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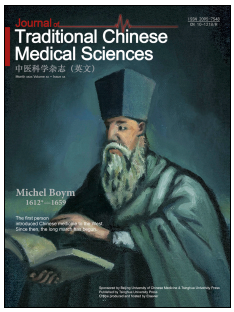

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Identification and molecular cloning of novel antimicrobial peptides from skin secretions of *Odorrana versabilis* and *Rana palustris*

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Abstract

Objective: Amphibian skin secretions are an abundant source of bioactive peptides, some of which could be developed as candidate drugs. Among these natural peptides, cytolytic peptides have attracted the most attention given that they might replace conventional antibiotics and help deal with the problem of microbial resistance. This study discovered two bioactive peptides, Brevinin-1-PLr and Nigrocin-2-OV, from two species frogs, *Rana palustris* and *Odorrana versabilis*, respectively. Their antimicrobial, anticancer and hemolytic activities were also investigated.

Methods: cDNA sequences encoding peptides were cloned from cDNA libraries constructed from the lyophilized secretions of *Rana palustris* and *Odorrana versabilis*. By reversed-phase HPLC and MS/MS fragmentation sequencing, the encoded novel peptides, named Nigrocin-2-OV and Brevinin-1-PLr, were identified in skin secretions and their structures were confirmed. Replicates of both peptides were produced through solid phase peptide synthesis. Their antimicrobial and anticancer activity was studied against three types of microorganisms (*S. aureus, C. albicans*, and *E. coli*) and five cancer cell lines (NCI-H157, PC-3, MDA-MB-435s, MCF-7, and U251MG). Their hemolytic activity was investigated using whole horse blood.

Results: In this research, cDNA sequences encoding two novel 24-mer peptides were cloned from cDNA libraries constructed from the lyophilized skin secretions of *Rana palustris* and *Odorrana versabilis*. Both of the peptides had the strongest inhibitory effect against *C. albicans*, and IC_{50} values against five cancer cell lines were all under 6 μM.

Conclusions: Nigrocin-2-OV and Brevinin-1-PLr had the strong ability to inhibit the proliferation of studied microorganisms and tumor cell lines, with slight hemolytic activity. Compared with Brevinin-1-PLr, Nigrocin-2-OV exhibited higher antimicrobial and anticancer activity but slightly higher hemolytic activity.

KEYWORDS

Amphibian; Peptide; Molecular cloning; Antimicrobial; Anticancer; Hemolysis

Introduction

Natural products have been gaining attention in recent years as a source of agents for treating many chronic diseases, especially in the development of anti-infection and anticancer drugs. Natural peptides with various activities have been found in many different amphibian species, and there is a clear relationship between frog skin peptides and their associated ability against pathogens.[1,2] Generally, frog skin peptides are divided into two types according to their different bioactivities: cytolytic peptides and peptides with pharmacological activities.[2]

With many lysine residues, antimicrobial peptides (AMPs) are cationic, which allows them to interact strongly with anionic phospholipids of bacterial membranes and eukaryotic cell membranes, such as cancer cells and erythrocytes.[3]

The Ranidae family has the widest distribution among all frog families. These frogs are found on nearly all continents, except for Antarctica, and their living conditions vary from aquatic environments to more arid terrain.[4]

Odorrana versabilis, the Chinese bamboo leaf odorous frog, is a species of frog in the Ranidae family that originates from China.[5]The North American pickerel frog (*Rana palustris*) is widely distributed in the United States and Canada.[6]

As Conlon et al. have stated, the taxonomic classification of Rana frogs is quite difficult because of slight morphological differences and overlapping distributions of several species. In consideration of AMP structural variations among species of ranid frogs, molecular heterogeneity might be helpful for taxonomic and phylogenetic purposes.[7] However, *O. versabilis* has been demonstrated to be the first major exception to this classification method. If the primary structures of frog skin peptides were used as a criterion for species classification, this species of frog should be counted as a North American species rather than Eurasian, according to Chen et al.[5] In this study, the two frog species were selected and their novel skin secretion peptides examined to confirm this conclusion and further study their related bioactivities.

Instead of a traditional animal-sacrifice method, secretions containing peptides were collected from frog skin surfaces in an animal-friendly manner, by the use of a pharmacological mediator or by a mild electrical shock, commonly applied in the laboratory.[8] Granular gland secretions that contain many peptides, cytosolic components, and genetic materials, are released by serous cells of frog skin compressed under adrenergic stimulation.[9]

Frog skin peptides are normally biosynthesized as prepropeptides. In amphibians, these precursors commonly display a tripartite structure, including a signal, spacer, and bioactive peptide.[2]The highly conserved features of the precursors of skin AMPs are most evident within the signal peptide domains.[10]The conserved region in the signal peptide sequence provides a basis for using a degenerate primer design in “shotgun” cDNA cloning, which is a method developed by Tianbao Chen’s laboratory.[11]

Given that multidrug resistant super bacteria (superbugs) having emerged across the world and microbial resistance development appearing to be almost unstoppable, there exists an urgent need for novel antimicrobial agents. Natural peptides from a

new biological source with antimicrobial activity might be regarded as a solution to the resistance problem and represent a class of new therapeutic agents.[12]

The mechanisms of anticancer and antimicrobial activities might be almost the same because the outer membrane surfaces of both cancer cells and pathogenic bacteria have above normal densities of negative charges, which make it easier for interactions with cationic peptides.[13] Cancer remains a major cause of death, but current therapeutic methods, such as chemotherapy and surgery, have relatively low success rates and the risk of reappearance. Anticancer peptides have been developed to be drugs with higher selectivity for cancer cells and lower harmful effects on healthy tissues.[14]

This study described the structural and functional characterization of two novel bioactive peptides with antimicrobial and anticancer activities from the defensive skin secretions of the Chinese bamboo leaf odorous frog (*O. versabilis*) and the North American pickerel frog (*R. palustris*), named Nigrocin-2-OV and Brevinin-1-PLr, respectively. The open-reading frames of cDNA encoding these peptides' biosynthetic precursor were deduced following RACE-PCR cloning from skin cDNA libraries. Once full structural characterization was achieved, the protein bioactivities were determined and bioinformatic analyses performed.

This study aimed to discover novel peptides with potential as candidates for antimicrobial and anticancer drugs. In addition, a goal was to discover the possible presence of similar primary structural peptides in these two frog species to confirm that peptide primary structures can be used to determine frog species relationships that might need to be better defined.

Materials and Methods

Specimen biodata and secretion collecting

Adult specimens of *O. versabilis* (*n*=3) were caught in Hainan Province, the People's Republic of China, and were settled into a vivarium at the Institute of Biotechnology, University of Fuzhou, for 4 months prior to secretion harvesting. Adult specimens of *R.palustris* (*n*=4) were purchased from the United States and raised in a purpose-designed amphibian facility at 18–25°C under a 12h/12h, light/dark cycle for 4 months prior to secretion harvesting. The frogs were fed multivitamin-loaded crickets 3 times per week. Mild electrical stimulation (5V, 100 Hz, and 140 ms pulse width; C.F. Palmer, UK) was used to induce glands in the skin to produce defensive secretions, by moving a platinum electrode over moistened frog dorsal skin for 10 s duration. The skin secretions were washed from the frogs' dorsal surface using deionized water, and the collections then frozen in liquid nitrogen, lyophilized in an Alpha1-2/LD freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany), and stored at −20°C before use. Sampling of skin secretion was performed under UK Animal (Scientific Procedures) Act 1986, project license PPL 2694, issued by the Department of Health, Social Service and Public Safety, Northern Ireland.

Nucleotide sequence determination of the bioactive peptide precursor using “shotgun” cloning

Through the process of shotgun cloning, the sequences of some of mRNAs present in lyophilized skin secretions were identified.

A Dynabeads® mRNA DIRECT™ Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was employed to isolate mRNA from lyophilized skin secretion. Five-mg samples from lyophilized skin secretions were separately dissolved in 1 mL of lysis/binding buffer. Polyadenylated mRNA was isolated using magnetic oligo-dT beads, as described by the manufacturer, and reverse-transcribed. The cDNA was subjected to 3’-RACE procedures to obtain full-length prepro-brevinin nucleic acid sequence data using a BD Smart RACE cDNA Amplification Kit (Clontech Inc., Mountainview, CA, USA), essentially as described by the manufacturer. Briefly, the 3’-RACE reactions employed a NUP primer (supplied with the kit) and a degenerate sense primer pool (S1:5’-GAWYYAYYHRAGCCYAAADATG-3’) that was designed to a highly conserved domain of the 5’-untranslated region of a previously characterized homologous peptide cDNA from *Rana* species.[15]In the first stage, samples were incubated at 96 °C for 1 minute, for initial denaturation. In the second stage, samples were incubated for 40 cycles of three steps: first, at 96 °C for 20 seconds for denaturation; then, 55 °C for 10 seconds for annealing; and finally, 60 °C for 4 minutes for extension. In the last stage, the sample was incubated at 72 °C for 10 minutes, as the final extension. The samples through these stages were incubated in the PCR machine at 4 °C. PCR products were gel-analyzed and purified applying an Omega E.Z.N.A.® Cycle Pure Kit (Omega Bio-tek, Inc., Norcross, GA, USA), cloned using a pGEM Easy Vector System II (Promega Corp., Madison, WI, USA), and sequenced usinga Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems/MDS SCIEX, Concord, ON, Canada).Finally, capillary electrophoresis and data analysis of the samples were performed.

Peptidomic studies and employed analytical techniques

Lyophilized skin secretions (5 mg) were dissolved in 1 mL of solution A (TFA/water, 0.05/99.95, v/v) and clarified of microparticulates by centrifugation. The supernatant was subjected to RP-HPLC using a Cecil Adept CE4200 HPLC system (Amersham Biosciences, Inc., Piscataway, NJ, USA) employing an HPLC column (LUNA C-5 preparative column, 250 × 10mm, Phenomenex Inc., Torrance, CA, USA) and Powerstream HPLC software, with the aid of a Waters 2489UV/Visible detector and 1525 Binary HPLC pump (Waters Corp., Milford, MA, USA). A gradient elution format was used, going from 100% solution A (TFA/water,0.05/99.95, v/v) to 100% solution B (TFA/water/acetonitrile, 0.05/19.95/80, v/v) over a period of 240 minutes at a flow rate of 1 mL/min and the detector wavelength set at λ = 214 nm to detect peptide bonds. The molecular masses of detected polypeptides in each chromatographic fraction were further analyzed using MALDI-TOF MS, in positive detection mode, using CHCA as the matrix. Internal mass calibration of the

instrument with known standards established the mass determination accuracy within a deviation of 0.1%.The peptide with a mass coincident with those predicted from cloned precursor cDNAs were subjected to primary structural analysis by MS/MS fragmentation sequencing using an LCQ™ Fleet ion-trap mass spectrometer (Thermo Fisher Scientific Inc.) with an ESI ion source.

Chemical synthesis of a novel peptide using solid phase peptide synthesis

The activity of Nigrocin-2-OV and Brevinin-1-PLr, the peptide sequence coincident with the precursor cloned cDNAs were chemically synthesized by solid-phase Fmoc chemistry using a PS4 automated solid-phase synthesizer (Protein Technologies, Inc., Tuscon, AZ, USA). The peptides were separated from the synthesized resin and side-chain protection groups using a cleavage cocktail (95% TFA, 2.5% TIPS, and 2.5% deionized water). Then, the peptides were treated with diethyl ether and stirring to form disulphide bonds. The synthesized peptides were analyzed by RP-HPLC and MALDI-TOF to verify their purity and structural reliability.

Antimicrobial susceptibility tests of two types of peptides

The antimicrobial activity of these novel peptides was assessed using three kinds of microorganisms, including Gram-negative bacterium *Escherichia coli* (NCTC 10418), Gram-positive bacterium *Staphylococcus aureus* (NCTC 10788), and yeast *Candida albicans* (NCPF 1467). The microorganisms were cultured in Muellar Hinton Broth (MHB) overnight (generally 16–20 hours) in an orbital incubator at 37 °C and 150–200 rpm. A 500 µL volume of a culture was transferred to 20 mL of pre-warmed MHB for subculture until the microorganism attained log phase growth. The log phase optical densities of *S. aureus*, *C. albicans*, and *E. coli* were 0.25, 0.15, and 0.4 respectively, detected at 550 nm using a UV spectrophotometer (CO7000 Medical Colorimeter, Biochrom Ltd., Cambridge, United Kingdom). Nigrocin-2-OV and Brevinin-1-PLr were prepared with PBS (Sigma-Aldrich, Inc., St. Louis, MO, USA) to reach a concentration of 512×10^2 mg/L (20 mM). This stock solution was then double diluted with the same solvent to achieve gradient peptide solutions. A specific concentration of peptide solution (1 µL) was added to a 96-well plate with seven replicates. Then, the bacterial subculture solution was diluted with sterilized MHB to reach a final concentration between 1×10^5 and 1×10^6 cfu/mL. This diluted subculture solution was used to dilute each peptide solution and added to growth control lines to produce 100 µLin each well. Sterilized MHB (100 µL) was used as negative control. The plates were incubated at 37°C overnight and the MIC values detected at $\lambda = 550$ nm. Peptides with inhibitory ability were further tested to observe their MBC values.

Hemolytic activity of novel peptides

Whole horse blood was used to test peptide hemolytic activity. Fresh whole horse blood (2–10 ml) was placed into a 50-mLcentrifuge tube and centrifuged to remove

the supernatant. The cell pellet was gently dispersed in 30 mL of PBS to wash the red blood cells (RBC) and recentrifuged until the supernatant ran clear. The clear supernatant was then discarded and 50 mL PBS added to the washed RBC to form a suspension. Each peptide was weighed to prepare a 512 × 2 mg/L (0.4 mM) peptide stock solution in PBS. Then, the stock solution was double diluted with sterilized PBS to produce gradient peptide solutions.

For each concentration, quintuplicate volumes of 200 μL of peptide solution were added into 1.5-mLtubes. Then, 200 μL of RBC suspension was added to each tube. PBS and 1% Triton X-100 (Sigma-Aldrich, Inc.) were regarded as negative and positive controls, respectively. All tubes were incubated at 37°C for 2 hours and then centrifuged at 930×g for 5 minutes. Supernatants were added into a 96-well-plate (ThermoFisher Scientific Inc.) and the absorbance detected at 550 nm with an ELx808TM Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

The half maximal hemolytic concentration (HC_{50}) values were analyzed with the aid of GraphPad Prism 6.0 software.

Cell culture experiment and MTT cell viability assay for assessing anticancer activity of novel peptides

Bioactivity screening of anticancer cell activity was performed using an MTT cell viability assay on five types of human cancer cell lines: NCI-H157, PC-3, MDA-MB-435s, MCF-7, and U251MG. The different cancer cell types were cultured using appropriate media, with RPMI 1640 Medium used to culture NCI-H157 and PC-3 and DMEM medium used to culture other three types of cancer cells.

These cancer cell lines were cultured with serum-containing medium (medium/FBS/P-S, 100/10/1, v/v) in a 37°C and 5% CO_2 incubator and the medium renewed every two days. When grown appropriately, cell lines should be subcultured to prevent cultures from dying. The peptide anticancer activity was tested by seeding cancer cells (100 μL) in 96-well plates, in quintuplicate, until they reached a concentration of 5000 cells/well, and the plates placed in a 37 °C incubator under 5% CO2 for 24 hours. Then, the culture medium was replaced with serum-free medium to starve the cancer cells for 6–12 hours. Peptide was dissolved in DMSO to obtain a 10^{-2}M stock solution, and gradient peptide concentration solutions (10^{-4} M to10^{-9} M) produced using serum-free medium. DMSO was 100-fold diluted by serum-free medium as a vehicle control, and serum-free medium used as a growth control. The cell medium was replaced by peptide and control solutions, and the plates incubated for 24 hours in a challenge peptide solution. Cell viability was determined by the reduction of MTT to its insoluble formazan. After 24 hours incubation, 10 μL of MTT (5 mg/mL in PBS) was added to each well and the plates further incubated for 4–6 hours. After removing the liquid, 100 μl of DMSO was added to each well to dissolve the developed formazan crystals and the plates placed in an orbital shaker for 10 minutes at maximum speed (250 rpm). The absorbance was measured using a Synergy HT plate reader (BioTek Instruments, Inc.) at 570nm. The half maximal inhibitory

concentration (IC_{50}) values were analyzed with the aid of GraphPad Prism 6.0 software.

Results

"Shotgun" cloning of novel peptide precursor-encoding cDNA

After carrying out "shotgun" cloning with the aid of the *Rana* genus-specific degenerate primer pool and bioinformatic analysis using the NCBI-BLAST program, two bioactive peptide precursor-encoding cDNAs were cloned from the skin secretion library of *O. versabilis* and *R. palustris* and separately encoded single copies of a bioactive peptide named Nigrocin-2-OV and Brevinin-1-PLr, respectively (Fig. 1A and 1B). Both translated open-reading frames of the novel cloned cDNAs consisted of 70 amino acid residues, including 22 amino acids of signal and spacer peptides, a processing site of Lys-Arg (K-R) for endoproteolytic cleavage, and a novel 24-mer of a mature peptide. The calculated mass and primary structures of these peptides are listed in Table 1.

A) Nigrocin-2-OV

```
      M  F  T  L  K  K  S  F  L  L  L  F  F  L  G  T  I  ·
  1   ATGTTCACCT TGAAGAAATC CTTCTTACTC CTTTTCTTCC TTGGGACCAT
      TACAAGTGGA ACTTCTTTAG GAAGAATGAG GAAAAGAAGG AACCCTGGTA
      ·  N  L  S  L  C  E  Q  E  R  N  A  E  E  E  R  R  D  ·
  51  CAACTTATCT CTTTGTGAGC AAGAGAGAAA TGCAGAGGAA GAAAGAAGAG
      GTTGAATAGA GAAACACTCG TTCTCTCTTT ACGTCTCCTT CTTTCTTCTC
      ·  E  P  D  E  M  N  V  E  V  E  K  R  F  L  P  A
 101  ATGAGCCAGA TGAAATGAAT GTTGAAGTGG AAAAACGATT CTTACCAGCT
      TACTCGGTCT ACTTTACTTA CAACTTCACC TTTTTGCTAA GAATGGTCGA
       I  A  G  L  A  A  K  F  L  P  K  I  F  C  A  I  T  ·
 151  ATTGCAGGCT TGGCCGCTAA ATTCTTGCCG AAAATATTTT GTGCAATAAC
      TAACGTCCGA ACCGGCGATT TAAGAACGGC TTTTATAAAA CACGTTATTG
      ·  K  K  C  *
 201  CAAAAAATGT TGAAAGTTTG GAAACTTGGA TTGGAAATCA TCTGATGGAG
      GTTTTTTACA ACTTTCAAAC CTTTGAACCT AACCTTTAGT AGACTACCTC
```

B) Brevinin-1-PLr

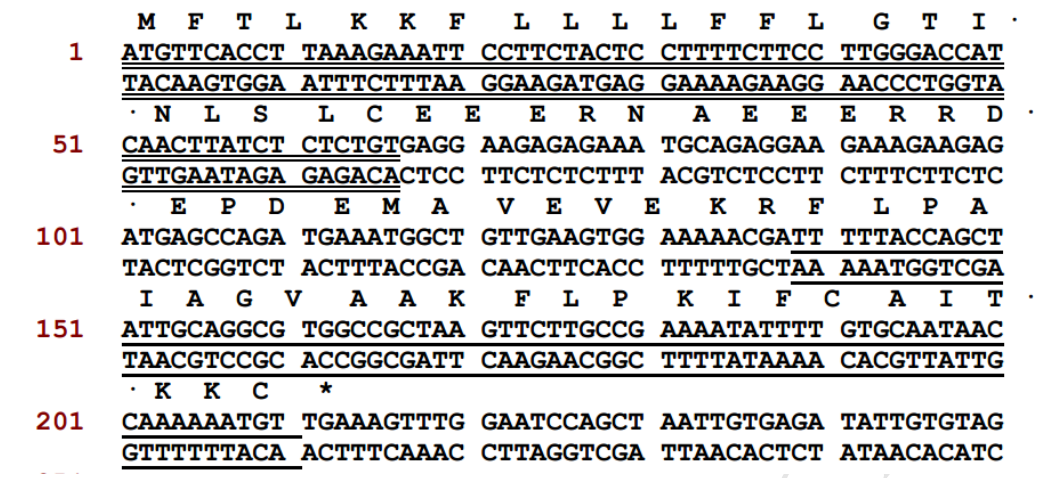

Figure1 Nucleotide and translated open-reading frame amino acid sequence of biosynthetic precursor-encoding cDNA of Nigrocin-2-OV and Brevinin-1-PLr cloned from skin secretions derived from a library of *O. versabilis* and *R. palustris*, respectively.

Note: The putative signal peptide was double-underlined, mature peptide was single-underlined, and stop codon was indicated by an asterisk.

Table 1 The calculated molecular mass data and primary structures for Nigrocin-2-OV and Brevinin-1-PLr.

Peptides	Mass calculated (ave., Da)	Primary structure
Nigrocin-2-OV	2563.246	FLPAIAGLAAKFLPKIFCAITKKC
Brevinin-1-PLr	2549.219	FLPAIAGVAAKFLPKIFCAITKKC

Identification and structural characterization of peptides in skin secretion

The dried skin secretions of two kinds of frogs were subjected to reversed-phase HPLC fractionation and peptides in the column effluent detected at214 nm. The observed peptide molecular masses were coincident with the calculated molecular masses of Nigrocin-2-OV and Brevinin-1-PLr, derived from cloned skin biosynthetic precursor-encoding cDNAs, identified using MALDI-TOF mass spectrometry. It was found that the target peptides, Nigrocin-2-OV and Brevinin-1-PLr, located in the 127th and 126thfractions, respectively (Fig. 2). These two fractions were analyzed by electrospray ion-trap MS/MS fragmentation and the primary structures of the two putative peptides confirmed by MS/MS fragmentation sequencing (Table 2A and B).

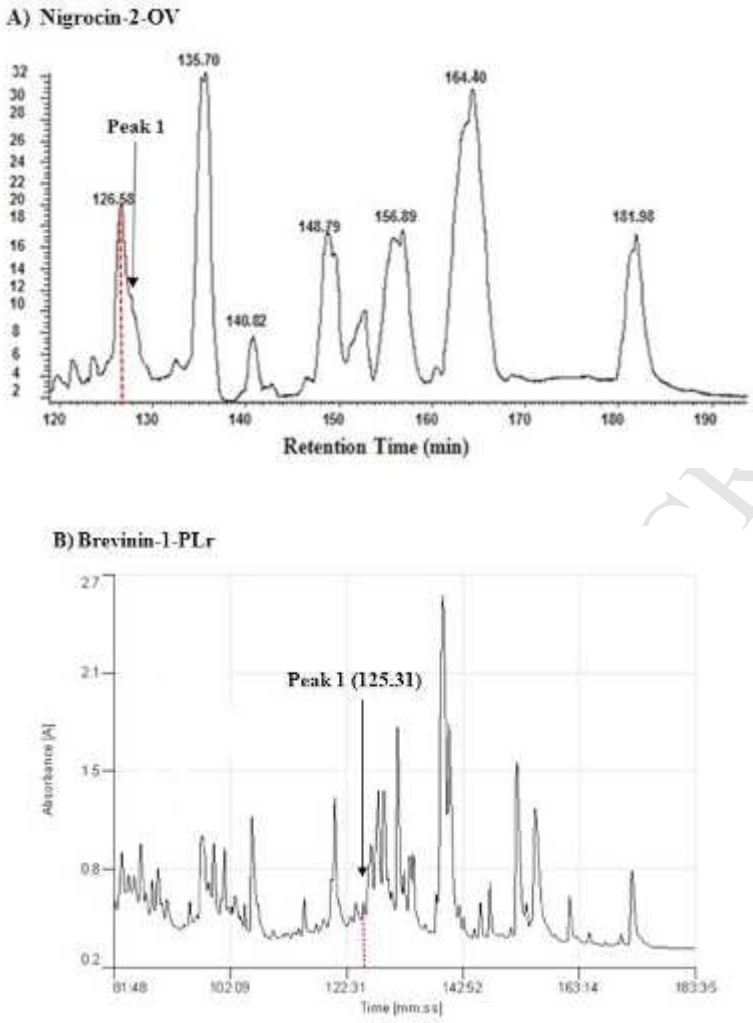

Figure 2 Region of RF-HPLC chromatogram of skin secretion peptides from O. *versabilis* and *R. palustris* indicating elution position/retention time (peak 1 arrow) of the target peptides, Nigrocin-2-OV and Brevinin-1-PLr.

Table 2 Electrospray ion-trap MS/MS fragmentation datasets derived from ions corresponding in molecular mass to the target peptides.

A) Nigrocin-2-OV						
#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	148.07570	74.54149	F			24
2	261.15977	131.08352	L	2417.41765	1209.21246	23
3	358.21254	179.60991	P	2304.33358	**1152.67043**	22
4	**429.24966**	215.12847	A	2207.28081	1104.14404	21
5	**542.33373**	271.67050	I	2136.24369	**1068.62548**	20
6	**613.37085**	307.18906	A	2023.15962	**1012.08345**	19
7	**670.39232**	335.69980	G	**1952.12250**	**976.56489**	18
8	**783.47639**	392.24183	L	**1895.10103**	**948.05415**	17
9	**854.51351**	427.76039	A	**1782.01696**	891.51212	16
10	**925.55063**	463.27895	A	**1710.97984**	**855.99356**	15
11	**1053.64560**	527.32644	K	**1639.94272**	820.47500	14
12	**1200.71402**	600.86065	F	**1511.84775**	756.42751	13
13	**1313.79809**	657.40268	L	**1364.77933**	**682.89330**	12
14	**1410.85086**	705.92907	P	**1251.69526**	**626.35127**	11
15	**1538.94583**	769.97655	K	**1154.64249**	577.82488	10
16	**1652.02990**	**826.51859**	I	**1026.54752**	**513.77740**	9
17	**1799.09832**	**900.05280**	F	**913.46345**	**457.23536**	8
18	**1902.10751**	951.55739	C	**766.39503**	**383.70115**	7
19	**1973.14463**	**987.07595**	A	**663.38584**	332.19656	6
20	2086.22870	**1043.61799**	I	592.34872	296.67800	5
21	2187.27638	**1094.14183**	T	**479.26465**	240.13596	4
22	2315.37135	**1158.18931**	K	378.21697	189.61212	3
23	2443.46632	**1222.23680**	K	250.12200	125.56464	2
24			C	122.02703	61.51715	1

B) Brevinin-1-PLr						
#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	148.07570	74.54149	F			24
2	**261.15977**	131.08352	L	2403.40200	1202.20464	23
3	**358.21254**	179.60991	P	2290.31793	**1145.66260**	22
4	**429.24966**	215.12847	A	2193.26516	**1097.13622**	21
5	**542.33373**	**271.67050**	I	2122.22804	**1061.61766**	20
6	**613.37085**	**307.18906**	A	2009.14397	**1005.07562**	19
7	**670.39232**	335.69980	G	1938.10685	**969.55706**	18
8	**769.46074**	385.23401	V	1881.08538	**941.04633**	17
9	**840.49786**	**420.75257**	A	1782.01696	**891.51212**	16
10	**911.53498**	**456.27113**	A	**1710.97984**	**855.99356**	15
11	**1039.62995**	520.31861	K	**1639.94272**	**820.47500**	14
12	**1186.69837**	**593.85282**	F	**1511.84775**	**756.42751**	13
13	**1299.78244**	**650.39486**	L	**1364.77933**	**682.89330**	12
14	**1396.83521**	**698.92124**	P	**1251.69526**	**626.35127**	11
15	**1524.93018**	**762.96873**	K	**1154.64249**	**577.82488**	10
16	1638.01425	**819.51076**	I	**1026.54752**	**513.77740**	9
17	1785.08267	**893.04497**	F	**913.46345**	**457.23536**	8
18	1888.09186	**944.54957**	C	**766.39503**	**383.70115**	7
19	1959.12898	**980.06813**	A	**663.38584**	**332.19656**	6
20	2072.21305	**1036.61016**	I	**592.34872**	**296.67800**	5
21	2173.26073	**1087.13400**	T	**479.26465**	240.13596	4
22	2301.35570	**1151.18149**	K	**378.21697**	189.61212	3
23	2429.45067	**1215.22897**	K	**250.12200**	125.56464	2
24			C	122.02703	61.51715	1

Note: Observed ions indicated in bold typeface and underlined.

Antimicrobial assay of the novel peptides against tested microorganisms

The antimicrobial activities of Nigrocin-2-OV and Brevinin-1-PLrwere tested by MIC assay on referenc estrains *E. coli*, *S. aureus*, and *C. albicans*. The growth of three types of microorganisms was inhibited by Nigrocin-2-OV and Brevinin-1-PLr to different extents, as shown in Table 3.

Table 3 The mean MIC and MBC values for Nigrocin-2-OV and Brevinin-1-PLr against the three different test organisms.

Organism	MIC (μM)		MBC (μM)	
	Nigrocin-2-OV	Brevinin-1-PLr	Nigrocin-2-OV	Brevinin-1-PLr
S.aureus	3.12	6.28	49.94	50.22
E.coli	12.49	25.11	99.88	50.22
C.albicans	3.12	3.14	24.97	12.55

Hemolytic activity of the novel peptides

The hemolytic activities of Nigrocin-2-OV and Brevinin-1-PLr were measured with the aid of whole horse blood. After triplicate experiments, both peptides were found to display hemolytic activity. The half maximal hemolytic concentrations (HC_{50}) of Nigrocin-2-OV and Brevinin-1-PLr were 16 and 85 μM, respectively, as calculated using GraphPad Prism 6.0 software.

MTT cell viability assay of novel peptides on tested cancer cell lines

Nigrocin-2-OV and Brevinin-1-PLrwere found to have dose-dependent inhibitory effects on all tested cancer cell lines, as assessed using the MTT assay treated with peptide solutions ranging from 10^{-9}M to 10^{-4} M and cultured over 24 hours. The half maximal inhibitory concentrations (IC_{50} values) of Nigrocin-2-OV and Brevinin-1-PLr against these types of cancer cells are shown in Table4.

Table 4 The IC_{50} values of Nigrocin-2-OV and Brevinin-1-PLragainst five tested cancer cells lines.

Cancer cell line	IC_{50} (μM)	
	Nigrocin-2-OV	Brevinin-1-PLr
NCI-H157	3.6	5.9
PC-3	4.0	5.9
MDA-MB-435S	4.3	3.5
U251MG	4.8	3.9
MCF-7	2.0	3.4

Discussion

By the means of NCBI BLAST, it was found that these two novel peptides, Nigrocin-2-OV and Brevinin-1-PLr, showed a high degree of similarity to peptides belonging to the brevinin-1 family, which is a typical antimicrobial peptide family given that they share some common features, such as a C-terminal disulphide-bridged cyclic heptapeptide, called “Rana box” cationic amino acids, and amphipathic α -helical structures.[1]Zhou et al. have compared the open-reading frames and nucleic acid sequences between *R. palustris* and *O. versabilis*, and analogous peptides with high degrees of structural similarity have been found in these two species of frogs from different continents.[10]

In this study, two novel bioactive peptides were discovered from the two frog species. The open-reading frames of skin AMPs were most evident within the signal peptide

domains, moderate within the acidic spacer peptide domains, and least within the mature peptide coding domains, which is in agreement with former studies. The primary structure of the mature peptides showed high identity, and there was only one different amino acid in the peptide primary structure, the 8th amino acid, which was lysine in Nigrocin-2-OV and valine in Brevinin-1-PLr, which indicated that using primary structure as criterion for taxonomy still needs to be improved. Both new peptides possessed a disulphide bridged cyclic heptapeptide (Cys^{18}-(Xaa)^{4}-Lys-Cys^{24}) which played a crucial role in their antibacterial activities.

Nigrocin-2-OV and Brevinin-1-PLr displayed an8-fold greater ability to inhibit *S. aureus* growth, compared to *E. coli*. Gehman has proposed that this preference might relate to higher amounts of negatively charged lipids in Gram-positive bacteria compared to Gram-negatives.[16]In addition to their binding affinity towards negatively charged membranes, other parameters also contribute to specific antibacterial effects, such as the complex ratio between surface charge and membrane order. In addition, bacterial membrane repair in nutrient-rich media and their ability to maintain against membrane damage might modulate growth inhibition potential.[17] Thus, Hurst has thought that differences in MIC values might derive from a general Gram-negative bacterium, such as *E. coli*, having more potential to sustain membrane homeostasis than a Gram-positive bacterium, such as *S. aureus.*[18]

Recently, some studies have illustrated that polycationic peptides possess synergistic properties with lipophilic and amphiphilic agents.[19] In addition, as it was found here that Nigrocin-2-OV has synergistic effects with Norfloxacin against *E. coli*, the synergistic interactions between these novel polycationic peptides have the potential to serve as an adjuvant for antimicrobial chemotherapy.[20]

Several AMPs have been reported to possess this obvious anticancer potential because of their cytotoxic activity against cancer cell growth. Previous research has indicated that cancer cells treated with frog skin peptides release the cytosolic enzyme lactate dehydrogenase, as a result of damage to plasma membrane integrity.[21] In addition, apoptosis and necrosis are the two major mechanisms causing celldestruction.[22]Considering research on anticancer activities, these new peptides might have the ability to trigger apoptosis or necrosis of cancer cells as well. However, the specific mechanisms of how AMPs penetrate the cell membrane and induce apoptosis in treated cancer cells remains unknown.[23]

As the peptide concentration that inhibits cancer cell growth might be lower than the concentration that inhibited the pathogen growth or RBC, this new peptide might have a relatively weaker ability for penetrating cell membranes. The ability to inhibit the growth of or to kill pathogens is determined by peptide structure, which is based on its amino acid sequence. The reason for the relatively higher cytolytic activity of Nigrocin-2-OV over Brevinin-1-PLr might be the different 8th amino acid, which produced different hydrophobicity and peptide spatial structure.

In conclusion, some bioactivities of both peptides were initially evaluated, but other aspects await further investigation. The mechanisms of these peptides in inhibiting cancer cell growth remain unclear.

Moreover, although these peptides were shown to have relatively low hemolytic

activity, this hemolytic ability would still be a negative factor for its use as a marketable drug. In this respect, more research is required to determine if modifications to the peptide can render it more cell-selective.

There are about 100 therapeutic peptides (mostly innovative synthetics) on the market in the United States, Europe, and Japan.[24] In 2012, there were 5 and 6peptides that gained market approval, respectively, in Europe and the United States, and there were128 peptide candidates in the clinical pipeline as drugs. Although great success has been achieved in the use of peptides in many clinical areas, the development of peptide drugs is still restricted by many factors.

The route of peptide drug administration is one problem that is difficult to solve, especially for AMPs, as AMPs are hemolytic at concentrations at which they kill pathogens, which makes them almost impossible to deliver by intravenous injection. Modification of peptide chemical structures is a possible solution and, indeed, some AMPs have been modified to be more selective.

Another problem is large-scale production. Peptide isolation from natural sources is not cost effective and can only be used for native peptide exploration. When it comes to manufacturing either native or modified cationic peptides, the natural approach is very costly.

Mature regulations for peptide drug registration are also needed. Only one guideline specifically addresses peptides in the field of manufacturing, which shows there is an urgent need to improve drug administration to involve more information and guidance in peptide drugs.

In addition, an increasing number of studies have illustrated that bacterial pathogens might resist AMPs.[25] AMPs and AMP-resistance mechanisms are the result of highly dynamic co-evolutionary processes, but it remains unclear how adaptations in resistant microorganisms have influenced AMP evolution.[26]Therefore, if AMPs are to be developed as an alternative therapy to traditional antibiotics, more research on the molecular basis of bacterial resistance to AMPs is required to improve the understanding of the process and avoid resistant cases.

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Abbreviation

Antimicrobial peptides (AMPs); matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS); α-cyano-4-hydroxycinnamic acid (CHCA); electro spray

ionization (ESI); fluorenyl methoxycarbonyl (Fmoc); trifluoroacetic acid (TFA), triisopropylsilane (TIPS); phosphate buffered saline (PBS); National Centre for Biotechnology Information (NCBI); Basic Local Alignment Search Tool (BLAST); colony forming units (cfu); non-small cell lung cancer (NCI-H157); human prostate carcinoma (PC-3); melanoma (MDA-MB-435s); breast cancer non-tumorigenic mammary gland (MCF-7); human neuronal glioblastoma (U251MG); Roswell Park Memorial Institute (RPMI); Dulbecco's Modified Eagle (DMEM); penicillin-streptomycin (P-S); fetal bovine serum (FBS); minimal inhibitory concentration (MIC); and minimal bactericidal concentration (MBC)

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