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Characterization of a bioactive acyclotide from *Palicourea rigida*.

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ABSTRACT

Here we report the extraction and purification of parigidin-br3, a cyclotide analog belonging to the 'bracelet' subfamily, from *Palicourea rigida* leaves. Unlike conventional cyclotides, parigidin-br3 has free N- and C-termini, as identified by MALDI-TOF/TOF analysis and confirmed by gene structure elucidation, and is one of a small number of acyclotides discovered over recent years. Parigidin-br3 showed cytotoxic activity against MCF-7 (breast cancer) and CACO2 (colorectal adenocarcinoma) cells, with IC_{50} values of ~2.5 μM, and less than 10% hemolytic activity. Overall, parigidin-br3 is a promising new molecule with cytotoxic properties against tumor cell lines and, unlike many synthetic acyclic analogues, demonstrates that cytotoxic activity is not limited to conventional (i.e. cyclic) cyclotides.

INTRODUCTION

Despite remarkable advances in biomedical research, cancer remains one of the major causes of mortality and morbidity worldwide.[1] Thus, there is a significant research focus on the discovery of molecules and the development of new treatments that are more selective and cause fewer side effects than conventional therapies.[2] Among molecules from natural sources, peptides are a promising class of drug leads.[3,4] In particular, cyclotides have attracted attention due to their ability to kill cancer cells through mechanisms involving membrane disruption.[5,6] These molecules have an ultra-stable structure resulting from a peptide link between their N- and C-termini and the presence of three highly conserved disulfide bonds, which together form a cyclic cystine knot (CCK) motif.[7]

Interestingly, several reports have recently described the discovery of backbone-linear cyclotide analogs[8–10] also known as uncyclotides[10] or acyclotides,[11] with the latter term our preference.[11] Acyclotides share the cystine knot motif and have high sequence homology with conventional cyclotides, but are biosynthetically unable to cyclize.[10–13] A common characteristic of acyclotides is the absence of a Asn or Asp residue at the C-terminal end of the mature peptide domain in the precursor protein; Asx residues are posited to be essential for recognition by asparaginyl endoproteinase enzymes implicated in backbone cyclization.[8] Acyclotides are relatively uncommon, with only 26 such sequences deposited in Cybase compared to more than 300 conventional cyclotides.[14–16]

Investigations into their functional properties have revealed that many cyclotides possess cytotoxic activity, including some cycloviolacins isolated from *Viola* sp. and varv peptides.[17] Moreover, vitri A, a cyclotide isolated from *V. tricolor*, showed

toxic activity toward lymphomas and myeloma cells with similar potencies to doxorubicin-containing chemotherapeutic drugs.[17,18] However, less is known about bioactivities of acyclotides.

Here, we report the identification and characterization of parigidin-br3 (PBR3), an acyclotide from *Palicourea rigida*. Elucidation of the partial gene structure encoding PBR3 revealed a premature stop codon at the position of the typically conserved Asn residue, which is essential for cyclization. PBR3's cytotoxic activity was evaluated against human breast cancer (MCF-7) and colorectal adenocarcinoma (CACO2) cell lines and it was found to be a promising molecule with selective toxicity for cancer cells.

RESULTS AND DISCUSSION

In a previous study the characterization of parigidin-br1 from *P. rigida* (Rubiaceae) leaves was reported.[19] In that study leaf extracts were subjected to multiple purification steps (Figure 1A) after MALDI-TOF analysis confirmed the presence of a cyclotide in Fraction 1, along with other peptides. In the current study, Fraction 1 was further purified by reversed-phase high performance liquid chromatography (RP-HPLC) and various fractions were collected (Figure 1B). MALDI-TOF MS analysis showed a peptide with a mass-to-charge ratio (*m/z*) of 3071.8 in RP-HPLC Fraction 2. To improve the purification, Fraction 2 was re-chromatographed using an analytical C18 Everest column yielding Fraction 3 (Figure 1C). A second round of reversed-phase purification was undertaken using ultrafast liquid chromatography (UFLC) (Figure 1D), where it was possible to separate the peptide of *m/z* 3071.8 from impurities. The purity

was confirmed by analytical HPLC analysis and the identity of the peptide was confirmed by MALDI-TOF mass spectrometry (Supplementary Figure 1).

MALDI-TOF MS analysis was employed for PBR3 sequence analysis. After purification, the peptide with an *m/z* of 3071.8 (Figure 2A) was reduced and alkylated, resulting in a modified *m/z* of 3419.9 (Figure 2B), as observed by MALDI-TOF MS. This represented an increase in mass of 348 Da, indicating the alkylation of six cysteine residues (carbamidomethylation of Cys = 57 Da increase). PBR3 was subsequently subjected to enzymatic digestion using endoproteinase Glu-C (endoGlu-C), which typically cleaves after the conserved glutamic acid residue in loop 1 of cyclotides and leads to a mass increase of 18 Da. However, following endoGlu-C digestion the predicted + 18 Da peak at *m/z* 3437.6 was absent, suggesting that PBR3 is not cyclic.

The peak at *m/*z 3419.9 was analyzed by MALDI-TOF/TOF (Figure 2C). Sequence analyses allowed the characterization of the 29 amino acids of PBR3 (PSPCGESCVFIPCISALIGCSCKNKVCYR), with a theoretical *m/z* 3071.8 for the oxidized peptide (monoisotopic mass). As MS/MS sequencing cannot distinguish between the isobaric residues Ile and Leu, amino acid analysis of the peptide was performed and revealed three Ile residues and one Leu. To determine the location of these residues, chymotrypsin digestion was employed, as chymotrypsin cleaves amide bonds C-terminal to Leu (as long as they are not followed by Pro). The position of the Leu in loop 3 was further confirmed by sequencing the PBR3 gene. The sequence contained the six cysteine residues that typically form the three disulfide bonds responsible for stabilizing the CCK motif of cyclotides. By comparing the residues present in loop 5 with those of known cyclotides, PBR3 was classified as belonging to the bracelet subfamily, as previously observed for parigidin-br1.[19] To obtain more information about PBR3, an alignment was carried out showing that PBR3 has at least

68.9% identity with parigidin-br1, the first characterized cyclotide from *P. rigida* and other cyclotides belonging to the bracelet subfamily (Figure 3).

Despite these similarities, it is important to note the lack of either an Asn or Asp residue in loop 6 of PBR3. These Asx residues are critical for the biosynthetic cyclization of the backbone in cyclotides.[9,13,20] Although the mechanism by which cyclotides are cyclized is not yet fully described, the N- and C-termini of the mature peptide domain excised from the precursor are connected in loop 6 at the highly conserved Asn or Asp residue,[12,13,20,21] via the action of an asparaginyl endopeptidase enzyme.[21] The first example of a linear cyclotide analog, violacin A, was reported in the plant *V. odorata.*[9] The gene for this peptide has a point mutation that introduces a stop codon, which inhibits the translation of an Asp residue essential for cyclization.[9,13,20,22] A number of other acyclotides have since been reported, including mram 1,[23] psyle C,[24] hedyotide B2,[8] panitide L1–L8,[16] chassatide C7, C8, C11 and C17.[10]

To confirm the PBR3 sequence, the partial coding sequence of the PBR3 gene was determined after cDNA amplification using specific forward and reverse primers and cloning into the pGEM®-T Easy vector (Promega). The PBR3 partial coding sequence displayed the expected amino acid residues and all conserved Cys residues, without signs of point mutations or internal DNA rearrangements, corroborating the proteomic mass spectrometry assays results supporting the presence of 29-residue peptide with five stabilizing loops (Figure 4).

A theoretical model of PBR3 suggested that the three-dimensional structure had the typical fold of a bracelet cyclotide subfamily member, stabilized by three disulfide bridges, but lacking the cyclic backbone. The structure incorporated a short 3_{10}-helix, comprising residues Ala^{16}-Ile^{18} and a β-sheet of two β-strands, comprising residues Ser^{21}-Lys^{23} and Tyr^{26}-Val^{28}, as illustrated in Figure 5. In general, the loops of cyclotides

have some highly conserved amino acid residues. The six cysteine residues, which delimit the six backbone loops of cyclotides are absolutely conserved and play an essential role in the preservation of the CCK motif.[22] Loop 1 shows the least variation of all the loops and the Glu8 residue in this loop (which is present in PBR3) is absolutely conserved, and seems to be involved in the stabilization of the cyclotide framework by forming hydrogen bonds with residues in loops 1 and 3.[25,26] In loop 3, Gly21 is also well conserved in most cyclotides.[27] Typically, loop 3 of bracelet cyclotides contains six to seven residues featuring a short helical segment,[28] as is seen in PBR3. Loop 4 is similarly highly conserved, always comprising just a single residue, in this case a serine, but threonine and lysine are also commonly observed in this site. These residues have been directly related to the connectivity of the disulfide bonds by making hydrogen bonds via their side chains.[9,29] Loops 1 and 4, which are embedded in the conserved CCK ring, have been shown to play a significant role in defining the cyclotide fold.[11,19,22,23,29] Moreover, loop 2 is preferentially composed of hydrophobic residues, in a region where an aromatic ring is conserved in bracelet cyclotides.[22] The most common residue at this position (residue 2 in loop 2) is tryptophan, but the parigidins, circulins B and C, cycloviolacin D and several other cyclotides have a phenylalanine instead.[30] Although loops 5 and 6 show extensive variations in their composition, there are highly conserved elements within them, most notably the Asn/Asp32 residue at the putative cyclization point in loop 6.[8,9]

Activity assays of PBR3 against CACO2 and MCF-7 cells were performed with 48 h of incubation for a range of peptide concentrations (0.33–10 μM), with the data represented by the mean of three independent experiments with triplicate analyses (n = 9). PBR3 showed cytotoxic activity against both cell lines, as evidenced by a 66% loss of CACO2 cell viability at 10 μM and an IC_{50} value of 2.5 μM (Figure 6A), and a 57%

loss of MCF-7 cell viability at the same concentration (10 μM) and an IC_{50} value of 2.5 μM (Figure 6B). By comparison, the fibroblast cell line L929 was less affected by PBR3, with an IC_{50} > 10 µM (Figure 6C).

Many studies have reported that native cyclotides are toxic towards several human cancer cell lines.[7,31–33] The most potent cyclotide with cytotoxic activity described to date is cycloviolacin O2, which is capable of complete inhibition of the growth of various cancer lines, including RPMI-8226, U-937, ACHN, CCRF-CEM and NCI-H69 in a dose-dependent manner.[18,34] The cycloviolacins and vitri-A have activity against lymphoma and myeloma cell lines at concentrations ranging from 0.96 to 5.0 μM, with potency similar to that of clinical chemotherapy drugs used in cancer treatments.[17,35] Cycloviolacin O2 has cytotoxic activity against ten different cancer cell lines (IC_{50} 0.1–0.3 μM).[34] In addition to cycloviolacin O2 and PBR3, psyle A (IC_{50} = 7.77 μM), psyle C (IC_{50} = 2.98 μM), and psyle E (IC_{50} = 0.64 μM) have been shown to have cytotoxic activity against MCF-7 cells.[36] The mechanism of cytotoxicity of cyclotides against tumor cells seems to involve membrane disruption, as supported by reports on cycloviolacin O2 and kalata B1.[37,38]

The hemolytic activity of PBR3 was evaluated, and only at the highest concentrations tested (21–42 μM) were low levels (~13-20%) of hemoglobin leakage observed after 1 h or 48 h (Supplementary Figure 2). Previous studies have suggested that the hemolytic activities of natural cyclotides are broadly correlated with hydrophobicity;[39] we believe that low hemolytic activity may be associated with the hydrophilic residues (38%) of PBR3, even though the peptide is more hydrophobic than other cyclotides, such as kalata B1. We note that in previous studies, synthetic linear versions of cyclotides were typically less hemolytic than cyclic versions.[8,10]

In summary, we have described the isolation, purification and characterization of PBR3, a novel acyclotide. PBR3 reduced the cell viability of two cancer cell lines, MCF-7 and CACO2. In addition, at the concentration at which this significant reduction was observed (10 μM) no significant hemolytic activity was observed, suggesting that this cyclotide may have a therapeutic index that can provide applications in cancer treatment.

EXPERIMENTAL SECTION

Purification of parigidin-br3. Leaves from *P. rigida* were ground in liquid nitrogen and extracted with dichloromethane and methanol (J.T. Baker) (1:1, v/v) for 12 h followed by filtration through cotton fibers. Subsequently, the sample was subjected to a liquid-liquid extraction and the aqueous phase was collected and lyophilized. The lyophilized material was acidified with 2% acetic acid (Sigma Aldrich) and applied onto an open column with polyamide resin (Fluka), followed by size exclusion chromatography using a Superdex^{MT} Peptide 10/300 GL column (GE Healthcare) equilibrated with 30% acetonitrile/0.1% TFA at a flow rate of 0.5 ml.min^{-1}. The peptide fraction was lyophilized and resuspended in 0.1% trifluoroacetic acid (TFA) (J.T. Baker) and further purified using HPLC on a C18 analytical column (2.6 x 150 mm) (Grace Vydac) with a nonlinear acetonitrile gradient (5–95%) at a flow rate of 1.0 mL.min^{-1}. Peptide elution was monitored at 216 and 280 nm. HPLC fractions were applied onto an UFLC and eluted with a linear gradient (5–95% acetonitrile) (J.T. Baker) at a flow rate of 400 μL.min^{-1} at 40°C using a C18 column (2.6 x 150 mm) (Grace Vydac).

Mass spectrometry analyses. The cyclotides were reduced, alkylated and digested according to methods previously described by Shevchenko and colleagues (1996),[40]

with minor modifications. The following solutions were prepared: Buffer A [1 g of guanidine hydrochloride (GdHCl) (Sigma Aldrich): 0.2 M Tris-HCl (Sigma Aldrich) pH 8]; Buffer B: [1 g guanidine hydrochloride (GdHCl): 37 mg of iodoacetamide: 1 mL Tris-HCl, pH 8] and 50 μM dithiothreitol (DTT) (Sigma Aldrich) in water. For the reduction reaction, 100 μg of the pure peptide was resuspended in 500 μL of Buffer A, after which 85 μL of the DTT solution was added and incubated at 70°C for 1 h. The material was then alkylated with addition of 450 μL of Buffer B, followed by incubation for another hour in the dark at room temperature. Purification of the sample was performed using an HPLC analytical C18. The fractions collected were analyzed by MALDI-TOF mixed with an α-cyano-4-hydroxycinnamic matrix (Bruker Daltonics) to check which fractions corresponded to the reduced peptide and alkylated peptide. Digestion of the pure peptide was performed using enzyme immobilized on agarose gel: endoGlu-C and trypsin (Thermo Scientific). MALDI-TOF analyses were conducted using an UltrafleXtreme TOF-TOF instrument (Bruker Daltonics). Linearized cyclotide containing crude plant extracts were analyzed using a QStar® Elite hybrid LC-MS/MS system (SCIEX, Redwood City, USA) equipped with a nano-electrospray ionization source. MALDI-TOF/TOF data were interpreted manually, and the peptide sequence was determined. LC-MS/MS data were searched against a custom cyclotide database using ProteinPilot (version4.0, SCIEX).

Sequence alignment. BLASTP[41] was used for identifying the sequences with the highest identity to PBR3 in the NCBI non-redundant protein database and PDB. The retrieved sequences were assembled with the sequences of parigidin-br[19] and cycloviolacin O2,[37] and a multiple sequence alignment was performed using Clustal Omega.[42]

RNA extraction and cDNA synthesis. Total RNA was extracted from fresh leaves of *P. rigida* with InviTrap® Spin Plant RNA Mini Kit (Invitek/STRATEC Molecular) and quantified using Qubit® RNA Assay Kit and Qubit® Fluorometer (Invitrogen). Single-stranded cDNAs were synthesized using the SuperScript® II Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions.

DNA amplification. The fragment containing the *P. rigida* cyclotide partial coding sequence was amplified by PCR using a specific primer pair based on the parigidin-br3 sequence determined by LC-MS/MS. Each amplification reaction contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 160 μM of each dNTP, 200 nM of each parigidin-specific primers (forward CCGAGCCCGTGCG and reverse TTAGCGATAGCACACTTTGTTTTT), 2U Taq polymerase (Invitrogen) and 20 ng of cDNA. The cDNAs in the PCR mixes were pre-denatured at 95°C for 5 min and amplified in a Veriti® Thermal Cycler (Thermo Fisher Scientific Inc.; Waltham, MA USA) for 35 cycles at 95°C for 1 min, 57°C for 1 min, and 73°C for 1 min, with a final cycle at 72°C for 5 min. Amplicons with the expected 90 bp were resolved by electrophoresis on 1% agarose gel containing 0.2 μg/mL ethidium bromide and visualized under UV light.

Gene cloning and sequencing. The PureLink® Quick Gel Extraction and PCR Purification Combo Kit® (Invitrogen) was used to purify the selected amplicons. DNA fragments were cloned into the pGEM®-T Easy cloning vector (Promega), following the manufacturer's instructions. Electro competent *E. coli* XL1-Blue cells were genetically transformed with 30 ng of DNA ligation products. Cloned fragments were sequenced using the same primers utilized for the initial gene amplification and also the universal SP6 and T7 primers, in the 3130 Genetic Analyzer® (Applied Biosystems/Life Technologies). The obtained sequences were analyzed using BioEdit.[43]

Molecular modeling. The structure of PBR3 was modeled using MODELLER 9.14.[44] Firstly, the structure of circulin B (PDB ID: 2ERI)[45] was identified by BLASTP[41] as the sequence with the highest identity to PBR3. The structure of circulin B was manually edited, placing the residues in their natural order. PBR3 was modeled using the default methods of automodel and environ classes from MODELLER. Then, 100 molecular models were constructed and the model with the minor DOPE score was selected. Structure validation was performed using Procheck[46] and PROSA[47] servers.

Cell viability tests using cancer cell lines. The colorectal adenocarcinoma cell line (CACO2, ATTC HTB-37) and breast cancer cell line (MCF-7, ATTC HTB-22) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibcon) supplemented with 10% fetal bovine serum (Gibcon), penicillin (100 U.mL-1) (Sigma Aldrich), streptomycin (100 mM.mL^{-1}) (Sigma Aldrich) and maintained at 37°C in an atmosphere of 5% CO_2. Cell cultures were maintained as described previously.[48] After reaching 80% confluence, the cells were removed with the aid of a plastic carrier (cell scraper). The cell concentration was adjusted to 1.10^5 cells.mL^{-1}. Cells were then incubated in a 96-well (TPP®) plate along with cyclotides at concentrations of 0.33; 0.65; 1.3; 2.6; 5.25 and 10.5 µM. The fibroblast cell line L929 was used as control and grown under the same conditions as the cancer cell lines. For each concentration of the peptide, three replicates were performed and the procedure was repeated three times. The MTT assay (Sigma Aldrich) was used for cell viability analysis, with readings taken at 48 h. For this assay, 155 μL of culture medium was removed and added to 10 μL of MTT; this was then incubated for 3 h at 37°C in the dark. Following incubation, 60 μL of dimethyl sulfoxide (DMSO; Sigma Aldrich) was added to each well to dilute the formazan crystals. The absorbance was determined using a microplate reader at 575 nm. Cell viability was expressed as a percentage compared to the untreated negative control cells

and the positive control cells, which were treated with the lysis solution (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, 0.1% Triton X-100).

Evaluation of hemolytic activity. Blood was collected from a healthy donor using heparin vacuum blood collection tubes, and used to determine the hemolytic activity of peptides, as described previously. Red blood cells were washed with phosphate buffered saline (PBS) (Sigma Aldrich) and centrifuged at 500 x *g*, after which the supernatant was removed. This procedure was repeated five times. Test peptides, at a range of concentrations (1.3, 2.6, 5.25 10.5, 21, and 42 μM), were mixed with 50 μL of the erythrocyte solution (1% of erythrocytes in PBS). For each concentration of the peptide, three replicates were performed and the procedure was repeated three times. A positive control (0.1% Triton X-100; Sigma Aldrich), and negative control (PBS) were included. After 1 h or 48 h, the tubes were centrifuged at 3500 x *g* for 15 min and 100 μL of each supernatant was applied to a 96-well microplate. Hemolysis was measured as absorbance at 540 nm using a microplate reader (Powerwave HT, Biotek). The percentage of hemolysis was calculated relative to the positive and negative controls: Triton X-100 was taken as 100% hemolysis and PBS as 0%.[49]

Statistical analyses. Data are presented as the mean ± standard error (S.E.) for all replicates (n = 9). GraphPad Prism software v6.0 (GraphPad Software, USA) was used for the determination of the IC_{50} value for the cell toxicity and haemolytic activity assays using a log(inhibitor) vs. response – variable slope regression model.

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Figure Legends

FIGURE 1. Purification profile of *P. rigida* cyclotides. (**A**) Size exclusion chromatography profile of the leaf extract from the wet season. The cyclotide-containing fraction is marked 1. (**B**) HPLC separation of Fraction 1 from size exclusion chromatography using a non-linear gradient of acetonitrile (ACN; 13–50%), represented by the diagonal line. The partially purified fraction is marked 2 on the chromatogram. (**C**) Repeat HPLC of Fraction 2 using a non-linear gradient of acetonitrile (ACN; 13–50%) on an Everest C18 analytical column. (**D**) UFLC purification of Fraction 3, where Fraction 4 corresponds to purified paragidin-br3. The HPLC trace represents absorbance at 216 nm.

FIGURE 2: MALDI-TOF MS of: (**A**) pure parigidin-br3 and (**B**) parigidin-br3 after reduction with dithiothreitol (DTT) and alkylation with iodoacetamide. (**C**) MALDI-TOF/TOF MS of the peak at *m/*z 3419 annotated with the sequence of parigidin-br3.

FIGURE 3. Multiple sequence alignment of parigidin-br3 (PBR3) and its homologs. Yellow boxes show the conserved Cys residues (represented by Roman numerals I–VI) and the connectivity between the disulfide bonds (represented by black lines). The Asp or Asn residues important for peptide cyclization are highlighted in pink. PBR3 has the typical cyclotide cystine knot arrangement and, despite its linearity, has a high level of sequence homology with other cyclotides.

FIGURE 4. Partial coding sequence and protein sequence of parigidin-br3. Letters highlighted in red indicate the translation stop codon. The annealing sites of the forward (CCGAGCCCGTGCG) and reverse (TTAGCGATAGCACACTTTGTTTTT) primers are highlighted in green and blue, respectively. The conserved Cys residues that stabilize the peptide structure are highlighted in yellow.

FIGURE 5. A molecular model of PBR3. This structure showed a DOPE score of -2206.44. In ProSA it showed a Z-Score of -5.29, and in the Ramachandran plot it showed 91.3% of residues in favored regions and 8.7% in allowed regions. The overall average of G-factor was -0.07.

FIGURE 6: Cell toxicity assays, wherein the cell media was used as a negative control (100% viability) and Triton X-100 detergent was applied as a positive control resulting in complete lysis (0% viability). Parigidin-br3 toxicity against (**A**) CACO2, (**B**) MCF-7 and (**C**) L929 fibroblast cell lines.

SUPPLEMENTARY FIGURE 1: (A) UFLC chromatography of parigidin-br3, linear gradient of acetonitrile (5-95%) represented by the diagonal line. Absorbance at 216 nm. (B) Spectrum of parigidin-br3 showing an ion at *m/z* 3071.8, representing pure PBR3, and small peaks at *m/z* 3093.3 and 3109.1, representing sodium and potassium adducts.

SUPPLEMENTARY FIGURE 2: Hemolytic activity of PBR3 at various concentrations after: (**A**) 1 h; or (**B**) 48 h.

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