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HAZ, a novel peptide with broad-spectrum antibacterial activity

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ABSTRACT

Objective: The growing microbial resistance to antibiotics is a global public concern, which creates serious needs for newer antimicrobial agents. Antimicrobial peptides (AMPs) are increasingly exploited in drug development as therapeutic candidates. Here, we aimed to design and characterize a novel peptide with broad spectrum antimicrobial activity.

Methods: Hybridization and sequence modification approaches were used to design the novel peptide, named HAZ, aiming at optimizing the physicochemical parameters involved in antimicrobial activity. Peptide activities were assessed alone or combined with different selected antibiotics against various sensitive and drug-resistant bacterial strains. In addition, the hemolysis and the cytotoxic activities of HAZ peptide were evaluated on human red blood cells and epithelial adenocarcinoma cells (A549), respectively.

Results: HAZ peptide was sequentially modified to result in favored physicochemical parameters (helicity 95.24 %, hydrophobic ratio 47 %, and net charge of 8 +). Functional assessment of HAZ revealed significant antimicrobial activity, with MIC values of 15 – 20 μM against tested bacterial strains. It also exhibited biofilm eradication activity at slightly higher concentrations. HAZ-antibiotics combinations exhibited a synergistic action mode that led to dramatic decrease in the MIC values for both HAZ peptide and the antibiotic. Such efficacy was accompanied with minimal hemolytic toxicity on human erythrocytes. Importantly, HAZ displayed promising anticancer activity against human lung cancer cells.

Conclusion: Rationally-designed antimicrobial peptides offer promising alternatives to the current antibiotics for management of infectious diseases. HAZ peptide is a broad-spectrum AMP, and a promising candidate for antimicrobial and anticancer drug development.

1. Introduction

Rates of bacterial resistance to existing antibiotics are escalating globally, threatening already-challenged health departments and hospitals, and placing extra pressure on pharmaceutical industry and health administrators to manage the consequences (Shah 2013). Antimicrobial resistance is attributed to the wide misuse of prescribed antibiotics, resulting in many species of pathogenic bacteria developing various forms of resistance machineries against wide numbers of antibiotics (Littmann and Viens 2015). Therefore, current research is directed toward exploring alternative approaches for management of antibiotic resistance, and to come up with new antimicrobial agents of improved safety and efficacy profiles.

Antimicrobial peptides (AMPs) are widely expressed among different species, such as animals, plants and bacterial species (Ageitos, Sánchez-Pérez et al. 2017). It has been that AMPs exert critical functions in hosts immunity as key players in defense mechanism against broad array of infectious pathogens (Schmidtchen, Pasupuleti et al. 2014). Typically, AMPs are short peptides of 10–40 residues (Mahlapuu, Håkansson et al. 2016), and characterized by moderate hydrophobicity, helicity and overall cationic net charge that account for their antimicrobial activity (Uggerhøj, Poulsen et al. 2015). AMPs can be categorized depending on their secondary structure into four subclasses: α-helical, flexible, β-sheet, and loop peptides (Wang, Shih et al. 2017). A

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majority of AMPs are amphipathic α-helices, including Maculatin, Citropin, and Mellitin (Kumar, Kizhakkedathu et al. 2018). AMPs exerts antibacterial activities through various mechanisms; such as destabilization of bacterial membranes, inhibition of essential cellular processes like cell wall, nucleic acids and protein synthesis, and various bio-distributive enzymatic activities (Moretta, Scieuzo et al. 2021). These effects, along with the potential cytotoxicity are largely influenced by peptide length, sequence, charge, hydrophobicity, amphipathicity and conformation or helicity.

AMPs are increasingly investigated as potential candidates for antimicrobial drug development because of their broad spectrum of activity, rapid killing kinetics and the unlikely predisposition for resistance development (Cruz, Rondon-Villarreal et al. 2018). Additionally, AMPs are being combined with existing antibiotics, showing synergistic effects, as well as reduced adverse events (Mohammad Alsaggar et al., 2021). Mounting research has shown accordingly that AMPs enhance the permeability and sensitivity of bacterial strains to antibiotics in combination by influencing the integrity of the bacterial membrane (Guilhelmelli, Vilela et al. 2013, Zhang, Liu et al. 2014). Therefore, current research is directed to design novel AMPs with promising potential for treating infections caused by pathogens resistant to conventional antibiotics. In this direction, several approaches have been utilized to design novel peptides, such as hybridization and/or sequence modification of peptide sequences of naturally existing peptides to overcome obstacles challenging fruitful applications of these peptides in drug development and clinical practice, particularly narrow spectrum of activity and hemolytic toxicity (Fjell, Hiss et al. 2012).

In this research, we relied on sequences of naturally-existing peptides, palustrin-1d and HP(2–20) to design a novel peptide, named HAZ peptide. Palustrin-1d is a natural AMP obtained from skin excretions of the North American pickerel frog *Rana palustris*. It is classified as narrow spectrum peptide because it exhibits activity against gram – negative bacteria only (Basir, Knoop et al. 2000). HP (2 – 20) is the anti-infectious bio-active region in the *N*-terminus of Helicobacter pylori ribosomal protein L1 (RPL1) with alpha helical conformation (Pütsep, Brändén et al. 1999). Our peptide HAZ displayed promising antimicrobial and antibiofilm effects on a wide range of Gram-positive and Gram-negative bacteria, and exhibited little toxicity toward red blood cells. Additionally, HAZ peptide displayed potential anticancer activity against human adenocarcinoma cell line.

2. Materials and methods

2.1. Bacterial strains and cell lines

Bacterial strains were obtained from the American type tissue culture collection (ATCC), including: Two Gram-positive bacteria, Methicillin-susceptible *Staphylococcus aureus* (ATCC 29215), and the methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41). There are also two Gram-negative strains (ATCC 25922) of susceptible *Escherichia coli*, in addition to the drug-resistant strain (ATCC BAA 2452). ATCC CCL-185 also provided human adenocarcinoma alveolar basal epithelial cells (A549).

2.2. Antibiotics

The following antibiotics were purchased from Sigma-Aldrich, China: Levofloxacin, chloramphenicol, and doxycycline, whereas ampicillin was purchased from Sigma-Aldrich, Canada. The Rifampicin was obtained from Biobasic, Canada. The stock solutions were formulated and stored according to the manufacturer's instructions.

2.3. Synthesis and purification of HAZ

HAZ peptide (NH2-GVKFAKRFWRFAKKAFKR-FEK-COOH) was supplied by GL Biochem (Shanghai, China). Synthesis of peptide was carried out using solid-phase methods and Fmoc chemistry, followed by reverse phase high-performance liquid chromatography to purify the peptide by 95.5 %. Using electrospray ionization mass spectrometry, the peptide's identification was determined.

2.4. Bioinformatic analysis

The estimated helicity of HAZ was predicted using NPS (Network Protein Sequence Analysis) software. HeliQuest software was used to calculate HAZ hydrophobicity and hydrophobic moment (μH), and to generate helical wheel plots. The net charge at neutral pH for the hybrid peptide was predicted using Innovagen's peptide calculator. I-TASSER software was used to verify model reliability and predict 3D structure.

2.5. Bacterial susceptibility assay

In order to determine the antibacterial activity of the HAZ peptide, we measured its minimum inhibitory concentration (Basir, Knoop et al. 2000) and its minimum bactericidal concentration (MBC) according to the guidelines of the Clinical and Laboratory Standards Institute.

In brief, different strains of bacteria at 10^6 CFU/mL concentration were prepared by diluting overnight cell culture using a fresh Muller Hinton Broth (MHB). In 96-well μl plates, 50 Âμl of diluted bacterial suspension were mixed with 50 Âμl of peptide concentrations. Bacterial growth was measured using optical density at λ = 600 nm. A 10 Âμl sample of each well was spread into Muller Hinton Agar (MHA; Oxoid ltd., Basingstoke, UK) and incubated at 37 °C for 24 h before reading the MBCs. MBC is the concentration of the peptide necessary to reduce survival bacterial cells to less than 0.1 % within 24 h after treatment.

2.6. Antibiofilm activity

As previously described, the attenuation of biofilm formation was investigated(Luca, Stringaro et al. 2013). In brief, 150 μL of the bacterial inoculum was added to each well of the 96-microtiter plate. Lids with pegs were placed in a 96-microtiter plate where biofilm-forming cells could accumulate, followed by orbital shaking at 125 rpm and incubation at 37 °C for 20 h. To remove planktonic cells, the plates were rinsed three times with 200 μL of phosphate-buffered saline. Following that, pegs-lids were placed in a "challenge 96-well microtiter plate" containing 200 μL of various peptide concentrations and incubated at 37 °C for 2 h before being transferred to a recovery plate. To determine the minimum biofilm eradication concentration, the absorbance of the plate at OD = 595 nm was measured to examine the growth of recovered bacteria (MBEC).

2.7. Synergistic checkerboard assay

Previous research (Dundar and Otkun 2010) were used to examine the synergistic effects of combinations of HAZ and selected antibiotics against four bacterial strains. In 96-well plates, 25 μL of each antibiotic concentration and 25 μL of each peptide concentration were added to 50 μL of diluted bacterial suspension.

After an 18-hour incubation at 37 °C, absorbance at 600 nm was measured. The fractional inhibitory concentrations (FIC) index was calculated according to the following equation (Dundar and Otkun 2010):

$$FICindex = \frac{MICofpeptideincombination}{MICofpeptidealone} + \frac{MICofantibioticincombination}{MICofantibioticalone}$$

The combination was considered synergistic, when the FIC index was ≤ 0.5. Additive effect, on the other hand is concluded when FIC value ranges as $0.5 < FICs \leq 1$. The combination effect is considered indifferent when $1 < FICs \leq 4$, and antagonistic when FIC values are greater than 4.

2.8. Hemolytic assay

2 mL of human blood (Sigma Aldrich, St. Louis, MO, USA) were mixed with 48 mL of phosphate-buffered saline to achieve a final concentration of 4 % RBC, then centrifuged at 2000 rpm for 5 min. The procedure was repeated twice, each time aspirating the supernatant and replacing it with a new buffer. 2 mL of various peptide solution concentrations were then added to 2 mL of erythrocyte suspension and incubated at 37 °C for 1 h. Triton X-100 (Santa Cruz Biotechnology, Dallas, TX, USA) was used as a positive control. After incubation, tubes were gently vortexed and 1 mL of each sample was transferred, then centrifuged at 2000 rpm for five minutes, then 200 μL of each supernatant was transferred into a 96-well plate and their absorbance was measured at 450 nm. The following equation was used to calculate the percentage of hemolysis (Au - Evans, Au - Nelson et al. 2013):

$$\%Hemolysis = \frac{A - AO}{AX - AO} * 100$$

Where: "A" is the absorbance value of the tested solutions, "A0″ is the absorbance value of the negative control, and "AX" is the absorbance value of the positive control.

2.9. MTT assay

Briefly, Adenocarcinoma human alveolar basal epithelial cells (A549) were cultured at 37 °C in F-12 K Medium supplemented with 10 % of fetal bovine serum and 1 % of penicillin–streptomycin solution. Cells were plated at a density of 7×10^3 cells/well in a 96-well plate and incubated overnight at 37 °C under 5 % CO_2 with various peptide concentrations (100, 80, 60, 40, 20, 10, 5, 1 μM). Then, 10 μL MTT solution (5 mg/mL in serum-free media) was added to each well, and the plates were incubated for 4 h at 37 °C, 5 % CO_2, and 95 % humidity. The media was then aspired, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. After shaking the plates for 1 min, the absorbance at 590 nm was measured. The viability percentage was calculated using the equation below (Kamiloglu, Sari et al. 2020):

$$\%\,\text{viability} = \frac{\text{sample abs}}{\text{negative control abs}}$$

The GraphPad Prism software was used for statistical analyses.

3. Results

3.1. Design and in-silico characterization of peptide

The design of our peptide relied on *in-silico* hybridization and characterization of peptide sequences comprising the bioactive helical parts of naturally existing peptides Palustrin-1d and HP (2–20) resulting in a hybrid peptide consisting of 11 amino acids from the first peptide (Palustrin-1d) and the 10 amine acids from the second peptide HP(2–20), as briefed in Table 1. Peptides helical wheel plots are shown in Fig. 1**A-1C**. *In silico* characterization of the hybrid peptide revealed suboptimal parameters essential for antimicrobial activity (Table 1). Therefore, we did a series of sequence modifications and amino acid replacement to improve the physicochemical parameters of the hybrid peptide. Indeed, a resulting peptide named HAZ, with 21 amino acids displayed predicted properties such as helicity, hydrophobicity and a helical wheel plot that potentially supports antimicrobial activity. (Table 1 **and** Fig. 1**D, 1E**).

3.2. Bacterial susceptibility assays

Upon characterization, we conducted functional assessment of HAZ peptide. The antibacterial effects of HAZ peptide were examined on four representative Gram-positive and Gram-negative, sensitive and multi-drug resistant *S. aureus* and *E. coli* bacterial strains. HAZ peptide displayed significant antimicrobial effects on the selected strains, with minimum inhibitory concentration values of 15–20 μM, as shown in Table 2. Interestingly, The MIC values were equal to minimum bactericidal concentration (MBC) values, suggesting a bactericidal activity for HAZ.

Various bacterial strains possess virulent factors enabling them to survive harsh environmental stress. In particular, biofilm formation has been a major challenge for efficient eradication of bacteria, especially in medical devices. Therefore, we investigated the potential of HAZ peptide to eradicate biofilms formed by the selected bacterial strains. HAZ peptide exhibited high efficacy in biofilm eradication, with minimum biofilm eradication concentrations (MBEC) values of 30–40 μM (Table 3). Interestingly, MBEC values for HAZ peptides vary among the strains as per structural differences, with higher potency against Gram-positive strains.

3.3. Synergistic antimicrobial activity of HAZ with selected antibiotics

Current treatment regimens rely on therapeutics combinations for the purposes of minimizing doses administered, maximizing efficacy and lowering unwanted side effects. In this direction, we examined the antimicrobial efficacy of HAZ peptide upon combination with representative antibiotics. As summarized in (Table 4), the calculated MICs of both HAZ and the selected antibiotics decreased dramatically in combinations compared to MICs of individual agents. While the MICs of the peptide reduced significantly in all combinations with all tested strains, the reduction percentages of MICs of individual antibiotics vary among different combinations, and most combinations displayed synergistic rather than additive effects on the studied strains, as demonstrated by the calculated FIC indices. Among all combinations, Levofloxacin, and to a lesser extent Doxycycline combination resulted in most dramatic reduction in MIC values of 66 % and more, with a synergistic effect against all tested strains. Apart from the MDR *E. coli* strain, Rifampicin-peptide combination has also displayed significant MIC reduction against tested bacteria. Ampicillin and Chloramphenicol combinations, on the other hand resulted in modest reduction in their MIC values, especially when tested against Gram-positive strains, with a common additive rather than synergistic action with the peptide. These findings showed that the beneficial antimicrobial activity of HAZ peptide could be further exploited to reduce drug resistance rates seen with other antibiotics.

3.4. Hemolytic toxicity evaluation

Cationic peptides are generally known for exhibiting toxicity on human erythrocytes. To evaluate the hemolytic toxicity profile of HAZ peptide on RBCs, blood samples were treated with various levels of HAZ peptide for measurement of the hemolytic activity. HAZ peptide displayed minimal hemolytic toxicity on RBCs even

Table 1
The physicochemical parameters of Palustrin-1d, HP (2–20), the hybrid peptide, and HAZ peptide. HR: Hydrophobic ratio, μH: Hydrophobic moment, z: Charge.

Peptide	Sequence	HR	<μH>	z	α-helical %
Palustrin-1d	ALSILKGLEKLAKMGIALTNCKATKKC	51 %	0.161	+5	59.26 %
HP 2–20	AKKVFKRLEKLFSKIQNDK	36 %	0.59	+5	84.21 %
Hybrid peptide	KGLEKLAKMGIAKKVFKRLEK	42 %	0.093	+6	90.48 %
HAZ peptide	GVKFAKRFWRFAKKAFKRFEK	47 %	0.631	+8	95.24 %

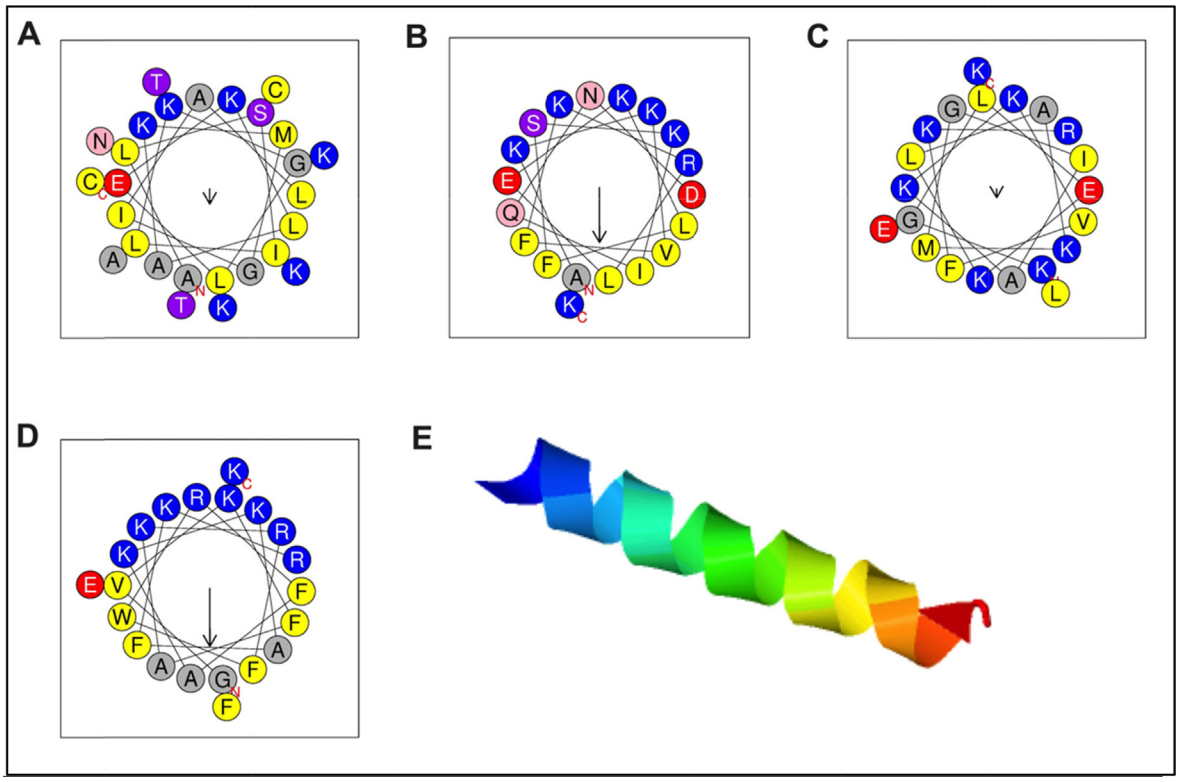

Fig. 1. The helical wheel plots of (A) Palustrin-1d, (B) HP(2–20), (C) hybrid peptide and (D) HAZ peptide. (E) The predicted 3D structure of HAZ peptide using I-TASSER software (TM score is 0.70 ± 0.12 while RMSD is 1.3 ± 1.3 Å).

Table 2
MIC and MBC results of HAZ peptide against selected bacterial strains. (Data represent triplicate).

Sensitive strains	ID	MIC (μM)	MBC (μM)
Staphylococcus aureus	ATCC-29215	15	15
Escherichia coli	ATCC-25922	20	20
Multidrug resistant strains	**ID**	**MIC (μM)**	**MBC (μM)**
Staphylococcus aureus	ATCC-BAA- 41	20	20
Escherichia coli	ATCC-BAA-2452	20	20

Table 3
MBEC values of HAZ peptide against the selected bacterial strains. (Data represent triplicates).

Gram-positive strains	ID	MBEC (μM)
S. aureus	(ATCC BAA41)	30
S. aureus	(ATCC 29215)	30
Gram-negative strains		**MBEC (μM)**
E. coli	(ATCC BAA-2452)	40
E. coli	(ATCC 25922)	40

at concentrations higher than therapeutic ones (Fig. 2**A**). Hemolytic reaction was only detected at peptide concentrations beyond 20 μM, and a hemolysis percentage around 20 % and beyond was detected at concentrations higher than 120 μM. These results suggested a minimal hemolytic toxicity profile of HAZ at therapeutic concentrations required for antimicrobial activity.

3.5. Cytotoxicity evaluation

Antimicrobial peptides are gaining increasing interest as potential anticancer therapeutics against various cancer types. In this direction, the potential anticancer activity was investigated using viability assessment of HAZ-treated adenocarcinomic alveolar epithelial cells (A549). As shown in Fig. 2**B**, HAZ displayed potent antiproliferative effects on A549 cells, with an IC50 around 5.44 μM. Even 1 μM was sufficient to inhibit cell proliferation by around 20 %, with more than 95 % viability inhibition achieved by treatment with 10 μM.

4. Discussion

The growing crisis of antimicrobial resistance to existing antibiotics exemplifies one of the most challenging health problems nowadays. Thus, it becomes urgent to bring together research efforts for development of antimicrobial therapies with particular efficacy on infections produced by drug-resistant strains. Here, we rationally designed a new antimicrobial peptide called HAZ,

Table 4
Minimum inhibitory concentrations (μM) of HAZ and antibiotics, and the calculated FIC. LVX, levofloxacin; CHL, chloramphenicol; RIF, rifampicin; AMP, ampicillin; DOX, doxycycline; S, synergistic; and A, additive; I, indifferent.

B. species	Drug				HAZ			FIC Index	
		MIC (μM)	MIC (μM) in combination	decrease in MIC%	MIC (μM)	MIC (μM) in combination	decrease in MIC%		
***E. coli* 25,922**	LVX	0.0025	0.000625	75	20	2.5	87.5	0.375	S
	CHL	12	2	83.3		0.3125	98.4	0.182	S
	RIF	5	0.625	87.5		2.5	87.5	0.25	S
	AMP	7.5	5	33.3		5	75	0.917	A
	DOX	5	0.625	87.5		2.5	87.5	0.25	S
***E. coli* BAA-2452**	LVX	70	5	92.85	20	0.625	96.87	0.103	S
	CHL	35	10	71.42		0.375	98.1	0.304	S
	RIF	0.625	0.625	–		17.5	12.5	1.875	I
	AMP	1500	1250	16.67		0.625	96.87	0.865	A
	DOX	30	10	66.67		2.5	87.5	0.458	S
***S. aureus* 29,215**	LVX	0.02	0.005	75	15	0.625	95.83	0.292	S
	CHL	9.0	7.5	16.67		0.3125	97.92	0.854	A
	RIF	1.25	0.125	90		5.0	66.67	0.433	S
	AMP	7.5	5.0	33.3		2.5	83.3	0.833	A
	DOX	5.0	1.25	75		2.5	83.3	0.417	S
***S. aureus* BAA-41**	LVX	100	25	75	20	5	75	0.5	S
	CHL	35	27.5	21.43		0.375	98.1	0.804	A
	RIF	2.5	0.625	75		2.5	87.5	0.375	S
	AMP	100	75	25		0.3125	98.4	0.766	A
	DOX	30	10	66.67		1.25	93.75	0.396	S

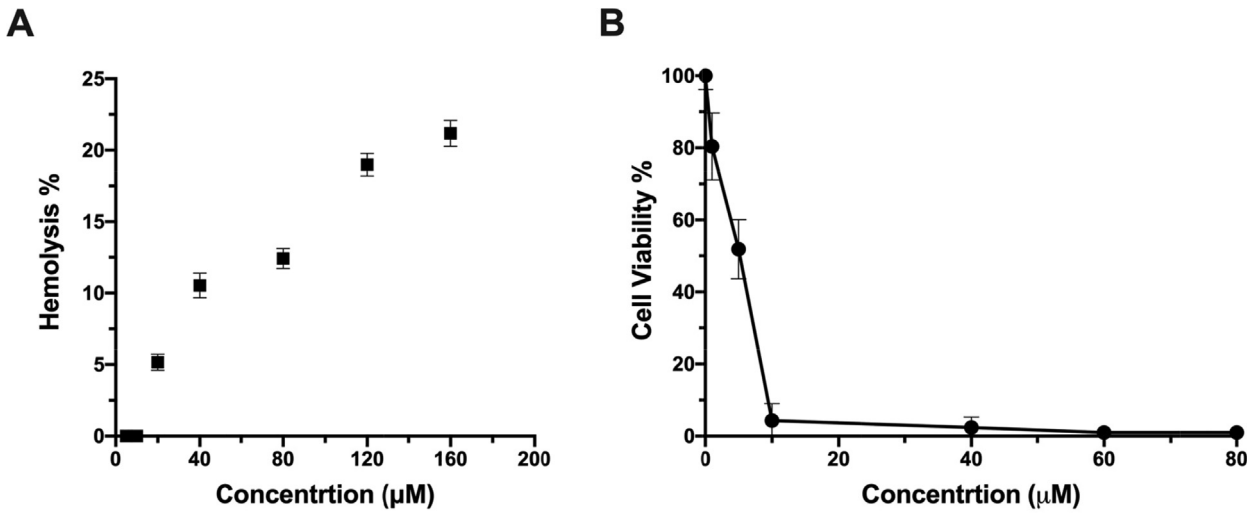

Fig. 2. HAZ hemolysis effect on human RBCs upon exposure time of 60 min at different peptide concentrations. Values are the means (±SD) of three different trials. A549 cell growth curve after treatment with various concentrations of HAZ peptide. Values are the means (±SD) of six different trials.

relying on sequences of existing AMPs. Functional characterization of HAZ peptide revealed promising antimicrobial activity against sensitive and multidrug resistant bacterial strains. Importantly, this beneficial activity was accompanied with minimal hemolytic toxicity on RBCs, along with potential anticancer activity against human lung cancer cells.

Various approaches have been developed to harness the activity of naturally-existing AMPs as drug development leads (Wang, Wang et al. 2022). Strategies such as point mutation of a given peptide sequence, hybridization of active moieties of different peptides, chemical modification of peptide backbone and/or combination of these approaches together aim to maximize their activity, broaden the spectrum and/or minimize off-target toxicity. In this direction, we combined the bioactive helical parts of naturally existing peptides Palustrin-1d and HP 2–20 to result in a hybrid peptide (KGLEKLAKMGIAKKVFKRLEK). Given the well-established essential physicochemical properties like higher helicity, intermediate level of hydrophobicity and a cationic net charge of around (+7) for biologically active peptide (Huang, Huang et al. 2010, Palermo and Kuroda 2010), the hybrid peptide was sequentially modified to fine-tune these properties. Sequence optimization as per calculated physicochemical properties resulted in novel peptide named HAZ, with a sequence of (GVKFAKRFWRFAK-KAFKRFEK). The net charge of the peptide was purposely increased to + 8 to enhance preliminary interaction of the cationic peptide with the bacterial membranes comprising anionic phospholipids, and hence increasing its antimicrobial effects, minimizing the cytotoxicity by increasing the selectivity (Ebenhan, Gheysens et al. 2014). The overall charge represents a sum of 9 positively charged amino acids (six lysine residues and three arginine residues) and one negatively charged glutamic acid residue. Besides polar residues, HAZ contains ten hydrophobic residues (five phenylalanine residues, three alanine residues, one tryptophan and one glycine), bringing the hydrophobic percentage to 47.6 % and hydrophobic momentum to 63.1 %. The enhanced helicity and optimized hydrophobicity of HAZ peptide resulted in a facially amphiphilic structure (Fig. 1D), wherein hydrophobic residues are segregated in one helical face, while polar cationic residues into the other

one; as commonly observed in biologically active peptides (Chrom, Renn et al. 2019).

For functional assessment of the peptide, the antibacterial activity was examined against four different bacterial strains, representing different Gram-stain, and drug-sensitivity phenotypes. HAZ peptide exhibited high activity against these strains, with MIC values of 15–20 μM. The equal MIC and MBC values for HAZ peptide suggest a bactericidal activity against tested strains. Interestingly, it demonstrated equipotent antimicrobial activity in spite of distinct structural components in different bacterial strains. Several theories have been proposed for the lethal effects of cationic AMPs. In Gram-negative bacteria, cationic AMPs initially hit the anionic phospholipids in the outer membranes, leading to their destabilization, followed by access to cell membrane. On the other hand, cationic peptides have components of peptidoglycan thick layer in Gram-positive bacteria as initial targets (Lee and Lee 2015). It is increasingly debated whether destabilization of bacterial structures via these mechanisms is lethally sufficient to trigger cell content leakage, or whether it is an intermediate event in the peptide uptake into bacterial cytoplasm where essential cell processes are inhibited, such as protein and DNA synthesis. Nonetheless, our results are in support for the later theory wherein cationic peptides exerts antibacterial effects irrespective of outer structures, i.e., via binding to intracellular targets, which has been proved for many peptides (Le, Fang et al. 2017). In contrast, the situation was different for biofilm eradication activity, as there was superior potency of HAZ peptide against Gram-positive strains in comparison to Gram-negative ones. The unique biochemistry of *E. coli*'s outer environment explains this, at least in part. Indeed, it was shown that *E. coli* secretes alginate, an anionic polysaccharide that can interact with cationic AMPs, resulting in the capture of these peptides, and subsequent protection of *E. coli* in the biofilm from their bactericidal actions (Yasir, Willcox et al. 2018).

Combined therapies are new treatment approaches that are increasingly applied in medical practice to maximize therapeutic benefits and minimize toxicity and/or costs. In this study, the antibacterial activity of HAZ peptide was examined upon combination with five different antibiotics for synergistic studies. These antibiotics were selected with diverse mechanisms of action (Kapoor, Saigal et al. 2017), specifically cell wall synthesis (AMP), protein synthesis (CHL and DOX) and DNA synthesis (RIF and LVX). Clearly, all antibiotics have intracellular activities except extracellularly-acting AMP, which explains the additive, rather than synergistic HAZ – AMP combination activity. On the other hand, HAZ displayed synergistic activity when combined with intracellularly-acting antibiotic. This is attributed to the aforementioned peptide destabilization of bacterial membranes that results in increased permeability and uptake of antibiotics into bacterial intracellular compartment, and consequently enhanced bactericidal effects.

The promising antimicrobial effects of HAZ was accompanied with potent anticancer effect on lung adenocarcinoma cells. Such anticancer activity of cationic peptides is attributed to cell membrane destabilization and cell content leakage, or indirectly through suppression of membrane-linked growth signaling, as clearly demonstrated with model peptides melittin and defensins (Moreno and Giralt 2015, Hanaoka, Yamaguchi et al. 2016). The promise of cationic peptides as alternative anticancer therapies is further augmented by their well-established selectivity to cancerous over normal cells. This selectivity is due to the more abundant negatively charged phosphatidylserine and O-glycosylated mucins on cytoplasmic membrane of cancer cells, resulting on preferential accumulation of peptide on cancer cell surface (Deslouches and Di 2017). For broader overview of HAZ bioactivity, and for clinical application consideration, peptide toxicity on normal cells has been investigated. Hemolysis is the most common side effect for cationic peptides, and is largely linked to peptide hydrophobicity and amphipathicity. Indeed, it's been shown that more hydrophobicity results in enhanced bioactivity but increased hemolytic activity. Inversely, the higher polarity is correlated to a lesser toxicity but diminished bioactivity (Greco, Molchanova et al. 2020). Our peptide demonstrated balanced physicochemical parameters that result in beneficial antimicrobial activity at minimal hemolytic toxicity levels. At therapeutic MIC levels, HAZ displayed minimal hemolytic activity of around 5 %. This could be attributed to the abundance of polar amino acids distributed throughout the HAZ sequence, which could contribute to breaking the nonpolar side of the helix when binding the RBC membrane, a feature that has been deemed essential for peptide-mediated hemolytic activity (Molchanova, Hansen et al. 2017).

5. Conclusion

In summary, we present in this study a novel peptide (HAZ) displayed powerful antimicrobial effects against a broad range of both Gram-positive and Gram-negative bacteria, including multi-drug resistant isolates. The antimicrobial activity of HAZ, coupled with its minimal hemolytic profile and promising anticancer activity indicate that the peptide has dual activities, and it is worth to be subjected to further investigation in order to optimize its pharmacokinetic and pharmacodynamic profiles. Besides management of infectious diseases, the beneficial antibiofilm activity of HAZ would suggest extending its use for non-autoclavable inanimate surfaces.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Ageitos, J., Sánchez-Pérez, A., Calo-Mata, P., Villa, T., 2017. Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria. Biochem. Pharmacol. 133, 117–138.

Basir, Y.J., Knoop, F.C., Dulka, J., Conlon, J.M., 2000. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, Rana palustris. Biochim. Biophys. Acta (BBA)-Protein Struct. Mol. Enzymol. 1543 (1), 95–105.

Chrom, C.L., Renn, L.M., Caputo, G.A., 2019. Characterization and antimicrobial activity of amphiphilic peptide ap3 and derivative sequences. Antibiotics 8 (1), 20.

Cruz, J., Rondon-Villarreal, P., Torres, R.G., Urquiza, M., Guzman, F., Alvarez, C., Abengozar, M.A., Sierra, D.A., Rivas, L., Fernandez-Lafuente, R., Ortiz, C.C., 2018. Design of bactericidal peptides against Escherichia coli O157: H7, Pseudomonas aeruginosa and methicillin-resistant Staphylococcus aureus. Med. Chem. 14 (7), 741–752.

Deslouches, B., Di, Y.P., 2017. Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications. Oncotarget 8 (28), 46635.

Dundar, D., Otkun, M., 2010. In-vitro efficacy of synergistic antibiotic combinations in multidrug resistant Pseudomonas aeruginosa strains. Yonsei Med. J. 51 (1), 111–116.

Ebenhan, T., Gheysens, O., Kruger, H.G., Zeevaart, J.R., Sathekge, M.M., 2014. Antimicrobial peptides: their role as infection-selective tracers for molecular imaging. Biomed. Res. Int. 2014, 867381.

Au - Evans, B. C., C. E. Au - Nelson, S. S. Au - Yu, K. R. Au - Beavers, A. J. Au - Kim, H. Au - Li, H. M. Au - Nelson, T. D. Au - Giorgio and C. L. Au - Duvall (2013). "Ex Vivo Red Blood Cell Hemolysis Assay for the Evaluation of pH-responsive Endosomolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs." JoVE(73): e50166.

Fjell, C.D., Hiss, J.A., Hancock, R.E., Schneider, G., 2012. Designing antimicrobial peptides: form follows function. Nat. Rev. Drug Discovery 11 (1), 37–51.

Greco, I., Molchanova, N., Holmedal, E., Jenssen, H., Hummel, B.D., Watts, J.L., Håkansson, J., Hansen, P.R., Svenson, J., 2020. Correlation between hemolytic activity, cytotoxicity and systemic in vivo toxicity of synthetic antimicrobial peptides. Sci. Rep. 10 (1), 1–13.

Guilhelmelli, F., Vilela, N., Albuquerque, P., Derengowski, L., Silva-Pereira, I., Kyaw, C., 2013. Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. Front. Microbiol. 4, 353.

Hanaoka, Y., Yamaguchi, Y., Yamamoto, H., Ishii, M., Nagase, T., Kurihara, H., Akishita, M., Ouchi, Y., 2016. In vitro and In vivo anticancer activity of human β-Defensin-3 and its mouse homolog. Anticancer Res. 36 (11), 5999–6004.

Huang, Y., Huang, J., Chen, Y., 2010. Alpha-helical cationic antimicrobial peptides: relationships of structure and function. Protein & Cell 1 (2), 143–152.

Kamiloglu, S., Sari, G., Ozdal, T., Capanoglu, E., 2020. Guidelines for cell viability assays. Food Front. 1 (3), 332–349.

Kapoor, G., Saigal, S., Elongavan, A., 2017. Action and resistance mechanisms of antibiotics: a guide for clinicians. J. Anaesthesiol. Clin. Pharmacol. 33 (3), 300–305.

Kumar, P., Kizhakkedathu, J., Straus, S., 2018. Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. Biomolecules 8 (1), 4.

Lee, J., Lee, D.G., 2015. Antimicrobial peptides (AMPs) with dual mechanisms: membrane disruption and apoptosis. J. Microbiol. Biotechnol. 25 (6), 759–764.

Littmann, J., Viens, A.M., 2015. The ethical significance of antimicrobial resistance. Public Health Ethics 8 (3), 209–224.

Luca, V., Stringaro, A., Colone, M., Pini, A., Mangoni, M.L., 2013. Esculentin (1–21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen Pseudomonas aeruginosa. Cell. Mol. Life Sci. 70 (15), 2773–2786.

Mahlapuu, M., J. Håkansson, L. Ringstad and C. Björn (2016). "Antimicrobial Peptides: An Emerging Category of Therapeutic Agents." Frontiers in Cellular and Infection Microbiology **6**.

Molchanova, N., Hansen, P.R., Damborg, P., Nielsen, H.M., Franzyk, H., 2017. Lysine-based α-peptide/β-peptoid peptidomimetics: Influence of hydrophobicity, fluorination, and distribution of cationic charge on antimicrobial activity and cytotoxicity. ChemMedChem. 12 (4), 312–318.

Mohammad Alsaggar, A. A.-T., Yara Al Tall, Mohammad Al-Hazabreh (2021). SAMA peptide, a rationally designed antimicrobial peptide, J App Pharm Sci. 2022; 12 (1): 182-189

Moreno, M., Giralt, E., 2015. Three valuable peptides from bee and wasp venoms for therapeutic and biotechnological use: melittin, apamin and mastoparan. Toxins 7 (4), 1126–1150.

Moretta, A., Scieuzo, C., Petrone, A.M., Salvia, R., Manniello, M.D., Franco, A., Lucchetti, D., Vassallo, A., Vogel, H., Sgambato, A., Falabella, P., 2021. Antimicrobial Peptides: A New Hope in Biomedical and Pharmaceutical Fields. Frontiers in Cellular and Infection Microbiology, p. 11.

Palermo, E.F., Kuroda, K., 2010. Structural determinants of antimicrobial activity in polymers which mimic host defense peptides. Appl. Microbiol. Biotechnol. 87 (5), 1605–1615.

Pütsep, K., Brändén, C.-I., Boman, H.G., Normark, S., 1999. Antibacterial peptide from H. pylori. Nature 398 (6729), 671–672.

Schmidtchen, A., Pasupuleti, M., Malmsten, M., 2014. Effect of hydrophobic modifications in antimicrobial peptides. Adv. Colloid Interface Sci. 205, 265–274.

Shah, A., 2013. Tackling the crisis of antibiotic resistance. South Asian J. Cancer 2 (01), 003–004.

Uggerhøj, L.E., Poulsen, T.J., Munk, J.K., Fredborg, M., Sondergaard, T.E., Frimodt-Moller, N., Hansen, P.R., Wimmer, R., 2015. Rational design of alpha-helical antimicrobial peptides: Do's and Don'ts. ChemBioChem. 16 (2), 242–253.

Wang, C.-K., Shih, L.-Y., Chang, K.Y., 2017. Large-scale analysis of antimicrobial activities in relation to amphipathicity and charge reveals novel characterization of antimicrobial peptides. Molecules 22 (11), 2037.

Wang, L., Wang, N., Zhang, W., Cheng, X., Yan, Z., Shao, G., Wang, X., Wang, R., Fu, C., 2022. Therapeutic peptides: current applications and future directions. Signal Transduct. Target. Ther. 7 (1), 48.

Yasir, M., Willcox, M.D.P., Dutta, D., 2018. Action of antimicrobial peptides against bacterial biofilms. Materials 11 (12), 2468.

Zhang, Y., Liu, Y., Sun, Y., Liu, Q., Wang, X., Li, Z., Hao, J., 2014. In vitro synergistic activities of antimicrobial peptide brevinin-2CE with five kinds of antibiotics against multidrug-resistant clinical isolates. Curr. Microbiol. 68 (6), 685–692.