in green and subunit B in yellow, showing the insertion of loop 2 of α-BTX into the interface of the two subunits. Hap2, which overlaps the 182–193-loop of AChBP is shown in blue. A positively charged HEPES molecule (*black stick figure*) shows the location of the acetylcholine binding site and the blockage of passage to this site caused by binding of the toxin. The figure is reprinted from Neuron 32, M. Harel, R. Kasher, A. Nicolas, J.M. Guss, M. Balass, M. Fridkin, A.B. Smit, K. Brejc, T.K. Sixma, E. Katchalski-Katzir, J.L. Sussman, S. Fuchs, The binding site of acetylcholine receptor as visualized in the X-ray structure of a complex between α-bungarotoxin and a mimotope peptide, 265–275, Copyright (2001), with permission from Elsevier Science.

position 189 (the corresponding position of Tyr3 in Hap2). It should be noted that there are no significant differences in the binding properties of AChRs containing Tyr or Phe at position 189. However, replacement of Tyr3 with Phe in the library lead peptide resulted in a decrease in potency by one order of magnitude [15] (see Table 2). Muscle AChRs of animal species that are resistant to α-BTX (e.g. snake and mongoose), or neuronal AChRs that do not bind α-BTX, have a non-aromatic residue at this position [13]. The receptor complexes containing these subunits do not bind α-BTX. In contrast, the neuronal α_7 receptor, which has a Tyr at this position, does bind α-BTX. The X-ray structure shows that Tyr3 cannot be replaced by Lys without clashes in the α-BTX binding cavity. This explains the resistance of neuronal α_2, α_3 and α_4 AChRs to α-BTX. This is also supported by our previous findings that the Tyr3Lys replacement in the library lead peptide or in the corresponding α_1 and α_7 peptides prohibits them from binding α-BTX [9].

Finally, it is pertinent to note that detailed X-ray analysis of the Hap2/α-BTX complex reveals the importance of Trp1 and Pro10 (Trp187 and Pro196 in AChR) in determining the high affinity of Hap2 towards the toxin. Trp1 at the N-terminus of the peptide adds two side-chain interactions with the toxin between the indole N^{ε} atom and the carbonyl oxygen of residues 6 and 7 of α-BTX. Indeed, residue 187 is Trp in the α-subunit of *Torpedo* AChR. The substitution of Ser10 of the lead peptide to Pro in Hap2 adds to the stability of the binding conformation of the peptide. Proline fits well in a hydrophobic pocket. It is situated at the core of the Hap2 intramolecular β-sheet, and makes stacking interactions with Tyr3 and edge-on interactions with Trp1.

7. Concluding remarks

In view of our success in the design and synthesis of peptides that bind α-BTX with high

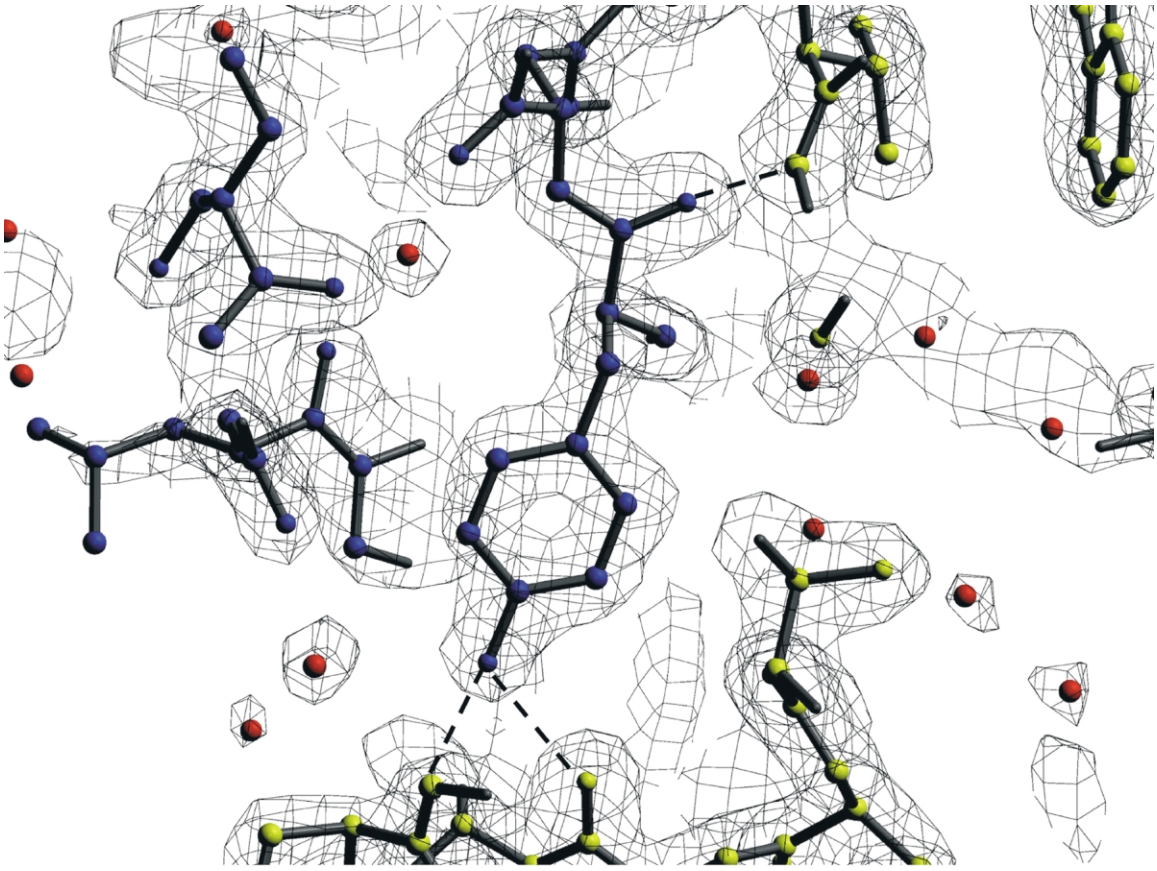

Fig. 7. The snug fit of Tyr3 of Hap2 (*cyan*) into a loop region of α-BTX (*yellow*), in the α-BTX/Hap2 complex determined by X-ray diffraction. Short interactions are shown as dashed lines and water molecules are shown in magenta. The composite omit map [27] is drawn at 1.7σ. The figure is reprinted from Neuron 32, M. Harel, R. Kasher, A. Nicolas, J.M. Guss, M. Balass, M. Fridkin, A.B. Smit, K. Brejc, T.K. Sixma, E. Katchalski-Katzir, J.L. Sussman, S. Fuchs, The binding site of acetylcholine receptor as visualized in the X-ray structure of a complex between α-bungarotoxin and a mimotope peptide, 265–275, Copyright (2001), with permission from Elsevier Science.

affinity, we proposed a general approach that we termed 'systematic residue replacement' (SRR strategy) for the design and synthesis of high-affinity peptides in any biochemical system that contains a ligand and a receptor molecule. The following steps should be carried out to achieve the desired goals:

1. By use of combinatorial chemical or biological random-peptide libraries, a lead peptide should be detected that binds specifically, even with moderate affinity, to a receptor molecule.
2. 3D structure determination (by NMR or X-ray) of the complex between the receptor molecule and the lead peptide should then be carried out. If NMR or X-ray data are not available, then computer modeling could be used to provide structural information.
3. Restricted systematic-residue replacement of the lead peptide should follow thereafter, to yield secondary libraries of peptides. Residues that contribute significantly to inter- or intra-molecular interactions in the peptide/receptor complex should be conserved, while all other amino acid residues may be systematically replaced. To minimize the number of peptides to be prepared, the general categorization of the 20

natural amino acids suggested in Fig. 2 may be used.

4. Finally, the amino acid sequences of natural ligand-binding sites should be analyzed to identify the amino acid residues essential for binding with the ligand. The task can be accomplished by the synthesis of new, secondary peptide libraries incorporating the information acquired in steps 2–4.
5. Once high-affinity peptides are obtained, it is desirable to determine their three-dimensional structure when complexed with the ligand. Tight binding between the peptide and the ligand might often suggest mimicry of the binding site of the receptor.
6. Obviously, the in vivo activity of the high-affinity peptides may be tested to examine their applications as potential drug lead compounds.

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A personal note: One of us (EK-K) had the pleasure of working in Prof Edsall's laboratory at Harvard University for about a year (1949–1951). Edsall taught EK-K the use of light scattering techniques to determine the size and shape of biopolymers in solution, and it was by these techniques that EK-K was able to determine the kinetics of dimerization of mercaptalbumin in the presence of mercury chloride. It was a most exciting period in the life of EK-K, a young Israeli scientist who was introduced by Edsall to a galaxy of outstanding scientists interested in the structure and function of proteins. The encouragement provided by Edsall, his lucid mind, his readiness to be of help whenever asked, encouraged EK-K to dedicate a good part of his coming academic years to study the physico-chemical properties of amino acids, peptides and proteins. The friendly relations established between Edsall and EK-K were strengthened and continued up to his passing. Edsall was not only an outstanding scientist, but also a wonderful human being, personified in his decency, wisdom and moral integrity. As a matter of fact, EK-K and many of his colleagues greatly respected Edsall and considered him as a symbol of a perfect scientist and human being worthy of being emulated.

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