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Research Article

The design and functional characterization of a novel hybrid antimicrobial peptide from Esculentin-1a and melittin

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Abstract

Antimicrobial agents are one of the most widely used drugs in medicine. In the last fifty years, the misuse of these agents caused the emergence of resistant strains of bacteria that led to an increase in life-threatening infections. The need to develop new agents has become a priority, and antimicrobial peptides attained high consideration. The antimicrobial activities of a novel In-house designed hybrid cationic peptide (BKR1) were studied against different strains of Gram-negative bacteria. This was done using the broth dilution method as outlined by the Clinical and Laboratory Institute (CLSI). Checkerboard assy was employed to investigate the synergistic activity of BKR1 peptide with four antibiotics (Levofloxacin, chloramphenicol, rifampicin, and ampicillin). Finally, the cytotoxicity of BKR1 was evaluated against human blood cells and mammalian kidney cells (Vero cells). BKR1 displayed bactericidal activity against tested strains of Gram-negative bacteria, with zero hemolytic effects. It also acts as a strong adjuvant with levofloxacin, chloramphenicol, and rifampicin against resistant strains of *P. aeruginosa* and *E. coli*. This study represents the design and elucidation of the antimicrobial activities of a novel hybrid antimicrobial peptide named (BKR1). Our results indicate thar BKR1 is a promising candidate to treat resistant infectious diseases individually or as an adjuvant with conventional antibiotics.

Keywords

Antimicrobial resistance, hybrid peptide, antibiotic adjuvant, bactericidal activity, hemolytic activity

Introduction

Infectious diseases are one of the leading causes of mortality all around the world (Waldman et al. 2016). Although many antimicrobial agents have been developed in previous decades, the misuse of these treatments led to severe resistance among several microorganisms, which are now called multi-drug resistant bacteria (MDRB) (Morens and Fauci 2012). These microorganisms not only gained resistance with time but gained the ability transfer their acquired resistance to other susceptible bacteria (Levy 2005). The development of resistance among bacteria challenges the progress of modern antimicrobial medicine, and this problem is now considered one of the most urgent dilemmas faced by humanity (Alekshun et al. 2007). The risk of death from resistant strains is twice higher than non-resistant ones and this has caused a stressful impact on the medical and public community (Khabbaz et al. 2014).

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The combination antimicrobial therapy approach has gained interest to treat resistant bacterial infections, including antibiotic-antibiotic or antibiotic-adjuvant combinations (Worthington and Melander 2013). The antibiotic-antibiotic combinations for some Gram-negative infections were successfully reported in literature (Petrosillo et al. 2008). Multi-drug resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli* infections have all benefited from this type of combination therapies (Garnacho-Montero et al. 2007; Daikos et al. 2009; Samonis et al. 2012). On the other hand, the antibiotic-adjuvant pairing strategy did not receive significant attention as the first type. The rate of developing and discovering new drugs has not met the urgent clinical need to halt the emergence of microbial resistance (Lerminiaux and Cameron 2019). Therefore, the development of antibiotic adjuvants gained significant attention to circumvent the expiration of exiting antibiotics (Wright 2017). Adjuvants empower the activity of antibiotics by blocking resistance mechanisms (Melander and Melander 2017), such as the β-lactamase inhibitors (Bassetti et al. 2011), antibiotic-modifying enzymes (Labby and Garneau-Tsodikova 2013), and efflux pump inhibitors (Sun et al. 2014).

Cationic antimicrobial peptides (AMPs) are considered potential antimicrobial agents and/or adjuvants to antibiotics (Geitani et al. 2019). The combination of antibiotics with AMPs displayed synergistic or enhanced activities with different antimicrobial agents such as rifampicin, clindamycin, levofloxacin, amoxicillin, chloramphenicol, itraconazole, amphotericin, 5-fluorocytosine and others (Giacometti et al. 2000; Desbois et al. 2010; Zhang et al. 2014; Almaaytah et al. 2018). Natural AMPs have been found and isolated from almost all living organisms, including invertebrates, insects, and humans (Luna-Ramirez et al. 2017). They constitute a critical part of the immune system in most organisms and act as the first line of defense in many species (Erdem Büyükkiraz and Kesmen 2022).

AMPs are generally short, less than 50 amino acids in length (Gabere and Noble 2017). They exhibit a cationic nature and form amphipathic structures when in contact with cell membranes or membrane mimetics (Schmidt and Wong 2013). AMPs interact with bacterial membranes depending on their physiochemical properties such as helicity, length, and net charge (Ahmed and Hammami 2019). They create pores in cytoplasmic membrane, which causes the loss of the bacterial cell wall integrity and eventually causing cellular death (Pasupuleti et al. 2012).

In this study, we have designed a novel hybrid peptide based on the amino acid sequence of both Esculentin-1a and melittin. Esculentin-1a exhibits strong antimicrobial activity and was found in the skin of many amphibians as a type of an innate defense mechanism (Simmaco et al. 1993). All groups of Esculentins are composed of 46 amino acids (Simmaco et al. 1998); Esculentin-1a displayed a broad spectrum against bacteria and low toxicity against human erythrocytes. The first 18 amino acids control the antimicrobial activity feature of the peptide (Mangoni et al. 2008). On the other hand, melittin is an AMP that was isolated from the venom of the honeybee *Apis mellifera* (Condie and Quay 1983). Melittin is composed of hydrophilic end hydrophobic regions with a positive net charge (Tender et al. 2021). In this study, we have employed the peptide hybridization strategy as a tool for designing a novel antimicrobial peptide with enhanced antimicrobial activity and reduced toxicity.

The newly designed hybrid peptide named BKR1 carries a higher positive charge when compared with the parent peptides; we deduced that this modification would enhance BKR1's antimicrobial activity against bacterial strains (Jiang et al. 2008). Additionally, the antimicrobial activities of this modified peptide were studied alone and in combination with four conventional antibiotics (levofloxacin, chloramphenicol, rifampicin, and ampicillin) against proposed models of planktonic Gram-negative bacteria. The previously named antibiotics were chosen due to their different mechanisms of action and good toxicity profile.

Materials and methods

Materials, chemicals and bacterial strains

All strains used in this study were obtained from American Type Tissue Culture Collection (ATCC; Manassas, VA, USA). The four strains used in this study are *Pseudomonas aeruginosa* (ATCC 27853 and BAA-2114), *Escherichia coli* (ATCC 25992 and BAA-2452). All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

Peptide design, molecular modelling and *in silico* analysis

Rational design of the new hybrid peptide was performed using online bioinformatics software to achieve the optimal range in physiochemical properties. The Hierarchical Neural Network software (HNN) from the Network Protein Sequence Analysis (NPS) server was used to predict the secondary structure and calculate the helicity percentages of the parent peptides and the hybrid peptide. The physicochemical properties were predicted using ProtParam/ExPASy server (Wilkins et al. 1999) and the Antimicrobial Peptide Database (APD3) (Wang and Wang 2016). The properties include the molecular weight and the number of amino acids, the instability index, the aliphatic index, and finally, the hydrophobic ratio. Moreover, the HHpred (Zimmermann et al. 2018) and the MODELLER software (Webb and Sali 2016) were used for homology modeling. The validation method, the RAMPAGE (Lovell et al. 2003) was used to assess the Ramachandran Plot, and the I-TASSER (Zhang 2008) was used to confirm the helical structure and the quality of the model.

Peptide design, synthesis, and purification

BKR1, a hybrid peptide of 21 amino acids (NH_2-VSK-LAKKIKNLKNVKKSWKRQ-COOH) was synthesized following standard Fmoc solid-phase protocols on Wang resin. Peptide elongation was performed using standard HBTU coupling chemistry in dimethylformamide (DMF) solvent with a fourfold molar excess of diisopropyl ethylamine (DIEA) in *N*-methyl-2-pyrrolidone (NMP) and a threefold molar excess of each Fmoc-protected amino acid or 2-(6-methoxynaphthalen-2-yl) propanoic acid. BKR! was cleaved from the resin, using 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane, and 2.5% thioanisole (3 h, room temperature), and precipitated using cold (−20 °C) diethyl ether. Reverse phase high-performance liquid chromatography (RP-HPLC) was used for purification of BKR1 using a C18 internsil ODS-SP column, the column was eluted with acetonitrile / H_2O-TFA gradient at flow rate of 1.0 ml/minute. The identification of BKR! was confirmed by mass analysis and through the employment of electrospray ionization mass spectrometry (ESI-MS).

Bacterial susceptibility assay

Antimicrobial activities of the peptide alone, the antibiotics alone and in combination were tested using the broth dilution method as described by the Clinical and Laboratory Standards Institute guidelines and as performed previously (Almaaytah et al. 2014).

Briefly, four bacterial strains including a control and resistant clinical isolate of *P. aeruginosa* (ATCC 27853 and ATCC BAA-2114) in addition to a control and resistant clinical isolate of *E. coli* (ATCC 25922 and ATCC BAA-2452) were grown in Muller Hinton broth (MHB) medium. The initial concentration of the bacterial culture (1.5×10^8 CFU/mL) were adjusted using 0.5 McFarland turbidity standard, and spectrophotometry at $\lambda = 600$ nm. The used concentration was diluted 100-fold to reach a value of 10^6 CFU/mL. The bacterial suspension of the diluted concentration was distributed over 96-well plates. Each plate had a range different concentration of one of the four tested antibiotics (LVX, CHL, RIF, and AMP) that were determined according to previous analysis of the MIC values of each antibiotic against the target strain ranging from (1–100 µM), or different concentrations of the hybrid peptide. The growth control consisted of bacterial suspension without any concentration of an antimicrobial agent, while the negative control consisted of MHB broth alone.

The plates were incubated overnight, and the bacterial growth was evaluated by measuring the absorbance at $\lambda = 600$ nm using an Enzyme-Linked Immunosorbent Assay (ELISA) microplate reader (Epoch™; BioTek, Winooski, VT, USA). The minimum inhibitory concentration (MIC) values of the antimicrobial agent (antibiotics or hybrid peptide) were assessed by the bacterial growth at this stage since the MIC value is defined as The minimum inhibitory concentration (MIC) is the lowest antimicrobial concentration at which no visible microbial growth can be detected with the naked eye or where there is no difference in absorbance between the negative control and the test concentration (Omran et al. 2018).

Additonally, the minimum bactericidal concentration (MBC) which is defined as "the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic free media" (Mohseni et al. 2014), was determined by taking samples of 10 µL from turbidity-free wells, and spreading them over agar plates, followed by incubation for 24–48 h. Colonies were then counted and compared with growth controls.

The MIC and the MBC values for the four antibiotics and the hybrid peptide were tested alone and in combination against different bacterial strains as mentioned above. All experiments were made in triplicates. The checkerboard assay was adopted to check the synergistic activities between the hybrid peptide and the antibiotics.

Synergistic checkerboard assay

The checkerboard assay employs different concentrations of antimicrobial agents to check their synergistic activities against a specific bacterial strain. Herein, samples of 25 µL of eight different concentrations of BKR1 peptide were distributed horizontally on 96-well plates, and each column had the same volume and the same concentration of the peptide. On the other hand, 25 µL of six different concentrations of a single antibiotic was added to different rows. Each row had the same concentration of the antibiotic. Meanwhile, two columns were reserved for testing the growth and negative controls. On each well, a fresh aliquot of 50 µL of bacterial suspension (10^6 CFU/mL) was added. The plates were then incubated overnight (18 h), at 37 °C, and the MIC values were then measured by reading the absorbance at $\lambda = 600$ nm throygh an ELISA plate reader (Epoch™; BioTek, Winooski, VT, USA).

The act of synergism was measured by calculating the Fractional Inhibitory Concentration Index (FICI) according to the following equation:

$$\text{FICI} = \frac{\text{MIC of BKR1 in combination}}{\text{MIC of BKR1 alone}} + \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

The results were then interpreted as synergistic if FIC value was £ 0.5, additive if FIC was in the range between 0.5–1, and indifferent if FIC was higher than 1 and lower than 4 (Sheikholeslami et al. 2016).

Hemolytic assay

2 mL of human blood (Sigma Aldrich, St. Louis, MO, USA) was added to 48 mL of sterile phosphate buffer saline (PBS) at pH 7.4. The suspension was centrifuged three times at 2000 rpm for 5 min. Each time the supernatant was discarded and replaced by a fresh PBS buffer. The final concentration of human blood (RBCs) suspension was 4%. Eight tubes were then prepared, in which six of them

contained 2 mL RBCs suspension and 2 mL of 6 different peptide concentration, one of them was for the positive control (2 mL of RBCs suspension mixed with 5μL of 0.1% Triton X-100), and the final tube represents the negative control (2 mL of RBCs suspension). Subsequently, all tubes were incubated at 37 °C for 60 min, then centrifuged at 2000 rpm for 5 min. After that, 1 mL was taken from each tube, and their absorbance was checked using (ELISA) microplate reader (Epoch^{TM}; BioTek, Winooski, VT, USA) at λ = 450 nm.

Cell culture

The cell lines used in the present study were Vero cells (ATCC CCL-81). The Vero lineage was isolated from kidney epithelial cells extracted from an African green monkey. The cells were grown in an RPMI media that consist of 10% fetal bovine serum and 1% v/v antibiotics (ampicillin, streptomycin), and antifungal agent (Amphotericin B), which were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Cells were seeded in 75 cm^2 flasks with 24 mL of prepared media, incubated in 5% CO_2 incubator at 37 °C. The media was changed every 24 h until the confluence reached 70%. The media was then discarded, leaving the adherent cells in the flask. A volume of 5–7 mL of trypsin 1X was added, and the flask was then returned to the CO_2 incubator for 5–10 min. The trypsin was removed, and the detachment of the cells was confirmed through the use of an an inverted microscope. The trypsinization process was repeated until sufficient cell detachment. Finally, 5 mL media was added to neutralize the low pH of trypsin, followed by centrifugation at 2500 rpm for 5 min. The supernatant was discarded and replaced with 10 mL media and mixed by a vortex.

Cells were mixed with an equal amount of trypan blue (4%). The cells that were stained by trypan blue stain were considered dying cells, whereas live cells looked like stars. The cells were then counted using hemocytometer.

MTT cell proliferation assay

This assay was performed by seeding the cells at 5×10^5 cells/well in a 96-well plate flat bottom. The plates were incubated in a CO_2 incubator at 37 °C for 18 h, to reattached cells to the bottom. The media was then discarded from the plates, and six different concentrations of peptide were dissolved in prepared RPMI media. The plates were incubated again in a CO_2 incubator at 37 °C for 18 -24 hours. A volume of 25 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) suspension (4 mg/mL) was added, and the plates were further incubated for 4–6 hours. The MTT-Peptide solution was then removed, and 100 μL of DMSO was placed in each well followed by continuous pipetting to dissolve the formed Formazan crystals. The absorbance was then measured using ELISA microplate (Epoch^{TM}; BioTek, Winooski, VT, USA) at λ = 540 nm (Almaaytah et al. 2019).

Results

Peptide design, synthesis, molecular modelling and *In silico* analysis

The sequence of the peptide was designed by fragmenting the helical parts of the parent peptides; Esculentin-1a and Melitten with some modifications as shown in Table 1; the bold underlined letters represent the parts which were taken from the parents, and valine was added at the beginning of the sequence to increase the half-life of the peptide as suggested by ProtParam/ExPasy. The design of BKR1 peptide was adopted after obtaining the highest helicity percentage and acquiring the most optimum *in silico* physicochemical properties as suggested by ProtParam/ExPASy server and the Antimicrobial Peptide Database (APD3). BKR1 is a 21 amino acid hybrid peptide with 85.71% helicity (Fig. 1), which is higher than both parents as shown in the table below.

The physicochemical properties of BKR1 mentioned above were tested by ProtParam/ExPASy server and the Antimicrobial Peptide Database (APD3). The peptide is expected to display a stable form in the test tube as suggested by the instability index (>40), and the aliphatic index which provides an indication of the peptide's thermostability. The peptide is considered hydrophilic (33% hydrophobic ratio) with high protein interaction ability (Boman index 2.81 kcal/mol). The charge of BKR1 peptide is +9 compared with +5 and +6 for Esculentin-1a and Melitten, respectively (Table 2). Finally, the I-TASSER results confirmed the helicity of BKR1 peptide with C-score -0.05, TM score 0.71 ± 0.12, and RMSD 1.2 ± 1.2 Å.

Table 1. Prediction of secondary structure for the parent peptides and the hybrid peptide (BKR1) using Hierarchical Neural Network software (HNN).

Name of Peptide	Sequence	# of a.a[1]	α- helix %	b- sheet %	Random coils %
Esculentin-1a	GIF**SKLA**G**KKIKNL**LISGL**KNVGK**EVGMDVVRTGIDIAGCKIKGEC	46	45.00	19.00	34.78
		26	42.31	26.92	30.77
		21	85.71	0.00	14.29
Melitten	GIGAVLKVLTTGLPALI**SWIKR**KRQQ				
BKR1	VSKLAKKIKNLKNVKKSWKRQ				

[1] # of a.a.; the number of amino acids.

Table 2. The physicochemical properties of the parent peptides and BKR1 peptide calculated by ProtParam from ExPASY and APD3.

Peptide	MWT	*pI*	Instability index	Aliphatic index	Hydrophobic ratio	Total net charge	Boman index (kcal/mol)
Esculentin-1a	4802.82	9.63	-8.14	114.35	43%	5	0.62
Melitten	2847.49	12.02	44.73	135.00	46%	6	0.57
BKR1	2525.31	11.51	16.68	88.10	33%	9	2.81

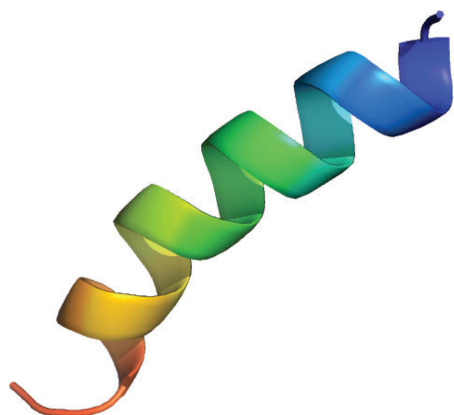

Figure 1. Three-dimensional structure of BKR1 generated by homology modelling using MODELLER and the figure was prepared using PyMol.

BKR1 displays a net positive charge of +3 and a molecular weight of 1144.34 Da. BKR1 was purified to >98% purity using RP-HPLC (Suppl. material 1) and its identity was confirmed by electrospray ionization mass spectrometry (ESI-MS) with the synthetic peptide displaying major peaks in the +3, +4 and +5 charge state of 842.6, 632.2 and 506.0 Daltons (Fig. 2).

Bacterial susceptibility assay

The minimal inhibitory concentrations (MIC) and the minimal bactericidal concentrations (MBC) for BKR1 displayed high antimicrobial activity against different Gram-negative bacteria. The average MIC value for all strains in this study was in the range of 25–30 µM, against the control strains and the resistant strains of the bacterial strains employed in the study (Table 3). For example, the MIC values were 25 µM for *P. aeruginosa* ATCC 27853 (control strain) and ATCC BAA-2114 (resistant strain), and the MIC values were also the same against *E. coli* ATCC 25992 (control strain) and BAA-2452 (resistant strain). On the other hand, the MBC values were equal to the MIC values in all experiments, which indicates the bactericidal behavior of the hybrid peptide.

Table 3. The minimum inhibitory concentrations (MIC) against different Gram-negative bacterial strains.

Bacteria strain	ATCC[1]	MIC (mM[2])
Pseudomonas aeruginosa	27853	25
Pseudomonas aeruginosa	BAA-2114	25
Escherichia coli	25992	25
Escherichia coli	BAA-2452	25

[1]ATCC: American Type Tissue Culture Collection.
[2]mM: Micromolar.

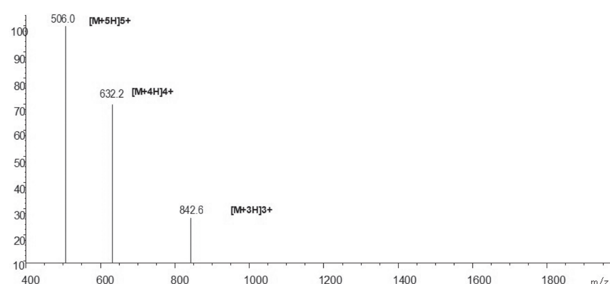

Figure 2. Positive electrospray ionization mass spectrometric (ESI-MS) analysis of the BKR1. The peptide shows major peaks in the +2, +3 and +5 state of 842.6, 532.2 and 506.0 Daltons.

Synergistic checkerboard assay

BKR1 peptide displayed significant antimicrobial activity against different strains of Gram-negative bacteria as shown in the previous section. However, we tested its potential of BKR1 to act as an antimicrobial adjuvant with the four suggested antibiotics (levofloxacin, LVX; chloramphenicol, CHL; ampicillin, AMP; and rifampicin, RIF) against different strains of *P. aeruginosa* (control strain ATCC 27853 and a resistant strain ATCC BAA-2114) in addition to *E. coli* (control strain ATCC 25922 and resistant strain ATCC BAA-2452). The act of synergism was checked using Checkerboard assay, and the results are summarized in Table 4.

To calculate the percentage reduction in MIC, the following equation was used:

$$\text{Percentage of MIC reduction} = \frac{\text{MIC alone} - \text{MIC combination}}{\text{MIC alone}} \times 100\%$$

Interestingly, the combination of LVX/BKR1 displayed additive activity against control strains of both *P. aeruginosa* and *E. coli* but expressed synergistic activities against the resistant strains. The synergism of this combination against the tested strains caused a huge reduction in the MIC values of the combined agents (LVX: BKR1 = > 75%: > 90%).

CHL/BKR1 combination, on the other hand, displayed synergism with all tested strains with ≥ 80% reduction in the CHL MIC values and ≥ 85% in the MIC values of BKR1 (except against the control strain of *E. coli*, which showed additive effect). Whereas the combination of RIF/BKR1 always displayed strong synergism with more than 70% reduction in the MIC of both antibiotics, and more than 90% reduction in the MIC of BKR1 in all cases. AMP/BKR1 combination showed no synergism, but the outputs showed that their combinations decreased the MIC values for both agents in all cases as shown in Table 4. The strongest synergism was found with CHL against the resistant strain of *P. aeruginosa* with a reduction percentage of 88% and 95% for CHL and BKR, respectively.

Table 4. Summation of Fractional inhibitory concentration indices (FICI) for the synergistic Checkerboard Assay. LVX, levofloxacin; CHL, chloramphenicol; AMP, ampicillin; RIF, rifampicin; C.S., control strain; R.S. resistant strain; The percentage reduction in MIC was calculated using equation (1).

Bacterial strain		Antibiotic			Peptide (BKR1)			FICI /Synergism	
		MIC alone (mM)	MIC in combination (mM)	Percentage reduction in MIC	MIC alone (mM)	MIC in combination (mM)	Percentage reduction in MIC		
P. aeruginosa	LVX	0.25	0.083	67	25	6.25	75	0.58	Additive
C.S. (27853)	CHL	25	5.25	79	25	3.75	85	0.36	Synergistic
	AMP	12.5	7.75	38	25	10	60	1.02	Indifferent
	RIF	15	2.5	83	25	2.5	90	0.27	Synergistic
P. aeruginosa	LVX	12	3	75	25	1.25	95	0.30	Synergistic
R.S. (BAA-2114)	CHL	200	25	88	25	1.25	95	0.18	Synergistic
	AMP	>500	187.5	>63	25	3.75	85	>0.53	Additive
	RIF	50	5	90	25	2.5	90	0.20	Synergistic
E.coli	LVX	0.25	0.188	25	25	2.5	90	0.85	Additive
C.S. (25922)	CHL	10	5	50	25	1.25	95	0.55	Additive
	AMP	12.5	10	20	25	10	60	1.20	Indifferent
	RIF	8	2.5	69	25	1.25	95	0.36	Synergistic
E.coli	LVX	25	3.75	85	25	2.5	90	0.25	Synergistic
R.S. (BAA-2452)	CHL	25	5	80	25	2.5	90	0.30	Synergistic
	AMP	>500	200	>60	25	6.25	75	>0.65	–
	RIF	5	1.25	75	25	1.25	95	0.30	Synergistic

Hemolytic assay

The toxicity of antimicrobial peptides against human red blood cells has been a significant obstacle against their development into clinically useful drugs (Yeung et al. 2011). Therefore, we tested different concentrations of BKR1 against human RBCs to evaluate its toxicity against mammalian cells. The concentrations used were 200, 160, 120, 80, 40, 20, and 10 μM), which were incubated with 4% human erythrocytes suspension. BKR1 peptide showed no hemolysis activity at its MIC values. Doubling the minimum inhibitory concentrations did not cause significant hemolysis against RBCs as the hemolysis did not exceed 8% as shown in Fig. 3.

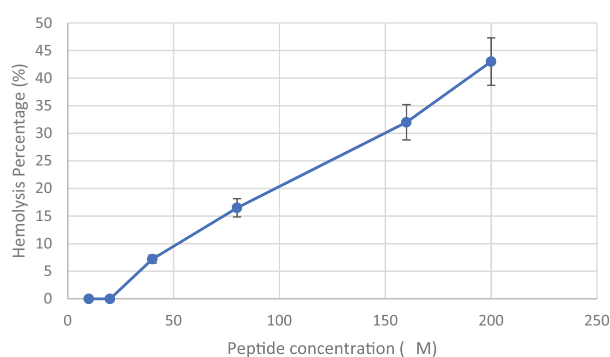

Figure 3. The hemolysis effect of different concentration of BKR1. The data are representative of three independent experiments.

Mammalian cell cytotoxicity assay

The MTT assay evaluated the toxicity profile of BKR1 peptide against Vero cell line. The selectivity of the new antimicrobial agent towards bacteria is a major issue when designing novel antimicrobial agents. Therefore, different concentrations of the hybrid peptide were tested, and the outputs are represented in Fig. 4. The IC_{50} of BKR1 was 43.1 μM, and unfortunately, the MIC values of the peptide when used alone put 40% of the viable cells at risk of damage. Therefore, this peptide can be used safely in combination with other antibiotics rather than being used indivdiually.

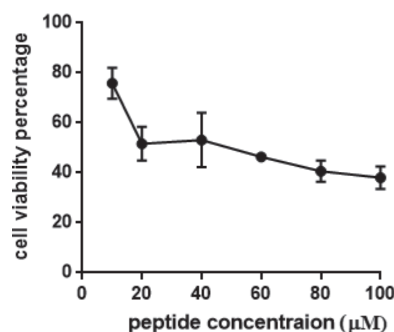

Figure 4. The MTT analysis of BKR1 peptide alone against Vero cell line at different concentration range. Error bars represent the standard deviation (±SD). Values represent the means of six different experiments.

Discussion

One of the major issues facing public health during last decade is the sustained emergence of bacterial resistance (Jasovský et al. 2016). Historically, the number of antibiotics families was big enough to treat different infectious diseases, and the diversity in the mechanisms of action of known antibiotics allowed the medical sector to comfortably deal with such diseases. The drug discovery process was faster and cheaper than nowadays, and the pharmaceutical sector was heavily interested in the discovery and development of new antimicrobial agents.

During the 21st century, several reports of the emergence of different multi-drug resistant bacteria (MDRB) were reported all around the world (Arias and Murray 2009). It is expected that MDRB will cause the death of 10 million patients annually by 2050 unless new strategies are mounted to counteract the threat of microbial resistance (Morrison and Zembower 2020). Bacterial resistance is now represented as the “silent tsunami” that faces modern

medicine, and therefore, there is an urgent need to develop new antimicrobial agents (Lata et al. 2007).

Antimicrobial peptides (AMPs) represents promising potential candidates for treating microbial infections (Zasloff 2002). AMPs display a unique mode of action; they initiate electrostatic interaction with the cell membrane and induce perforation which can destruct the plasma membrane (Wimley 2010). AMPs show a broad spectrum of activity against microorganisms, including viruses, bacteria (Gram-positive, and Gram-negative), and fungi (Seo et al. 2012; Al Tall et al. 2019).

In this work, we report the design and antimicrobial activity of a novel hybrid peptide (BKR1) based on two natural AMPs; Esculentin-1a and melittin. These peptides were chosen because they were cationic and displayed significant antimicrobial activity against Gram-negative bacteria (Pfalzgraff et al. 2018). The selectivity of cationic peptides toward bacterial cells is fundamentally derived from the electrostatic attraction of these agents towards the anionic bacterial membranes (Pouny et al. 1992). The anionic phospholipids and the lipopolysaccharides in the Gram-negative bacterial membranes attract positively charged peptides. Therefore, herein we increased the positive net charge of the hybrid peptide compared with the parent peptides. We also increased the helicity of the peptide and chose the best physiochemical properties *in silico* to create a new antimicrobial agent with enhanced physicochemical properties.

The design strategy employed first melittin for the process of hybridization as this peptide is an attractive antimicrobial agent of 26 residues (Leveritt et al. 2015). It contains five cationic residues in which four of them are located in the C-terminal part of the peptide. These last six amino acids are reported to be responsible for the antibacterial and the hemolytic activities of the peptide (Asthana et al. 2004). It is also well known that this peptide has low selectivity toward bacterial cells (Ostroumova et al. 2015). Esculentins, on the other hand, are isolated from *Rana esculenta*; a European frog. They are cationic peptides with the tendency to adopt helicity in a lipophilic environment, and this tendency is believed to be responsible for interaction with bacterial membrane causing their cell death (Conlon et al. 2007). The first 18 amino acids from the N-terminal region is known to have anti-pseudomonal activity and exhibit higher potency compared with human cathelicidin (LL-37) (Di Grazia et al. 2015).

The novel hybrid peptide, BKR1 is a 21 amino acids peptide that was designed by joining truncated fragments from the N-terminal region of esculentin-1a and a modified part of the C-terminal of melittin. The MIC of BKR1 peptide was 25 μM against all tested strains with this study. The potency of the peptide against tested bacteria was the same regardless of the resistance degree. The MIC values of the parent peptides were reported in the literature; the MIC of melittin alone was recorded higher than 22.5 μM and 5.6 μM against *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 25922), respectively (Dosler et al. 2016)

Melittin causes 100% lysis of erythrocytes at only 10 μM (Oren and Shai 1997). On the other hand, the MIC for the first 21 amino acid of Esculentin-1a against *P. aeruginosa* was reported to be 16 μM, yet it showed cytotoxicity at a concentration close to its MIC value (Casciaro et al. 2017). Meanwhile, BKR1 displayed bactericidal behavior against planktonic Gram-negative bacteria, without any differences between the wild type and the resistance strains and showed no hemolytic effects at effective inhibitory concentrations which indicates that the design strategy was successful in generating a novel peptide with an enhanced selectivity index compared with its parent peptides. BKR1 peptide also potentiates the activity of four conventional antibiotics with different mechanisms of action: cell wall inhibitor (AMP), topoisomerase inhibitor (LEV), RNA polymerase inhibitor (RIF), and protein synthesis inhibitor (CHL). Most combinations displayed synergetic effects. The best synergistic combinations were with CHL followed by RIF and LVX against the resistant strain *P. aeruginosa*, knowing that this strain is resistant to these antibiotics mainly due to efflux pumps (Fetar et al. 2011). Synergism was also seen with these antibiotics against the resistant strain of *E. coli* which also shares the same mechanism of resistance with *P. aeruginosa* (Ghisalberti et al. 2005). We assume that BKR1 disrupts the integrity of the bacterial membrane, jeopardizing the permeability barrier, and that gives our investigated antibiotics more accessibility to their target sites, however, the exact mechanism of action has yet to be studied in future studies. In conclusion, these combinations could provide potential candidates for battling infections caused by the tested bacteria.

Conclusion

In conclusion, we report the design of a novel hybrid peptide named BKR1 based on the amino acid sequence of Esculentin-1a and melittin. The hybrid peptide displayed strong antimicrobial activities against planktonic Gram-negative bacterial strains including resistant clinical isolates. BKR1 displayed bactericidal activity and showed similar potency against bacteria regardless of its resistance degree. The hemolytic activity of BKR1 at its antimicrobial concentrations were negligible however, it still risks the viability of the mammalian cells as indicated in the cell proliferation assays. The synergism studies were performed to decrease the MIC values of the peptide to make it safer to use in mammalian cells, and to decrease any possibilities for future resistance against this peptide. The synergism was the strongest with chloramphenicol and rifampicin against *P. aeruginosa* resistant strain, even though these antibiotics are not effective when used alone. On the other hand, the synergism with levofloxacin was the strongest against the *E. coli* resistant strain, regardless of its resistance against this antibiotic when used alone.

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Supplementary material 1

RP-HPLC analysis Chromatogram of BKR1 peptide

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