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Research Paper

Design and synthesis of peptides that bind α-bungarotoxin with high affinity

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Abstract

Background: α-Bungarotoxin (α-BTX) is a highly toxic snake venom α-neurotoxin that binds to acetylcholine receptor (AChR) at the neuromuscular junction, and is a potent inhibitor of this receptor. We describe the design and synthesis of peptides that bind α-BTX with high affinity, and inhibit its interaction with AChR with an IC_{50} of 2 nM. The design of these peptides was based on a lead peptide with an IC_{50} of 3×10^{-7} M, previously identified by us [M. Balass et al., Proc. Natl. Acad. Sci. USA 94 (1997) 6054] using a phage-display peptide library.

Results: Employing nuclear magnetic resonance-derived structural information [T. Scherf et al., Proc. Natl. Acad. Sci. USA 94 (1997) 6059] of the complex of α-BTX with the lead peptide, as well as structure–function analysis of the ligand-binding site of AChR, a systematic residue replacement of the lead peptide, one position at a time, yielded 45 different 13-mer peptides. Of these, two peptides exhibited a one order of magnitude increase in inhibitory potency in comparison to the lead peptide. The design of additional peptides, with two or three replacements, resulted in peptides that exhibited a further increase in inhibitory potency (IC_{50} values of 2 nM), that is more than two orders of magnitude better than that of the original lead peptide, and better than that of any known peptide derived from AChR sequence. The high affinity peptides had a protective effect on mice against α-BTX lethality.

Conclusions: Synthetic peptides with high affinity to α-BTX may be used as potential lead compounds for developing effective antidotes against α-BTX poisoning. Moreover, the procedure employed in this study may serve as a general approach for the design and synthesis of peptides that interact with high affinity with any desired biological target.

Keywords: α-bungarotoxin; Acetylcholine receptor; Systematic residue replacement

1. Introduction

The nicotinic acetylcholine receptor (AChR) is a ligand-gated ion channel and is activated by binding of acetylcholine. α-Neurotoxins such as α-bungarotoxin (α-BTX) bind specifically to AChR and exhibit high toxicity due to inhibition of the AChR function at the neuromuscular junction. Synthetic peptides that bind α-BTX may yield competitive inhibitors of α-BTX binding 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 binding is obviously of considerable importance in the study of the ligand-binding site of AChR.

The ligand-binding site of AChR is located mainly at the α-subunit, in the vicinity of Cys192 and Cys193 [3–5] and partially overlaps with the binding domain for α-BTX (*Torpedo* AChR residues 185–196) [6]. A synthetic peptide corresponding to the sequence of *Torpedo* α185–196 was found to bind agonists specifically and to inhibit the binding of α-BTX to AChR with an IC_{50} of 10^{-4} M [7]. A 13-mer peptide corresponding to residues 187–199 exhibited higher inhibition potency with an IC_{50} of 10^{-6} M [1].

The aim of the present study was to design and prepare

Abbreviations: AChR, acetylcholine receptor; α-BTX, α-bungarotoxin

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peptides that bind α-BTX with high affinity and inhibit its interaction with AChR, by taking into consideration the available structural data (nuclear magnetic resonance, NMR) and functional information on the system studied. It was expected that the information acquired during this procedure would shed new light on the nature of the AChR residues that are responsible for binding α-BTX and for determining the biological specificity of this interaction. Moreover, such an approach might lead to a general procedure for the design and synthesis of peptides having a desired highly specific biological activity.

In a previous study [1] we employed a combinatorial phage-display peptide library and identified a library-lead peptide (MRYYESSLKSYPD) that binds specifically α-BTX and inhibits its binding to AChR with an IC_{50} in the low micromolar range. This peptide contains the motif YYXSS that is homologous to the AChR consensus motif YYXCC, located at the ligand-binding site. The structure of the complex between α-BTX and the library-lead peptide was determined using two-dimensional ^{1}H-NMR [2]. 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 and/or interact internally with other residues in the peptide were identified. These are Arg2, Tyr3, Tyr4, Glu5, Ser6, Ser7, Leu8, Lys9, Ser10 and Tyr11 [2].

Additional information as to amino acid residues 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 [8–10]. Such an analysis demonstrated major amino acid differences between sensitive and resistant animal species, in four residues at positions 187, 189, 194 and 197 of the AChR α-subunit [9]. A more recent study [11], employing point-mutations in the α-subunit of mongoose (resistant to α-BTX) and comparing them to the α-subunit of mouse (sensitive to α-BTX) led to the definition of two subsites at the AChR-binding domain that are important for binding of α-BTX: the proline subsite, which is critical for binding and includes Pro194 and Pro197, and the aromatic subsite, which includes residues 187 and 189.

In the present study we describe the design and synthesis of a new peptide library based on adequate amino acid residue replacements of some of the residues of the library-lead peptide. In the design of this library, both structural and functional available data were taken into consideration. This study resulted in peptides that bind α-BTX with high affinity and inhibit its binding to AChR with an IC_{50} of 2 nM. The inhibition potency of the high affinity peptides is stronger by at least two orders of magnitude than the inhibitions obtained by the original phage library-lead peptide and also by a 13-mer peptide derived from the binding site domain of AChR (α187–199). The paradigm employed for this particular study can be used as a possible general procedure for the design and synthesis of desired biologically active peptides, that might present affinities and specificities even better than those of peptides derived from naturally occurring proteins.

2. Results

2.1. Systematic single amino acid residue replacement

In an attempt to design peptides with increased affinity to α-BTX we first built a new peptide library derived from the phage library-lead peptide (MRYYESSLKSYPD) [1] by systematic single amino acid residue replacement. In preparing this library we first took into consideration the information available from the NMR analysis of the complex of α-BTX with the library-lead peptide [2]. Table 1 summarizes the external and internal interactions of the library-lead peptide in its complex with α-BTX [2]. As

Table 1
Inter- and intra-molecular interactions in the complex of the library-lead peptide and α-BTX[a]

Lead-peptide residue	Contact area with α-BTX (Å^2)	Interacting α-BTX residues	Atom type involved in intra-peptide interactions[b]
M1	–		–
R2	46	V39	bb+sc ($^{\beta}CH_2$)
Y3	135	T6, A7, I11, V39, H68	bb+sc (Ar)
Y4	125	D30, R36, V39, V40	bb+sc ($^{\beta}CH_2$)
E5	104	K38, V39, V40, H68	bb
S6	–		bb
S7	32	H68, R72	bb+sc ($^{\gamma}OH$)
L8	97	I11, H68, K70, Q71	–
K9	–		bb
S10	–		bb
Y11	–		sc (Ar)
P12	–		–
D13	–		–

[a]The data were derived from the NMR solution structure of the complex [2].
[b]sc = side chain, bb = backbone, Ar = aromatic.

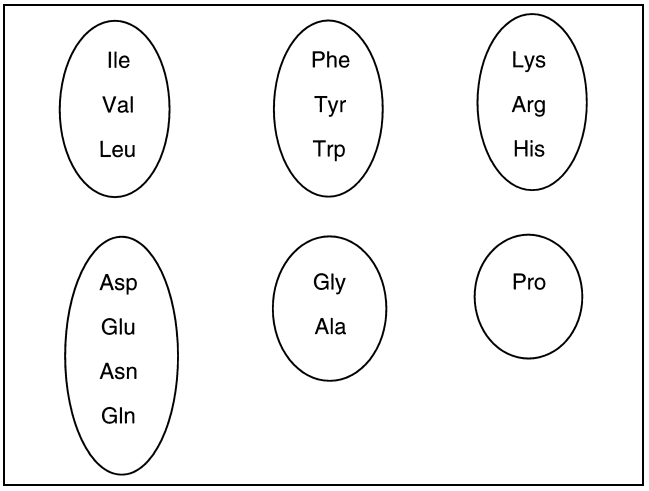

Fig. 1. Suggested categorization of natural amino acids according to the chemical nature of their side-chain.

shown in Table 1, residues Arg2, Tyr3, Tyr4, Glu5, Ser7 and Leu8 of the lead-peptide form large contact areas with α-BTX and were therefore conserved. In addition, in order to stabilize the conformation of the bound peptide in the complex, we retained residue Tyr11, which participates in intra-molecular interactions within the peptide. Residues Ser6, Lys9 and Ser10 contribute to intra-peptide interactions only by backbone atoms, and therefore these positions were not conserved. Thus, in this series of peptides, replacements were introduced at residues Met1, Ser6, Lys9, Ser10, Pro12 and Asp13.

With the object of reducing the number of prepared peptides to a few tens, the 20 natural amino acids were categorized according to the chemical nature of their side chains, as well as their classification as proposed elsewhere [12,13]. Six groups of amino acids were suggested (see Fig. 1) 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), 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, to reduce the number of peptides that is adequate to the multi-peptide synthesizer (see Section 5).

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 (Table 2). Three replacements were introduced also in position Ser7, despite its participation in α-BTX binding. Experiments on inhibition of the binding of α-BTX to *Torpedo* AChR by the newly synthesized peptides were carried out according to standard procedures (see Section 5). The IC_{50} values were calculated and compared with that of the original library-lead peptide that exhibited an IC_{50} value of 3.3×10^{-7} M. All peptides were first analyzed in their crude form (40–90% purity); peptides of special interest were purified by reversed-phase high performance liquid chromatography (HPLC), and their inhibition potencies were determined also in their purified form. Although replacements were made one position at a time, large differences in inhibitory potencies were observed between some of the peptides. As shown in Table 2, seven peptides retained the same or slightly higher inhib-

Table 2
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[a]

Position / *Peptide*	1	2	3	4	5	6	7	8	9	10	11	12	13	*IC_{50} (M)*
Library	M	R	Y	Y	E	S	S	L	K	S	Y	P	D	4.9×10^{-7}; $3.3\times10^{-7\#}$
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	1×10^{-6}
3	K	R	Y	Y	E	S	S	L	K	S	Y	P	D	5×10^{-6}
4	D	R	Y	Y	E	S	S	L	K	S	Y	P	D	5×10^{-6}
5	G	R	Y	Y	E	S	S	L	K	S	Y	P	D	5×10^{-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	5×10^{-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	5×10^{-6}
10	M	R	Y	Y	E	D	S	L	K	S	Y	P	D	No inhib.
11	M	R	Y	Y	E	T	S	L	K	S	Y	P	D	5×10^{-6}
12	M	R	Y	Y	E	G	S	L	K	S	Y	P	D	5×10^{-6}
13	M	R	Y	Y	E	P	S	L	K	S	Y	P	D	No inhib.
14	M	R	Y	Y	E	S	T	L	K	S	Y	P	D	1×10^{-5}
15	M	R	Y	Y	E	S	Y	L	K	S	Y	P	D	No inhib.
16	M	R	Y	Y	E	S	N	L	K	S	Y	P	D	1×10^{-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	5×10^{-6}
19	M	R	Y	Y	E	S	S	L	R	S	Y	P	D	1×10^{-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	5×10^{-6}
22	M	R	Y	Y	E	S	S	L	G	S	Y	P	D	5×10^{-6}
23	M	R	Y	Y	E	S	S	L	P	S	Y	P	D	No inhib.
24	M	R	Y	Y	E	S	S	L	K	L	Y	P	D	1×10^{-5}
25	M	R	Y	Y	E	S	S	L	K	Y	Y	P	D	5×10^{-6}
26	M	R	Y	Y	E	S	S	L	K	K	Y	P	D	1×10^{-5}
27	M	R	Y	Y	E	S	S	L	K	D	Y	P	D	5×10^{-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	6.1×10^{-8}; $3.2\times10^{-8\#}$
30	M	R	Y	Y	E	S	S	L	K	S	Y	L	D	5×10^{-6}
31	M	R	Y	Y	E	S	S	L	K	S	Y	Y	D	5×10^{-6}
32	M	R	Y	Y	E	S	S	L	K	S	Y	K	D	5×10^{-6}
33	M	R	Y	Y	E	S	S	L	K	S	Y	D	D	5×10^{-6}
34	M	R	Y	Y	E	S	S	L	K	S	Y	G	D	5×10^{-6}
35	M	R	Y	Y	E	S	S	L	K	S	Y	P	F	5×10^{-6}
36	M	R	Y	Y	E	S	S	L	K	S	Y	P	K	5×10^{-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	5×10^{-6}

[#]HPLC purified peptide.
[a]Amino acid residues that were replaced in the library-lead peptide are outlined.

Table 3
Alignment of amino acid sequence of the library-lead peptide in comparison with the sequence of the α-BTX-binding region of muscle AChR (position 187–200 in the α-subunit) from various animal species, and with the amino acid sequence of neuronal α-subunit (α_7) [11][a]

		1	2	3	4	5	6	7	8	9	10	11	12	13
Library-lead peptide		M	R	Y	Y	E	S	S	L	K	S	Y	P	D
Muscle AChR														
α-BTX binding	Source	187		189			192	193	194			197		199 200
++	*Torpedo*	**W**	V	**Y**	**Y**	T	C	C	**P**	D	T	**P**	Y	L D
++	Chicken	**W**	.	**Y**	**Y**	A	.	.	**P**	.	.	**P**	.	. .
++	Mouse	**W**	.	**F**	**Y**	S	.	.	**P**	T	.	**P**	.	. .
+	Human	S	.	T	**Y**	S	.	.	**P**	.	.	**P**	.	. .
-	Hedgehog	R	.	I	**Y**	A	.	.	**P**	S	.	**P**	.	. .
-	Cobra	S	.	N	**Y**	S	.	.	L	.	.	**P**	.	. .
-	Mongoose	N	.	T	**Y**	A	.	.	L	T	.	H	.	. .
Neuronal AChR		187		189			192	193	194			197	198	199 200
+	Rat α_7	E	K	F	Y	E	C	C	K	E	P	Y	P	D

[a]Dots represent amino acid residues that are identical to the corresponding positions in *Torpedo* AChR.

itory activity than that of the library-lead peptide (IC_{50} in the range of 1×10^{-7}–3.0×10^{-7} M). The activity of some peptides was lower by 1–2 orders of magnitude, and four peptides (No. 10, 13, 15 and 23) did not exhibit any detectable inhibition. One peptide (No. 29) exhibited one order of magnitude increase in the inhibitory activity (IC_{50} of 3.2×10^{-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).

It should be noted that replacement of Ser6 by Leu, Lys, Thr or Gly yielded peptides with inhibitory potencies of only one order of magnitude lower than that of the original peptide, indicating that this position does not contribute significantly to α-BTX binding. However, a replacement of this position by Asp or Pro resulted in peptides No. 10 and 13 with no detectable inhibition. The latter replacement (peptide 13) may result from alteration of the backbone conformation. Finally, substitution of Ser6 by Tyr (peptide 8, Table 2) resulted in a peptide having inhibitory potency similar to that of the library-lead peptide. It is of interest that the replacement of Ser7 by Tyr (No. 15, Table 2) abolished the inhibition. This effect might be explained by a steric hindrance caused by repulsions between the side chains of Tyr7 and Tyr11, since the side chains of the corresponding residues of the library-lead peptide (Ser7 and Tyr11) are directed towards the core of the peptide in its complex with α-BTX.

2.2. Single amino acid residue replacements based on amino acid sequences of the AChR ligand-binding site

Additional 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 an increased affinity for α-BTX. Table 3 summarizes the amino acid sequences of residues 187–200 of the α-subunit of several AChR species that are sensitive or resistant to α-BTX [10]. The library-lead peptide is described in alignment with the receptor sequences. As reported earlier [8–10] and depicted in Table 3, Trp187 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 focused on the corresponding position of the library-lead peptide (Met1). This methionine in the library-lead peptide does not participate in either external or internal molecular interactions when the peptide is in complex with α-BTX [2] (Table 1). Therefore, in an attempt to increase the affinity of the library-lead peptide for α-BTX, we prepared a peptide with Trp at position 1 (peptide 39, Table 4), replacing Met. Peptide 39 indeed showed an IC_{50} of 3.5×10^{-8} M, that is one order of magnitude better than that of the original library-lead peptide (3.3×10^{-7} M; Table 4).

Val188 of the α-subunit is a conserved residue in all receptor sequences (Table 3). This valine residue is probably not important for α-BTX binding, as it is present in both α-BTX-sensitive and -resistant species. To verify this point, we prepared a peptide that has Val at position 2 (peptide No. 40, Table 4). The inhibitory activity of peptide No. 40 was decreased by one order of magnitude in

Table 4
Inhibition of the binding of α-BTX to *Torpedo* AChR by peptides 39–47[a]

Peptide														*IC_{50} (M)*
Library	M	R	Y	Y	E	S	S	L	K	S	Y	P	D	4.9×10^{-7}; $3.3\times10^{-7\#}$
39	**W**	R	Y	Y	E	S	S	L	K	S	Y	P	D	7.9×10^{-8}; $3.5\times10^{-8\#}$
40	M	**V**	Y	Y	E	S	S	L	K	S	Y	P	D	5×10^{-6}
41	M	R	**F**	Y	E	S	S	L	K	S	Y	P	D	5×10^{-6}
42	M	R	**W**	Y	E	S	S	L	K	S	Y	P	D	No inhib.
43	M	R	Y	Y	E	S	S	**P**	K	S	Y	P	D	5×10^{-5}
44	M	R	Y	Y	E	S	S	**E**	K	S	Y	P	D	5×10^{-5}
45	M	R	Y	Y	E	S	S	**K**	K	S	Y	P	D	No inhib.
29	M	R	Y	Y	E	S	S	L	K	**P**	Y	P	D	6.1×10^{-8}; $3.2\times10^{-8\#}$
46	M	R	Y	Y	E	S	S	**P**	T	T	P	Y	L D	No inhib.
47	**W**	**V**	**F**	**Y**	**S**	**C**	**C**	**L**	**K**	**S**	**Y**	**P**	**D**	No inhib.
Torpedo	W	V	Y	Y	T	C	C	P	D	T	P	Y	L D	

[#]HPLC purified peptide.
[a]Amino acid residues that were replaced in the library-lead peptide are outlined.

Table 5
Inhibition of binding of α-BTX to AChR by peptides 48–56 obtained by two or more amino acid residue substitutions[a]

Peptide														*IC_{50} (M)*
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}; 1.0×10^{-8}#
49	W	R	Y	Y	E	S	S	L	D	P	Y	P	D	3.8×10^{-9}
50	W	R	Y	Y	E	S	S	L	E	P	Y	P	D	2.8×10^{-9}; 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	2.8×10^{-9}; 1.6×10^{-9}#
53	W	R	Y	Y	E	S	S	L	D	P	Y	P	E	4.8×10^{-9}
54	W	R	Y	Y	E	S	S	L	L	P	Y	P	D	3.8×10^{-9}; 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	2.9×10^{-9}; 1.9×10^{-9}#

#HPLC purified peptide.
[a]Amino acid residues that were replaced in the library-lead peptide are boxed.

comparison to the library-lead peptide, suggesting that Val at this position in AChR is probably not important for α-BTX binding, and verifying that Arg2 in the peptide does contribute to α-BTX binding, as reported previously from NMR studies [2].

As reported earlier, position 194 in the AChR α-subunit is occupied by proline in animal species sensitive to α-BTX, whereas it is occupied by leucine in both snake and mongoose, that are resistant species [8,9,11] (Table 3). Surprisingly, the phage library chose Leu in position 8 (correlated to AChR position 194), similar to that in species resistant to α-BTX. Moreover, Leu8 of the library-lead peptide had been shown to contribute significantly to α-BTX binding [2] (Table 1). We assumed that the similarity of position 8 in the peptide with position 194 in muscle AChR sequences of species that are resistant to α-BTX (both snake and mongoose have Leu at this position) is a coincidence. To verify this assumption, we prepared a peptide with Pro at position 8 (peptide 43, Table 4). The inhibitory activity of this peptide was two orders of magnitude lower than that of the library-lead peptide. Replacement of Leu8 by Lys (peptide 45, Table 4) yielded a peptide with no detectable binding, probably because of electrostatic repulsion, as this position faces Lys70 of α-BTX. Electrostatic repulsion might also be caused by the presence of two adjacent positively charged residues in peptide 45 (Lys8 and Lys9; see Table 4).

Alignment of residues 194–199 of the *Torpedo* muscle AChR with the C-terminal part (residues 8–13) of the library-lead peptide shows a poor sequence similarity (see Table 3). Furthermore, a peptide whose N-terminus contains seven amino acid residues of the library-lead peptide, followed by seven residues corresponding to amino acid residues 194–200 of the *Torpedo* muscle AChR, showed no inhibitory effect (peptide 46, Table 4). On the other hand, alignment of residues 8–13 of the lead peptide with residues 194–199 of the neuronal α_7 AChR that binds α-BTX, indicates a high sequence similarity (Table 3). We therefore decided to rely for this part of the peptide on the information available from neuronal α-BTX-binding AChRs (Table 3). Indeed, as mentioned above (Table 2), peptide 29, which contains Pro at position 10 replacing Ser in the library-lead peptide, exhibited an inhibition of α-BTX binding ($IC_{50} = 3.2\times10^{-8}$ M) that is 10-fold stronger in comparison to the library-lead peptide. Peptide 29 is characterized by four C-terminal residues (PYPD) identical to residues at positions 196–199 of the neuronal rat α_7 type AChR (Table 3). The high affinity of peptide 29 (Tables 2 and 4) may indicate that Pro196 of the neuronal rat α_7 AChR plays an important role in its interaction with α-BTX. Therefore, Pro196 and Pro198 might represent the proline subsite [11] of the neuronal α-BTX-binding AChRs.

2.3. Design of peptides having two or more replacements

In an attempt to further increase the inhibitory potency of the peptides, we prepared a third series of peptides based on the information obtained from peptides studied thus far. This third series was based on the assumption that a replacement of two residues or more in a single peptide might yield peptides with a further increase in affinity. Among the 47 peptides described in Tables 2 and 4 (the second series), peptides 29 and 39 were the most potent inhibitors of α-BTX binding to AChR. These peptides (No. 39 and 29) were obtained by single replacements of Met1Trp and Ser10Pro, respectively. Accordingly, we prepared a peptide in which the corresponding two amino acid replacements were carried out concomitantly. As shown in Table 5, the resulting peptide (peptide 48) with the double replacement has an IC_{50} of 1×10^{-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 three-fold increase in the affinity of either of the peptides with single replacements (peptides 29 and 39). It thus shows that the two replacements together, namely of Met1Trp and of Ser10Pro, did not interfere with each other, and suggests that the affinity of the peptide for α-BTX could possibly be further im-

Table 6
Inhibition of binding of α-BTX to *Torpedo* AChR by peptides derived from the α-subunit of muscle AChR (α_1), neuronal AChR (α_7) and combinations of the two

Peptide		IC_{50} (M)	Source type
57	W V Y Y T CCP D T P Y L D	2.6×10^{-8}	α_1 (muscle)
58	E K F Y E CCK E P Y P D	1.8×10^{-5}	α_7 (neuronal)
59	E K F Y E S S K E P Y P D	$>10^{-3}$	α_7 with Ser
60	E K F Y E S S P D T P Y L D	no inhibition	$\alpha_7+\alpha_1$
61	W V Y Y T S S K E P Y P D	1.5×10^{-5}	$\alpha_1+\alpha_7$
50	W R Y Y E S S L E P Y P D	2.0×10^{-9}[a]	third series

[a]HPLC purified peptide, see Table 5.

proved by introducing numerous replacements concomitantly into the library-lead peptide.

We then prepared more peptides derived from peptide 48, with additional replacements. The list of peptides synthesized in this series and their IC_{50} values for the inhibition of α-BTX binding to AChR are described in Table 5. As shown, several peptides with increased affinity for α-BTX (IC_{50} in the low-nanomolar range) were obtained. Peptides 49, 50, 52, 53 and 56, in all of which Lys9 in the lead peptide was replaced by Asp or Glu (which are the residues present at this position in neuronal α_7 AChR), exhibited an IC_{50} of $1.6–3.8\times10^{-9}$ M. This inhibition potency is more than two orders of magnitude better than that of the library-lead peptide. Interestingly, replacing Lys9 by Leu (Table 5, peptide 54) resulted also in increased affinity ($IC_{50} = 1.9\times10^{-9}$ M). Notably, introducing two adjacent cysteine residues replacing Ser6 and Ser7 (peptide 56) did not result in a binding coefficient different from that of peptide 49 (Table 5). On the other hand, replacing these two serine residues of the library-lead peptide yielded a peptide (peptide 55, Table 5) with a binding activity considerably higher than that of the library-lead peptide ($IC_{50} = 3.3\times10^{-7}$ M).

2.4. Comparison of the binding coefficients of the synthetic peptides with those of peptides derived from AChR

The synthetic peptides depicted in Table 5 with IC_{50} in the nanomolar range represent the best obtained 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 ([1]; Table 6). It should be noted that even a 14-mer peptide corresponding to residues 187–200 of the muscle AChR α-subunit that includes Asp200 ($IC_{50} = 2.6\times10^{-8}$ M [Balass, personal communication] and peptide 57, Table 6) has an IC_{50} that represents a one order of magnitude weaker inhibitory potency than that of peptides 49, 50, 52, 53, 54 and 56 (nanomolar range; Table 5). Asp200 probably aligns with Asp199 in the neuronal AChR and Asp13 in the lead peptide. This result shows that the systematic residue replacement approach yielded peptides with high affinities that could not have been achieved by the preparation of peptides corresponding to the amino acid sequence of the AChR-binding site.

Additional peptides were designed to represent various combinations of muscle and nerve α-subunits (peptides 58–61, Table 6). Peptides 59–61 have AChR sequences in which Cys192 and Cys193 are replaced by two Ser residues, as in the case of the library peptides. Peptide 59 is derived from the neuronal α_7 AChR (residues 187–199); peptide 60 is a combination of the amino-end of the neuronal α_7 (residues 187–191) and the carboxyl-end of muscle AChR (residues 194–200), and peptide 61 is a reversed combination of peptide 60. All of these combinations yielded peptides with lower affinities than either the AChR-derived peptides (Table 6) and much lower than those of the peptides obtained by the systematic residue replacement (Table 5).

2.5. Biological activity of a high affinity peptide

Peptides that bind toxins and inhibit binding to their targets may be used as lead compounds for potential antidotes against such poisons. It was therefore interesting to examine if the high affinity peptides designed in this work would protect against α-BTX lethality in mice. Peptide No. 50 was incubated with α-BTX for 1 h, and the mixture was injected into mice. The peptide (5 mg per mouse) conferred full protection from α-BTX (5 μg) toxicity, whereas control mice injected with α-BTX alone died 2 h after injection. A dose of 1 mg peptide per mouse protected 25% of the mice, and 0.1 mg peptide did not confer any protection.

3. Discussion

In this study we designed and prepared a series of peptides that interact with high affinity with α-BTX, and succeeded in obtaining peptides that bind α-BTX and inhibit its binding to AChR with an IC_{50} of ~2 nM. This inhibition represents an affinity that is more than two orders of magnitude better than that of a previously described 13-mer peptide, which was selected from a phage-display peptide library and used as a lead-peptide.

Replacements of three positions in the library-lead peptide resulted in the most significant increases in binding. These included replacement of Met1 with Trp, Ser10 with Pro, and Lys9 with Asp, Glu or Leu. A combination of these three replacements resulted in peptides with an IC_{50} in the nanomolar range (Table 5). The replacement of Met1 by Trp was introduced based on information obtained from sequence data of muscle AChR. This Trp, which corresponds to Trp187 in the muscle AChR α-subunit, was shown to be important for α-BTX binding [7,10,11]. Thus, this element in the high affinity library peptides comes exclusively from sequence data of muscle AChR. It may be noted in this respect that the NMR-derived structure of the library-lead peptide with α-BTX [2] shows that Met1 of the peptide is located near Arg36 of α-BTX; it is therefore possible that the indole ring of Trp at this position interacts with the side chain of Arg36 in α-BTX.

The replacement of Ser10 with Pro is based on sequence alignment with the neuronal α_7 AChR. The presence of Pro10 and Pro12 in the high affinity peptides represents the proline subsite of the neuronal α_7 AChR (Pro196 and Pro198; see Table 3), and appears to be of critical importance in establishing the affinity of this receptor to α-BTX. It should be mentioned that systematic residue replace-

ment based on NMR information (Table 2) also led to this replacement. This highlights the possible general use of the approach described in this work, since structural data can be utilized in many biochemical systems, and is not unique to toxin–receptor interactions.

The third replacement that was significant in increasing the binding potency is Lys9 to Asp or Glu. Interestingly, the library-lead peptide had a basic residue at this position, whereas in both muscle and neuronal (α_7) AChRs there is an acidic residue (or sometimes a hydroxyl-side chain residue Thr or Ser) at this position (Table 3). Thus, this replacement incorporates sequence information from both muscle and neuronal (α_7) AChRs. Finally, it should be noted that a substitution of Lys9 of the lead peptide by Leu also enhanced the inhibitory effect. In most cases proline was found to affect extremely the inhibitory potency of the peptides: in positions that Pro contributes for binding or enables correct folding of the peptide, a replacement by Pro resulted in a significant increase in inhibitory potency (peptide 29), whereas in other positions (6 and 9) no inhibition was observed (peptides 13, 23).

The high affinity peptides (Table 5) have the motif EPYPD at their carboxyl-terminal end (residues 9–13). This motif is identical to residues 195–199 of the neuronal α_7-subunit. The carboxyl-terminal acidic residue (Asp13) of the library-lead peptide, as well as that of all high affinity peptides, was demonstrated to be close to Arg36 of α-BTX [2]. Replacement of this Asp with a positively charged residue (Lys, peptide 36, Table 2) decreased the inhibitory potency by two orders of magnitude. This result may indicate the importance of Asp200 in muscle AChR and of Asp199 in neuronal AChR in binding to α-BTX, by forming electrostatic interactions with Arg36 of α-BTX.

The library lead peptide has Leu at position 8, which was shown by NMR analysis to interact with α-BTX [2]. Interestingly, this residue is different from either Pro or Lys, that are present at this position in the muscle and in neuronal AChR that bind α-BTX (Table 3). Replacement of this Leu by either Pro or Lys resulted in a significant decrease in the affinity (peptides 43 and 45, Table 4).

The increase of activity in the synthetic peptides was obtained in this study by exploiting information available from both muscle and neuronal AChR types, as well as from the library-lead peptide itself. The peptides obtained by the third series preparation (Table 5) inhibit the binding of α-BTX to AChR significantly better than any peptide derived entirely from the receptors (see Table 6). The higher affinity suggests that the systematic residue replacement is based on independent and different sources of information, all of which lead to the required results.

In order to reduce the number of peptides to be prepared, we made a general categorization of the 20 natural amino acids. A systematic residue replacement of the lead peptide was performed by using one representative amino acid residue from each group. A larger library of peptides, that includes more members of each category to replace each position, might therefore yield peptides with better inhibition potencies.

Peptide No. 50 had a protective effect on mice in-vivo against α-BTX lethality. As linear peptides undergo proteolytic degradation in blood, an increase of the peptide stability to proteolysis may improve its in vivo activity. This may be achieved by cyclization through a disulfide bond, as previously described for other peptides with anti-α-BTX in vivo activity [Balass et al., unpublished results] or by other chemical modifications.

In designing the peptides described in this study, we applied the relevant available knowledge of the system studied, including NMR-derived structural information and functional information. The increase in activity was achieved mainly by integrating data obtained from the NMR solution structure of the complex between α-BTX and the lead target peptide, together with information from analysis of the AChR-binding domain in animal species that are sensitive or resistant to α-BTX. By rationally integrating these data in a systematic residue replacement of the lead peptide, we were able to optimize the activity using only a limited number of peptides. Additional information, such as computer-assisted modeling, point-mutations and X-ray structure determination, can in principle be integrated in the procedure.

4. Significance

The present study describes rational design and synthesis of peptides that bind α-BTX with high affinity and inhibit α-BTX binding to AChR with an IC_{50} of 2 nM. These peptides may be used as lead compounds for the development of antidotes against α-BTX poisoning, taking into consideration the in-vivo protection of mice against α-BTX poisoning observed with peptide No. 50. Based on the success in obtaining peptides with high affinity, we propose a novel approach (shown below), termed systematic residue replacement, that may be used as a general procedure for obtaining high affinity peptides in any biochemical system that contains a ligand and a receptor molecule.

(1) Use of combinatorial chemical or biological random-peptide libraries to detect a lead peptide that binds specifically, even with a moderate affinity, to a receptor molecule. (2) Structure determination (by NMR or X-ray) of the complex formed between the receptor molecule and the lead peptide. If NMR and X-ray data are not available, computer modeling can be used to provide structural information. (3) Systematic residue replacement of the lead peptide, to yield a secondary library of peptides. To minimize the number of peptides to be prepared by this procedure, the general categorization of the 20 natural amino acids suggested in this work (Fig. 1) may be used. (4) Analysis of the amino acid sequences of natural ligand-

binding sites, to identify the amino acid residues essential for binding with the ligand. (5) Synthesis of new secondary peptide libraries based on the new information acquired in steps (2)–(4).

In conclusion, the procedure employed in this study offers a promising method for obtaining high affinity peptides, by integration of a random substitution technique with structural and functional information. The random techniques utilize combinatorial libraries to yield lead peptides that bind specifically to the relevant receptor. The structural data acquired should provide information on the three-dimensional structure of the receptor–lead peptide complex. In addition, the functional biological information, if available, should provide complementary data of considerable importance.

5. Materials and methods

5.1. General peptide synthesis

All protected amino acids and coupling reagents were purchased from Novabiochem (Laufelfingen, Switzerland). Solvents for peptide synthesis were of synthesis-grade (Labscan; Dublin, Ireland). The peptides were prepared by conventional solid-phase peptide synthesis, using an ABIMED AMS-422 automated solid-phase multiple peptide synthesizer (Langenfeld, Germany). The 9-fluorenylmethoxycarbonyl (Fmoc) strategy was used throughout the peptide chain assembly [14], following the company's commercial protocols. Wang resin, which contains the first covalently bound corresponding *N*-Fmoc C-terminal amino acid (12.5 μmol in the preparation of peptides 1–47 and the library-lead peptide, and 25 μmol in all other preparations) was used in each reaction vessel (polymer loading of 0.55–0.76 mmol/g). Side chain protecting-groups were *tert*-butyloxycarbonyl for Lys and Trp; trityl for Asn and Cys; *tert*-butyl-ester for Asp and Glu; *tert*-butyl ether for Ser, Tyr and Thr and pentamethyldihydrobenzofuran-5-sulfonyl for Arg.

Coupling for the preparation of 12.5 μmol peptides was carried out by using two successive reactions with 50 μmol (4 equiv.) of the corresponding *N*-Fmoc amino acid, 50 μmol (4 equiv.) of benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate reagent, and 100 μmol (8 equiv.) of 4-methyl-morpholine, all dissolved in dimethylformamide for 20–45 min at room temperature. For the preparation of 25 μmol peptides the same equivalents of the reagents were used.

Cleavage of the peptides from the polymer was performed usually by reacting the resin with 1.8 ml trifluoro-acetic acid (TFA)/H_2O/triethylsilane (TES) at volume ratios of 90/5/5 for 2 h at room temperature. Peptides that contain Cys or Met were treated with TFA/H_2O/TES/thioanisole (85/5/5/5, v/v), whereas peptides that contain both Trp and Arg were treated with TFA/H_2O/thioanisole/ethane-dithiol/phenol (87.5/5/5/2.5/trace amount, v/v). The cleavage mixtures were cooled down to 4°C, the peptides were precipitated with ice-cold di-*tert*-butyl ether and centrifuged for 10–15 min, 3000 rpm at 4°C. The pellet was washed and centrifuged three times with di-*tert*-butyl ether, dissolved in 30% acetonitrile in water, and lyophilized.

5.2. Reversed-phase HPLC

Purity validation of synthetic peptides was performed by analytical reversed-phase HPLC using a prepacked Lichrospher-100 RP-18 column (4×250 mm, 5 μm bead size) employing a binary gradient of 0.1% TFA in water (solution A) and 0.1% TFA in 75% acetonitrile in water (solution B) eluted at $t = 0$ min, $B = 5\%$; $t = 5$ min, $B = 5\%$; $t = 45$ min, $B = 95\%$ (flow rate 0.8 ml/min). The chromatographic separation was performed using a Spectra-Physics P200 liquid chromatography system equipped with a Spectra-Physics UV100 variable wavelength absorbance detector and AS100 auto sampler. The chromatograms were recorded on a PE Nelson integrator model 1022. The purities of the crude peptides were in the range of 40–90%.

Peptides of special interest (the library-lead peptide and peptides No. 29, 39, 48, 50, 52, 54 and 56) were purified by using a prepacked LiChroCart RP-18 column (10×250 mm, 7 μm bead size) using the same buffer system as described above except that the gradient was of $t = 0$ min, $B = 20\%$; $t = 15$ min, $B = 20\%$; $t = 50$ min, $B = 60\%$ (flow rate 5 ml/min). Peptide purifications were performed using a Spectra-Physics SP8800 liquid chromatography system equipped with SP8500 dynamic mixer and an Applied Biosystems 757 variable wavelength absorbance detector. The chromatograms were recorded on a Chrom-Jet integrator. HPLC-purified peptides were $>98\%$ pure. All column effluents were monitored by UV absorbance at 220 nm; solvents and HPLC columns were obtained from Merck (Darmstadt, Germany).

5.3. Mass spectrometry

The HPLC-purified peptides were analyzed by time-of-flight (TOF) mass spectrometry using a VG matrix-assisted laser desorption ionization TOF mass spectrometer (VG Fisons, Altrincham, UK), and were found to exhibit the calculated mass. Insulin was used as a standard.

5.4. Biotinylation of α-BTX

HPLC-purified α-BTX from the snake venom of *Bungarus multicinctus* was purchased from Sigma (product number T3019). For biotinylation, 100 μg of α-BTX in 100 μl of 0.1 M $NaHCO_3$ pH 8.6, were incubated for 1 h at room temperature with 2.5 μg 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 overnight at 4°C against phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M phosphate buffer, pH 7.4).

5.5. Inhibition of the binding of α-BTX to Torpedo AChR by the peptides

AChR from the electric organ of *Torpedo californica* was solu-

bilized with Triton X-100 and purified as described [15]. Inhibition of the binding of α-BTX to AChR by the peptides was carried out as follows: *Torpedo* AChR (0.25 μg/ml, in PBS) was coated on an enzyme-linked immunosorbent assay plate (50 μl per well) overnight at 4°C. After washing with 0.05% Tween-20 in PBS (PBS/T), the wells were blocked with a solution of 1.5% bovine serum albumin (BSA) and 1.5% hemoglobin (in PBS), incubated for 2 h at room temperature and then washed with PBS/T. Stock solutions of the individual peptides (1 mg/ml water adjusted to pH 7 with 0.5 N NaOH and 0.2 N HCl) were diluted to various concentrations with 1% hemoglobin in PBS, and incubated with biotinylated α-BTX (50 ng/ml) for 30 min at 37°C, prior to their addition to the receptor-coated wells. After washings (PBS/T), the wells were incubated with alkaline phosphatase conjugated extravidin (BioMakor, Israel; 1:2000, in 1% BSA/PBS) for 50 min at room temperature. The non-bound conjugate was washed (PBS/T), and the bound biotinylated α-BTX was monitored by the enzymatic reaction of alkaline phosphatase, using the substrate *p*-nitrophenyl-phosphate (1 mg/ml in ethanol-amine buffered saline; 33.9 g/l NaCl, 0.104 g/l $MgCl_2$, 0.6 ml/l mono ethanol amine, pH 9.5). The color developed after 50 min at room temperature was determined, using a tunable OPTImax microplate reader (Molecular devices, Sunnyvale, CA, USA) at 405 nm. The concentration that resulted in 50% inhibition of α-BTX binding to AChR (IC_{50}) was determined for each peptide. All peptides were first analyzed in their crude form (40–90% purity). Peptides of special interest were purified by reversed-phase HPLC, and their inhibition potencies were determined also in their purified form (see Tables 2 and 4–6).

5.6. Inhibition of the lethal effect of α-BTX in mice by peptide No. 50

The in-vivo experiments were carried out on groups of four mice (Balb/c, 3 weeks old). A solution of α-BTX (5 μg) and peptide No. 50 in 1 ml PBS was preincubated for 1 h at room temperature and injected subcutaneously into each mouse. A control group of mice that were treated with 5 μg α-BTX alone became paralyzed and died 2 h after the injection.

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