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Manuscripts

A Venomics approach coupled to high-throughput toxin production strategies identifies the first venom-derived melanocortin receptor agonists

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3 **ABSTRACT:** Animal venoms are rich in hundreds of toxins with extraordinary biological activities.
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5 Their exploitation is difficult due to their complexity and the small quantities of venom available from
6
7 most venomous species. We developed a Venomics approach combining transcriptomic and proteomic
8
9 characterization of 191 species and identified 20,206 venom toxin sequences. Two complementary
10
11 production strategies based on solid-phase synthesis and recombinant expression in *E. coli* generated
12
13 a physical bank of 3,597 toxins. Screened on hMC4R, this bank gave an incredible hit rate of 8%.
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15 Here, we focus on two novel toxins: N-TRTX-Preg1a, exhibiting an inhibitory cystine knot (ICK)
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17 motif, and N-BUTX-Ptr1a, a short scorpion-CS α β structure. Neither N-TRTX-Preg1a nor N-BUTX-
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19 Ptr1a affect ion channels, the known targets of their toxin scaffolds, but bind to four melanocortin
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21 receptors with low micromolar affinities and activate the hMC1R/Gs pathway. Phylogenetically, these
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23 two toxins form new groups within their respective families and represent novel hMC1R agonists,
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25 structurally unrelated to the natural agonists.
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33 **Introduction**

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35 It is estimated that more than 200,000 venomous species exist on Earth, found in almost all animal
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37 subgroups across a range of diverse habitats¹. Many are terrestrial, such as some vertebrates (e.g.,
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39 snakes, platypus, lizards) or arthropods (e.g., scorpions, spiders, centipedes, ticks, ants), some marine,
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41 such as cnidarians (e.g., sea anemones and jellyfish) or some mollusks (e.g., cuttlefish, octopus, marine
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43 cone snails, terebrids, squids), and some aerial including insects (e.g., mosquitoes, bugs, wasps, bees)
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45 and vampire bats. Venoms are used for defense and/or predation and are composed, among other
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47 components, of peptide toxins. Toxins are small proteins up to one hundred residues in size, reticulated
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49 by disulfide bridges. Number of toxins present in venoms depends of the species and of the method
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51 used to identify them. In the 1990's, publications mentioned few tens of toxins in *Conus* venom.
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53 Recently, almost 6,000 toxins were reported in the species *Conus lividus*². All in all, we can estimate
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55 that at least 40 million toxins exist on the planet and of these, less than 5,000 have been
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3 pharmacologically characterized. Despite this very small number, a plethora of activities have been
4 reported. Toxins interacting with membrane receptors like voltage- and ligand-gated ion channels^{3,4}
5 and G-Protein Coupled receptors (GPCR)⁵⁻¹⁰ have been frequently described whereas other toxins
6 inhibit enzymes or bind membranes to exert hemolytic and/or antimicrobial actions¹¹. Toxins are very
7 often selective (with the exception of some lytic toxins) and make excellent pharmacological tools to
8 better understand the physiological or pathophysiological roles of their targets. In some cases, the
9 toxins themselves or their derivatives have applied clinical uses, such as exenatide from the beaded
10 lizard), a treatment against type-1 diabetes, ziconotide from a cone snail, a treatment for chronic pain,
11 captopril derived from a snake toxin and widely used to treat hypertension, but also some other snake
12 toxins e.g. batroxobin with clotting properties used to stop bleeding¹². In addition, more than 20 other
13 toxins are currently investigated in clinical trials as biopharmaceutical and therapeutic tools.

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Venoms are a very promising source of selective bioactive molecules and considerable effort is being
undertaken to discover new toxins with exploitable activities^{13,14}. Classically, toxins are identified by
bioguidage, i.e. successive cycles of venom deconvolution and screening tests against a given target.
After defining its sequence, the novel toxin of interest must be produced to allow in-depth
characterization. This strategy is long, costly and risky and requires large amounts of venoms,
unavailable for most venomous animals which are very small in size. This strategy is not adapted for
the vast majority of this fantastic biodiversity.

New sequencing strategies that appeared in the 21st century are revolutionizing the toxinological field.
These technologies have been commonly applied to whole genome sequencing, however, eukaryote
genomes are typically large with tandem repeats and only few genomes from venomous species were
reported (several snakes, two scorpions, a handful of spiders and dozens of Hymenopteras) but none
from cone snails. By contrast, many studies have taken advantage of these same strategies for the
sequencing of various venom gland transcriptomes. Toxins are produced as precursor proteins by
venom-apparatus specific cells and undergo several maturation steps before being secreted¹⁵. The

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3 formation of disulfide bonds is the most important post-translational modification (PTM) and their
4 arrangement is commonly used to classify toxins¹⁶. Sequenced transcripts are translated into their
5 cognate amino acid sequences allowing identification of the endoplasmic reticulum signal peptide, the
6 propeptide and mature peptide regions. While tools like SignalP often accurately predict the
7 endoplasmic reticulum signal peptide boundary, relying on transcriptomics alone, it is far more
8 complicated to obtain robust information regarding further processing to the mature peptide due to the
9 lack of information on most pro-protein convertase specific cleavage sites. Moreover, it is important
10 to note that using a purely transcriptomic strategy, the physical presence in the venoms of most of the
11 sequences described in databases is almost never demonstrated. Alternatively, proteomic strategies
12 study only secreted toxins and provide the opportunity to characterize post-translational modifications
13 (PTMs). Thus, only the combination of these complementary proteomic and transcriptomic strategies
14 leads to a higher level of confidence for mature peptide sequences. Consequently, it is expected that
15 the toxin sequences present in these types of databases will become more and more accurate.

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33 The last and most complicated challenge remains the biological characterization of these natural
34 peptides. This step cannot be done by a simple sequence analysis, especially for peptides with a low
35 sequence similarity with previously characterized toxins. It is necessary to build a physical bank of
36 peptides ready to be screened. The FP7 Venomics Project is part of the biotechnology program “Small-
37 to-Medium Enterprise (SME)-targeted research for developing tools and technologies for high-
38 throughput research”. The total project budget was close to 10 million euros (equivalent to 11 million
39 USD) shared by eight partners from five countries. The vision associated with the Venomics Project
40 is not only to investigate the diversity of venom peptides through the development of “omics”
41 technologies but also to replicate toxin diversity *in vitro* to generate novel peptide banks to be used in
42 drug discovery programs¹⁷. This unique toxin bank was generated and screened on G-Protein Coupled
43 Receptor (GPCR) families for which no toxin modulators have previously been described.

Results

A large venomous animal collection. In total, 323 animal samples from 191 different species were gathered (Table 1). Almost every terrestrial and marine venomous clade was represented by at least one species such as ray (*Potamotrygon motoro*) or venomous lizard (*Heloderma horridum exasperatum*). Small terrestrial arthropods represented 95 species *i.e.* half of the sourcing. 55 species (29% of the total sourcing) were studied for the first time, including 44 new species from the most infamous venomous clades (Araneae, Conus, Scorpiones and Serpentes). For instance, 12 previously-unexplored species of Neogastropods were sourced including *Conus canonicus* from the Conidae family, the most studied group of Neogastropoda. The Turridae and Terebridae groups also belong to the Neogastropoda order but have been largely neglected compared to their counterpart¹⁸. For instance, among over 4,000 described species of Turridae, venom secretions of only twelve species have been studied so far. One of them, *Unedogemmula bisaya* was included in the sourcing. Concerning the Terebridae family, only seven of around 350 described species has been subject of venom analysis. In Venomics sourcing, two of them, *Terebra subulata* and *Terebra guttata*, were chosen. A third species, *Terebra maculata*, never explored before, completed the Terebridae species of the sourcing. Sourcing also included totally unexplored groups such as Mitridae. Two species considered non-venomous, the *Silurus glanis* fish and the *Trachycephalus resinifictrix* frog, but known to produce toxic skin secretions, were also part of the collection. The diverse collection of source animals encompassed various habitats, morphologies and animal sizes ranging from millimeters (*Manica rubida* ant) to several meters (*Ophiophagus hannah* snake).

Table 1. Selected taxa for sourcing.

Taxa	Main families	Total N° of species	Never studied species
Neogastropoda	<i>Conidae</i>	38	10
	Other (<i>Turridae</i> , <i>Terebridae</i> & <i>Mitridae</i>)	5	2
Octopoda	<i>Octopodidae</i>	3	0
Chilopoda	<i>Scolopendridae</i>	6	2
Scorpiones	<i>Buthidae</i> , <i>Scorpionidae</i> , <i>Vaejovidae</i> , <i>Scorpionidae</i> , <i>Hemiscorpiidae</i> & <i>Euscorpiidae</i>	40	7
	<i>Theraphosidae</i> , <i>Ctenidae</i> , <i>Agelenidae</i> , <i>Araneidae</i> , <i>Dipluridae</i> , <i>Lycosidae</i> , <i>Nephilidae</i> , <i>Sparassidae</i> & <i>Theridiidae</i>	41	23
Hymenoptera	<i>Apidae</i> , <i>Vespida</i> , <i>Formicidae</i> & <i>Myrmicinae</i>	8	4
Chondrichthyes	<i>Potamotrygonidae</i>	1	0
Teleostei	<i>Synanceiidae</i> , <i>Plotosidae</i> & <i>Siluridae</i>	8	2
	Serpentes (<i>Elapidae</i> , <i>Viperidae</i> & <i>Atractaspididae</i>)	39	4
Squamata	Anguimorpha (<i>Helodermatidae</i>)	1	0
	Anura	<i>Hylidae</i>	1

A huge sequence toxin diversity in animal venoms. *De novo* transcriptomics opens the door to the study of Nature without any previous knowledge of it and with a very high resolution, allowing the unbiased identification and quantification of transcripts in a broad spectrum, even for those of low expression. Total RNA was extracted from venom glands. In order to include species diversity, sample requirements were decreased from 2 µg to 100 ng of RNA, being even as low as 60 ng in specific samples. Illumina specific 2x100 pair-end libraries were constructed from each RNA following manufacturer's protocols. Over 60 million reads were obtained per sample using Hiseq 2000 sequencing platform with an average length of 500 nucleotides.

Two proteomic strategies were used according to the size of the toxins¹⁹. The first one, for venoms presenting most of toxins with less than 25 amino acid residues (like Neogastropoda, Hymenoptera, Anura), analyzed the peptides post-disulfide-reduction by liquid chromatography coupled to tandem mass spectrometry. The second strategy, dedicated to the sequencing of the long venom toxins (> 25 amino acid residues (like Chilopoda, Scorpiones, Araneae, Squamata) required additional alkylation and trypsin digestion (short time and low enzyme concentration) steps after disulfide treatment leading to the generation of overlapping peptides (+/- 15 amino acids)¹⁹. These resulting peptides were then analyzed with the same mass spectrometry platform. The number of sequences varied between fifty to several hundred, depending on the venom. A high number of sequences are observed in Conidae venoms by proteomics. This diversity relies on the presence of post-translational modifications at different sites in a single toxin and has been discussed in previous publications^{2,20}. A total of 21,300,000 sequence tags were obtained.

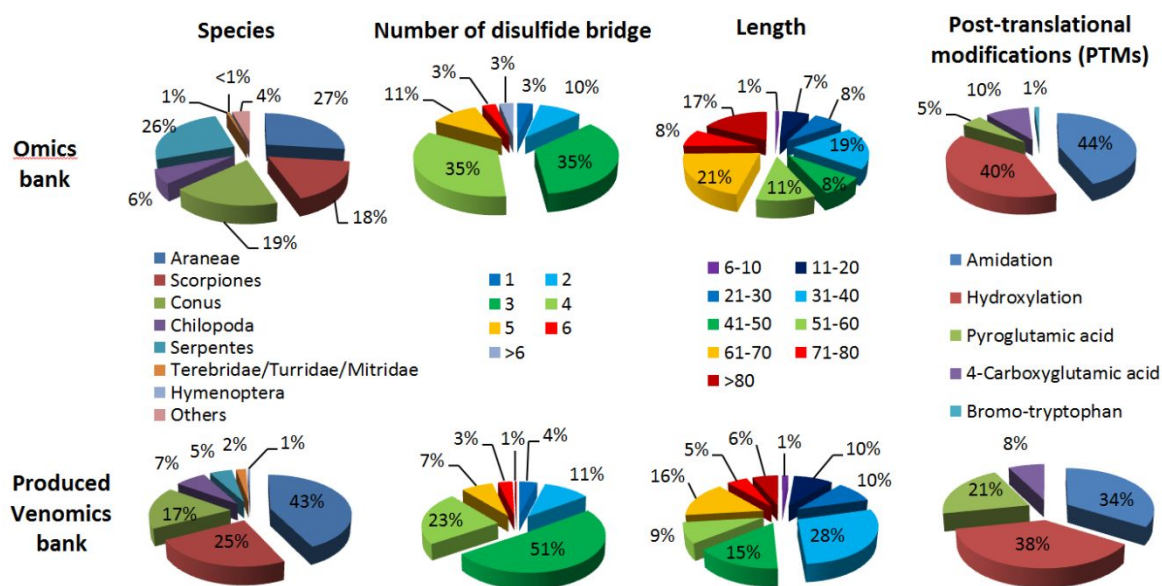


Figure 1. Richness of animal origin and structural features (number of disulfide bridges, length and post-translational modification) of Venomics bank (lower panel) compared to the original Omics bank (upper panel). Omics bank represents 20,206 sequences identified both in transcriptomic analysis of

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3 the venom glands and proteomic analysis of the venoms. From it, 3,597 peptides were produced to
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5 build the Venomics bank which closely replicates the origin and structural characteristics of the
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7 primary Omics bank.
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12 Proteomic and transcriptomic data generated from the same animal were cross-validated to extract
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14 only sequences present in both the venom and venom gland transcripts, resulting in 20,206 validated
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16 toxin sequences (defined here as peptide up to 110 residues with at least one disulfide bridge). Figure
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18 1 describes these sequences in term of origins, disulfide bridges (1 to 9), sizes (6 to 110) and PTMs.
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20 The quasi-totality of Conus sequences (98%) bears at least one PTM, in addition to disulfide bridges.
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22 Amidation is found in 2,889 sequences (14%) from all groups studied except Terebridae, Turridae,
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24 Mitridae and Teleostei. Hydroxylation on Pro, Met, Lys or Val residues was found in 2,630 sequences
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26 (13%) from various groups with a great majority from Conus (70%). In our study, 4-carboxyglutamic
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28 acid, pyroglutamic acid and bromo-tryptophan appeared to be Conus specific. Strangely, we did not
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30 find pyroglutamic acid in Arachnid venoms (Scorpiones and Araneae), although its presence has been
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32 described previously in many other studies^{21,22}. The 20,206 toxin sequences were divided into 137
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34 different cysteine patterns, 97 with an even number of cysteines. Among these 97 patterns, 38 present
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36 new cysteine patterns never described in any venomous species. Regarding the 59 known patterns, C-
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38 C-CC-C-C framework is the most represented comprising 19% of the bank (Table S1). It is related to
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40 Inhibitory Cystine Knot Toxins (ICK), which is known to be particularly abundant in spider, snail and
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42 scorpion venoms²³⁻²⁶. For the first time, ICK peptides were identified at protein level from Mitridae,
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44 Turridae, *Manica rubida* ant, Chilopoda and the octopus *Hapalochlaena lunulata*.
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48 Interestingly, 874 sequences covering 40 cysteine patterns display an odd number of cysteine residues
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50 (Table S2). Only thirteen were previously described. Two patterns (both lacking vicinal cysteine
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52 residues) are particularly abundant: C-C-C-C-C-C-C with 130 sequences (Scorpiones, Serpentes,
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3 Octopoda, Conus) and C-C-C-C-C specific to the Araneae taxon with 179 sequences. We did not
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5 identify any sequences from Conus sharing the latter pattern, despite it has been previously described²⁷.
6
7 Among these 20,206 sequences, 782 (4%) display a new cysteine pattern and 4,393 (22%) share less
8
9 than 50% sequence identity with any described peptide or protein in the UniprotKB/SwissProt database
10
11 using default settings (e-value max =1). It must be noted that the cysteine connectivity of the native
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13 toxins can not be explicitly determined using this pipeline.
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17 **From sequences to a high throughput screening compatible toxin bank.** In order to allow screening
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19 and characterization of peptide activity, we developed two high-throughput production protocols.
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21 5,923 sequences representing the broad diversity of the sequence database were selected and produced
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23 in the dedicated time of 18 months. Coming from 64 different species, they cover 91 different cysteine
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25 patterns, including 14 with an odd number of cysteine residues (Tables S1 and S2).
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29 We selected 931 peptides shorter than 35 residues, with or without PTMs, to be produced by solid-
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31 phase chemical synthesis²⁸. The crude synthetic product was cleaved from the resin, desalted and
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33 subjected to an optimized oxidation process, whatever the number or pattern of cysteines²⁸. After a
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35 final desalting step, liquid chromatography and mass analysis were performed to validate the presence
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37 of the expected oxidized mass (Figure S1). 893 toxins from 9 to 34 residues with 1 to 3 disulfide
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39 bridges were successfully produced, representing a success rate of 96%.
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43 The remaining 4,992 sequences containing more than 34 residues were selected for heterologous
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45 expression in *E. coli*²⁹⁻³¹. All synthetic genes encoding toxin sequences were subcloned into an
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47 optimized periplasmic expression plasmid containing an N-terminal *E. coli* Disulfide bond Isomerase
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49 C (DsbC) chaperone fusion tag, a 6xHIS tag for purification and a tobacco etch virus (TEV) protease
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51 cleavage site to release the native peptide from the fusion protein. Expressed fusion proteins are
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53 purified on a Nickel resin, subjected to overnight TEV cleavage and finally desalted. The correct mass
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55 was confirmed by mass analysis (Figure S2). 2,704 toxins, from 35 to 110 residues with 1 to 9 disulfide
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57 bridges were successfully produced representing a success rate of 54%.
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3 Determining disulfide connectivity in native toxins is a challenge and the Venomics project did not
4 have the resources to carry out this task. In the absence of this information, cysteine residues were
5 randomly oxidized and the vast majority of toxins presented a unique peak in LC, suggesting that they
6 adopted only one conformation, hopefully the natural one. In the case where LC profiles presented two
7 or three peaks with the good expected oxidized mass, we assumed that it could be due to the generation
8 of peptide isomers³² (Figures S1 and S2).
9

10 Together, these two strategies resulted in a bank of 3,597 peptides composed of 71 different cysteine
11 frameworks (Tables S1 and S2). In addition, 158 toxins (4%) display a novel cysteine pattern and
12 1,026 (29%) share less than 50% sequence identity with any previously described peptide or protein
13 in the UniprotKB/SwissProt database. All together, we have successfully translated the high
14 complexity of the omics bank into a physical toxin bank via high throughput methods.
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17 **First animal toxins on melanocortin receptors.** Animal toxins are mostly known for their bioactivity
18 on ion channels. Few but very interesting toxins active on GPCRs have also been identified³³. For
19 example, the commercially available type 2 diabetes treatment, exenatide derived from a venomous
20 lizard, targets a class A GPCR. Belonging to the same class, human melanocortin 4 receptor (hMC4R)
21 regulates food intake and its genetic invalidation is responsible of morbid obesity. Screening of the
22 Venomics bank on hMC4R gave an amazing high hit rate of 8%, 163 coming from chemical synthesis
23 and 123 from recombinant expression. We decided to further investigate two hits displaying cysteine
24 patterns related to two of the largest structural families in animal toxins, ICK-related pattern (-C-C-
25 CC-C-C-) and the CS α β -related cysteine patterns (-C-C-C-C-C-K-C-), and primary renowned for their
26 activities on ion channels. The ICK-related N-TRTX-Preg1a comes from the spider *Poecilotheria*
27 *regalis* while the CS α β -related N-BUTX-Ptr1a from the scorpion *Parabuthus transvaalicus*. N letter
28 (uppercase nu greek letter) has been chosen as the activity descriptor for modulators of hMCRs. In
29 primary screening on hMC4R, N-TRTX-Preg1a and N-BUTX-Ptr1a both showed strong inhibition of
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^{125}I -NDP- α -MSH, 97% and 86% respectively (Figure 2). Further pharmacological investigations were then done with newly synthetically produced toxins of more than 95% purity (Figures 3 and S3).

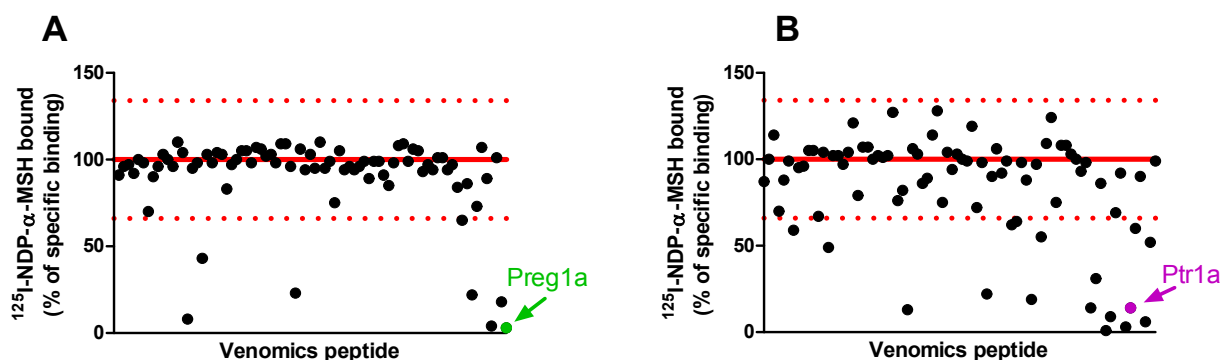


Figure 2. Screening results on hMC4R from the two plates containing N-TRTX-Preg1a (A) and N-BUTX-Ptr1a (B) peptides in the Venomics bank. Screening on hMC4R, stably expressed in CHO cells, was based on competitive binding on the receptor of the tested product against the radio-labeled ligand, ^{125}I -NDP- α -MSH. Full red line is the median and red dash lines are median plus or minus 3 median absolute deviation. Dots above or under dash lines are hits. Each dot corresponds to a tested Venomics peptide (80 peptides by plate). Coloured dot represents either N-TRTX-Preg1a (in green) or N-BUTX-Ptr1a (in purple). Preg1a: N-TRTX-Preg1a. Ptr1a: N-BUTX-Ptr1a.

N-TRTX-Preg1a and N-BUTX-Ptr1a display low micromolar affinities on membrane preparations of every studied subtype of melanocortin receptors stably expressed in CHO-K1 cells for hMC1R, hMC4R, hMC5R and HEK293 cells for hMC3R (Table 2, Figure 4A). A final check was performed by measuring the affinities of the linear toxins with cysteines alkylated by iodoacetamide treatment. Up to 100 μM (1, 10 and 100 μM concentrations were tested), unstructured N-TRTX-Preg1a and N-BUTX-Ptr1a did not inhibit the binding of ^{125}I -NDP- α -MSH on hMC1R and hMC4R, demonstrating that the three-dimensional structures of the toxins are essential to their activities (Figure 4A). Their agonistic and antagonistic activities on the signalling pathways hMC1R/Gs and hMC4R/Gs were then assessed on HEK293 cells. As control, it was demonstrated that neither toxin induced any effect on

control hMCR-free HEK293. Both toxins tested at 100 μ M induced cAMP production on hMC1R, similar to 1 nM of α -MSH, while no effect was observed on hMC4R (Figure 4B). In the presence of 1 nM of α -MSH, the toxins slightly enhance hMC1R related cAMP production but did not reduce the cAMP production related to hMC4R (Figure 4B). Both toxins were also tested at 100 μ M on several human sodium channels (Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6, Nav1.7 and Nav1.8), potassium channels (Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv3.1, Kv4.3, Kv7.1, Kv7.3, Kv10.1, Kv11.1(hERG), GIRK1), a voltage-gated calcium channel (Cav3.3) and ligand-gated ion channels nAChR α ₁ β 1 γ δ and nAChR α 7. No activity could be detected (Figure S4 and S5).

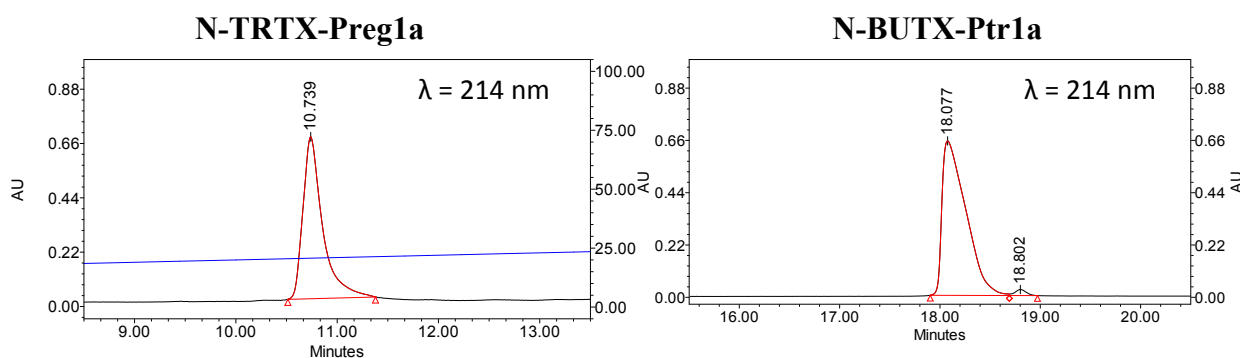


Figure 3. Analytical HPLC chromatogram of the final purified products of N-TRTX-Preg1a and N-BUTX-Ptr1a. Between 10 and 15 μ g of each toxin were analyzed on a X Bridge BEH 300 \AA 5 μ m 4.6 x 250 mm with a gradient of 10-50% CH₃CN/TFA 0.1% in 40 min at a flow rate of 1 mL/min. A spontaneous transformation of the N-terminal glutamine (Q form) residue of the peptide N-BUTX-Ptr1a into a pyroglutamic acid (Z form) can be detected after crude peptide deprotection. According to LC-MS analysis performed on Elute Sp Pump HPG 700 (Bruker) coupled with AmaZon SL LC/MS (Bruker), the peak at 18.077 min corresponds to the Q form (average mass of 3795.70 m/z) and represents 96.5% of the total area. The peak at 18.802 min with an average mass of 3778.70 m/z seems to correspond to the Z form of the peptide and represent 3.5% of the total area.

Table 2. Inhibition constant (K_i) values of N-TRTX-Preg1a, N-BUTX-Ptr1a on melanocortin receptors, mean (μM) \pm SEM (n=3-10).

	hMC1R	hMC3R	hMC4R	hMC5R
N-TRTX-Preg1a	1.8 \pm 0.3	19.8 \pm 3.5	7.1 \pm 1.5	10.0 \pm 4.4
N-BUTX-Ptr1a	2.9 \pm 0.6	3.9 \pm 0.7	2.6 \pm 0.4	2.2 \pm 0.8

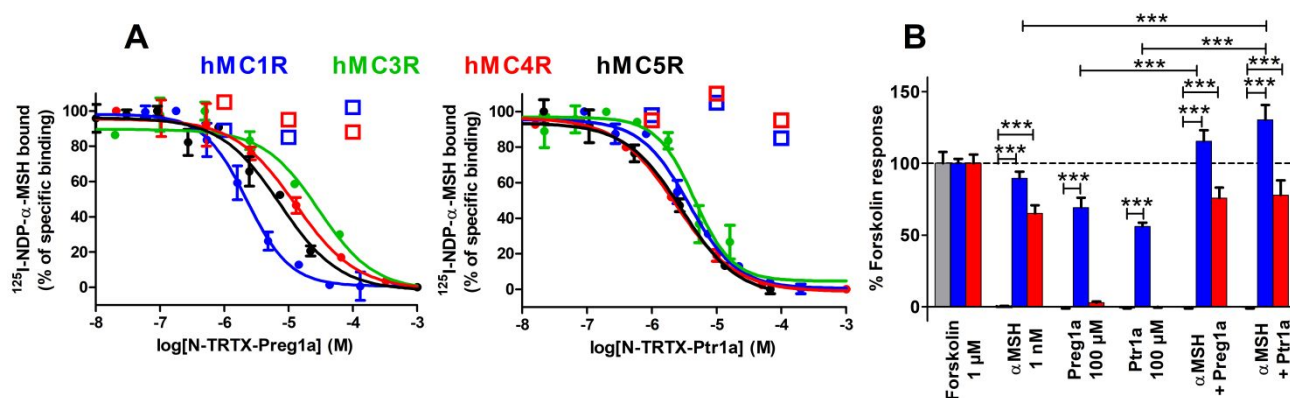


Figure 4. Pharmacological characterization of N-TRTX-Preg1a and N-BUTX-Ptr1a. (A) Representative curves of binding of ^{125}I -NDP- α -MSH on membrane preparations of hMC1R, hMC4R and hMC5R (stably expressed in CHO cells) and on hMC3R (stably expressing in HEK293 cells) in the presence of N-TRTX-Preg1a (left panel) or N-BUTX-Ptr1a (right panel). Mean (%) \pm SD (n=2-3). Open squares represent peptides with alkylated cysteines by iodoacetamide treatment, tested on hMC1R and hMC4R. (B) Functional assays on HEK293 (grey), HEK293 stably expressing hMC1R (blue) or HEK293 transitory expressing hMC4R (red). cAMP production expressed as % of the positive control (1 μM forskolin). Preg1a: N-TRTX-Preg1a. Ptr1a: N-BUTX-Ptr1a. Mean (%) \pm SEM (n = 3), ***: p<0.001; one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Are N-TRTX-Preg1a and N-BUTX-Ptr1a real ICK-related and CS α -related scaffold toxins?

The two N-TRTX-Preg1a and N-BUTX-Ptr1a toxins have a cysteine pattern corresponding to the ICK-

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3 related and the CS α β -related structure with the following pairing C1-C4, C2-C5, C3-C6. As both
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5 toxins display original pharmacological activities, we characterized both structures by NMR and
6
7 determined experimentally their cysteine connectivity. Both peptides are well-folded, illustrated by
8
9 their good dispersion of resonances. All the ^1H (except for the methyl groups of Met2 and Met28 of
10
11 N-BUTX-Ptr1a), the ^{15}N chemical shifts and the ^{13}C resonances of C α and C β were assigned.
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13 Depositions in the BioMagResBank were done with the entry code 34117 for N-TRTX-Preg1a and
14
15 34118 for N-BUTX-Ptr1a.
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19 A set of 546 NOEs for N-TRTX-Preg1a were used in the final ARIA run, which includes 454
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21 unambiguous restraints (Table S3). Additional constraints were introduced during the iterative ARIA
22
23 procedure: 38 dihedral angle restraints, 10 H-bond constraints observed within the β -strands and 3
24
25 covalent bonds between sulphur atoms (C2-C19, C9-C22, C18-C27). Indeed, when enough
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27 experimental data had been introduced into the first runs of the iterative ARIA process, the C18-C27
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29 disulfide bond is formed in two-thirds of the N-TRTX-Preg1a structures. The fine analysis of NOE
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31 constraints and the evaluation of distance between sulfur atoms allowed discrimination between the
32
33 two remaining possibilities: C2-C19, C9-C22, C18-C27 (favorable) or C2-C22, C9-C19, C18-C27
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35 (excluded). Therefore, the corresponding covalent bonds were added between sulfur atoms to refine
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37 the N-TRTX-Preg1a structure in the last runs of the calculations. The 10 models representative of the
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39 solution structure (Figure 5A) display a very low RMSD, small potential energy values, respect all the
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41 experimental data and are in perfect agreement with standard covalent geometry with 64.8% of the
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43 residues in the most favored regions of the Ramachandran plot, and 35.2% in additional allowed
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45 regions (Table S3). The N-TRTX-Preg1a structure is very well defined, organized into the ICK motif
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47 without ambiguities regarding cysteine pairing. It is composed of a short antiparallel β -sheet made up
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49 of two short strands (22–23 and 26–27). The very short turn between these two strands, containing two
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51 arginine residues (R24 and R25), is highly constrained. Note the presence of an extended region (7–
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53 9), which faces the two-stranded β -sheet, resembling a third quasi- β -strand. Proline residue 12 is in a
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majority trans configuration (about 80%). N-TRTX-Preg1a sequence produced only a handful of significant hits in public databases and shares only 61% identity with the closest toxin with a known activity, Hs1a from *Cyriopagopus schmidti* (Figure 5C), a calcium channel blocker³⁴.

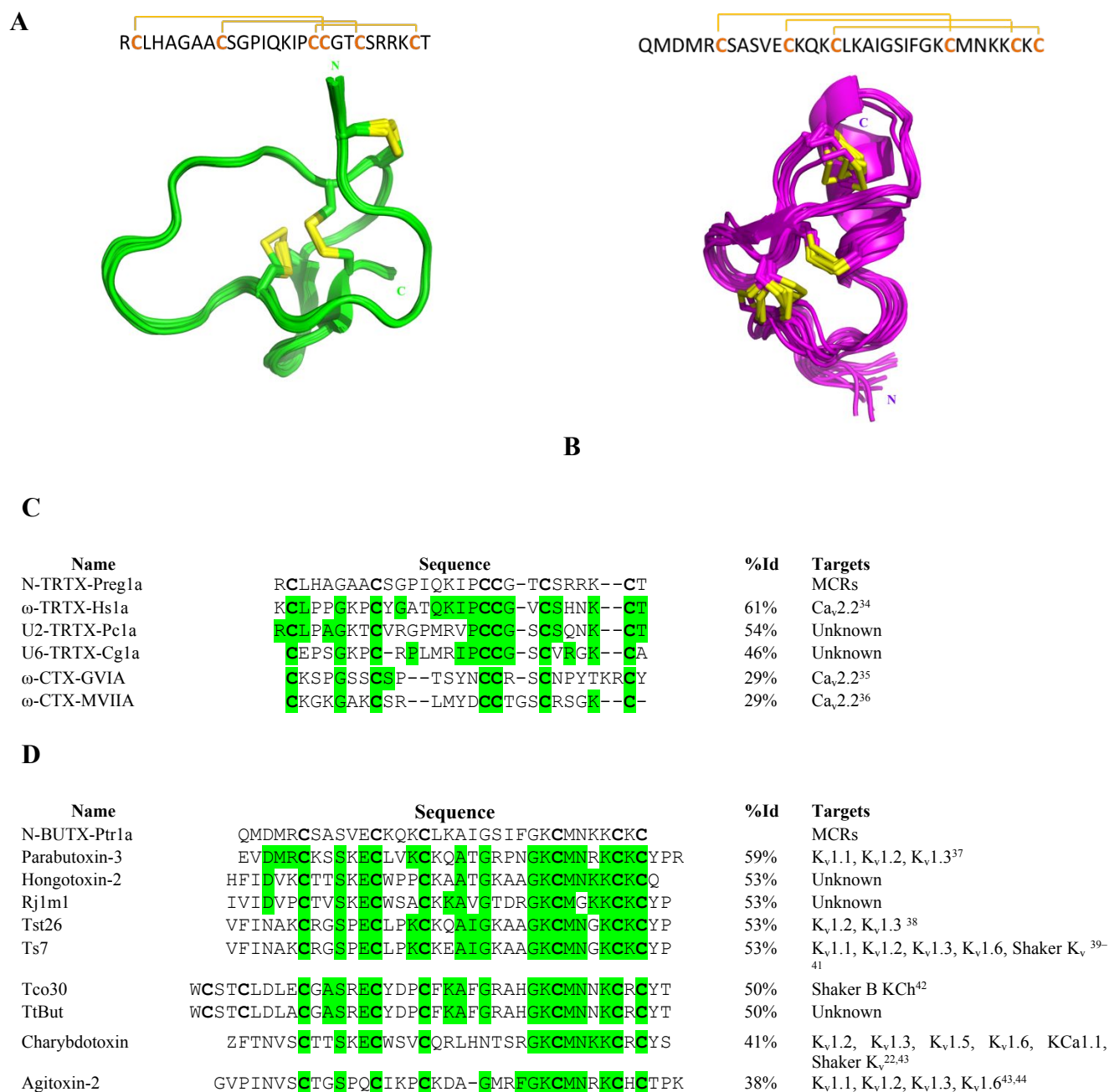


Figure 5. Structural characterization of N-TRTX-Preg1a and N-BUTX-Ptr1a. Primary structure and overlay of the 10 NMR models representative of the solution structure of N-TRTX-Preg1a (A) and N-

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3 BUTX-Ptr1a (B). N and C termini are indicated. Comparison of amino acid sequences between N-
4 TRTX-Preg1a (C) or N-BUTX-Ptr1a (D) and the most similar toxins obtained with NCBI Protein Blast
5 program (default settings). Cysteine residues are in bold, conserved residues from N-TRTX-Preg1a or
6 N-BUTX-Ptr1a are in green. Sequence alignment was performed with Clustal Omega (version 1.2.4
7 from Emboss programs, EBlosum62 matrix for two pair alignment).
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17 Regarding structural determination of N-BUTX-Ptr1a, 839 NOEs (including 558 unambiguous
18 distance restraints) and 4 hydrogen bonds present within the β -sheet were used in the final ARIA run
19 (Table S4). 92% of the 250 final structures display a C1-C4, C2-C5, C3-C6 disulfide bridge array,
20 without adding any constraint on the bonds. The 10 models representative of the solution structure
21 (Figure 5B) display low root mean square deviations (RMSD), small potential energy values, and
22 respect all the experimental data, as attested by the absence of residual violations (Table S4). The
23 Ramachandran plot exhibits 53% of the (ϕ, ψ) angles in the most favored regions, and 40% in the
24 additional allowed regions. The N-BUTX-Ptr1a structure scaffold is a well-known motif within
25 scorpion toxins⁴⁵, plant defensins⁴⁶ and insect defensins⁴⁷, and was designated as a cysteine-stabilized
26 α -helix β -sheet motif (CS $\alpha\beta$ motif)⁴⁸. The structure is composed of an α -helix (11–19) and a 2-stranded
27 short β -sheet (26–28; 31–33), stabilized by three disulfide bridges (C6-C27, C12-C32, C16-C34)
28 unambiguously assigned. N-BUTX-Ptr1a shares the same sequence pattern as the majority of α -KTx
29 toxins known to block potassium channels: C-x(4,5)-C-x(3)-C-x(6,9)-[GAS]-K-C-[IMQT]-x(3)-C-x-
30 C (Prosite [PS01138](http://www.pdb.org)). Yet, N-BUTX-Ptr1a shares only 59% identity with the closest toxin with a known
31 activity, Parabutoxin-3 from the same species *Parabuthus transvaalicus* (Figure 5D), a potassium
32 channel blocker³⁷. The coordinates of structures have been deposited in the Protein Data Bank
33 (<http://www.pdb.org>) with the entry codes 6SAA and 6SAB for N-TRTX-Preg1a and N-BUTX-Ptr1a,
34 respectively.
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3 While N-TRTX-Preg1a and N-BUTX-Ptr1a display the canonical ICK and CS $\alpha\beta$ structure,
4 respectively, their sequences diverged so much from most described toxins that homology searches or
5 phylogenetic reconstruction failed to assign them into known toxin groups. On one hand, N-TRTX-
6 Preg1a matched only a handful of spider toxins that could not be linked to the main group of spider
7 ICK toxins. On the other hand, phylogenetic reconstruction with all known alpha-KTx (UniProt)
8 placed N-BUTX-Ptr1a as sister to a clade consisting of several monophyletic alpha-KTx subfamilies
9 (alpha-KTx 2, 4 and 12).
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21 **Discussion and conclusions**

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23 To our knowledge, this is the first time that a thorough ‘-omic’ analysis of venoms has been carried
24 out on such a wide number and diversity of venomous species from various habitats (128 terrestrial,
25 55 aquatic and 8 aerial) including two European species (*Manica rubida* ant and *Buthus occitanus*
26 scorpion). The *Manica rubida* ant was the smallest species of the project, studied for the first time and
27 35 sequences were validated. The number of validated sequences ranges from 0, due to experimental
28 failures (some collecting conditions were not compatible with proper mRNA extraction), to 1,163, in
29 the venom of the Mediterranean scorpion *Buthus occitanus*. Finally, no validated toxin sequences
30 could be detected in the skin secretions of *Silurus glanis* fish and the *Trachycephalus resinifictrix* frog.
31 An average of 106 toxin sequences per venom were validated, much less than what could be expected
32 according to the assumed richness of venoms. This difference highlights the difficulties in obtaining
33 full and unambiguous toxin sequences. The gotten sequence diversity also highlights our very limited
34 state of knowledge of the toxin world. Indeed, almost one quarter of the toxins share less than 50%
35 sequence identity with known toxin sequences, suggesting unpredictable and original biological
36 activities. Furthermore, 65 new naturally occurring cysteine patterns have been found, suggesting 65
37 new structural organizations. This is an opportunity to discover peptides with attractive biophysical
38 properties in terms of engineering and thermodynamic stability for optimal drugability. Another level
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3 of complexity, not investigated here, is the dimerization (homo- and/or hetero-) of toxins containing
4 an odd number of cysteines. A specific proteomic analysis should be designed to address this point.
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7 Converting a sequence database into physical peptides is challenging, in particular for the oxidation of
8 disulfide bridges. Our recombinant expression strategy was able to directly produce oxidized peptide
9 and has proven to be effective in terms of high throughput production. The two-step chemical synthesis
10 strategy was also extremely efficient. This is mainly thanks to the use of a universal folding strategy
11 applied on all toxins regardless of size or number of cysteines. These production strategies provided
12 peptides with impurities, as no high-efficiency purification step was performed. While the presence of
13 these contaminations could have caused numerous false positive results during the screening tests⁴⁹
14 we shown this was not the case as 90% of the tested hits were confirmed.
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18 GPCRs constitute one of the largest and most versatile families of receptors. They are involved in a
19 wide spectrum of physiological functions. Since the late 20th century, pharmaceutical companies have
20 significantly diminished their natural product programs, partly because of the advances in both HTS
21 and combinatorial synthesis, thereby the creation of large synthetic libraries of small but artificial
22 molecules. Natural product libraries consist of extracts and partially purified fractions and are
23 perceived to be incompatible with HTS. Unfortunately, this loss of interest in natural products
24 correlated with the overall reduction in new leads in the drug development pipeline and the substantial
25 decline in new drug approval⁵⁰. The Venomics bank reconciles venoms, a particularly difficult
26 exploitable resource of natural peptides, with HTS. The screening of the Venomics bank provided an
27 extraordinary hit rate of 3%, much higher than the one reported in literature concerning artificial
28 molecules and estimated at around 0.05%^{51,52}. To our knowledge, this is the first large-scale
29 demonstration of the power of natural peptide toxins to provide GPCR ligands. This strategy opens up
30 an unexplored avenue for the discovery of new ligands on receptors linked to diseases with unmet
31 medical needs such as obesity.
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3 MC4R is mainly expressed in the paraventricular nucleus of the hypothalamus. Its activity is finely
4 regulated by both anorexigenic agonists, the melanocyte stimulating hormones (MSH), and the
5 orexigenic antagonist, Agouti-related protein (AgRP)⁵³. Loss-of-function mutations on hMC4R are the
6 primary cause of monogenic obesity (<http://www.orpha.net/>) making it a therapeutic target in eating
7 disorders. Currently, only one MC4R ligand is in clinical trials (Setmelanotide,
8 <https://clinicaltrials.gov/>). Subtype 1 (MC1R) is also of therapeutic interest with its protective role
9 against melanoma. It elicits eumelanin production, a dark, UV radiation-absorbing, photoprotective
10 pigment⁵⁴ and promotes nucleotide excision repair of DNA damaged by UV radiation⁵⁵. There is an
11 urgent need for development of innovative ligands to better understand MCR functions and to generate
12 new medicines. N-TRTX-Preg1a and N-BUTX-Ptr1a are the first MCR venom-peptide ligands,
13 structurally unrelated to natural endogenous MCR agonists, while similarities and differences exist
14 with other natural ligands such as human Agouti-related protein (hAgRP) or human β -defensin 3
15 (HBD3). As N-TRTX-Preg1a, hAgRP displays an ICK fold in its C-terminal part (hAgRP 83-132)
16 representing its hypothesized mature form⁵⁶ with two additional disulfide bridges. As presented in
17 Figure 6A, cysteine residues involved in ICK fold in N-TRTX-Preg1a and hAgRP 83-132 are well
18 aligned with similarly β -sheets involved the two last cysteine residues of the ICK pattern. Composition
19 and loop sizes are quite divergent between the two peptides especially for the third loop which is
20 almost two times longer in hAgRP 83-132 (C3-C9). This loop contains the Arg-Phe-Phe
21 pharmacophore of AgRP^{56,57} that is lacking in N-TRTX-Ptr1a. This difference suggests that in spite of
22 sharing the same structural ICK family, N-TRTX-Preg1a and hAgRP 83-132 have a different mode of
23 action on MCRs. Regarding N-BUTX-Ptr1a, we noticed that it shared with HBD3 interesting features.
24 They both display similar secondary structures composed of a short α -helix and several antiparallel β -
25 sheets reticulated by three disulfide bridges (Figure 6B). In addition, they are both rich in cationic
26 residues with high basic pIs of 10 for HBD3 and 9.3 for N-BUTX-Ptr1a. This last remark was of
27 particular interest as in a previous study, Nix *et al.* demonstrated the role of large patches of positively
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3 charged residues in the binding of HBD3 on melanocortin receptors⁵⁸. In the same study, the authors
4 described nanomolar affinities on melanocortin receptors (42 nM on hMC1R and 110 nM on hMC4R),
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6 but more recently, Ericson *et al.* published micromolar affinities, 7 μM on hMC1R and 100 μM on
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8 hMC4R⁵⁹. According to Ericson *et al.*, this difference may be explained by a discrepancy between
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10 protocols. We used herein the same methodology as Ericson *et al.* but with a longer incubation time
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12 (3 hours *versus* 1 hour). Without testing HBD3 in our conditions, we cannot be sure that HBD3 and
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14 N-BUTX-Ptr1a share or not similar affinities on melanocortin receptors. Nevertheless, the micromolar
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16 affinity of HBD3 on hMC1R was supported by functional results who described the peptide as a
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18 micromolar agonist of the Gs pathway on hMC1R ($\text{EC}_{50} = 0.40 \mu\text{M}$)⁵⁹, a feature shared with N-BUTX-
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20 Ptr1a. Despite these similarities, important differences between the two peptides have also to be
21
22 noticed. Firstly, they do not share global three-dimensional structures, surely linked to the difference
23
24 in cysteine pattern (Figure 6B). Secondly, Nix *et al.* showed that the absence of disulfide bridges, by
25
26 cysteine replacement in serine in HBD3 sequence, decreased HBD3 affinities by less than 10 times ⁵⁸
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28 contrary to the linear N-BUTX-Ptr1, which lost at least 100 times in affinity. Therefore, the structural
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30 constraints induced by disulfide bridges may be more important in N-BUTX-Ptr1a binding than in
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32 HBD3. These two structural differences may be linked in distinct interaction profile of the two peptides
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34 with melanocortin receptors.
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42 N-TRTX-Preg1a and N-BUTX-Ptr1a also lack the HFRW pharmacophore, described in endogenous
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44 agonists as the minimal active sequence^{62,63}, suggesting that they activate hMC1R in a different way,
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46 that would be interesting to deeply study. In addition, both toxins promote a synergistic effect with
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48 endogenous agonist α -MSH. This activity deserves to be finely studied as both ligands may activate
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50 hMC1R by a new molecular mechanism. N-TRTX-Preg1a and N-BUTX-Ptr1a inhibit ¹²⁵I-NDP- α -
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52 MSH binding on hMC4R but are not able to modulate activate or antagonize the hMC4R/Gs pathway,
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54 under our experimental conditions. This discrepancy can come from our experimental procedure. We
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56 perhaps did not incubate enough time as the toxins display low affinities and may need longer time to
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bind the receptor. Indeed, functional tests are designed according to the pharmacological characteristic of the agonist, which is nM. Another possibility is that these toxins display biased functions, with activities on other pathways than the Gs one. A deeper exploration of the functions of the two toxins on various MC4R signaling pathways (Gq, β -arrestin, Kinases, etc.) will be performed to better understand their modes of action.

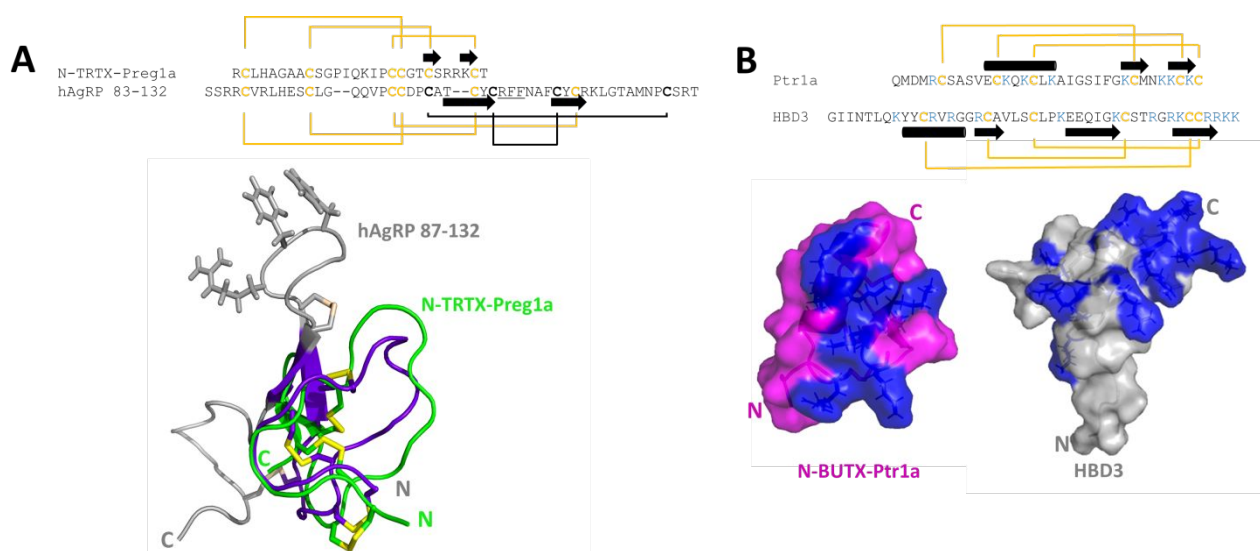


Figure 6. Structure comparison of the spider and scorpion toxins with melanocortin receptor structured peptide ligands. (A) N-TRTX-Pregla *versus* human Agouti-related protein 83-132 (hAgRP 83-132). Upper panel: primary (Needl, version 6.6.0 from Emboss programs, EBlosum62 matrix) and secondary structure (arrow: β -sheet) alignment. Cysteine residues and associated disulfide connectivity are in bold and orange when implied in the ICK motif or in bold and black otherwise. The hAgRP Arg-Phe-Phe is underlined. Lower panel: Overlay of N-TRTX-Pregla and hAgRP 87-132 (grey, 1HYK⁶⁰) NMR structures. Aligned ICK part of hAgRP is represented in colors. The hAgRP pharmacophore residues (Arg-Phe-Phe) have developed side-chains. (B) N-BUTX-Ptr1a (Ptr1a) *versus* human β -defensin 3 (HBD3). Upper panel: primary and secondary structures (arrow: β -sheet, tube: α -helix). Cysteine residues and associated disulfide connectivity are in orange. Basic residues are in blue. Lower panel: tertiary NMR structure comparison of N-BUTX-Ptr1a and HBD3 (1KJ6⁶¹). Structures

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3 display the solvent exposed surface area with basic residues in blue. “C” and “N” correspond to the N-
4 and C-termini of the peptides.
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10 N-TRTX-Preg1a exhibits a common ICK fold only described so far for activity on ion channels. Its
11 sequence is close to calcium channel toxins but does not possess the tyrosine residue in position 16
12 (N-TRTX-Preg1a numbering), described to be essential for blocking $Ca_v2.2$ channel conductivity⁶⁴.
13
14 N-BUTX-Ptr1a, close in sequence to potassium channel toxins, possesses a dyad composed of a basic
15 and an aromatic residue^{65,66} (F24 and K26, N-BUTX-Ptr1a numbering). This dyad, stated as essential
16 for potassium activity^{65,66} but we showed that is clearly not sufficient for N-BUTX-Ptr1a to bind
17 potassium channels. N-TRTX-Preg1a and N-BUTX-Ptr1a sequences diverge so much within their own
18 group that it was even impossible to phylogenetically anchor them into known toxin families. Thus,
19 they constitute new clades for ICK and $CS\alpha\beta$ families. Several other toxins of the produced bank
20 sharing more than 80% of sequence identity with these two toxins are present in the Venomics
21 database, suggesting that N-TRTX-Preg1a and N-BUTX-Ptr1a are not the only members of these new
22 families. Production and pharmacological characterization of these natural variants should provide
23 interesting insights with regard to the mode of action of these toxins on hMCRs. This may aid the
24 generation of ligands with improved affinity and selectivity for development of new hMC1R agonists
25 with applications in the treatment and/or prevention of melanoma.
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45 To our knowledge, the Venomics bank is the largest one ever built. We demonstrated that it represents
46 a real opportunity to identify ligands with unusual structures and pharmacological properties. Its high
47 hit rate shows that quality can beat quantity. Two toxins, with classical toxin structures but original
48 pharmacological activities highlight the diversity of toxin properties and open the door to the
49 development of new hMCR modulators.
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58 **Experimental Section**

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3 **Sourcing.** We paid important caution to respect the natural resources to which we had access. Thus,
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5 we limited the number of collected specimens to the strict number necessary to ensure the amounts
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7 and volumes of biological samples were compatible with the experiments to be carried out within the
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9 Venomics consortium. For instance, during the Mayotte and Polynesia expeditions, supernumerary
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11 cone snails collected during the day, were systematically dropped back to water. In the case of the
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13 venomous lizard specimen (*Heloderma horridum exasperatum*), the lizard was anaesthetized while a
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15 veterinary surgeon performed surgery to remove only one of the venomous salivary glands of the
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17 animal. The animal fully recovered. This demonstrates the applicability of such a strategy of tissue
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19 extraction that could certainly be applied with success to rare snakes too.
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24 **Transcriptomic analysis.** The Illumina paired-end NGS platform was selected to assure sequence and
25
26 data quality. 203 RNA samples from different venomous species were sequenced through a low-input
27
28 method. Quality and quantity of total RNA was determined using a Bio-analyzer 2100 (Agilent,
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30 California), and Qubit 2.0 (ThermoFisher, Massachussetts). The poly(A)+ mRNA fraction was
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32 isolated from total RNA and cDNA libraries were obtained following Illumina's recommendations.
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34 Briefly, poly(A)+ RNA was isolated on poly-T oligo-attached magnetic beads and chemically
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36 fragmented prior to reverse transcription and cDNA generation. cDNA fragments then went through
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38 an end repair process, the addition of a single 'A' base to the 3' end and then ligation of the adapters.
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40 Finally, the products were purified and enriched with PCR to create the indexed final double stranded
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42 cDNA library. Library quality was analyzed via a Bioanalyzer 2100 High Sensitivity assay and
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44 quantity was determined by real-time PCR using a LightCycler 480 (Roche, Basilea, Suiza). The pool
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46 of libraries was sequenced by paired-end sequencing (100 x 2) on an Illumina HiSeq 2000 sequencer
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48 (Illumina, California). Bioinformatic analysis was a crucial step of the process to identify peptide
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50 sequences. This analysis started with NGS raw data quality control, followed by exhaustive cleaning
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52 of primers, adapters, indexes and low-quality bases. The next step consisted of joining the paired-end
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54 reads and dereplicating all reads before entering the assembly step itself. To assure the quality of the
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3 assembly two different assemblers were used; Velvet (v1.2.10)⁶⁷ and Trinity (v2.1.1)⁶⁸, creating a
4
5 robust and comprehensive *de novo* assembly pipeline.
6
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8 **Proteomic analysis.** Venoms predominantly containing toxins with less than 25 amino acid residues
9
10 such as cone snails and wasps, were treated with dithiothreitol (10 mM final concentration, 1 hour at
11
12 56 °C) and alkylated with iodoacetamide (20 mM final concentration, 1 hour at room temperature).
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14 Reduced and alkylated peptidic toxins were separated by reverse phase chromatography (nanoLC-
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16 UPLC Acquity, Waters, Milford, Massachusetts, United States) and analyzed by tandem mass
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18 spectrometry (Q-Exactive, ThermoFisher, Waltham, Massachusetts, United States). This “top-down”
19
20 proteomics approach has the advantage of measuring the mass of the intact full-size toxins but also
21
22 allows for sequence determination using collision-based fragmentation mass spectrometry
23
24 experiments. Venoms containing large toxins, such as snake, spider and scorpion venoms, were, after
25
26 reduction and alkylation, enzymatically cleaved (by using a ratio of 1/50 protein/trypsin, with
27
28 incubation at 37°C over 1 hour). These cleavage conditions yield truncated peptides cleaved at
29
30 different positions, with several overlapping peptides covering the full toxin sequence. The peptides
31
32 were then analyzed with the same nanoUPLC-MS/MS platform, in the same way as the smaller toxins
33
34 permitting the full toxin sequences to be determined. As large toxins have been transformed into a
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36 collection of smaller peptides, the fragmentation yield is then much better and MS/MS spectra can
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38 provide useful sequence information.
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44 **Sequence validation.** For the transcriptomics data, different databases were generated: (1) the first
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46 one including genes coding for peptidic toxins by using predictive bioinformatics tools and (2) the rest
47
48 of the venom gland transcriptome translated into the six possible open reading frames. The
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50 bioinformatics data analysis has been supported by Peaks software (Bioinformatics Solutions Inc.,
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52 Waterloo, Canada). In addition to classical data analysis, Peaks was also used to *de novo* sequence
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54 peptides not identified from the databases, and to predict mutations with Spider algorithms. All the
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3 peptides discovered was classified and only those with a false discovery rate of less than 0.1% were
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5 considered for inclusion in the Venomics database.
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8 **High-throughput peptide production by solid-phase synthesis.** The high-throughput chemical
9
10 production of toxins up to 34 residues with or without post-translational modifications (PTMs) was
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12 undertaken by solid-phase peptide synthesis on an automated Prelude synthesizer (Protein
13
14 Technologies, USA) equipped with six reactions vessels using the Fmoc strategy. Amino acids were
15
16 coupled twice for 10 minutes using five or twenty equivalents for toxins up to 25 residues or between
17
18 26 and 34 residues, respectively, on a ChemMatrix® or pre-loaded Wang resins using HCTU as a
19
20 coupling reagent. Both strategies lead to toxins with excellent purities higher than 50%. After a
21
22 desalting step on homemade C18 columns and a drying step, the refolding of the different toxins was
23
24 performed directly on the crude material. A universal folding protocol was applied for each toxin as
25
26 already described²⁸ using 50 mM HEPES buffer pH 7.5, 20% acetonitrile, 1 mM cysteine, 0.1 mM
27
28 cystine, over 48 hours at 4 °C. After a desalting step and analytical characterizations by analytical
29
30 HPLC on a Waters e2695 separation module equipped with a XBridge BEH300 C18 column (5µm ,
31
32 4.6x250mm, Waters) and by mass spectrometry on a MALDI-TOF spectrometer (4800 MALDI TOF-
33
34 TOF Analyser from Applied Biosystems, USA), the toxins were distributed in 96 well plates at a
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36 concentration between 2-120 µM.
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42 **High-throughput peptide production by recombinant expression.** Optimization of gene synthesis
43
44 and expression clone constructs for venom-peptide production have been published previously³¹, in
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46 addition to the final protocols used in this project²⁹. Briefly, genes encoding animal venom peptides
47
48 were designed with codon optimization for expression in *E. coli* using the ATGenium codon
49
50 optimization algorithm (NZYTech genes & enzymes, Portugal). Synthetic genes were produced in a
51
52 high-throughput pipeline and purified PCR products were subsequently cloned into the pHTP4
53
54 expression vector using the NZYEasy Cloning kit (NZYTech genes & enzymes, Portugal), according
55
56 to ligation independent cloning (LIC) technology protocols. In pHTP4, peptide genes are placed under
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3 the control of a T7 promoter, and are produced as fusion proteins in the *E. coli* periplasmic space.
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5 Fusions contain an N-terminal Disulphide bond isomerase C (DsbC) fusion, an internal hexa-histidine
6
7 tag for purification and a TEV protease recognition sequence to allow cleavage and isolation of the
8
9 native peptides. Optimization of expression and purification conditions^{30,31} and the final recombinant
10
11 production protocols used herein²⁹ have been described previously. All steps were carried out in 24-
12
13 deep-well plates or in 96-deep-well plates on a Tecan Freedom EVO 200 robot (Switzerland)⁶⁹. pHTP4
14
15 expression plasmids were transformed into BL21 (DE3) pLysS *E. coli* and cultured overnight at 25 °C
16
17 before harvesting. Cell pellets were lysed enzymatically and by sonication. DsbC-His-peptide fusions
18
19 were isolated from the soluble cell lysates by nickel affinity purification. Concentrations of the 96
20
21 purified DsbC-His-fusion proteins were estimated by UV absorbance at 280 nm in a microtiter plate
22
23 reader (GENios Plus, TECAN, Switzerland). TEVsh protease was added (1/10 w/w) directly to the
24
25 eluted fusion proteins. The mixture was supplemented with a final concentration of 0.1 mM DTT then
26
27 incubated overnight at room temperature in a Microtron shaking incubator (INFORS-HT, Switzerland)
28
29 (200 rpm). Finally, samples were acidified for 1 h with a final concentration of 5% acetonitrile and
30
31 0.1% formic acid (FA) at room temperature with mild agitation. TEV protease were precipitated during
32
33 this acidification process and the 96 recombinant toxins per plate were subjected to a final purification
34
35 step on C18 chromatographic resin by Solid-Phase Extraction (SPE). The target peptides were eluted
36
37 from the C18 resin, and analyzed by UHPLC and MS to confirm the identity and the quantity of the
38
39 toxin (Figure S2). The toxins were distributed in 96 well plates at a concentration between 1-10 μM.

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47 **Production of N-TRTX-Preg1a and N-BUTX-Ptr1a toxins.** Linear peptide was obtained by solid-
48
49 phase chemical synthesis following the same protocol described above using Fmoc-Thr(tBu)-Wang
50
51 resin LL (Novabiochem[®]) for N-TRTX-Preg1a and H-Cys(Trt)-Trityl NovaPEG resin
52
53 (Novabiochem[®]) for N-BUTX-Ptr1a. After resin cleavage and ether precipitation, linear peptide was
54
55 then purified on Waters Preparative HPLC 600 with an XBridge BEH 300Å Prep C18 column (5 μm,
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57 19 x 250 mm, Waters). If disulfide bridges were to be prevented from forming, linear peptide was
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3 treated in Guanidinium chloride 6M with iodoacetamide (ten times in excess) for 2h under agitation at
4
5 room temperature. The oxidation step was performed using Venomics oxidation conditions²⁸.
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7 Resulting oxidized peptide was finally purified on a Waters 600 controller using an XBridge BEH
8
9 300Å Prep C18 column (5 µm, 10 x 250 mm, Waters). Purity of the final product was above 95% and
10
11 was assessed separately by MALDI-TOF spectrometry (4800 MALDI TOF-TOF Analyser (Applied
12
13 Biosystems, USA)) and analytical HPLC on Waters e2695 separation module with an XBridge BEH
14
15 300Å C18 column (5 µm, 4.6 x 250 mm, Waters). Final quantity was measured by amino-acid
16
17 quantification using the PicoTag kit (Waters Associates, Milford, MA).
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21 **Sequence analysis and phylogenetic reconstruction.** Sequences were queried against UniProt
22
23 (release 2019_02) and refseq_protein (release-96) with blastp 2.29.1. Searching parameters were
24
25 adjusted for short-length sequences. Spider and scorpion venom proteins in UniProt were annotated as
26
27 in VenomZone (<https://venomzone.expasy.org/> release May, 2019). Multiple sequence alignments
28
29 were performed with mafft 7.310 --auto option. Phylogenetic trees were obtained with RAxML 8.2.11
30
31 --PROTGAMMAAUTO --# 100 options, and MrBayes v3.2.0 ngen=1000000 nchains=4 nruns=2.
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35 **NMR structures.** Solutions of 0.5 mM N-TRTX-Preg1a, and 1.2 mM N-BUTX-Ptr1a were prepared
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37 in 3 mm tubes (respectively, 315 µg of N-TRTX-Preg1a and about 1 mg of N-BUTX-Ptr1a were
38
39 dissolved in 220 µL of H₂O/D₂O (95/5), at pH 4.5). All NMR spectra were acquired on a BRUKER 700
40
41 MHz NMR spectrometer equipped with a 5 mm TCI cryoprobe, at 298K. The standard set of 2D ¹H-
42
43 NMR experiments (COSY, 80 ms TOCSY, and 160 ms NOESY) were completed with natural
44
45 abundance ¹⁵N- and ¹³C-HSQC spectra. Processing, analyses, and calculations were successively
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47 performed with Bruker's TopSpin3.2, CcpNMR⁷⁰ and Aria2.3⁷¹ software. Chemical shift assignments
48
49 were carried out following the classical procedure, distance constraints were deduced from the volume
50
51 integration of NOE correlations and introduced into the standard iterative calculation process of the
52
53 Aria (ambiguous restraints for iterative assignment) software. For each Aria run, 500 structure
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55 calculations were performed per iteration, and 250 structures were calculated in the final iteration in
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3 water. For both samples, the first runs were performed allowing all combinations of disulfide pairing
4 (ambiguous intersulfur constraints option). This option was kept for the N-BUTX-Ptr1a, for which the
5
6 calculations converged towards a unique array of disulfide bridges, and the only additional constraints
7
8 were the H-bonds observed in the β -sheet already formed in the first runs. For the N-TRTX-Preg1a
9
10 sample, (φ, ψ) angular restraints deduced by the TALOS software⁷² from ^{13}C and ^1H chemical shifts
11
12 were added to NOE constraints. Covalent bonds between sulphur atoms involved in each defined
13
14 bridge were introduced in the last runs of the calculation. Finally, for each peptide, ten of the structures
15
16 refined in water in the final iteration were selected according to their agreement with experimental
17
18 data. The final sets of structures were displayed and analyzed using PyMol (The PyMOL Molecular
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20 Graphics System, Version 2.0 Schrödinger, LLC), and their quality were evaluated using Promotif⁷³
21
22 and PROCHECK3.5⁷⁴ software.

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28 **Screening.** The ^{125}I -NDP- α -MSH and membranes expressing receptors were purchased from Perkin
29
30 Elmer (Courtaboeuf, France). Screening conditions were optimised in order to get a Z'-factor value
31
32 superior to 0.5. Z'-factor is defined as: $1 - \frac{3\text{MAD}_{max} + 3\text{MAD}_{min}}{\text{Median}_{max} - \text{Median}_{min}}$, where MAD equals Median
33
34 Absolute Deviation. On each 96-well plate, eight wells were dedicated to negative controls and
35
36 correspond to total binding of the radioligand in absence of any other ligand. Nonspecific binding was
37
38 measured in the presence of 1 μM NDP- α -MSH (Tocris Bioscience) in eight other wells per plate.
39
40 Following screening, peptides with an associated binding value bigger or lower than Median –
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42 $3 \times \text{MAD}_{\text{median}}$ ⁷⁵ were classified as hits. Peptides were screened at concentrations between 0.1 to 100
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44 μM according to their production yields.

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49 **Radioligand binding assays.** The ^{125}I -NDP- α -MSH and melanocortin receptors were purchased from
50
51 Perkin Elmer (Courtaboeuf, France). Binding experiments were performed using radioligand at 50 pM
52
53 in a 100- μL reaction mixture at room temperature in buffer composed of 25 mM Hepes pH 7.4, 1 mM
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55 MgCl_2 , 1 mM CaCl_2 , 0.2 g/L BSA with various concentrations of competitors. Controls were the same
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3 as for the primary screening on hMC4R. After 3 hours of incubation time, samples were filtered
4 through 96 well GF/C filter plates preincubated with 0.5% polyethylenimine. 25 μ L of MicroScint 0
5
6 was added onto each dry filter and the radioactivity was quantified on a TopCount beta counter (Perkin
7
8 Elmer, Courtaboeuf, France) with 45% yield. Competition binding data were fitted to a one-site/state
9
10 inhibition mass action curve using Prism software, v5.0.0 (GraphPad, La Jolla, CA, USA). IC₅₀ values
11
12 were converted to K_i, using the Cheng–Prusoff equation⁷⁶. K_d values were 0.34 nM, 0.47 nM, 0.25
13
14 nM and 0.88 nM, respectively, for hMC1R, hMC3R, hMC4R and hMC5R. Data represent at least
15
16 three independent experiments performed in duplicate and are presented as mean \pm SEM.
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21 **Functional assays.** Human embryonic kidney cells (HEK293, DSMZ, ACC305, authentication: full-
22 matching STR reference profile of 293 in the database, authentic) and HEK293-MC1R (human
23 embryonic kidney cells expressing the human melanocortin 1 receptor with an N-terminal HA-tag and
24 C-terminal FLAG-tag due to stable transfection, selection marker: hygromycin, AG Beck-Sickinger,
25 University Leipzig, Leipzig, Germany) were grown in Dulbecco's modified Eagle's medium (DMEM)
26 with 4.5 g/L glucose and L-glutamine and Ham's F12 (1:1, v/v; Lonza) supplied with 15% (v/v) heat-
27 inactivated fetal calf serum (FCS; Lonza) in a humidified atmosphere at 37 °C and 5% CO₂. HEK293
28 or stably transfected HEK293-HA_MC1R_FLAG cells were transfected in solution with 0.15 μ L
29 Lipofectamine 2000 (Invitrogen) per well and 30 ng Plasmid pGL4.29[luc2P/CRE/Hygro] (Promega)
30 per well and additionally 30 ng pcDNA3-hMC4R per well when needed. Lipofectamine and plasmid
31 solutions were prepared in 6 μ L Opti-MEM medium (Gibco) per well. After 5 min incubation at RT
32 the Lipofectamine solution was slowly added to the DNA solution and incubated for 30 min at RT.
33 Transfection of 20,000 cells/well in 30 μ L medium (DMEM/Ham's F12/ 15% FCS) without antibiotics
34 was performed in solution by slowly adding the DNA/Lipofectamine solution to the cells. After careful
35 mixing, 42 μ L per well was plated on a 384-well microplate (Greiner bio-one, F-Bottom, white) and
36 incubated overnight. The day after transfection, medium was removed and cells were stimulated with
37 15 μ L peptide solution in serum free medium (1 nM α -MSH (AG Beck-Sickinger, University Leipzig,
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3 Leipzig, Germany) corresponding to \sim EC₈₅ at hMC1R or \sim EC₇₅ at hMC4R, 100 μ M N-TRTX-Preg1a
4 or 100 μ M N-BUTX-Ptr1a) for 3 h at 37 °C, 5% CO₂. As a positive control 1 μ M forskolin (Sigma-
5 Aldrich) was used, while serum-free medium was used as the negative control (baseline). For
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Luciferase detection 15 μ l OneGlo Substrate (Promega) was added at RT to each well. The 384-well
plate was transferred directly to the plate reader (Tecan Infinite 200 PRO) and luminescence was
measured after shaking for 5 sec (double orbital, amplitude: 3.5 mm) and 5 min incubation with an
integration time of 1000 ms at room temperature. Data analysis was performed with GraphPad Prism.
After baseline subtraction, data were normalized to stimulation with 1 μ M Forskolin. Data represent
the mean \pm SEM from three independent experiments performed in triplicates. Statistical analysis was
performed with GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, California, USA).
Statistical significance was determined by one-way analysis of variance (ANOVA) followed by
Dunnett's multiple comparison test. *** p < 0.001.

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Electrophysiology. For the expression of Na_v channels (human (h)Na_v1.1, rat (r)Na_v1.2, rNa_v1.3,
rNa_v1.4, hNa_v1.5, mouse (m)Na_v1.6, hNa_v1.7, rNa_v1.8 channels and the auxiliary subunits r β 1, h β 1),
K_v channels (rK_v1.1, rK_v1.2, hK_v1.3, rK_v1.4, rK_v1.5, rK_v1.6, rK_v2.1, hK_v3.1, rK_v4.3, hK_v7.2,
hK_v7.3, hK_v10.1, hK_v11.1), Ca_v channels (rCa_v3.3), hGIRK1 and nAChRs (h α 1 β 1 γ δ and h α 7)
in *Xenopus laevis* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMACHINE-
mMACHINE transcription kit (Ambion, Carlsbad, CA). The harvesting of stage V–VI oocytes from
anesthetized female *Xenopus laevis* frogs was previously described⁷⁷. Oocytes were injected with 50
nl of cRNA at a concentration of 1 ng/nl using a microinjector (Drummond Scientific, Broomall, PA).
The oocytes were incubated in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM
MgCl₂, and 5 mM HEPES pH 7.4, supplemented with 50 mg/L gentamycin sulfate.

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Two-electrode voltage-clamp recordings were performed at room temperature (18–22 °C) using a
Geneclamp 500 amplifier (Molecular Devices, Downingtown, PA) controlled by a pClamp data
acquisition system (Axon Instruments, Union City, CA). Whole cell currents from oocytes were

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3 recorded 1–4 days after injection. Bath solution composition was 96 mM NaCl, 2 mM KCl, 1.8 mM
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5 CaCl₂, 2 mM MgCl₂, and 5 mM HEPES pH 7.4. Toxins were applied directly to the bath. Resistances
6
7 of both electrodes were kept between 0.8 and 1.5 MΩ. The elicited currents were filtered at 2 kHz
8
9 (Na_v), 0.5 kHz (K_v & Ca_v), 0.05 kHz (GIRK & nAChR) and sampled at 20 kHz (Na_v), 2 kHz (K_v &
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11 Ca_v) and 0.1 kHz (GIRK & nAChR) using a four-pole low-pass Bessel filter. Leak subtraction was
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13 performed using a -P/4 protocol. For the electrophysiological analysis, a number of protocols were
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15 applied from a holding potential of -90 mV. K_v1.1–K_v1.6 currents were evoked by 250 ms
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17 depolarizations to 0 mV followed by a 250 ms pulse to -50 mV. K_v2.1, K_v3.1, and K_v4.3 currents
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19 were elicited by 250 ms pulses to +20 mV from a holding potential of -90 mV. K_v7 and K_v10.1
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21 currents were evoked by 2 s depolarizing pulses to 0 mV from a holding potential of -90 mV. Current
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23 traces of K_v11.1 channels were elicited by applying a +40 mV prepulse for 2 s followed by a step to
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25 -120 mV for 2 s. Sodium current traces were evoked by 100 ms depolarization to the voltage
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27 corresponding to maximal sodium current in control conditions. For GIRK measurements, the oocytes
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29 were clamped at -70 mV and superfused with a high K⁺ solution (96 mM KCl, 2 mM NaCl, 1 mM
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31 MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES pH 7.5). Ca_v channel currents, carried by 10 mM Ba²⁺, were
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33 elicited by 200 ms test depolarizations applied from -90 mV to voltages ranging from -50 mV or +30
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35 mV. For nAChRs, oocytes were clamped at a holding potential of -70 mV throughout all recordings.
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37 To minimize interference of endogenous Ca²⁺-activated Cl⁻ channels during the experiments, a Ca²⁺-
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39 free bath perfusion solution was used, containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2.5
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41 mM BaCl₂, and 5 mM HEPES pH 7.4. Current responses were evoked by 10 s applications of
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43 increasing concentrations of acetylcholine (starting at 200 μM). All data were analyzed using pClamp
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45 Clampfit 10.1 (Molecular Devices) and Origin 7.5 software (OriginLab, Northampton, MA).
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53 **Toxins nomenclature.** Newly characterized toxins were named according to the rational nomenclature
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55 proposed by King *et al.*⁷⁸ with a greek letter associated with a biological function followed by the
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57 generic name indicating from which taxon the toxin is derived, genus and species indicated
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3 respectively by uppercase and lowercase letters, a number to distinguish different toxins with similar
4 activity and a lowercase letter for isoforms. In this study, uppercase nu greek letter (ν) was chosen as
5 new descriptor of toxins active on melanocortin receptors.
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10 11 12 **ASSOCIATED CONTENT**

13 14 **Supporting Information**

15 The Supporting Information is available free of charge on the ACS Publications website at DOI:
16
17 LC-MS analysis of five representative toxins with their cystein patterns, produced by solid phase
18 chemical synthesis; analysis of 4 representative toxins with their cystein patterns, produced by
19 recombinant expression; quality assessment at each stage of N-TRTX-Preg1a and N-BUTX-Ptr1a
20 production in Fmoc solid-phase chemical synthesis; activity of 100 μ M N-TRTX-Preg1a on ion
21 channels; activity of 100 μ M N-BUTX-Ptr1a on ion channels; cysteine pattern diversity for toxins
22 containing an even number of cysteines identified in the Venomics bank; cysteine pattern diversity for
23 toxins containing an odd number of cysteine residues identified in the Venomics bank; experimental
24 NMR data used for the calculations, and final structural statistics for the ten models representative of
25 the solution structure of N-TRTX-Preg1a; experimental NMR data used for the calculations, and final
26 structural statistics for the ten models representative of the solution structure of N-BUTX-Ptr1a
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44 45 **AUTHOR INFORMATION**

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ABBREVIATIONS

ANOVA, one-way analysis of variance; BUTX, butantoxin; Cav, voltage-gated calcium channel; CHO, chinese hamster ovary cells; COSY, correlation spectroscopy; CS $\alpha\beta$, cysteine-stabilized α -helix β -sheet; DsbC, Disulfide bond Isomerase C; DMEM, Dulbecco's modified Eagle's medium; Fmoc, fluorenylmethoxycarbonyl; GIRK, G protein-coupled inwardly-rectifying potassium channel; GPCR, G-Protein Coupled receptors; HCTU, H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEK, human embryonic kidney cells; HEPES, hydroxyethyl piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; HT, high-throughput; HT, high-throughput screening; ICK, inhibitory cystine knot; Ki, inhibitory constant; Kv, voltage-gated potassium channel; LC, liquid chromatography; hMC1R, human melanocortin 1 receptor; hMC3R, human melanocortin 3 receptor; hMC4R, human melanocortin 4 receptor; hMC5R, human melanocortin 5 receptor; MALDI-TOF, Matrix Assisted Laser Desorption Ionisation - Time of Flight; MAD, median absolute deviation; MS, mass spectrometry; MSH, melanocyte stimulating hormone; N $^{\circ}$, number; Nav, voltage-gated sodium channel; NDP- α -MSH, [Nle⁴,D-phe⁷]- α -melanocyte stimulating hormone; NGS, next-generation sequencing; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PTM, post-translational modification; RMSD, root-mean-square deviation; RT, room temperature; SD, standard deviation; SEM, standard error of the mean; Seq, sequence; TOCSY, total correlation spectroscopy; TRTX, theraphotoxin; UPLC, ultra-performance liquid chromatography; UV, ultraviolet.

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