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Novel Kunitz-like Peptides Discovered in the Zoanthid *Palythoa caribaeorum* through Transcriptome Sequencing

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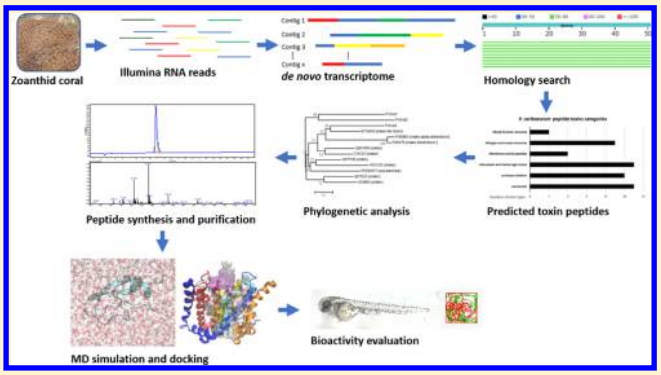
Supporting Information

ABSTRACT: *Palythoa caribaeorum* (class Anthozoa) is a zoanthid that together jellyfishes, hydra, and sea anemones, which are venomous and predatory, belongs to the Phyllum Cnidaria. The distinguished feature in these marine animals is the cnidocytes in the body tissues, responsible for toxin production and injection that are used majorly for prey capture and defense. With exception for other anthozoans, the toxin cocktails of zoanthids have been scarcely studied and are poorly known. Here, on the basis of the analysis of *P. caribaeorum* transcriptome, numerous predicted venom-featured polypeptides were identified including allergens, neurotoxins, membrane-active, and Kunitz-like peptides (PcKuz). The three predicted PcKuz isotoxins (1−3) were selected for functional studies. Through computational processing comprising structural phylogenetic analysis, molecular docking, and dynamics simulation, PcKuz3 was shown to be a potential voltage gated potassium-channel inhibitor. PcKuz3 fitted well as new functional Kunitz-type toxins with strong antilocomotor activity as *in vivo* assessed in zebrafish larvae, with weak inhibitory effect toward proteases, as evaluated *in vitro*. Notably, PcKuz3 can suppress, at low concentration, the 6-OHDA-induced neurotoxicity on the locomotive behavior of zebrafish, which indicated PcKuz3 may have a neuroprotective effect. Taken together, PcKuz3 figures as a novel neurotoxin structure, which differs from known homologous peptides expressed in sea anemone. Moreover, the novel PcKuz3 provides an insightful hint for biodrug development for prospective neurodegenerative disease treatment.



KEYWORDS: *soft coral, Transcriptome, Kunitz-like peptides, protein docking, zebrafish, neurotoxin, zoanthids*

INTRODUCTION

The Phylum Cnidaria comprises approximately 13 000 species distributed in five different classes: Anthozoa (sea anemones, corals, zoanthids), Scyphozoa (true jellyfishes), Cubozoa (box jellyfishes), Hydrozoa (hydras, hydroids, hydromedusae, and siphonophores), and Staurozoa (stalked jellyfish).[1,2] In general, all of them are considered to be toxic.[3] The distinguishing feature of cnidarian is the presence of cnidocytes (cnidoblasts or nematocytes) in the body tissues for prey capture and survival. Along the sting, the cnidocytes fire the cnidocysts (cnidae) that convey numerous toxins such as neurotoxins, cytolysins, toxic phospholipases.[4]

Zoanthids are hexacorallian anthozoans belonging to the order Zoantharia.[2] They are characterized by colonies of clonal polyps comprising two rows of tentacles and a single ventral siphonoglyph linked together by a coenenchyma.[5] Conservatively, the toxic secretion (venom) stored in the nematocysts of tentacles is a complex of substances, composed of a diversity of molecules including large proteins, peptides, polyamines, and salts, among others. In combination, these components disrupt the physiological homeostasis of prey animals upon cnidarian-venom injection.[6] Over the past decade, several toxins such as cytolysins and protease inhibitors have been mostly characterized, in their basic and applied aspects, from the sea anemones and jellyfishes.[7,8] For instance, from the beadlet anemone *Actinia equina* Equinatoxin II (EqT II), a pore-forming toxin was purified and shown to have a significant cytotoxicity against Ehrlich ascites tumor, L1210 leukemia cell lines,[9] and diploid lung fibroblasts of the Chinese hamster.[10]

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Another *A. equine* toxin, namely Equistatin, was shown to be a potent inhibitor of papain-like cysteine proteinase and aspartic proteinase cathepsin D.[11] Interestingly, the overexpression of papain-like cysteine proteases and cathepsin D has been reported in diseases of central nervous system[12] and breast carcinoma,[13] which open a window of medical applications for inhibitors. Progress on "omics" research has allowed the improvement of our knowledge about the components of the toxic content of cnidarians.[14,15] However, with exception of a recent report on the transcriptome of *P. variabilis*,[16] the comprehension of venom-related polypeptides and peptide toxins from anthozoans other than sea anemones have received less attention. In fact, only extracts and some countable isolated compounds have been characterized from zoanthids, of which they exhibit several and interesting biological activities, such as, neurotoxicity,[17] cardiotoxicity,[18] citotoxicity, and antioxidant, hemolytic, and antimicrobial activity.[19]

Therefore, the disclosure of novel toxins from zoanthids will have a significance to offer a range of molecular tools to study cell physiology and provide promising sources of pharmacological lead or active agents to include in the therapeutic arsenal to investigate and fight human pathologies.

In the present work, we aimed to analyze the transcriptome of the zoanthid *Palythoa caribaeorum* (class Anthozoa, subclass Hexacorallia, order Zoantharia, family Sphenopidae) to investigate the presence of venom-related polypeptides and peptide toxins that have the potential to be developed into a lead-compound in pharmaceutical biotechnology. A diverse array of predicted venom toxin polypeptides was found and now reported. Three Kunitz-type peptide isotoxins, namely PcKuz1 to 3, were identified in the transcriptome data set of *P. caribaeorum*, and they were selected for in silico structural and binding studies, including protein−protein docking, as well as *in vivo* toxicity test in zebrafish larvae. Additionally, the 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in zebrafish model[20−22] was employed to examine the neuroprotective effect of the most neuroactive PcKuz peptides, the PcKuz3.

EXPERIMENTAL PROCEDURES

Sampling and Processing of Zoanthid *P. caribaeorum*

Description of *P. caribaeorum* sampling can be found elsewhere.[23] According to previously described, all specimens were quickly washed in distilled water, chopped with scissors, and dipped immediately into 10 volumes of RNAlater (Life Technologies, USA) for ribonucleic acid preservation. After storage at 4 °C for 48 h, the RNA-preserving solution was drained and the tissue maintained at −80 °C until processing. The minced tissue was powdered with a porcelain mortar and pestle under liquid nitrogen and total RNA was purified using TRIzol reagent (Life Technologies, USA) following the manufacturer's protocol.

Preparation of RNA Library and RNA-Sequencing

The library for whole RNA sequencing was prepared through a standard protocol established by the Beijing Genomic Institute, BGI (Shenzhen, China). Initially, polyadenylated RNA sequences were isolated using oligo (dT). Single-stranded 5′ RNA adaptors were ligated to mRNA fragments using T4 RNA ligase (Ambion, Austin, TX, USA) and then reversely transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). 3′ DNA adaptor was ligated to the digested DNA fragments after digestion with Mmel, and the products were amplified using PCR. Finally, RNA deep sequencing (RNA-seq) was performed on a HiSeq 2500 automatic sequencing platform at the BGI (Shenzhen, China). The transcriptome shotgun assembly (TSA) project was previously deposited at DDBJ/EMBL/GenBank under the accession GESO00000000, associated with the BioProject PRJNA320984 and bioasmple SAMN04961660, SAMN04961665.

Bioinformatic Processing

RNA sequencing, assembly, and assessment of *P. caribaeorum* were as described in our recently published article.[23] The raw reads were processed to obtain clean reads by removing the adapter sequences and low-quality sequences (i.e., reads in which more than 50% of the bases had quality value $s \leq 5$) by an in-house C++ script. Next, de novo transcriptome assembly was carried out using the short-read assembly program Trinity.[24] The TIGR Gene Indices Clustering Tools (TGICL) software[25] was used to obtain the longest and most complete consensus transcripts by clustering the assembled data sets of the two samples. Raw data and clean data were subjected to FastQC[26] to evaluate sequence quality. Systematically similarity analyses were performed on the transcriptome using BLASTX tools against UniprotKB database.[27] The standard cutoff E-value of <1e-5 was used. The best matches were selected to predict ORFs using TransDecoder v2.0.1 tool.[24] The predicted proteome was searched against PFAM database to identify the functional domains.[28−30] On the basis of the functional annotation, the peptides were classified into different toxin categories by an in-house Perl script. Phylogenetic analysis was performed using the program MEGA version 6[31] with the MUSCLE[32,33] algorithm as the multiple alignment method. Reliability of the tree was assessed by the bootstrap method and the node support was determined using 500 bootstrap replicates.

Structures of the candidate peptide toxins were predicted using SWISS-MODEL server.[34,35] The modeled structures were subjected to energy minimization and molecular dynamics (MD) simulations with CHARMM27 all-atom force field using the GROMACS 5.1 simulation software.[36,37] Each system was each system was solvated with TIP3P water then subjected to energy minimization of 5×10^7 steps, equilibration in 310 K for 10 ns, and production run for 10 ns, with 2 fs time step and van der Waals interaction cutoff at 1.2 nm. Particle-meshed Ewald was employed for the long-range electrostatics, 310 thermostat and 1.0 barostat were used to generate the NPT ensemble. The equilibrated structures were compared to dendrotoxins (PDB 1DTX and 1DEM), which were considered as homologous to the candidate peptides. Molecular visualization and structure alignment were achieved using the PyMOL program (version 1.8, Schrödinger, LLC).

The atomic coordinates of the potassium voltage-gated channel subfamily A channels including member 1 (UniProt ID: Q09470, Kv1.1) and member 2 (UniProt ID: P16389, Kv1.2) were homology-modeled in the SWISS-MODEL server taking the Kv1.2 crystal structure (PDB ID: 2R9R) as the template. Only the transmembrane regions of the modeling channels were retained for simulation studies. The protein−POPC bilayer complexes were constructed using the Membrane-builder tool[38,39] in the CHARMM-GUI server.[40] Then each system was fully solvated with TIP3P water and ions were added to reach the physiological conditions of 150 mM. Each system was subjected to energy minimization of 5×10^7 steps, equilibration in 310 K for 10 ns, and production run for

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10 ns. Each final protein−membrane system consisted of about 315 POPC lipids and 29143 water molecules with the box dimension of 12.35 × 12.35 × 10.31 nm^3. The simulations were run with 2 fs time step, with van der Waals interaction cutoff at 1.2 nm. Particle-meshed Ewald was employed for the long-range electrostatics, 310 thermostat and 1.0 barostat were used to generate the NPT ensemble. Molecular interactions were described by the CHARMM27 force field and simulations were performed using GROMACS 5.1.

According to the annotations from Uniprot database, S4 to S6 domains of the Kv1.1 and Kv1.2 channels that form transmembrane regions were retained for docking prediction (Figure 6a and Figure 6b). The fast Fourier transform (FFT)-based, initial-stage rigid-body molecular docking algorithm ZDOCK[41−43] was applied to model the interactions between *P. caribaeorum* kunitz toxin and the ion channels. All structures visualization was achieved using the VMD program v1.9.2.[44]

Peptide Sequences and Synthesis

All peptides in this study were *a priori* linear without preformed disulfide bonds, namely PcKuz1 to 3, were synthesized by solid phase chemistry and obtained at a purity grade over 90% and confirmed by the presence of a single peak in analytical reverse-phase HPLC (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS) analysis (Cellmano Biotech Limited, Hefei, China). Complete deprotection and cleavage was carried out essentially with trifluoroacetic acid in water. The crude peptides were precipitated out by the addition of chilled ether. Then the crude peptide was purified by HPLC, freeze-dried, and retested by HPLC to make sure that it is qualified (Figure S1). The peptide was solubilized in 7% dimethyl sulfoxide to make a 1 mM stock solution and stored at −20 °C until required.

Toxicity Experiments toward Zebrafish Larvae

Zebrafish Maintenance. Wild-type zebrafish, which were used in this study, was manipulated as described in the Zebrafish Handbook.[45] Briefly, the zebrafish was maintained in standard conditions at the temperature of 28 °C with a 14 h/10 h light/dark cycle. The zebrafish was fed twice daily with brine shrimp and occasionally with general tropical fish food. The embryos were generated by natural pairwise mating (3−12 months old) and were raised at 28.5 °C in embryo medium (13.7 mM NaCl, 540 μM KCl, 25 μM Na_2HPO_4, 44 μM KH_2PO_4, 300 μM $CaCl_2$, 100 μM $MgSO_4$, and 42 μM $NaHCO_3$, pH 7.4). Ethic approval for the animal experiments was granted by the Animal Research Ethics Committee in University of Macau.

Assessment of Survival Rate. Zebrafish larvae at six-day postfertilization (6-dpf) were separated into a 24-well plate and exposed to 2-logs (from 5 to 100 μM) of *P. caribaeorum* Kunitz-like (PcKuz) peptides. The acute toxicity and mortality of zebrafish exposed to PcKuz peptides were determined by monitoring the absence of a heartbeat, as observed under a light microscope.

Assessment of Locomotion Toxicity. Zebrafish embryo at 6-dpf were incubated with different concentration of only PcKuz3 at 5 μM, 10 μM, and 15 μM for indicated durations. Zebrafish larvae at 6-dpf were transferred into 96-well plates (one larva per well and 12 larvae per group). Zebrafish showing signs of excessive stress upon handling (such as rapid and disorganized swimming or immobility for 2 min) were discarded. The experiments were performed in a calm, sealed area. The swimming behavior was monitored by an automated video tracking system (Viewpoint, ZebraLab, LifeSciences, France). The 96-well plates and camera were housed inside a Zebrabox, and the swimming pattern of each fish was recorded in five sessions of 10 min each. The total distance traveled was calculated as the distance that zebrafish larvae was capable of swimming during one session (10 min). A statistical analysis of the total distance traveled by each zebrafish larva in the different treatment groups was performed using an ANOVA and Dunnett's test.

Assessment of Neuroprotective Effect. Zebrafish larvae at 3dpf were treated with indicated concentrations of the peptides in the absence or in the presence of 250 μM 6-OHDA for 4 days, and then zebrafish at 7 dpf were transferred into 96-well plates (1 fish/well). Zebrafish behavior was monitored by a digital video tracking system (Viewpoint, ZebraLab, LifeSciences), as described above. The total moved distances and swimming patterns were recorded in 10 min-long session. The larvae were allowed to habituate to the environment of the system for 30 min before the start of the data acquisition.

Protease Inhibitory Activity. The inhibitory activity of PcKuz peptides toward serine proteases included trypsin, and elastase. The test was conducted as suggested by Hergenhahn et al.[46] Trypsin, alpha-chymotrypsin, substilisin, elastase, the chromogenic susbstrates N-benzoyl-Phe-Val-Arg-para-nitroanilide, N-succinyl-Ala-Ala-Pro-Phe-para-nitroanilide, and N-succinyl-Ala-Ala-Ala-para-nitroanilide were from Sigma-Aldrich. Briefly, increase concentrations (50 μM, 100 μM, 200 μM) of PcKuz peptides were incubated with protease (62.5 μM trypsin and 1.25 μM elastase) at 30 °C for 10 min. After incubation, the respective substrates (500 μM of N-benzoyl-Phe-Val-Arg-para-nitroanilide as substrate for trypsin, 125 μM N-succinyl-Ala-Ala-Pro-Phe-para-nitroanilide for alpha-chymotrypsin, and 125 μM of N-succinyl-Ala-Ala-Ala-para-nitroanilide for elastase) diluted in 0.1 mM Tris-HCl buffer (pH 8.0) were added into the mixture and again incubated at 30 °C for additional 10 min. The total reaction volume was 80 μL. Then, the reaction was stopped by adding 20 μL of 50% molecular grade acetic acid. Finally, the extinction of para-nitroanilide formation was determined at 405 nm using UV-spectrophotometer (NanoView). The percentages of remaining activity were calculated and plotted against the molar ratios of protease inhibitor to protease. The assay was performed in three replicates and the average was considered.

RESULTS

RNA sequencing data processing, assembly, and assessment of *P. caribaeorum* transcriptome were conducted as detailed in our previous study.[23] Briefly, RNA sequencing of pair-end 90 bp was conducted with sample from whole bodies of healthy colonies and from colonies undergoing bleaching, resulted in a total of 63 914 343 and 55 523 043 reads, respectively. The transcripts of each sample were first assembled using Trinity and then clustered together using TGICL to obtain the consensus transcript sequences. A data set of 136 654 transcripts, with a mean sequence length of 874 bp and N50 equaling 1391 bp, was obtained for the combined transcriptomes of healthy *P. caribaeorum* tissue and tissue undergoing bleaching. All these unigenes were blasted against the Tox-Prot database in the SWISS-PROT. A total of 1350 unigenes was selected after e-value-stringent filtration and 2279 predicted ORFs were got after proteome prediction. Finally, a total of 47 predicted toxin-related polypeptides were found.

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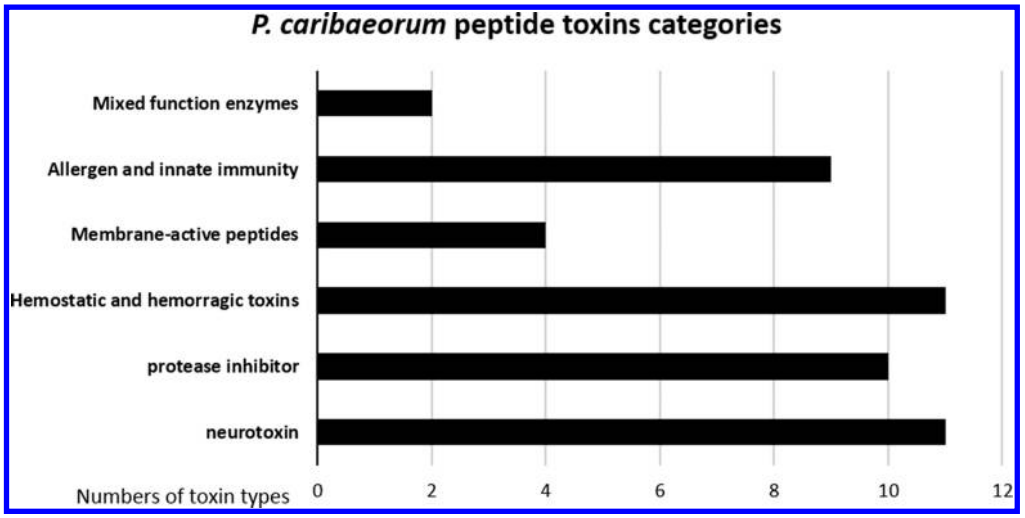

Figure 1. Classification of putative *P. caribaeorum* toxins according to PFAM domain database scan. Two categories of toxins, including neurotoxin, hemostatic, and hemorrhagic toxins, comprise 11 peptides each, respectively. These contain the most abundant numbers of all putative peptides. Within neurotoxin, there are three toxin types: Kunitz-like peptides, cysteine-rich secretory peptides, and ShK-like peptides. The third most abundant toxins were protease inhibitors contain Kazal-type serine protease inhibitor domain. The fourth most abundant toxins were peptides related to allergens and innate immunity components. The left two categories were membrane-active peptides and mixed function enzymes.

These predicted toxic peptides were classified into six functional categories (Figure 1, Table S1).

After toxin classification, Kunitz-type isotoxins (PcKuz peptides) were initially annotated as inhibitors of serine proteases, which exert their action on living processes, such as hemostasis,[47,48] neurotransmission,[49] or both.[50−52] These PcKuz peptides were selected for multiple sequence comparison and phylogenetic analysis. From the Neighbor-joining tree, the candidate PcKuz peptides were phylogenetic related to the snake-type Kunitz-like toxins. The PcKuz peptides have similar sequence characteristics with snake-type Kunitz-like toxins, that is, they are basic peptides with 50 to 60 amino acids, containing six predicted cysteine residues, that could fold by means of three highly conserved disulfide bridges (C1−C6, C2−C4, C3−C5) (Figure 2). Indeed, the PcKuz peptide sequences splitted in two phylogenetic branches. One comprises PcKuz1 and PcKuz2 and the other the PcKuz3, which group together with other known Kunitz-like toxins mostly originated from snakes, indicated that PcKuz3 is more evolutionary related to snake-type Kunitz-like toxins. Notably, PcKuz3 grouped very close with the Mamba dendrotoxin, which has been validated as a peptide ligand of several members of potassium voltage-gated channel subfamily A channels such as Kv1.1 or Kv1.2.[49,53]

After obtaining the homology models of the candidate peptides, MD simulations were employed to refine the modeling structures. As shown in Figure 3, the root-mean-squared deviation (rmsd) values after 10 ns simulation of PcKuz1 and PcKuz2 reached a plateau at about 0.2 and 0.6 Å respectively, while PcKuz3 remains at or below 0.2 Å over the course of simulation. These indicate that structures of all three peptides obtained from the homology modeling are quite stable.

The sequences of PcKuz peptides (PcKuz1, PcKuz2, and PcKuz3), their molecular weights, pI values, and LD_{50} values for zebrafish larvae are listed in Table 1. In a biological activity screening (Figure 4) of PcKuz1, PcKuz2, and PcKuz3, no significant effect on the survival rate was observed in zebrafish groups that were exposed to PcKuz2. Treatment of zebrafish with 30 μM of PcKuz1 after 1 h resulted in a survival rate of 10%. None of the zebrafish survived when the PcKuz3 concentrations was 20 μM or higher. Therefore, PcKuz3 showed the highest acute lethal toxicity to the zebrafish larvae, exhibiting a LD_{50} value between 10 to 20 μM, which was recognized as the most potent peptide. The bioactivity validation was consistent with the phylogenetic analysis, seen that PcKuz3 grouped with potent snake-type Kunitz-like animal toxins, like mamba dendrotoxin and taicotoxin (Figure 2).

From survival test, LD_{50} value of PcKuz3 is the lowest among the three *P. caribaeorum* candidate Kunitz peptides within 1 h-window of treatment, which indicated PcKuz3 is able to cause acute toxicity at very low concentration. Therefore, PcKuz3 was selected for the test of locomotion using zebrafish as model of screening (Figure 5). The locomotion test showed that the total distance traveled by zebrafish larvae decreased significantly after exposure to 5 μM of PcKuz3 for 20 min. The traveled distance proportionally decreased, while concentration increased, indicating that PcKuz3 disrupted the zebrafish neurophysiology in a dosage-dependent manner. Interestingly, the distance began to increase after 40 min of exposure, which indicated the toxicity might be reversible. These data suggest that the PcKuz3 figures as a potent neurotoxin that severely disturbed the locomotion of zebrafish at concentration as low as 5 μM.

In neuroprotective effect assessment on zebrafish larvae, injury of dopaminergic (DA) neurons affects mobility. As shown in Figure 8, 6-OHDA significantly reduced the swimming distance of zebrafish larvae (from 813 mm to 373 mm), compared with the control group. Under the same conditions, PcKuz3 peptide (2.5 and 5.0 μM) inhibited the 6-OHDA-induced movement, which was then decreased in a concentration-dependent manner (530 mm, 639 mm, respectively). These data suggest that PcKuz3, at low concentration, can suppress 6-OHDA-induced deficits in the locomotive behavior of zebrafish.

Again, the PcKuz3 model superposes spatially well with known Kunitz-like toxins, particularly from snakes, like α-dendrotoxin and dendrotoxin I, with rmsd value is 0.924 and 1.698 Å, respectively (Figure 6c,d). There is experimental

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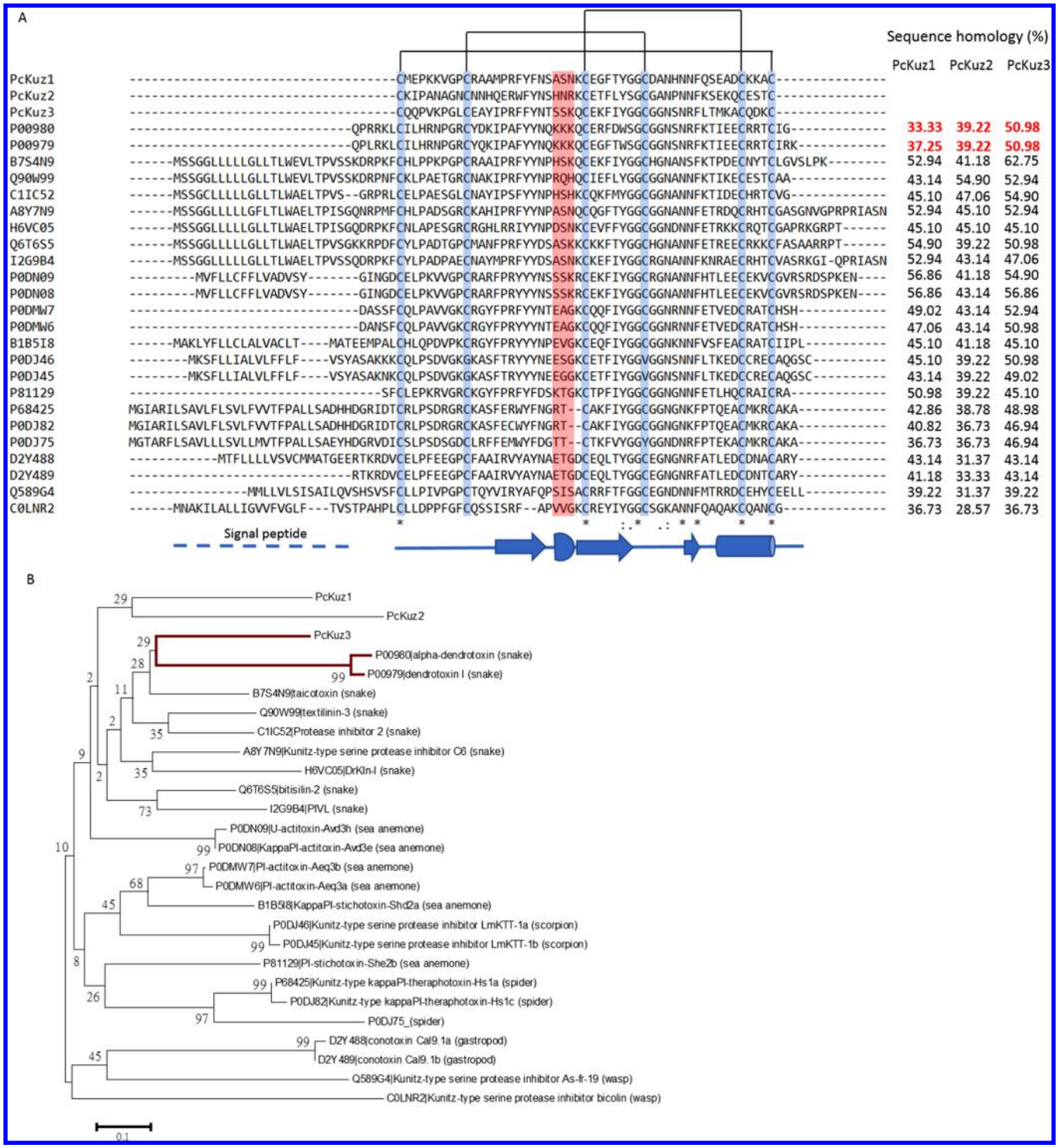

Figure 2. Multiple sequence alignment and Neighbor-joining tree of predicted Kunitz-like peptides of *P. caribaeorum*. (A) Multiple sequences alignment of *P. caribaeorum* Kunitz-like peptides and toxins originated from different species of marine and terrestrial organisms including snake, sea anemone, scorpion, spider, gastropod, and wasp. PcKuz peptides were different from sea anemone toxins that have Kunitz-domain in their structure, and also they cannot be clustered well with Kunitz-like toxins from scorpion, spider, gastropod, and wasp. Notably, PcKuz3 is most closely similar to neurotoxic dendrotoxin originated from *Dendroaspis* (Mamba) snakes. Residues highlighted in blue are cysteine and the region in red was marked as β-turn, which blocks the active sites of ion-channels. The arrow symbols represent β-sheet, semicircle is β-turn and cylinder, α-helix. Sequence homology were presented. The homology value highlighted in red were the templates used for modeling. (B) Neighbor-joining tree of phylogenetic analysis. The *P. caribaeorum* Kunitz-like peptides (PcKuz1 to 3) split in two branches. One groups together PcKuz1 and PcKuz2, the other PcKuz3 and most snake-type Kunitz-like toxins, indicating that PcKuz3 is more evolutionary related to Kunitz-like toxins from snake venom.

evidence that both dendrotoxins have the ability to block the activity of Kv1.1 and Kv1.2 subtypes of K-channels, which was confirmed in docking analysis (positive control). Through zebrafish locomotion test and the structurally homology with dendrotoxins, PcKuz3 was hypothesized to block potassium ion channels and, thus, to act as neurotoxin. The ZDOCK docking

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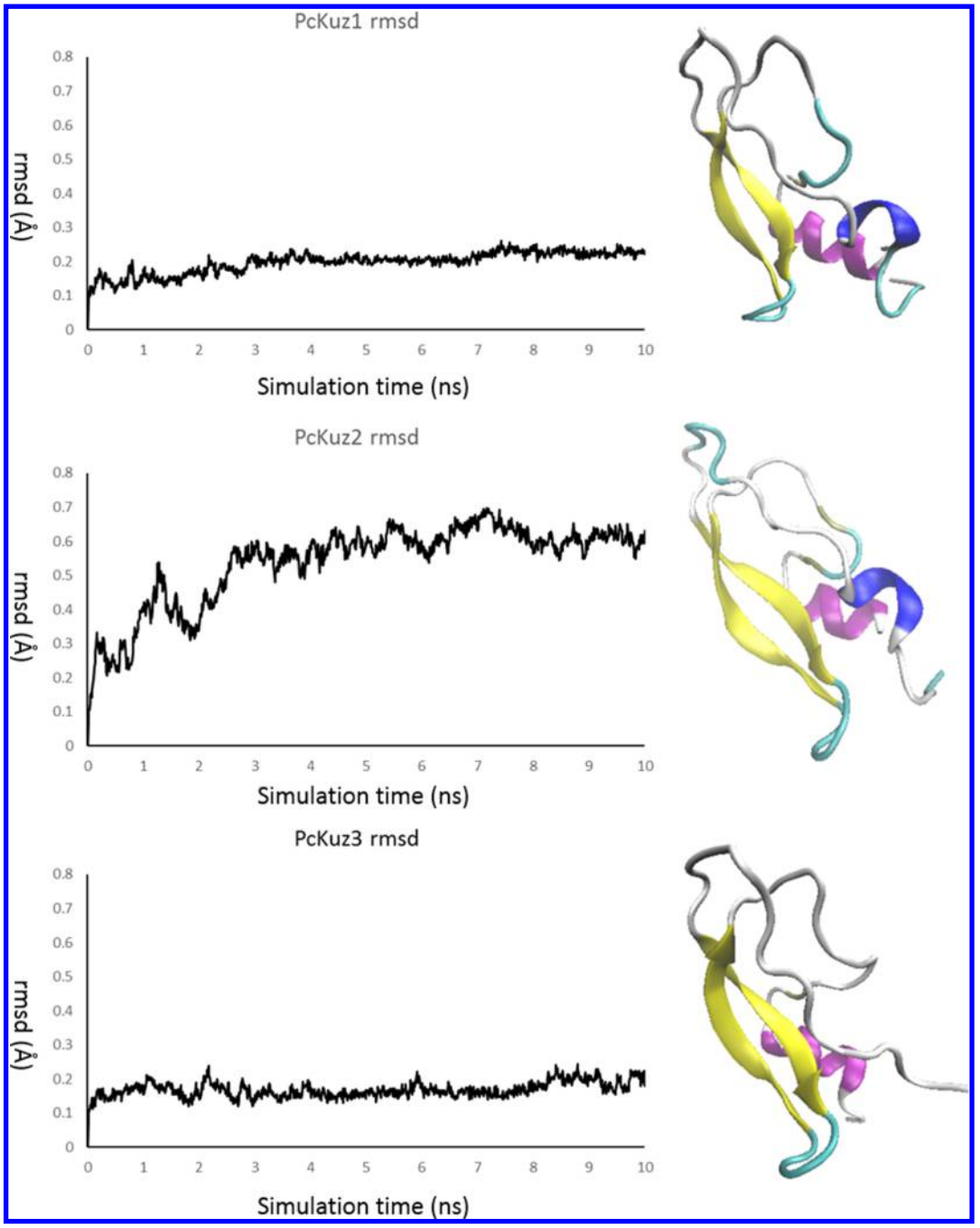

Figure 3. Structural model visualization of *P. caribaeorum* Kunitz-like peptides. Refinement was achieved by 10 ns simulation after minimization and equilibration steps. Structures were recognized as stable when rmsd value was lower than 0.3 Å. Molecules were displayed in the Cartoon style and colored according to their secondary structure: α-helix in purple, β-sheet in yellow, and coil in cyan.

Table 1. Primary Sequences, Physicochemical Characteristics, and Toxicity Levels of Kunitz-like Peptides from *P. caribaeorum*[a]

peptides	primary sequences	MW	pI	net charge (z)	LD_{50} (μM)
PcKuz1	CMEPKKVGPCRAAMPRFYFNSASNKCEGFTYGGCDANHNNFQSEADCKKAC	5628.35	8.3	1.7	25
PcKuz2	CKIPANAGNCNNHQERWFYNSHNRKCETFLYSGCGANPNNFKSEKQCESTC	5845.42	8.1	1.8	>100
PcKuz3	CQQPVKPGLCEAYIPRFFYNTSSKQCEKFIYGGCGGNSNRFLTMKACQDKC	5761.66	8.9	3.6	10−20

[a]LD_{50} denotes the concentration of Kunitz-like peptides that cause 50% of death in zebrafish larvae.

score of α-dendrotoxin to Kv1.1 and Kv1.2 is 1753.276 and 1524.983, respectively (Figure 6f,i). Meanwhile, the score of dendrotoxin I to Kv1.1 and Kv1.2 is 1680.996 and 1811.671, respectively (Figure 6g,j). After docking analysis of dendrotoxins, PcKuz3 was selected to dock with Kv1.1 and Kv1.2 and the score is 1604.786 and 1663.898, respectively (Figure 6e,h). These data confirmed what was experimentally observed in the bioactivity assays.

Finally, the protease inhibitor activity of the *P. caribaeorum* Kunitz-type peptides (PcKuz1, PcKuz2, and PcKuz3) was

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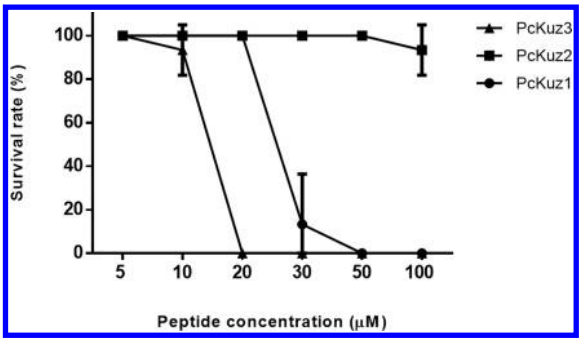

Figure 4. Survival rate of zebrafish larvae after 1-h exposure to three *P. caribaeorum* Kunitz-like peptides (PcKuz1, PcKuz2, and PcKuz3). The survival rate reached 10% when zebrafish larvae was exposed to PcKuz1 (30 μM) for 1 h. Any zebrafish larvae survived at higher concentration. The survival rate was higher than 80% after zebrafish larvae was exposed to PcKuz2 (100 μM). Notably, the death rate reached 100% when zebrafish larvae were exposed to PcKuz3 at 20 μM after 1 h.

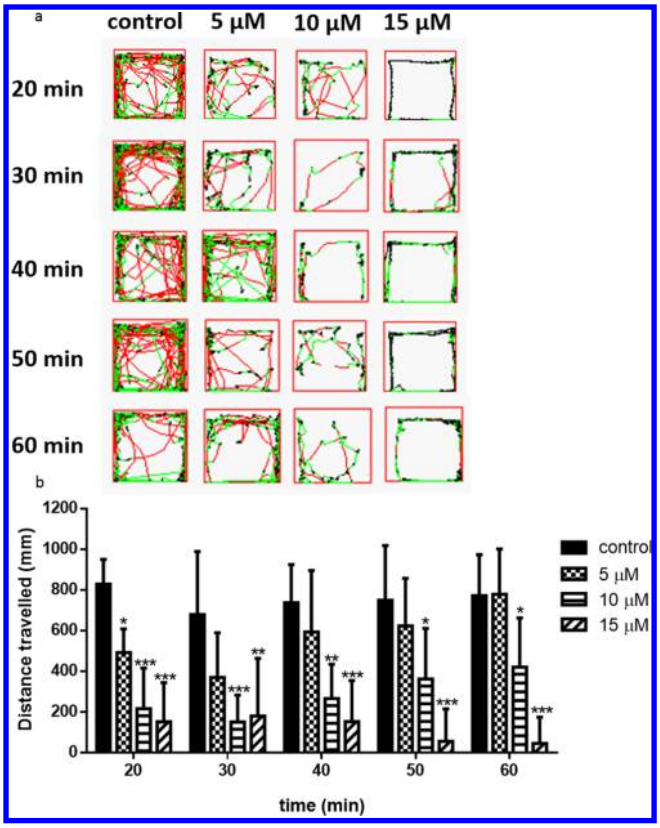

Figure 5. PcKuz3 disturbs the locomotion and swimming of zebrafish larvae. (A) Movement trajectory of zebrafish larvae was recorded every 10 min after 20 min of exposure to PcKuz3 at concentration of 5 μM, 10 μM, 15 μm. (B) Calculation of travel distance of zebrafish. The total traveled distance was computed at every 10 min. Each treatment group contained 12 larvae, and three independent trials were performed for each experimental set. The results represent the mean distance traveled by the larvae. The values are expressed as the means ± SD $*P < 0.05$ and $***P < 0.001$ versus control (no exposure to the peptide) were considered statistically significant.

assessed with trypsin and elastase and their respective chromogenic substrates, which by spectrophotometrically measuring the ability of remaining free protease (trypsin or elastase) to release para-nitroaniline. Figure 7 showed that the remaining activity was still higher than 50% even though the concentration of PcKuz peptides was as high as 200 μM (at a 3:1 and 160:1 ratio of peptide over trypsin and elastase, respectively). The results showed that the PcKuz peptides displayed a weak inhibitory trypsin and elastase activity, even with the increase of peptide concentration (Figure 7).

DISCUSSION

On the basis of transcriptomics analysis of the zoanthid *P. caribaeorum*, a soft coral that has a large distribution in bench-rocks of Atlantic coast, numerous predicted venom-featured polypeptides and toxin peptides were found. These putative peptides and proteins structurally and categorically fitted in different toxin families, comprising neurotoxins, protease inhibitors, hemostatic, and hemorrhagic toxins, membrane-active peptides, toxins related to allergen and innate immunity, as well as mixed function enzymes. Of particular interest in the present study, the novel anthozoan Kunitz-domain-containing peptide sequences were retrieved and they are related to proteins that can inhibit the activity of serine proteases[54] or can act as ion-channel blockers.[55]

Usually, venom-type Kunitz proteins play important roles in envenomation by inhibiting serine proteases, which play as role in vital living process like the hemostatic system[47,48] or by blocking potassium channels, in neurotransmission.[49] There are two families of venom Kunitz-like peptides that are classified based on the different cysteine frameworks. One family retains the typical Kunitz-domain architecture with three highly conserved disulfide bridges such as kappa-theraphotoxin-Hh1a[56,57] from spider, dendrotoxin-K[58,59] from snake, and kalicludines[50] from sea anemone. The other family has member with only four or five cysteine residues that result in loss of a conserved disulfide bonding such as conkunitzin-S1[60,61] from cone snail. On the basis of the topology of the phylogenetic tree (Figure 2), the *P. caribaeorum* Kunitz-domain containing peptides were evolutionary and closely related to snake-type Kunitz-like peptides, which display the three highly conserved disulfide bridges.

It is suggested that the genetic and functional evolutionary process of Kunitz-type toxins probably occurred in three stages which give rise to toxins that could be categorized in three different groups: "old functional proteins" with serine protease inhibition activity, which was suggested to represent all Kunitz-domain containing proteins; "bi-functional toxins" with both capabilities to inhibit the activity of serine proteases and potassium-channels; and "new functional toxins", which seemed to have lost their ability to act as serine protease inhibitor, but display selective action on the inhibition of K+-channel subtypes.[62,63] Thus, some Kunitz-type toxin structures have been selected in the course of the molecular evolution to possess neurotoxic function.

Herein, despite the disulfide bonds in PcKuz peptides were not experimentally annotated, the experimental data from the toxicity test with zebrafish larvae show that the LD_{50} value of PcKuz3 was below 20 μM, which pointed out that this *P. caribaeorum* Kunitz-type peptide might be characterized as neurotoxin. Moreover, the zebrafish locomotion test confirmed that PcKuz3 significantly decreased the zebrafish larvae swimming distance, at concentration as low as 5 μM. Both sequence and structure alignment analyses provide more evidence that PcKuz3 is phylogenetically and structurally homologous to dendrotoxins, like α-dendrotoxin and dendrotoxin I (1DTX and 1DEM). Therefore, PcKuz3, a positively

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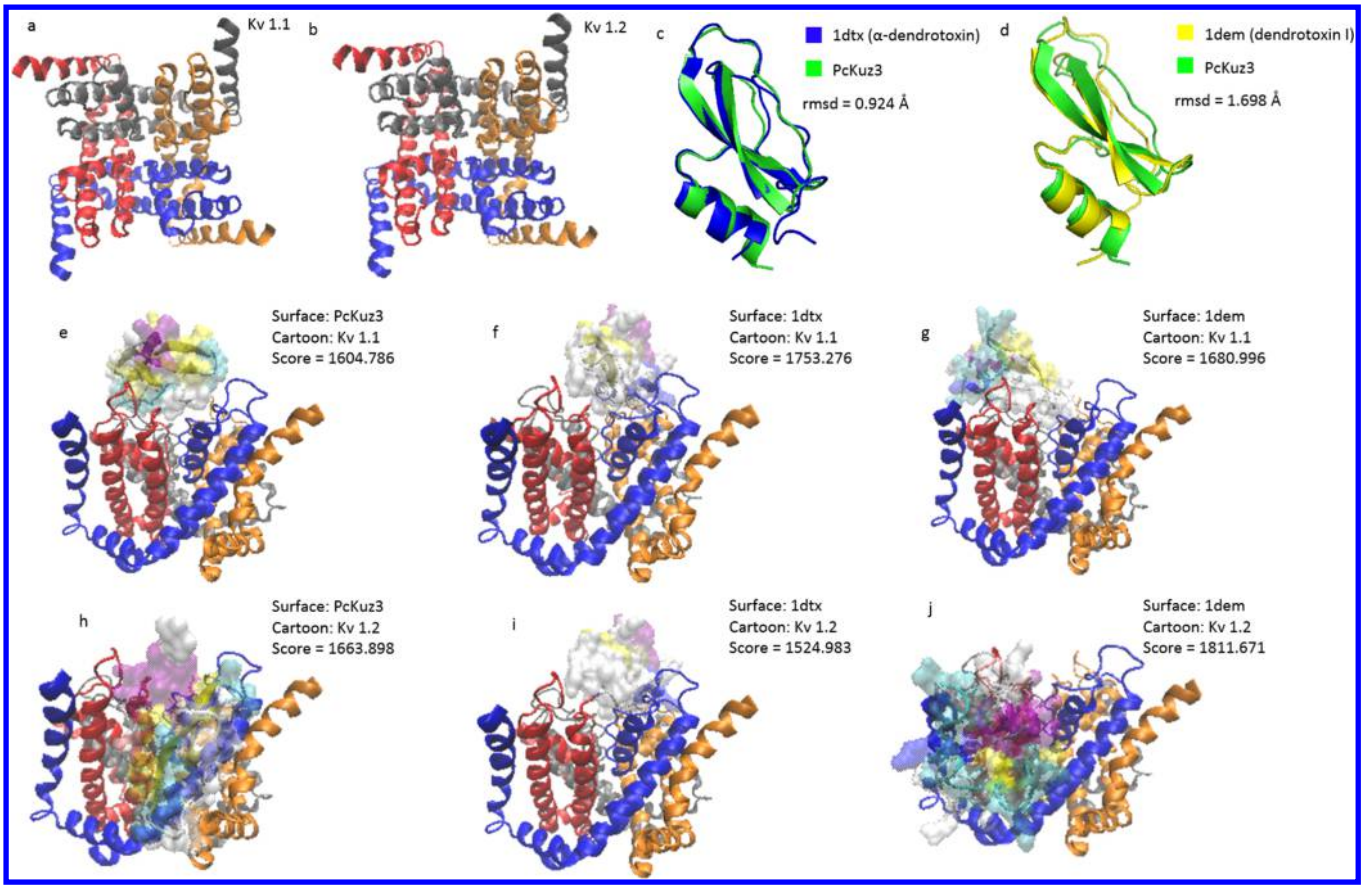

Figure 6. Spatial structural peptide overlap and peptide−protein docking visualization. (a) S4, S5, and S6 domain of Kv1.1; (b) S4, S5, and S6 domain of Kv1.2; (c) Structure overlap of PcKuz3 and α-dendrotoxin (1dtx), rmsd = 0.924 Å, PcKuz3 is colored as green and 1dtx as blue; (d) structure alignment of PcKuz3 and dendrotoxin I (1dem), rmsd = 1.698 Å, PcKuz3 is colored in green and 1dem in yellow; (e) PcKuz3 and Kv1.1 docking complex, ZDOCK score is 1604.786; (f) α-dendrotoxin and Kv1.1 docking complex, ZDOCK score is 1753.276; (g) dendrotoxin I and Kv1.1 docking complex, ZDOCK score is 1680.996; (h) PcKuz3 and Kv1.2 complex, ZDOCK score is 1663.898; (i) α-dendrotoxin and Kv1.2 complex, ZDOCK score is 1524.983; (j) dendrotoxin I and Kv1.2 complex, ZDOCK score is 1811.671.

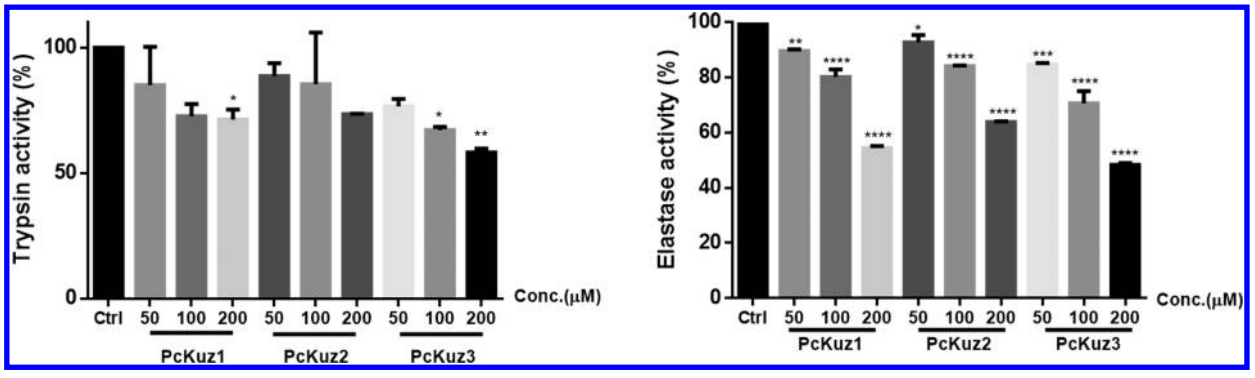

Figure 7. Inhibition assay of serine-protease with *P. caribaeorum* Kunitz-like peptides. Data are presented as mean ± SD. Asterisks indicate residual activity of protease that were statistical significantly ($p < 0.05$) in comparison with control group.

charged cationic peptide, appears to act as a neurotoxin by presumably interacting selectively with a potassium ion-channel region, which is composed of negative charged residues (the amino acid stretch −TTVGYG−). As a matter of comparison, mamba dendrotoxins are about 7 kDa proteins, consisting of a single peptide chain of approximately 57−60 amino acids and three disulfide bonds. Several homologues of α-dendrotoxin have been isolated, which possess slightly divergent sequences.[58,59,64,65] Dendrotoxin has been proved to block several subtypes of voltage-gated potassium channels in neuronal tissue.[65] The IC_{50} values for dendrotoxin I to block voltage-gated potassium channels (Kv1.1, Kv1.2, and Kv1.6) are known to be between 0.13 nM to 50 nM,[53] while the values for α-dendrotoxin lie in the range of 0.4 nM to 150 nM.[49,55] The active binding sites of dendrotoxin were residues Lys28, Lys29, Lys30 form narrow β-turn region. This region is believed to play a critical role in the interaction of toxin with the potassium channel.[66] Here, the scores from the docking analysis using ZDOCK server indicated that PcKuz3 has hypothetically a similar binding mode and configuration that dendrotoxins have.

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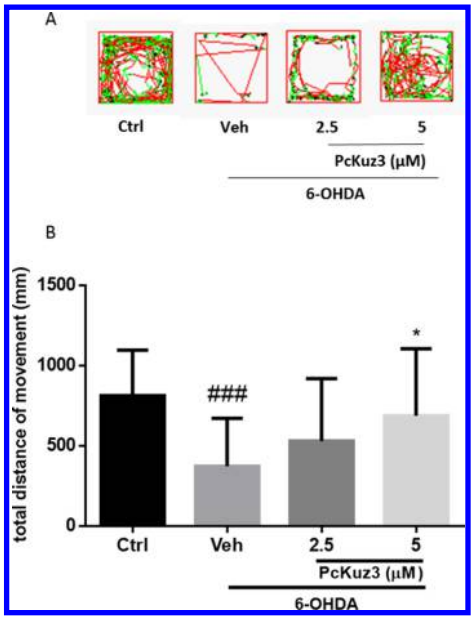

Figure 8. Effect of PcKuz3 on 6-OHDA-induced deficits in the locomotor behavior of zebrafish. Zebrafish at 3 days post fertilization were exposed to PcKuz3 with 250 μM 6-OHDA for 4 days. Then zebrafish were collected, and the locomotor activity of each group was monitored using the Viewpoint Zebrabox system; total distance traveled in 10 min was calculated. (A) Representative patterns of zebrafish locomotion traced from control and different treatment groups. (B) Statical analysis of total distance moved of different treatment groups, eight fish larvae per group from three independent experiments. ###$p < 0.005$ versus control group, *$p < 0.05$ versus 6-OHDA group.

Notably, dendrotoxin molecule associates reversibly with potassium channels, mediated by electrostatic interactions between the positively charged residues in the cationic domain and the negatively charged residues in the ion channel pore, to exert its inhibitory effect.[67] In case of PcKuz3 peptide, the swimming distance, in the experimental locomotion test (Figure 5b), increased after 40 min exposure, which indicated the toxicity exerted by PcKuz3 might be reversible. The inhibitory activity of Kunitz-like peptides, discussed above, is summarized in Table 2, particularly for PcKuz3, and compared with data from snake dendrotoxins and sea anemone actitoxin.

The protease inhibition ratios of all the three novel *P. caribaeroum* Kunitz-type peptides, using the serine proteases trypsin and elastase, showed that less than 50% of inhibition were reached, even when the peptide concentration was high (200 μM) (at a 3:1 and 160:1 ratio of peptide over trypsin and elastase, respectively). It presented the protease inhibitory activity of PcKuz peptide were as weak as that of dentrotoxins, which also could not inhibit the trypsin activity even at a 6:1 ratio of toxin over trypsin.[68] The weak inhibitory proteolytic activity of PcKuz peptides is suggestive that in *P. caribaeorum* Kunitz-like structure have been recruited to act as neurotoxins, especially PcKuz3.

Noteworthy, potent neurotoxins with Kunitz-type motif are particularly restricted, for unknown biological reasons, to snakes,[51,69] while sea anemone, scorpion and spider toxins have preserved the dual-function Kunitz-type toxins, frequently with weak potassium channel blocking activity.[54] Indeed, only potassium channel blockers with Kunitz-type motif have been characterized from snake venoms so far.[55] In our study, multiple sequence alignment, spatial overlapping and phylogenetic analysis indicated that predicted *P. caribaeorum* Kunitz-like peptide 3 (PcKuz3) shares structures that are evolutionary closer to Kunitz-domain toxins from snakes rather than from sea anemones. Also, molecular docking analysis has predicted that PcKuz3 peptide has the potential to block potassium ion-channels through the narrow β-turn region that selectively interact to the binding region (−TTVGYG−). Besides, experimental data from the bioactivity validation of both the antilocomotion test in zebrafish and the inhibitory protease assay, demonstrate that the synthesized *P. caribaeorum* peptides, particularly PcKuz3, act as a neurotoxin rather than a protease inhibitor. More importantly, PcKuz3 acts by a similar binding mode like dendrotoxin, which has a high potency to block Kv1, as observed through peptide−protein docking analysis. Notably, some evidence showed that some Kv blockers can display neuroprotective effects. For instance, Peng and colleagues[70] found ShK-170, which has the ability to block Kv1.3, could protect mice from radiation-induced brain injury. Ogita and collaborators[71] demonstrate that in vivo treatment with the K+-channel blocker 4-aminopyridine protects neural tissue against kainite induced neuronal cell death through activation of NMDA receptors in murine hippocampus. Furthermore, Taherian and co-workers[72] reported that 4-aminopyridine decreases MPTP-induced behavioral disturbances. Hence, in our study, we tested and found that PcKuz3 is additionally able to suppress 6-OHDA-induced neurotoxicity caused in the locomotive behavior of zebrafish. Interestingly, 6-OHDA can enhance the voltage-dependent potassium currents in dopaminergic neurons.[73] Meanwhile, 6-OHDA toxicity in dopaminergic neurons can be blocked by Stromatoxin, which is a Kv2.1 selective blocker. These facts and findings have allowed us to hypothesize that PcKuz3 may act by means of a similar mechanism to block the 6-OHDA toxicity, that is, via Kv-channel, and therefore exhibiting the neuroprotective effect at low concentration (<5 μM). To further confirm that *P. caribaeorum* Kunitz-like peptides act as potent ion-channel blockers, more refined molecular neurobiology methods, like patch clamp, for the assessment of electrophysiological response of subtypes of voltage-dependent K+-channel upon PcKuz3 peptide action will be further necessary. Altogether, the present study has reported the transcriptomic of *P. caribaeorum*

Table 2. Comparison of PcKuz3 Peptide and Known Kunitz-like Toxins from Snake Venom and Sea Anemone

		serine protease inhibitory activity			voltage-gated potassium ion channel blocker activity				
					Kv 1.1		Kv 1.2		
species	Kuntiz-like toxins	trypsin	α-chymotrypsin	elastase	IC_{50} (nM)	ZDOCK Score	IC_{50} (nM)	ZDOCK score	ref
snake	α-dendrotoxin (P00980)	−	−	−	0.4−150	1753.276	0.4−150	1524.983	49
	dendrotoxin I (P00979)	−	−	−	2.5	1680.996	10	1811.671	53
sea anemone	actitoxin (P0DMW7)	+	+	−	0.9 ± 0.1	1733.451		1729.802	68,74
P. caribaeorum	PcKuz3	±	−	±		1604.786		1663.898	this study

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and the functional data of novel Kunitz-like peptides. The overall data provide an insightful perspective to characterize neuroactive peptide sequences in zoanthid corals, with potential neurotoxic activity that will serve to develop molecular biotools for the investigation and prospective treatment of neurodegenerative diseases that arise from ion-channel dysfunctions.

In the present study, the synthetic peptides used biological evaluation are free folding form. As experimentally tested, the free folding peptide figures as a potent neurotoxin that severely disturbed the locomotion of zebrafish at concentration as low as 5 μM. It can suppress 6-OHDA-induced deficits in the locomotive behavior of zebrafish at concentration lower than 5 μM. A challenge in this study is that S−S bond connectivity of these novel peptides was only predicted by the sequence homology to the related peptides (Figure 2A) but the S−S bond attribution of each peptide was not determined. The S−S bond connectivity usually leads to specific 3-D structure, which is very important because of deep relation to biological activities. Thus, S−S bond pattern after synthesized the peptides will be validated by chemical evidence. Moreover, the peptide with preformed disulfide bonds and short derivates with dissected structures will be evaluated in terms of the bioactivity scanning in the future work.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00686.

Purification and characterization of PcKuz1 to 3 (PDF)
Identification of the putative toxin peptides from the *P. caribaeorum* transcriptome (XLSX)

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Notes

The authors declare no competing financial interest.

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