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ORIGINAL ARTICLE

Functional characterization of two novel peptides and their analogs identified from the skin secretion of *Indosylvirana aurantiaca*, an endemic frog species of Western Ghats, India

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Abstract
Two new antimicrobial peptides belonging to brevinin 1 (B1) and brevinin 2 (B2) families were identified from the skin secretion of *Indosylvirana aurantiaca*, an endemic frog of Western Ghats, India, through shotgun cloning. Antibacterial, antibiofilm and cytotoxic effects of these peptides were evaluated and compared with their C-terminally amidated forms. Both the amidated peptides (B1-NH_2 and B2-NH_2) showed significantly enhanced broad-spectrum activities against Gram-positive and Gram-negative bacteria and the latter was found to have potent biofilm inhibition properties against *Klebsiella pneumoniae* over other peptides. Both forms of B2 were less cytotoxic against human red blood cells (hRBC) and peripheral blood mononuclear cell (PBMC). Hence, B2 and its amidated analog have good therapeutic value and these peptides could be considered as potential lead molecules for further development.

Keywords Antibacterial · Antibiofilm · Brevinin · Frog skin peptide · Therapeutics

Introduction

The emergence of drug resistant microorganisms poses a major challenge to public health, which demands the search for novel effective antimicrobial compounds. Host defence peptides (HDPs) are a part of innate immune system, which act effectively against invading pathogens. HDPs of plant and animal origins have been widely studied to use as a supernumerary to conventional drugs. HDPs are considered as promising molecules effective against wide range of microorganisms including multidrug-resistant bacterial infection (Laverty et al. 2011) and biofilm formation (Park et al. 2011). Even though the mode of action of HDPs on microorganisms are relatively unknown, it is believed that the HDPs primarily act on bacterial membrane, which reduces the chance to induce bacterial resistance (Hancock 2001) and thereby makes them potential candidates for the development of novel therapeutics.

Among the various peptides having promising antimicrobial action, amphibian peptides are unique due to the rich structural diversity offered by their existing habitat and mode of life (König et al. 2015). Frog skin peptides are usually cationic with a molecular weight below 4KDa. Brevinins are the most ubiquitous broad-spectrum antimicrobial peptides identified from anuran skin secretion, which consist of two families: brevinin-1 and brevinin-2 (Savelyeva et al. 2014). Post-translational modification such as C-terminal amidation has been shown to enhance the bioactivity of brevinin peptides against microorganisms (Kumar et al. 2017). Developments in the molecular techniques enabled to determine the peptides from the skin secretion without killing the animal (Chen et al. 2006) and thus, promoting sustainable bio-prospecting.

In spite of the rich diversity of Asian frogs, research on their AMPs is sparse (Kumar et al. 2015). Endemic frogs from the Western Ghats of India offer a unique model system to explore many novel peptides with improved bioactivity (Kumar et al. 2017). In this study, we report the identification of two novel antimicrobial peptides (AMPs) that belong

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to brevinin 1 and brevinin 2 families from the skin secretion of *Indosylvirana aurantiaca* an endemic frog species of Western Ghats through shotgun cloning (Oliver et al. 2015). Analysis of the sequence structure revealed that these peptides belong to brevinin 1 and 2 and on further characterization of these peptides showed strong broad-spectrum bactericidal and biofilm inhibition potency, which was significantly enhanced on C-terminal amidation.

Materials and methods

Frog skin secretion harvesting

Frogs (*I. aurantiaca*, $n=5$) were collected from the hilly terrains of Thiruvananthapuram, Kerala, under a permit from the Kerala Forest Department. Secretion was harvested by rinsing the skin with Milli-Q water while applying a mild transdermal electric stimulation of 6 V DC, 4 ms pulse width, 50 Hz, for 10 s. Then frogs were released to the same habitat without causing any harm. Filtered secretion was snap frozen in liquid nitrogen, lyophilized and kept at − 20 °C prior to analyses.

Screening of cDNA-encoding peptides through shotgun cloning

Polyadenylated mRNAs were isolated from the snap-frozen skin secretion with oligo (dT) dyna beads® (Dynal Biotech, UK) as per the manufacturer's instructions. cDNA library was constructed with SMARTer™ cDNA Amplification Kit (Clontech, UK) and was subjected to RACE amplification with a sense (5′ATGTTCACCTTGAAGAAT) and anti-sense primer (5′-AGATGATTTCCAATTCCAT-3′) to obtain full-length peptide precursor nucleic acid under the following conditions: 94 °C for 2 min; followed by 30 cycles of 92 °C for 10 s, 47 °C for 30 s and 72 °C for 40 s; followed by an extension at 72 °C for 10 min. Purified amplicons were cloned into pGEMT-easy vector (Promega) and positive clones were amplified and sequenced (ABI DNA sequencer 3730). The nucleotide sequences were translated to amino acid sequence with EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and were subjected to homology searches using NCBI BLAST (http://w.w.w.ncbi.nlm.nih.gov).

Physico-chemical properties and structure of peptides

Net charge, molecular weight, grand average hydropathicity (GRAVY) and hydrophobicity values were calculated with Protparam (http://expasy.org/tools/protparam.html) and APD3 (http://aps.unmc.edu/AP/prediction/prediction_main.php). The secondary structure prediction of the peptides was done with self-optimized prediction method with alignment (SOPMA) (Combet et al. 2000). Arrangements of the amino acids on alpha helix were predicted by plotting helical wheel projection as per Don Armstrong and Raphael Zidovetzki (Version:Id: wheel.pl,v 1.4 2009-10-20 21:23:36 don Exp).

Peptide synthesis

The parent peptides (B1, B2) and their C-terminal amidated analogs (B1-NH_2, B2-NH_2) were synthesized using FMOC solid-phase chemistry and were purchased from Synpeptide, Shanghai, China. The peptides were dissolved in deionized water (2 mg/ml) after confirming their purity with RP-HPLC and MALDI-TOF mass spectroscopy.

Bacterial strains used for activity studies

Bacterial strains used for activity studies were *Staphylococcus aureus* ATCC25923, methicillin-resistant *Staphylococcus aureus* (clinical isolate), *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* (clinical isolate) and *Vibrio cholerae* N16961.

Antibacterial assay

Anti-bacterial assays were carried out in Mueller–Hinton broth (Hi-media, India) and were evaluated by broth microdilution method as per guidelines (Clinical and Laboratory Standards Institute 2012). Fifty microliters of bacterial suspension (10^6 CFU/ml) at its mid-logarithmic phase of growth was incubated with equal volume of twofold serially diluted peptides in sterile 96-well plates for 20 h at 37 °C. Then the bacterial growth was assessed by measuring the absorbance at 595 nm in a microplate reader (iMark microplate reader, Bio-Rad). The lowest concentration of peptide, which showed no visible growth, was taken as the minimum inhibitory concentration (MIC) of peptides. The experiment was conducted three times in triplicate along with serially diluted ampicillin for Gram-positive and kanamycin sulfate for Gram-negative bacteria as controls.

Cytotoxicity assay

Lactate dehydrogenase (LDH) release assay

Cytotoxic effect of the peptides on peripheral blood mononuclear cells (PBMC) was evaluated by LDH release assay (Van Dijk et al. 2009). For this, PBMCs were isolated from heparinised blood. PBS-diluted blood was layered above 5 ml of histopaque and centrifuged at 1200 rpm for 30 min at room temperature. PBMCs were collected carefully from the plasma–histopaque interface and washed with PBS

and re-suspended in RPMI medium. The PBMCs (50,000/well) were cultured in RPMI medium for 3 h and incubated with different concentrations of peptides (10–60 µM) for 18 h. The release of lactate dehydrogenase (LDH) into the medium was quantified by measuring the absorbance at 492 nm using a Cytotox96 non-radioactive cytotoxicity kit (Promega), following the manufacturer's instructions. The percentage LDH release was calculated with the equation $(Tr - Spo)/(Max - Spo) \times T100$, where Tr is the treatment LDH release, Spo is spontaneous LDH release and Max is maximum LDH release. The experiment was repeated thrice.

Hemolytic assay

Hemolytic assays of B1, B1-NH_2, B2 and B2-NH_2 were carried out as described (Yang et al. 2006) with slight modification. Fresh human RBCs collected from heparinized blood were re-suspended in PBS to make 4% (v/v) solution and exposed to serially diluted peptides in PBS for 1 h at 37 °C. Subsequently, the amount of hemoglobin released due to the lysis of RBC was estimated by measuring the absorbance of the supernatant at 415 nm. Hemolysis with Triton X-100 served as positive control. The experiment was performed in triplicates. Percentage hemolysis was calculated by the equation: $\{(As - A_0)/(Ac - A_0)\} \times 100$, where As is the absorbance of sample, A_0 is the absorbance of blank and Ac is the absorbance of positive control.

Therapeutic index

To evaluate the selectivity of the peptides against bacterial and eukaryotic cells, therapeutic indices (TI) were calculated as described (Ilić et al.2013) using the formula: $TI = HC_{50}/GM$, where HC_{50} represents the mean concentration of peptide causing 50% hemolysis of hRBC and GM is the geometric mean of the MIC values of the peptide against bacteria.

Killing kinetics assay

Killing kinetics assay of the peptides was performed on *S. aureus* and *E. coli*. Bacterial culture from its mid-logarithmic phase of growth was re-suspended in MHB (10^6 CFU/ml) and incubated with the peptides at their MIC and sub-MIC (1/4 MIC) along with the control. Aliquots drawn at different intervals of time were diluted and incubated on LB plates at 37 °C for 24 h for counting the number of colonies developed. Time–killing curves were generated by plotting $\log_{10}$ CFU/ml against time and the experiment was repeated thrice in triplicates. Cells without peptide treatment were taken as negative control.

Anti-biofilm activity

Biofilm inhibition assay was carried out as described (Zhang et al. 2016) with slight modifications. Fifty microliters of biofilm-forming *K. pneumoniae* (10^6 cells/ml) was incubated with equal volume of serially diluted peptides at their sub-MIC concentrations (1/2 MIC, 1/4 MIC and 1/8 MIC) in 96-well plates sealed with pegged TSP lids (NUNC, Roskilde, Denmark). The plates were incubated at 37 °C for 24 h without shaking. For estimating biofilm biomass, the pegs were removed from the plates and washed with 1X PBS twice and fixed with 1.2% glutaraldehyde (200 µl) for 30 min. Then the adhered cells on the pegs were stained with crystal violet (0.41%) for 10 min and the pegs were washed with PBS to remove excess dye. The cell-bound crystal violet was re-solubilized in 200 µl of dimethyl sulfoxide (DMSO) for 10 min and the absorbance was measured at 595 nm.

Statistical analysis

The experimental data were analyzed by one-way analysis of variance (ANOVA) with SPSS, version 16.00 (SPSS 2007). Statistical significance between the experimental groups was calculated by comparing multiple means with Turkey's LSD test.

Results

Screening of cDNA encoding peptides

On shotgun cloning, cDNA sequences encoding two novel peptides were identified from the skin secretions of *I. aurantiaca* and the amino acid precursor obtained from the translated transcript revealed similar structural organization as that of amphibian skin peptides with a highly conserved N-terminal signal region followed by an acid spacer, which terminates in dibasic cleavage site KR followed by a highly variable C-terminal mature peptide. The structural organizations of the two peptides deduced from cDNA library were subjected to NCBI BLAST search, which revealed that the first peptide exhibited 67% homology with brevinin-1 and the second peptide exhibited 79% homology with brevinin-2. The mature peptide domain of brevinin 1 has 24 amino acid residues in length, while that of brevinin 2 contains 29 amino acids (Table 1). The peptides were named as brevinin1 IDau 1 (B1) and brevinin 2 IDau1 (B2) as per the current nomenclature system of frog skin peptides (Thomas et al. 2012). Both brevinin1 IDau 1 and brevinin 2 IDau1 were synthesized in two forms with C-terminal acid (B1 and B2) and C-terminal amide (B1-NH_2 and B2-NH_2) for further characterization.

Table 1 Peptide sequences and physico-chemical properties of brevinin 1 and brevinin 2 peptides identified in this study

	Signal peptide	Spacer peptide	Mature peptide
Peptide sequences			
B1	MFTLKKPMLLLFFLGTINLSLC	QEERNAEEERRDGDEM-NAEVQEKR	FLGALLKIGAKVLPSVL-CGIFKKC
B2	MFTLKKSMLLLFFLGTISLSLC	EEERGADEDDGGEMTEEEKR	GIKSALLGIAKN-VGMSLLQKAQCKLSGSC

Physico-chemical properties of the peptides

Peptides	Sequence	NA	NC	MW	G	HR	Structure	
							α-Helix	β-Turn
B1	FLGALLKIGAKVLPSVLCGIFKKC	**24**	**+4**	2519.229	1.308	**62%**	**75%**	**16.7%**
B1-NH_2	FLGALLKIGAKVLPSVLCGIFKKC-NH_2	**24**	**+5**	2518.234				
B2	GIKSALLGIAKNVGMSLLQKAQCKLSGSC	**29**	**+4**	2919.544	0.469	**48%**	**82.7%**	**10%**
B2-NH_2	GIKSALLGIAKNVGMSLLQKAQCKLSGSC-NH_2	**29**	**+5**	291.559				

NA no. of amino acids, *NC* net charge, *MW* molecular weight, *G* GRAVY, *HR* hydrophobic ratio

Physico-chemical properties and secondary structure prediction

Physico-chemical properties such as net charge, hydrophobicity, GRAVY, helicity, molecular mass and peptide sequence of B1 and B2 are given in Table 1. Analysis of physico-chemical properties revealed that both the peptides have the same charge of +4 and their amidated analogs have +5. Comparison of hydrophobicity and GRAVY values indicated that B1 is more hydrophobic (62%) with a higher GRAVY value of 1.308 than B2 peptide (48% and 0.469, respectively). Secondary structure prediction of B1 and B2 with SOPMA method illustrated that the major portions of both the peptides are α-helical. B1 showed 75% α-helical structure and 16.7% β-turn, while B2 depicted 82.7% α-helix and 10% β-turn; hence, both the peptides exhibited trends for helical conformation, which is usually found in typical AMPs. Helical wheel projection (Fig. 1) revealed distinct demarcation of hydrophobic and hydrophilic residues in B1 than B2, confirming the strong amphipathic nature of B1. Moreover, B1 showed smaller polar angles due to less

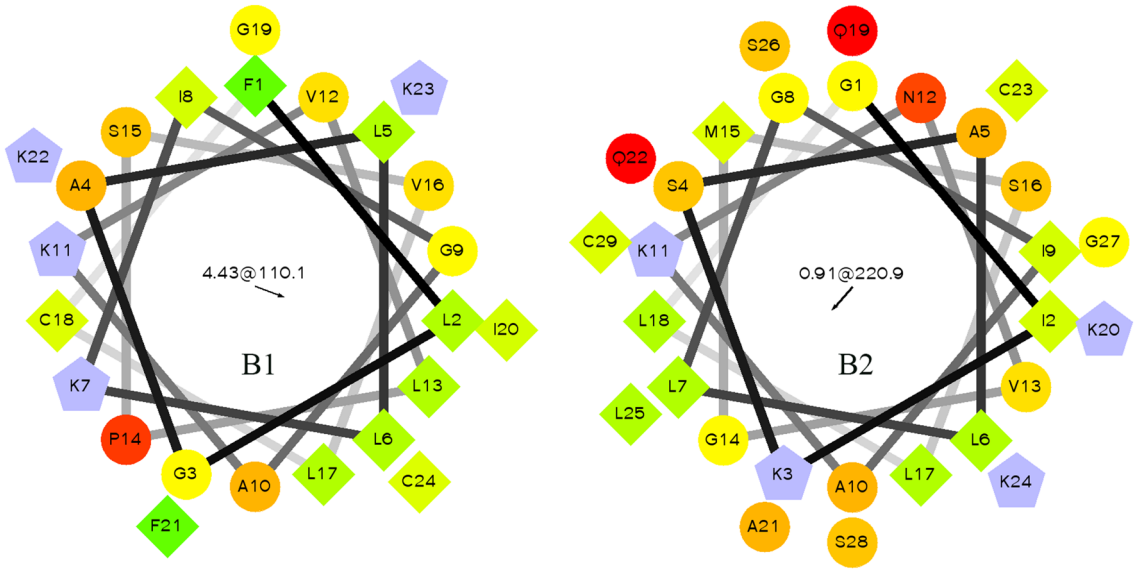

Fig. 1 Helical wheel projections of B1 and B2. Hydrophobic residues are depicted as green diamonds (with reduction in colour directly proportional to hydrophobicity), uncharged hydrophilic residues as red circles, (reduction in colour associated with reduction in hydrophilicity) and positively charged residues as pentagons with blue colour (colour figure online)

Table 2 MIC values of peptides against Gram-positive and Gram-negative bacteria

Bacteria	Peptide MIC (μM)				Antibiotics
Gram-negative bacteria	B1	B1-H_2	B2	B2-NH_2	K
E. coli ATCC25922	16 (> 100*)	7 (80*)	9 (86*)	8 (72*)	45
K. pneumonia (clinical)	20	15	13	8	60
V. cholerae N16961	12	4.5	15	10	20
Gram-positive bacteria	B1	B1-NH_2	B2	B2-NH_2	A
S. aureus ATCC 25923	5 (19*)	4 (15*)	12 (28*)	8 (22*)	10
MRSA (clinical)	6	4	20	16	NA

The data are drawn from three independent experiments

MIC (minimum inhibitory concentration)—lowest concentration of peptide, which showed no visible bacterial growth

K kanamycin sulphate, *A* ampicillin, *NA* not applicable

*MIC in 20% serum

Table 3 HC_{50}, GM and TI of the peptides

Peptides	HC_{50} (μM)	Gram-positive		Gram-negative	
		GM (μM)	TI	GM (μM)	TI
B1	16.620^{a}	5.477^{b}	3.034^{b}	15.659^{d}	1.061^{a}
B1-NH_2	16.630^{a}	4^{a}	4.157^{d}	7.789^{a}	2.221^{b}
B2	49.040^{c}	15.491^{d}	3.165^{c}	12.062^{c}	4.065^{d}
B2-NH_2	24.530^{b}	11.313^{c}	2.168^{a}	8.617^{b}	2.846^{c}

Values with same superscript in a column do not differ significantly

HC_{50} hemolytic concentration of peptide that induces 50% hemolysis on hRBC, *GM* geometric mean of the MIC values of peptides against Gram-positive and Gram-negative bacteria, *TI* therapeutic index (the ratio of HC_{50} to GM) of peptides against Gram-positive and Gram-negative bacteria

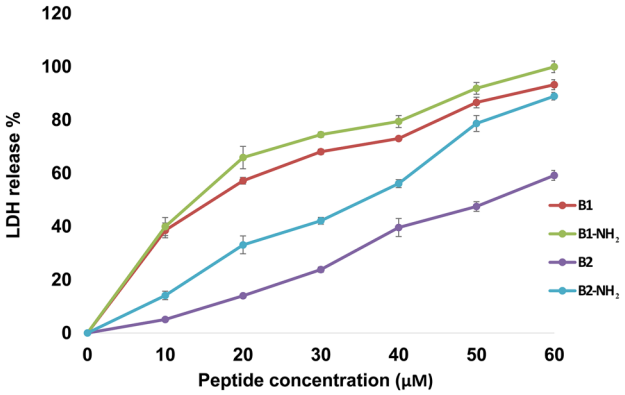

Fig. 2 LDH release of PBMC in the presence of peptides. Each data are representative of three independent tests, and the error bars indicate the standard deviations

segregation between domains and more number of hydrophobic residues than B2.

The antibacterial activities of parent peptides and their amidated derivatives against a set of Gram-positive and Gram-negative bacteria are shown in Table 2. Both the parent peptides showed considerable activity with MICs ranging from 5 to 20 μM across the bacterial strains tested. B1 showed maximum activity against *V. cholerae* (MIC 12 μm) and *S. aureus* (MIC 5 μm), while B2 was more active against *E. coli* (MIC 9 μm) and *S. aureus* (MIC 12 μm).We also observed that C-terminal amidation of B1 and B2 considerably augmented their anti-bacterial activity (4–16 μM) than parent ones. Geometric mean (GM) of MICs of each peptide against the Gram-positive and Gram-negative bacteria was calculated separately (Table 3),which showed significant variation across the peptides ($p < 0.01$).

Cytotoxicity assay

Lactate dehydrogenase (LDH) release assay

Percentage LDH release on treatment with B1, B1-NH_2, B2 and B2-NH_2 on PBMC showed a concentration-dependent release of LDH (Fig. 2). The peptide concentrations that caused 50% LDH release (LD_{50}) on human PBMCs were determined, which varied significantly among the peptides ($p < 0.01$). It is clear from the results that the highest cytotoxicity was observed for B1-NH_2 (LD_{50}-13.37 μM) followed by B1(LD_{50}-15.39), B2-NH_2, (LD_{50}-30.54 μM) and B2 (LD_{50}-52.71 μM), respectively.

Hemolytic assay

Hemolytic capacity of peptides against hRBC was estimated to assess the systemic toxicity of peptides against non-nucleated mammalian cells. Hemolytic concentration

of peptides that induce 50% hemolysis on hRBC (HC_{50}) is shown in Table 3. Hemolytic activities of all the peptides showed a direct relation with concentration and B2 showed lowest hemolytic activity (HC_{50}-49.04 µM) followed by B2-NH_2 (24.530 µM). B1 showed the highest hemolytic activity (HC_{50}-16.62 µM), which was on par with B1-NH2 (16.63 µM), but significantly differed from B2 and B2-NH_2 ($p < 0.01$). B2 and B2-NH_2 had not exhibited remarkable hemolytic activity in the range of its antibacterial concentrations (9–20 µM and 8–16 µM) against the tested bacteria.

Therapeutic index (TI)

TI values of all the peptides against both Gram-positive and Gram-negative bacteria are given in Table 3, which varied significantly among the peptides ($p < 0.01$). B2 showed the highest cell selectivity on the tested Gram-negative bacteria followed by B2-NH_2 due to its low hemolytic activity. B1-NH_2 showed the highest TI value against Gram-positive bacteria, due to its high bactericidal potential against Gram-positive bacteria, which compromises over its high hemolytic value.

Killing kinetics assay

Viability of *S. aureus* and *E. coli* on treatment with peptides was evaluated by killing kinetics assay and is illustrated in Fig. 3. Results of this assay revealed that both B1-NH_2 and B1 exhibited faster elimination of bacteria and 99.99% reduction in bacterial count (*E. coli*) took place within a short period (15 and 25 min, respectively) and B2-NH_2 and B2 showed 99.99% reduction within 35 and 50 min. Similarly killing kinetics assay against *S. aureus* revealed that B1 and B1-NH_2 showed 99.99% reduction of bacteria within 35 and 45 min and B2 and B2-NH_2 eliminated the bacteria within 65 and 85 min. From Fig. 3, it is also clear that the bactericidal action of the peptides started immediately after the addition of the peptides. On C-terminal amidation, both the peptide becomes capable of faster clearance of bacteria. Viability of bacteria at sub-MIC of the peptides was also evaluated to illustrate the growth pattern and it resembled that of negative control.

Biofilm inhibition

Studies were conducted to evaluate the biofilm inhibition capacity of B1 and B2 and their amidated analogs at sub-MICs (1/2 MIC, 1/4 MIC and 1/8 MIC) against *K. pneumoniae,* a strong biofilm-forming Gram-negative bacteria (Fig. 4). All the peptides showed a dose-dependent biofilm inhibition, which varied significantly among the peptides except for B1 and B1-NH_2 at 1/4 MIC and at 1/8 MIC ($p < 0.01$). B2-NH_2 showed a considerable biofilm inhibition compared to other peptides at all the concentrations tested. At 1/2 MIC B2-NH_2 showed a 6-fold reduction of biofilm

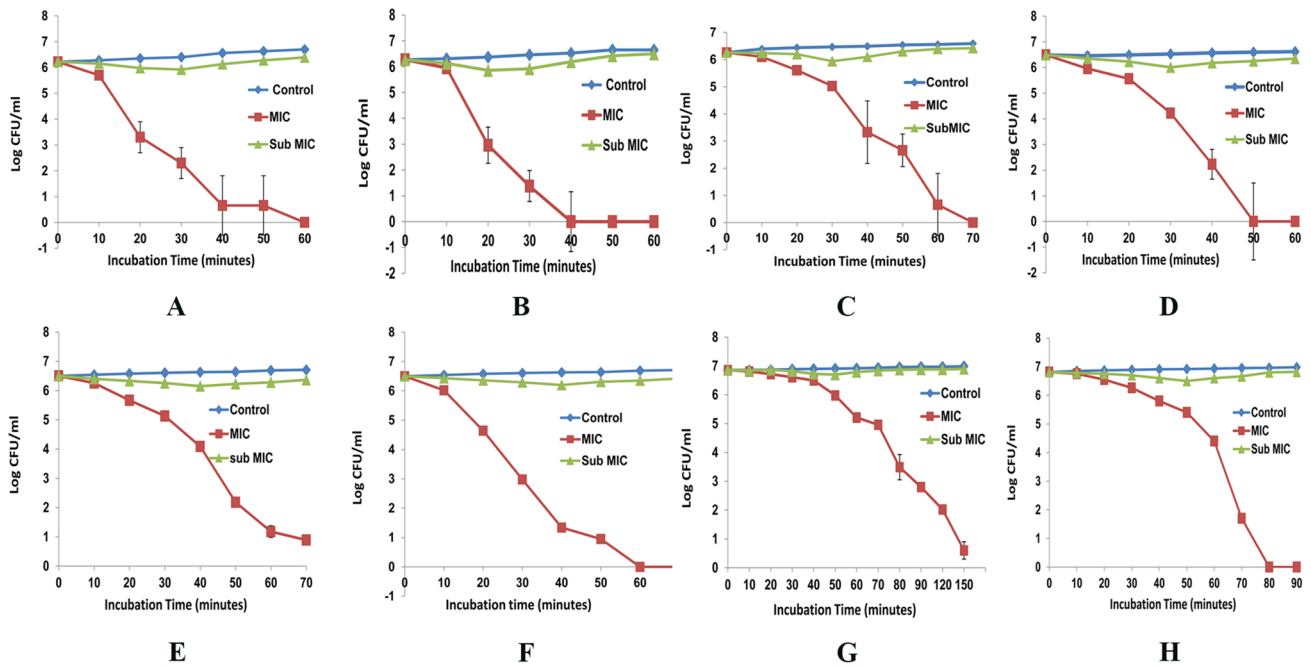

Fig. 3 Killing kinetics of B1, B1-NH_2, B2, and B2-NH_2 against *E. coli* (**a**–**d**) and *S. aureus* (**e**–**h**). Data are representative of three replications, and the error bars indicate the standard deviations

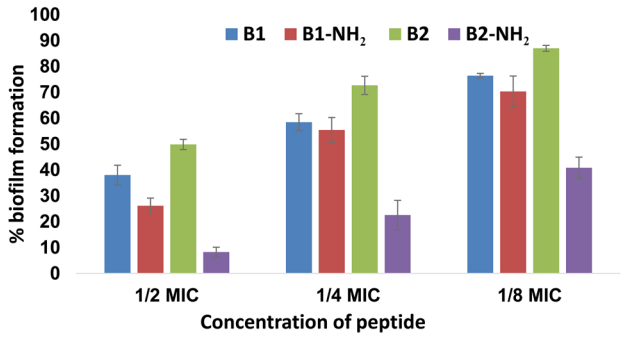

Fig. 4 Anti-biofilm activity of peptides against *K. pneumoniae*. Data are representative of three independent tests and the error bars indicate the standard deviations. All the treatments were statistically significant except B1 and B1-NH_2 at 1/4 MIC and at 1/8 MIC ($p < 0.01$)

formation than its parent peptide B2 followed by a 3.22-fold decrease (at 1/4 MIC) and 2.13-fold decrease at (1/8 MIC).

Discussion

The present study reports the identification of two novel peptides from *I. aurantiaca,* an endemic frog of the Western Ghats, India, and further characterization of their antibacterial and antibiofilm properties with and without C-terminal amidation. Shotgun cloning is used to derive the primary structure of the peptides from precursor genes thereby eliminating the need to kill the specimen, which favors conservation. B1 and B2 identified in this study are two novel peptides of brevinin 1 and brevinin 2 families, which are structurally similar to previously reported peptides of the same family with a conserved signal portion and acid spacer followed by a KR cleavage site. Mature peptide domain of B1 is moderately shorter than B2. Since structural modifications like amidation have been shown to increase the cationicity, cell permeabilization capacity and selectivity (Giangaspero et al. 2001; Kim et al. 2011; Kumar et al. 2017), the peptides used in this study were amidated at their C terminus and their activities were compared to the parent ones.

Antimicrobial properties of peptides are strongly correlated with the features such as hydrophobicity, amphipathicity and net positive charge (Giangaspero et al. 2001). Peptides with a charge more than +4 with higher hydrophobicity exhibit strong membrane disturbance (Zelezetsky and Tossi 2006) and can be powerful antimicrobial agents. Both the peptides used in our study showed +4 charge and elevation of charge by +1 on amidation. From structure prediction both the peptides showed helical domain. B1 showed more amphipathicity, while B2 is a larger peptide with comparatively less amphipathicity than B1. Helix stabilizing amino acids such as leucine, lysine and alanine (Conlon et al. 2007) are also present in both brevinin peptides particularly a leucine in the polar region. Hence, these cationic peptides (B1, B1-NH_2, B2, and B2-NH_2) can be predicted to be alpha helical anti-microbial peptides with strong hydrophobicity.

All bacterial strains tested in this study are highly susceptible to B1 and B2. B1 peptides are more potent against Gram-positive while B2 is specific to Gram-negative bacteria. The anti-bacterial potency of these peptides can be explained on the basis of their structure. The presence of proline in B1 is also thought to make a stable twist, which affects the linearity of the peptide. Though the effect of proline in peptides is confusing, many studies reported that the occurrence of proline augmented the biological activities and cell selectivity of peptides (Dempsey et al. 1991; Bobone et al. 2013). High hydrophobicity and stable helical structure with pronounced amphipathicity of B1 might have contributed to its selectivity towards Gram-positive bacteria. Since such a structural stringency is not a prerequisite against Gram-negative bacteria, the bactericidal action is well due to electrostatic interaction (Giangaspero et al. 2001).

C-terminal amidation imparts more cationicity (Shalev et al. 2002) and rigid structure to the peptides (Shahmiri et al. 2015) facilitating their perpendicular insertion into bacterial inner membrane and helping them to traverse the lipid bilayer (Kim et al. 2011).These can be the reasons for the reduction in time taken to eliminate bacterial cells. Faster rate of bacterial killing and permeabilization of B1-NH_2 can also be correlated with its smaller polar angles. These results emphasize the benefits of C-terminal amidation of the peptides to increase their activity.

Initial interaction of peptides on negatively charged bacterial membrane is electrostatic (Matsuzaki 1999), while it is hydrophobic on electrically neutral mammalian cell membrane (Lee and Hodges 2003). Thus, B2, which is less hydrophobic showed reduced cytotoxicity, when compared to B1. We also found that amidation also enhanced the cytotoxicity of the peptides by inducing strong hydrophobicity (Strandberg et al. 2007), which is in conformity with the previous report that C-terminal amidation enhances both antimicrobial and hemolytic activities (da Silva et al. 2014). Therapeutic index (TI) of each peptide against the bacteria was computed for testing the cell selectivity. The above results indicate that the peptides are more selective against bacteria rather than eukaryotic cells. Therefore, low cytotoxicity and strong anti-bacterial activity against Gram-negative bacteria confer a high therapeutic potential to B2 followed by B2-NH_2.

Bacteria within biofilm are more drug resistant than planktonic cells (de la Fuente-Núñez et al. 2013) as they are metabolically quiescent and produce protective polysaccharide matrix (Monroe 2007). In this study, we tried to evaluate the biofilm inhibition capacity of the peptides against a strong biofilm-forming clinical isolate of *K.*

pneumoniae, which is an opportunistic pathogen causing infections of urinary tract and respiratory tract as well as septicemia in immune compromised persons (Balaban et al. 2004). Our results clearly indicate that B2-NH_2 is a strong biofilm inhibitor at concentrations 4 μM or below (Fig. 4) with negligible cytotoxicity. We speculate that B2-NH_2 may regulate or modulate molecules or the receptors involved in quorum sensing pathways (Cady et al. 2012) leading to biofilm inhibition.

Conclusion

In summary, this work demonstrated antibacterial and biofilm inhibition properties of two novel brevinin peptides B1 and B2 identified from *I. aurantiaca* and the scope of optimization of activities on C-terminal amidation. The low cytotoxicity, strong antibacterial activity and augmentation of biofilm inhibition of C-terminal amidated B2 make it a promising peptide having therapeutic potential and a lead molecule to design a new drug. Bioprospection opens a new avenue for the utilization of novel structure of natural peptides for designing drug molecules and the scientific outcome of this work can also be used as templates for designing a new group of drugs with strong anti-biofilm activity with less cytotoxicity.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interests.

Ethical approval The protocols of the assays with human samples were approved by the human ethics committee of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala.

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