Venom Peptides Hot Paper

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Autocrine-Based Selection of Drugs That Target Ion Channels from Combinatorial Venom Peptide Libraries

Hongkai Zhang[+], Mingjuan Du[+], Jia Xie, Xiao Liu, Jingying Sun, Wei Wang, Xiu Xin, Lourival D. Possani, Kyungmoo Yea, and Richard A. Lerner**

Abstract: *Animal venoms represent a rich source of pharmacologically active peptides that interact with ion channels. However, a challenge to discovering drugs remains because of the slow pace at which venom peptides are discovered and refined. An efficient autocrine-based high-throughput selection system was developed to discover and refine venom peptides that target ion channels. The utility of this system was demonstrated by the discovery of novel Kv1.3 channel blockers from a natural venom peptide library that was formatted for autocrine-based selection. We also engineered a Kv1.3 blocker peptide (ShK) derived from sea anemone to generate a subtype-selective Kv1.3 blocker with a long half-life in vivo.*

Nature has evolved elaborate defense systems to neutralize predatory organisms that invade the host or are present in the environment. These can be divided into intrinsic and extrinsic systems. Intrinsic systems operate inside the host while extrinsic systems operate outside the host. The most thoroughly understood intrinsic system is the adaptive immune system, which generates its diversity by combining a large starting repertoire with selectability for improved binding.[1] This system has been harnessed in vitro by creating large synthetic combinatorial antibody libraries containing as many as 10^{11} members in a selectable format for binding,[1,2] or more recently, for phenotype screens.[3] An equally large extrinsic defense system consists of venoms and other toxic molecules. It is estimated that there are about 10 million uncharacterized venomous animals, each of which may produce 300–400 different toxins.[4] Rapid advances in proteomics, genomics, and transcriptomics have provided affordable technology platforms that allow in-depth exploration of the molecular diversity of venoms.[5] However, this natural resource is largely underexploited, and only a minute fraction of the worldwide venom repertoires have been functionally characterized. This is because the discovery of functional venom peptides has relied upon bioactivity-guided fractionation that is time consuming and requires large volumes of crude venom.[6]

Furthermore, although venom peptide toxins are naturally pre-optimized to be highly potent compounds, their pharmacological and pharmacokinetic properties still need to be optimized. This could, in principle, be achieved through either chemical synthesis or genetic methods. For example, more than 130 analogues of ShK were chemically synthesized to discover critical structural changes for higher target selectivity.[7] However, chemical synthesis of these cysteine-rich peptides has been challenging because of the difficulties involved in correct formation of multiple regioselective disulfide bonds. In the few cases where genetic methods were used to produce venom peptides, the absence of a selection system allowed only a limited entry into the repertoire, thereby eschewing the power that derives from the massive diversity of peptide toxins.[8]

In spite of the small number of pharmacological active venom peptides characterized, there are already seven drugs, and dozens of other venom peptides that are currently in development for use in man. An example is the analgesic drug ziconotide, which is an ω-conotoxin peptide derived from the cone snail. Its mechanism of action is selective inhibition of *N*-type voltage-gated calcium channel.[6] If the vast venom repertoire could be harnessed in the way that antibodies are, venom peptides could become a far more powerful component of the modern pharmacopeia than they are today.

Herein, we describe the construction of natural venom libraries in an autocrine-based format for the discovery of venom peptides that target ion channels. The format also allows the selected molecules to be easily refined. The key to this method is the autocrine-based format, which allows the powerful process of selection to be used to identify or improve active venoms in the library.[9]

For the initial proof of concept, interactions between the Kv1.3 channel and the characterized venom peptides were studied by using an autocrine proximity-based assay. To construct the system, the platelet derived growth factor receptor transmembrane domain (PDGFR-TM) was used to anchor the venom(s) of interest to the plasma membrane. The tobacco etch virus (TEV) protease was added to the cytoplasmic face of the PDGFR-TM domain. The reason for using the TEV protease is its high sequence specificity. The cytoplasmic face of the voltage gated potassium channel

[*] Dr. H. Zhang,[+] Dr. J. Xie, Dr. K. Yea, Prof. R. A. Lerner
Department of Molecular and Cellular Biology
The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
E-mail: hongkai@scripps.edu
rlerner@scripps.edu

Dr. M. Du,[+] X. Liu, J. Sun, Dr. W. Wang, X. Xin
The institute for Advanced Immunochemical Studies
Shanghai Tech University
99 Haike Road, Pudong, Shanghai, 201210 (P.R.China)

Prof. L. D. Possani
Departamento de Medicina MolecularyBioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México
Apartado Postal 510-3, Cuernavaca, Morelos, 62250 (México)

[+] These authors contributed equally to this work.

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Kv1.3 was coupled to an optimized TEV substrate sequence and the artificial transcription factor GAL4-VP16. The result is a system with venoms bearing a protease and target channels bearing a substrate for the protease. When the Kv1.3 channel and venom peptides interact, they bring the enzyme and its substrate into proximity, and catalytic cleavage of the substrate sequence occurs. The released transcription factor then enters the nucleus and activates the expression of the fluorescent protein reporter gene (Figure 1). The vectors is described are Figure S1 in the Supporting Information. The method is general and only requires proximity of venom peptides and the channel, such that the number of productive enzymatic interactions is increased.[10]

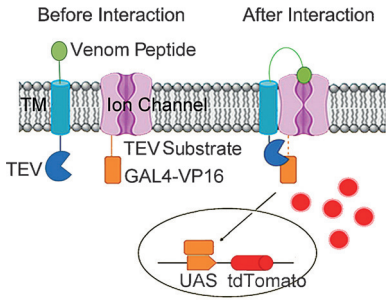

Figure 1. Identification of interactions between venom peptides and ion channel through a proximity-based assay. Membrane-tethered venom peptides are coupled to the TEV protease on its cytoplasmic side. The ion channel is fused to a TEV substrate sequence and transcription factor GAL4-VP16 on its cytoplasmic side. Interaction between the ion channel and the ligand brings TEV and its substrate sequence into close proximity, thereby resulting in the release of GAL4-VP16 and expression of the reporter gene (in this case, the fluorescent protein tdTomato).

We generated a Kv1.3 reporter cell line in which the Kv1.3 channel fusion protein was expressed at low levels and the reporter vector containing the upstream activation sequence (UAS) for transcription activation of the expression of the reporter gene tdTomato was integrated into the genome. The Kv1.3 target peptide ShK and Anuroctoxin were cloned into the pLigand vector and corresponding lentivirus particles were prepared. Lentiviral vectors encoding either peptide stimulated the Kv1.3 reporter cells to express the tdTomato fluorescent protein, while the negative control lentiviral vectors encoding the GLP-1 receptor, which bind exendin-4, or the $GABA_B$ receptor, which binds the conotoxin Vc1.1, exhibited no activity (Figure S2).

After initial experiments showing that the selection system was functional, we next selected Kv1.3-targeting peptides from a natural venom peptide library. Cysteine-knot peptides were identified from the ATDB[11] and ArachnoServer[12] databases based on three criteria (Figure 2a). First, the toxin should be less than 42 amino acids long. Second, the toxin peptides should contain at least six cysteine residues, which indicates that the peptide features a cysteine-knot structure. Finally, redundant peptides were removed. As a result, 589 natural venom toxins and their variants were extracted after data mining of the databases.

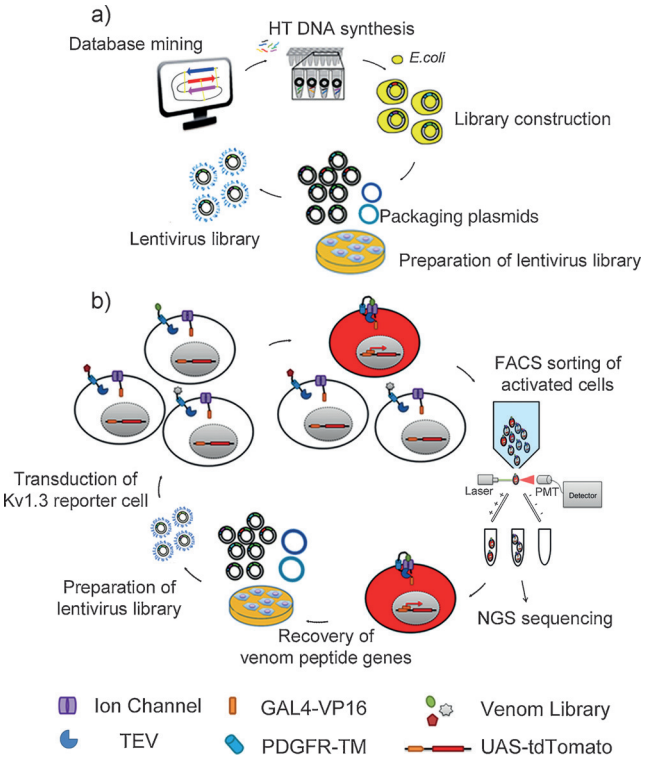

Figure 2. a) Construction of a lentiviral vector library for natural venom peptides. Cysteine-knot peptides are extracted from the venom database using bioinformatics. The corresponding oligonucleotides are synthesized through high-throughput DNA synthesis. Toxin genes are amplified from these oligonucleotides and cloned into the lentiviral vector pLigand to produce a lentiviral library. b) Autocrine-based selection from the venom peptide lentiviral library through a proximity-based assay. Lentiviral particles are prepared from the lentiviral plasmids and used to transduce Kv1.3 reporter cells. Cells expressing tdTomato (see Figure 1) are sorted and venom-peptide-encoding genes are amplified from the sorted cells to make an enriched lentiviral library for the next round of selection. After iterative rounds of selection, enriched peptide sequences are analyzed by NGS.

The key to our method is that it utilizes autocrine-based selection and each cell becomes a reporter system unto itself. Kv1.3 reporter cells were transduced with a lentiviral library of natural venom peptides at a multiplicity of infection (MOI) equal to 1. After transduction, a venom peptide fusion protein and the Kv1.3 fusion protein were coexpressed in the plasma membrane of the same cell. Only when the venom peptide interacted with Kv1.3, were the TEV protease and its substrate sequence brought into proximity, thereby resulting in release of the transcription factor and expression of tdTomato. The tdTomato-positive cells were sorted by FACS and the toxin genes were recovered by PCR and cloned into the pLigand vector to prepare lentiviral particles for the next round of selection (Figure 2b). Significant enrichment was observed during three rounds of selection of the natural venom peptide library (Figure S3a).

To go beyond flow cytometry data and to understand the selection process in more detail, next-generation sequencing (NGS) was done and the enrichment factor of the individual toxin in each round was calculated. The NGS results are

summarized in Table S1 in the Supporting Information. Twenty five out of the twenty seven most enriched sequences are known blockers of the Kv1.3 channel (Table 1).

Table 1: 27 top hits from the natural venom library following selection against Kv1.3 ($R_3/R_0 > 5$).

Name	R_1/R_0	R_2/R_0	R_3/R_0	Known Kv1.3 Blocker?
NxTx I3A	3.52	12.09	29.26	Y
NxTx V5A	3.75	11.68	20.56	Y
NxTx I2A	3.75	10.66	15.98	Y
NxTx K6A	2.48	7.66	13.72	Y
NxTx S14W	3.42	9.40	13.27	Y
TsTx-Kα	2.37	7.20	12.53	Y
TsTx-Kα R8A	3.37	8.12	12.43	Y
ClITx1	2.05	5.89	12.16	N
TsTx-Kα K27R	2.86	6.93	10.16	Y
NxTx N4A	2.03	6.23	9.13	Y
AgTx 2 G10V	2.49	6.08	8.63	Y
ChTx V16E	2.83	5.58	8.26	Y
Aek	2.58	3.82	7.48	N
AgTx 2	1.86	5.13	7.35	Y
NxTx Δ25A	2.26	5.74	7.21	Y
Tc30	2.00	4.52	7.20	Y
NxTx-IbTx I	1.45	3.77	7.02	Y
AgTx 2 R31A	2.09	5.41	7.01	Y
ChTx-c	2.63	5.57	6.71	Y
AgTx 2 T9A	1.77	4.53	6.46	Y
Ce2	1.22	4.23	6.41	Y
ChTx V5E	2.70	4.53	5.54	Y
AgTx 2 R24A	1.81	3.81	5.50	Y
Ce4	1.31	3.75	5.44	Y
ChTx W14M	1.72	3.60	5.40	Y
Lq2 R25A	2.88	4.75	5.39	Y
OsK1	4.33	4.24	5.03	Y

The count for each toxin was normalized to the total read number from the same round. The enrichment factor (R_x/R_0) was defined as the normalized count for a toxin in round x divided by the normalized count for the same toxin in the original library (Round 0).

There remained two peptides of unknown specificity. We tested one of them, the ClITx1 peptide, for its ability to block the Kv1.3 channel. Because of the difficulties encountered when attempting the chemical synthesis of cysteine-knot peptides, we fused the ClITx1 peptide to the N-terminus of the human IgG1 Fc fragment and generated the fusion protein through recombinant expression. Immunofluorescence staining of Kv1.3-expressing HEK293F cells showed that the ClITx1–Fc fusion protein bound to the Kv1.3 channel (Figure 3). The peptide was also purified from crude venom of the scorpion *Centruroides limpidus limpidus* by HPLC as described before.[13] This authentic venom peptide strongly blocked the Kv1.3 channel (Figure 3). The other toxin, Aek, was previously classified as a potassium channel toxin based on its sequence similarity to AsKS, BgK, and ShK.[14] The selection method was thus not only validated by the identification toxins known to bind to the input target but was also able to identify new toxins with previously unknown target specificity.

An equally important aspect of this method is that it allows selection from venom peptide based combinatorial

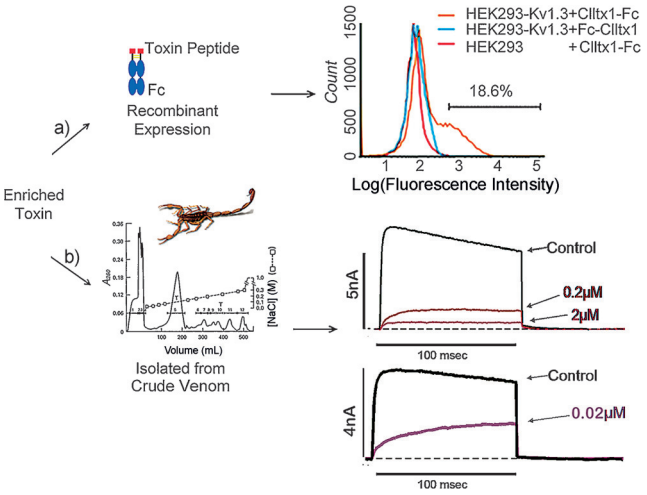

Figure 3. Characterization of the selected peptide ClITx1. a) Kv1.3-expressing cells were stained with recombinant ClITx1–Fc fusion protein and the immunostained cells were analyzed by flow cytometry. b) Authentic ClITx1 peptide isolated from scorpion venom potently blocked Kv1.3 current in patch-clamp studies.

libraries to find variants with improved pharmacological properties. We demonstrated the utility of this method by generating potent and selective Kv1.3 blockers with long in vivo half-lives.

ShK is the most widely studied Kv1.3-targeting venom peptide, and its synthetic analogue ShK-186 has completed phase 1 clinical trials for active-plaque psoriasis. However, this molecule has a short in vivo half-life.[7,8,15] Fusion to an IgG1 Fc is an established strategy to extend the half-life of a therapeutic protein. We thus engineered the venom peptide ShK as a fusion protein to the C terminus of the human IgG1 Fc fragment. Although the N terminus of the venom faces away from the channel pore, the potency of a toxin peptide may be decreased when it is fused to the C terminus of the Fc fragment because of steric hindrance.[8] To overcome this problem, the first 7 positions of ShK were randomized in order to re-orientate the Fc portion of the construct in a variety of ways (Figure S4). The diversity of this library was about one million members. The Kv1.3 reporter cell line was transduced with the combinatorial library of Fc–ShK variants, and the variants that retained Kv1.3 binding ability were enriched by three rounds of selection (Figure S3b). Variants from each round of selection were analyzed by NGS and the frequencies of the variants were calculated.

The NGS results are summarized in Table S2. The variegated region of each variant was extracted based on the known flanking sequences. The ratio between number of unique toxins and the count of all reads represents the diversity of a selected library. Highly enriched variants versus those that were not enriched were chosen and their affinity to Kv1.3 were assessed and ranked. In general, the enrichment factor of a variant is positively correlated with its affinity to Kv1.3 (Figure 4).

Patch-clamp studies showed that the highly enriched variant S1-2 (Figure 5a) retains its potency as a blocker of the Kv1.3 channel ($IC_{50} = 158$ pM; Figure 5b). To test the critical

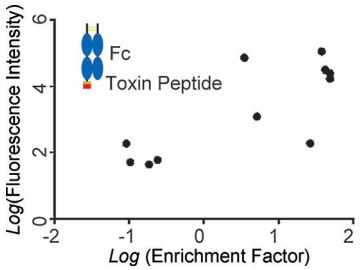

Figure 4. Correlation between Kv1.3 binding ability and enrichment factor. HEK293F cells transiently transfected with Kv1.3 were stained with the variants and analyzed with flow cytometry. The fluorescence intensities were plotted against the enrichment factors of the variants as calculated from NGS results.

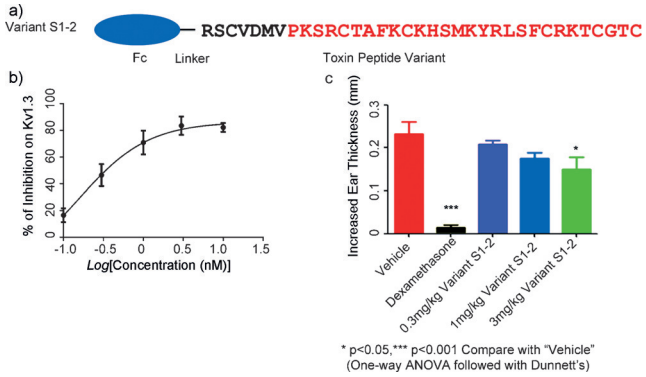

Figure 5. Characterization of the Fc–ShK variant S1-2. a) The sequence of S1-2. The randomized region is shown in black. The linker is GGGGS×2. b) IC_{50} of S1-2 for Kv1.3 current determined by patch-clamp electrophysiology. c) Efficacy of S1-2 in DNFB-induced delayed-type hypersensitivity in Lewis rats. Lewis rats were sensitized with DNFB on shaved dorsum on day 0 and challenged with DNFB on both sides of the right pinnae on day 5. Ear thickness changes were measured 24 h after challenge. 16 h and 1 h before DNFB challenge, rats were subcutaneously dosed with S1-2, and positive control rats were treated with oral dexamethasone 10 mg kg^{-1} once daily.

property of specificity, patch-clamp analysis of S1-2 was also carried out on HEK293 cells expressing other Kv1 family members, including Kv1.1 through Kv1.7 and the hERG channel. Wild-type ShK exhibited comparable inhibition of Kv1.1, Kv1.6, and Kv1.3, whereas ShK variant S1-2 had no effect on any of the other channels (Table S3).

Because the Kv1.3 channel is known to be involved in the activation of effector memory T-cells, blocking it presents a novel therapeutic opportunity for autoimmune diseases such as psoriasis and rheumatoid arthritis.[15] We studied the efficacy of the selected channel blocker in the rat dinitrofluorobenzene (DNFB)-induced delayed-type hypersensitivity model. Lewis rats were dosed subcutaneously with the Fc–ShK variant S1-2 (0.3,1 and 3.0 mg kg^{-1}) and changes in ear thickness were measured following DNFB challenge. S1-2 showed a dose-dependent reduction in inflammation. At the maximum dose of 3 mg kg^{-1}, S1-2 achieved 40% decrease in ear thickness after DNFB challenge (Figure 5c).

Many of the problems plaguing venom research can be circumvented by the method described in this report. As with antibodies, venoms are collected in a combinatorial matrix that allows expanded diversity, and selection is used to identify functional molecules. Since the method relies only on bioinformatics and genetics for generation of the repertoire, the capture of venomous organisms is not required and synthetic problems are avoided. In addition, selection from a library of venom peptides by flow cytometry can significantly reduce the number of molecules that must be subjected to expensive and time-consuming electrophysiology measurements. Another important feature of autocrine-based selection system is that it allows the target to be studies in the natural milieu of the plasma membrane.

Relative to antibodies, libraries of venoms have advantages and disadvantages. The molecules contained in the venom repertoire have been selected by evolution to bind to certain targets such as ion channels, and their mode of binding thus shows precision and mechanisms that only evolution can deliver. On the other hand, their target space is limited. By contrast, the germline repertoire of antibodies as a collective is largely target agnostic and thus the interaction space is infinite. Therefore, depending on the target, each method should have its place.

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[1] R. A. Lerner, *Angew. Chem. Int. Ed.* **2006**, *45*, 8106–8125; *Angew. Chem.* **2006**, *118*, 8284–8305.
[2] a) W. D. Huse, L. Sastry, S. A. Iverson, A. S. Kang, M. Alting-Mees, D. R. Burton, S. J. Benkovic, R. A. Lerner, *Science* **1989**, *246*, 1275–1281; b) J. McCafferty, A. D. Griffiths, G. Winter, D. J. Chiswell, *Nature* **1990**, *348*, 552–554; c) C. F. Barbas III, A. S. Kang, R. A. Lerner, S. J. Benkovic, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7978–7982; d) H. R. Hoogenboom, A. D. Griffiths, K. S. Johnson, D. J. Chiswell, P. Hudson, G. Winter, *Nucleic Acids Res.* **1991**, *19*, 4133–4137.
[3] a) H. Zhang, I. A. Wilson, R. A. Lerner, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 15728–15733; b) H. Zhang, K. Yea, J. Xie, D. Ruiz, I. A. Wilson, R. A. Lerner, *Chem. Biol.* **2013**, *20*, 734–741.
[4] a) J. D. Romano, N. P. Tatonetti, *Sci. Data* **2015**, *2*, 150065; b) B. L. Sollod, D. Wilson, O. Zhaxybayeva, J. P. Gogarten, R. Drinkwater, G. F. King, *Peptides* **2005**, *26*, 131–139.
[5] a) K. Luna-Ramírez, V. Quintero-Hernández, V. R. Juárez-González, L. D. Possani, *PloS one* **2015**, *10*, e0127883; b) Y. Zhang, J. Chen, X. Tang, F. Wang, L. Jiang, X. Xiong, M. Wang, M. Rong, Z. Liu, S. Liang, *Zoology* **2010**, *113*, 10–18.
[6] A. L. Harvey, *Toxicon* **2014**, *92*, 193–200.
[7] J. K. Murray, Y. X. Qian, B. Liu, R. Elliott, J. Aral, C. Park, X. Zhang, M. Stenkilsson, K. Salyers, M. Rose, H. Li, S. Yu, K. L. Andrews, A. Colombero, J. Werner, K. Gaida, E. A. Sickmier, P. Miu, A. Itano, J. McGivern, C. V. Gegg, J. K. Sullivan, L. P. Miranda, *J. Med. Chem.* **2015**, *58*, 6784–6802.

[8] W. Edwards, W. P. Fung-Leung, C. Huang, E. Chi, N. Wu, Y. Liu, M. P. Maher, R. Bonesteel, J. Connor, R. Fellows, E. Garcia, J. Lee, L. Lu, K. Ngo, B. Scott, H. Zhou, R. V. Swanson, A. D. Wickenden, *J. Biol. Chem.* **2014**, *289*, 22704 – 22714.
[9] H. Zhang, E. Sturchler, J. Zhu, A. Nieto, P. A. Cistrone, J. Xie, L. He, K. Yea, T. Jones, R. Turn, P. S. Di Stefano, P. R. Griffin, P. E. Dawson, P. H. McDonald, R. A. Lerner, *Nat. Commun.* **2015**, *6*, 8918.
[10] H. Zhang, J. Xie, R. A. Lerner, *Biochem. Biophys. Res. Commun.* **2014**, *454*, 251 – 255.
[11] Q. Y. He, Q. Z. He, X. C. Deng, L. Yao, E. Meng, Z. H. Liu, S. P. Liang, *Nucleic Acids Res.* **2008**, *36*, D293-297.
[12] V. Herzig, D. L. Wood, F. Newell, P. A. Chaumeil, Q. Kaas, G. J. Binford, G. M. Nicholson, D. Gorse, G. F. King, *Nucleic Acids Res.* **2011**, *39*, D653-657.
[13] B. M. Martin, A. N. Ramirez, G. B. Gurrola, M. Nobile, G. Prestipino, L. D. Possani, *Biochem. J.* **1994**, *304 (Pt 1)*, 51 – 56.
[14] S. Minagawa, M. Ishida, Y. Nagashima, K. Shiomi, *FEBS Lett.* **1998**, *427*, 149 – 151.
[15] a) C. Beeton, M. W. Pennington, R. S. Norton, *Inflammation Allergy: Drug Targets* **2011**, *10*, 313 – 321; b) R. S. Norton, M. W. Pennington, H. Wulff, *Curr. Med. Chem.* **2004**, *11*, 3041 – 3052.

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