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Cobra cytotoxins: determinants of antibacterial activity

Peter V. Dubovskii,*[a] Olga V. Vorontsova,[a,b] Yuri N. Utkin,[a] Alexander S. Arseniev,[a] Roman G. Efremov[a,c] and Alexei V. Feofanov[a,b]

[a] *M. M. Shemyakin–Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117997 Moscow, Russian Federation. Fax: +7 495 335 5033; e-mail: peter@nmr.ru*
[b] *Department of Biology, M. V. Lomonosov Moscow State University, 119234 Moscow, Russian Federation*
[c] *Higher School of Economics, 101000 Moscow, Russian Federation*

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The investigation of antibacterial activity of three-finger cobra cytotoxins towards Gram-negative and Gram-positive bacteria showed no activity against the former species, whereas *M. luteus* from the latter ones was the most susceptible to cytotoxins. A correlation was revealed between this activity and hydrophobicity of the toxins (HTL scores), total charge and its distribution over the toxin molecule: the absence of Glu-16 residue and the presence of positively charged residues (Lys30/His31) in the tip of the loop 2.

A specific feature of cytolytic peptides from the venom of insects and snakes is the presence of hydrophobic regions bordered with Lys and Arg residues. Such peptides can be linear,[1,2] containing disulfide bonds[3] or a combination of the above two types.[4] Cytotoxins (CTs or, otherwise, cardiotoxins) belong to the second of these groups. CTs are β-structural three-finger toxins from cobra venom stabilized with four disulfide bonds.[3,5] They are toxic to cancer cells[6] and possess antibacterial activity.[7] The goal of this work was to study the antibacterial activity of some CTs (Table 1) towards Gram-positive and Gram-negative bacteria and to interpret the results from the structural viewpoint.

The antibacterial activity of CTs was studied by a method of serial dilutions in a liquid medium (broth microdilution assay) as described earlier.[1,‡]

We found that CTs do not show antibacterial activity towards Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus* at concentrations up to 10 μM for CT2No and CT3Nk and through 40 μM for CT1No, CT1Nh and CT2Nh (Table 1). CT2No and

Table 1 Cobra CTs[a] and their properties.

Cobra species	Toxins[†]	SWISSPROT code	Type (P/S)[b]	Total charge (pH 7)[c]	Charge of the tip of the loop 2 (residues 29–31)	Charge of the residue no. 4	Absence of the residue Glu16	HTL[d]	Minimum inhibitory concentration (activity)[e]	
									M. luteus	*E. coli* C-600/ *S. aureus* 209-P
N. haje	CX1_NAJHA (CT1Nh)	P01455	S	+8 (14/11/3)	0	+1	No (0)	9.5	80 (0.0125)[f]	>40
	CX2_NAJHA (CT2Nh)	P01462	P	+7 (15/11/4)	0	+1	No (0)	6.1	40 (0.025)	>40
N. kaouthia	CX3_NAJKA (CT3Nk)	P01446	S	+9 (13/11/2)	+1	0	Yes (1)	7.0	2.5 (0.4)	>10
N. oxiana	CX1_NAJOX (CT1No)	P01451	S	+6 (14/10/4)	–1	0	No (0)	8.9	80 (0.0125)[f]	>40
	CX2_NAJOX (CT2No)	P01441	P	+10 (14/12/2)	+1	+1	Yes (1)	16.3	1.0 (1.0)	>10

[a] Aligned sequences of CT (residue numbers are given above, charged residues are shown in boldface letters, sequences are grouped according to their homology extent, see the respective homology tree to the right of the sequences, CT names and their abbreviations are shown to the left), web-server used: http://www.uniprot.org/align/.

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                  |1        |10       |20       |30       |40       |50       |60
CX3_NAJKA (CT3Nk) LKCNKLIPLAYKTCPAGKNLCYKMFMVSNKTVPVKRGCIDACPKNSLLVKYVCCNTDRCN
CX1_NAJOX (CT1No) LKCNKLVPIAYKTCPEGKNLCYKMFMMSDLTIPVKRGCIDVCPKNSLLVKYVCCNTDRCN

CX2_NAJOX (CT2No) LKCKKLVPLFSKTCPAGKNLCYKMFMVAAPHVPVKRGCIDVCPKSSLLVKYVCCNTDKCN

CX1_NAJHA (CT1Nh) LKCHKLVPPVWKTCPEGKNLCYKMFMVSTSTVPVKRGCIDVCPKNSALVKYVCCSTDKCN
CX2_NAJHA (CT2Nh) LKCHKLVPPFWKTCPEGKNLCYKMYMVATPMLPVKRGCIDVCPKDSALVKYMCCNTDKCN
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[b] CT of the P-type are featured by the presence of Pro30-residue, those of S-type – with Ser28 residue, these residues are underlined. [c] The total number of charged residues/number of positively/negatively charged residues are given in parentheses. [d] Index of Hydrophocity of the Tips of the Loops (HTL score, in the Kyte–Doolittle hydrophobicity scale[16]), the residues accounting for are: 5–11, 24–37, 46–50. [e] Minimum Inhibitory Concentration (MIC, μM), the reciprocal value, given in the parentheses, stands for activity (μM^{-1}). [f] The given value, satisfying the formal criterion of being >40 μM, was used to calculate the coefficients in Table 2.

[†] Abbreviations: CT1Nh, cytotoxin 1 from venom of *N. haje*; CT2Nh, cytotoxin 2 from venom of *N. haje*; CT3Nk, cytotoxin 3 from venom of *N. kaouthia*; CT1No, cytotoxin 1 from venom of *N. oxiana*; CT2No, cytotoxin 2 from venom of *N. oxiana*.

[‡] Peptides were added to a suspension of bacterial cells grown up to the beginning – middle of exponential phase of growth in low salt LB medium (Sigma, 5 g dm^{-3} of NaCl) and incubated for 20 h (at 37 °C, 100% humidity, mixing speed of 400 rpm). Activity of the peptides was tested in triplicate. The concentrations of the peptides were 0.039–10 μM for CT2No and CT3Nk and 0.156–40 μM for CT1No, CT1Nh and CT2Nh. The experiments were carried out using *Micrococcus luteus* bacteria (a strain from a collection of the Faculty of Microbiology of Biological Department of the Moscow State University; $(1.4\pm0.6)\times10^7$ col ml^{-1}), *Escherichia coli* C-600 and *Staphylococcus aureus* 209-P [$(0.8\pm0.2)\times10^5$ col ml^{-1}].

CT3Nk suppress the growth of Gram-positive *M. luteus* in the micromolar concentration range. CT2Nh is active at 40 μM, and CT1No and CT1Nh are inactive even at a concentration of 40 μM (Table 1).

It was reported earlier that CT A3 from *N. atra* possesses antibacterial activity towards *E.coli* and *S. aureus*.[7] The activity increases considerably when a lypopolysaccharide layer of *E. coli* is destroyed by the addition of EDTA.[7] Similar effect is observed if bacteria are treated with lysozyme.[8] For *S. aureus* the corresponding effect occurs when the biosynthesis of a lipoteichoic layer is blocked by the addition of the antibiotic rifampin.[7] The presence of anionic phospholipids in plasma membranes of both Gram-positive, and Gram-negative bacteria makes them rather vulnerable to CT, as shown in the experiments with model membranes of the corresponding composition.[7,9,10] Therefore, the structural features and composition of the cell wall/envelope, most likely, are responsible for the inhibition of the antibacterial activity of CTs for *E. coli* and *S. aureus* (Table 1).

Let us consider in detail, which factors determine the activity of CTs towards *M. luteus*. For this purpose, we calculated correlation coefficients (Table 2) between the row of activity of CTs and their characteristics given in Table 1. CTs are classified into P- and S-types that determine their interaction with model lipid membranes.[9] In a new classification,[3] each CT is ascribed with the index of hydrophobicity of the tips of the loops (HTL) (Table 1). It is seen (Table 2) that the activity row and HTL feature a high positive correlation coefficient of 0.81. Probably, this indicates that the death of *M. luteus* is caused by the incorporation of the tips of the three loops of CTs into the plasma membrane of bacteria followed by its destabilization. From the experiments with model lipid membranes, we know that membrane destabilization is strengthened when the charge of the tip of the loop becomes zero.[11] However, Table 2 demonstrates the activity increases, if the tip of the loop 2 is charged. The corresponding correlation coefficient is 0.76. Probably, this factor is responsible for the penetration of CTs through a peptidoglycan layer of bacteria. Likely, the absence of Glu16 residue facilitates this process because this residue is far distant from the penetration interface at the toxin–lipid bilayer boundary. According to the Goy–Chapman theory, a contribution of this residue to the toxin–membrane interaction is small.[12] The total charge of CTs might be equally important for the interaction with membrane and penetration through the peptidoglycan layer. It is expected that the total negative charge of CT and activity exhibit a negative correlation coefficient of –0.79. Becoming neutral for His4 residue is known to be important for interaction with model membranes.[3] The correlation coefficient between the activity and charge of the fourth residue is 0.18. Apparently, it means that the contributions of this charge into penetration through the peptidoglycan layer and interaction with model lipid membranes are of opposite sign. Thus, the activity of CTs toward Gram-positive bacteria of *M. luteus* is determined by (i) the HTL index, (ii) total positive charge (>8) and (iii) charge distribution over the toxin molecule, specifically, the absence of Glu16 residue, and the presence of a cationic residue in the tip of the loop 2 (Lys30/His31). Note that *S. aureus* are resistant to the tested CTs (Table 1). This indicates that the peptidoglycan of *S. aureus* has a more basic nature, as compared to that of *M. luteus*.[13]

Table 2 Correlation coefficients between the row of activity of CTs towards Gram-positive bacteria *M. luteus* and their molecular properties.

Property of CT[a]	Correlation coefficient in the activity/property row
HTL	0.81
Charge (total)	0.86
Absence of Glu16 residue	0.87
Net charge of (29–31) residues[b]	0.76
Charge of the residue no. 4	0.18
Sum of negatively charged residues	–0.79

[a] See Table 1. [b] The tip of the loop 2.

The design of antibacterial peptides is frequently accompanied by testing the membrane activity of the peptides in model lipid membranes, which are close in the lipid composition to one of the plasma membrane of the target bacteria. This work discloses the fact that, to forecast a structure–activity relationship, it is necessary to take into account interaction of the peptides with the outer membrane of bacteria and/or their peptidoglycan layer incorporating anionic glycopolymers. For linear cationic peptides, this strategy allows one to achieve practically significant results.[14,15]

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References

1 S. A. Kozlov, A. A. Vassilevski, A. V. Feofanov, A. Y. Surovoy, D. V. Karpunin and E. V. Grishin, *J. Biol. Chem*., 2006, **281**, 20983.
2 G. Corzo, E. Villegas, F. Gomez-Lagunas, L. D. Possani, O. S. Belokoneva and T. Nakajima, *J. Biol. Chem*., 2002, **277**, 23627.
3 P. V. Dubovskii, A. G. Konshina and R. G. Efremov, *Curr. Med. Chem.*, 2014, **21**, 270.
4 P. V. Dubovskii, A. A. Vassilevski, O. V. Samsonova, N. S. Egorova, S. A. Kozlov, A. V. Feofanov, A. S. Arseniev and E. V. Grishin, *FEBS J.*, 2011, **278**, 4382.
5 A. G. Konshina, P. V. Dubovskii and R. G. Efremov, *Curr. Protein Pept. Sci*., 2012, **13**, 570.
6 A. V. Feofanov, G. V. Sharonov, M. A. Dubinnyi, M. V. Astapova, I. A. Kudelina, P. V. Dubovskii, D. I. Rodionov, Y. N. Utkin and A. S. Arseniev, *Biochemistry (Moscow)*, 2004, **69**, 1148 (*Biokhimiya*, 2004, 1410).
7 L. W. Chen, P. H. Kao, Y. S. Fu, S. R. Lin and L. S. Chang, *Toxicon*, 2011, **58**, 46.
8 P. Shashidharan and L. K. Ramachandran, *Indian J. Biochem. Biophys.*, 1983, **20**,132.
9 P. V. Dubovskii, D. M. Lesovoy, M. A. Dubinnyi, A. G. Konshina, Y. N. Utkin, R. G. Efremov and A. S. Arseniev, *Biochem. J.*, 2005, **387**, 807.
10 M. A. Dubinnyi, D. M. Lesovoy, P. V. Dubovskii, V. V. Chupin and A. S. Arseniev, *Solid State Nucl. Magn. Reson.*, 2006, **29**, 305.
11 P. V. Dubovskii, D. M. Lesovoy, M. A. Dubinnyi, Y. N. Utkin and A. S. Arseniev, *Eur. J. Biochem.*, 2003, **270**, 2038.
12 M. A. Dubinnyi, P. V. Dubovskii, Y. N. Utkin, T. N. Simonova, L. I. Barsukov and A. S. Arseniev, *Russ. J. Bioorg. Chem*., 2001, **27**, 84 (*Bioorg. Khim*., 2001, **27**, 102).
13 W. Vollmer, D. Blanot and M. A. de Pedro, *FEMS Microbiol. Rev*., 2008, **32**, 149.
14 H. Mohanram and S. Bhattacharjya, *Antimicrob. Agents Chemother.*, 2014, **58**, 1987.
15 A. S. Kuznetsov, P. V. Dubovskii, O. V. Vorontsova, A. V. Feofanov and R. G. Efremov, *Biochemistry (Moscow)*, 2014, **79**, 459 (*Biokhimiya*, 2014, 583).
16 J. Kyte and R. F. Doolittle, *J. Mol. Biol.*, 1982, **157**, 105.

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