KOMODO-DRAGON INSPIRED CATHELCIDIN PEPTIDES ARE ANTIBACTERIAL AGAINST CARBAPENEM-RESISTANT KLEBSIELLA PNEUMONIAE

by

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DEDICATION

To all the friends that helped me through this

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LIST OF ABBREVIATIONS AND SYMBOLS

Carbapenem-resistant *Klebsiella pneumoniae* ..... CRKP
Microliter ..... µL
Microgram ..... µg
Multidrug Resistance ..... MDR
Minimum inhibitory concentration ..... MIC
Clinical & Laboratory Standards Institute ..... CLSI
Cation-adjusted Mueller Hinton Agar ..... CAMHA
Cation-adjusted Mueller Hinton Broth ..... CAMHB
3,3'-dipropylthiadicarbocyanine iodide ..... $DiSC_3(5)$
Phosphate buffered saline ..... PBS
Micromolar ..... mM
Nanometer ..... nm
Celsius ..... C°
Optical Density ..... OD
Ethidium Bromide ..... EtBr
Circular Dichroism ..... CD
Trifluoroethanol ..... TFE
Sodium Dodecyl Sulfate ..... SDS
Colony Forming Units ..... CFU
Cationic Antimicrobial Peptides ..... CAMP
Grand Average Hydropathy ..... GRAVY
Dulbecco Phosphate buffered saline ..... DPBS
Isoelectric Point ..... pI

ABSTRACT

KOMODO-DRAGON INSPIRED CATHELCIDIN PEPTIDES ARE ANTIBACTERIAL AGAINST CARBAPENEM-RESISTANT KLEBSIELLA PNEUONMIAE

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The rise of carbapenem-resistant enterobacteriaceae (CRE) is a growing crisis that requires development of novel therapeutics. To this end, cationic antimicrobial peptides (CAMPs) represent a potential source of new potential therapeutics to treat difficult pathogens such as *Klebsiella pneumoniae* (CRKP), which has gained resistance to many if not all currently approved antibiotics making treatment difficult. In this paper we examined the anti-CRKP antimicrobial activity of the predicted cathelicidins derived from *Varanus komodoensis* (Komodo dragon) as well as synthetic antimicrobial peptides that we created. We did not observe significant anti-CRKP activity of for the predicted native Komodo cathelicidin peptides. We found significant antimicrobial activity of that the novel peptides DRGN-6, -7 and -8 were antimicrobial against CRKP with MICs between 4-8 μg/mL. DRGN-6 peptide was the most effective peptide against CRKP. We characterized the abilities of these peptides to disrupt the hyperpolarization of the

bacterial membrane as well as their ability to form pores in the membrane. After further testing, these peptides showed higher than desired levels of hemolysis although *in vivo* testing in the waxworm *Galleria mellonella* showed no mortality associated with treatment by the peptide; however, CRKP-infected waxworms treated with peptide did not show an improvement in survival. Given the challenges of treating CRKP, identification of peptides with activity against it represents a promising avenue for further research. Given DRGN-6's similar level of activity to colistin, DRGN-6 is a promising template for the development of novel antimicrobial peptide-based therapeutics.

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative rod-shaped bacterium belonging to the family Enterobacteriaceae, and it has been associated with a range of human diseases, including urinary tract infections, bacteremia and pneumonia, both in community and hospital-associated infections [1, 2]. *K. pneumoniae* has also been shown to cause pyogenic liver abscesses [1, 2]. It is a member of the ESKAPE family of pathogens, a group of bacteria that are responsible for the majority of nosocomial infections [3]. Moreover, many strains of ESKAPE pathogens are multidrug resistant (MDR), and they are thus of particular concern [3]. Of greatest concern are carbapenem-resistant strains of Enterobacteriaceae, which are considered one of the most urgent threats. CRE are associated with 9000 infections and 600 deaths annually in the US, with 7900 infections being linked to CRE *K. pneumoniae* specifically [4].

Carbapenem-resistance can be conferred via several mechanisms, including the overproduction of AmpC β-lactamases or extended spectrum β-lactamases with porin defects that lead to lowered membrane permeability [5, 6]. Typically, three types of carbapenemases, *K. pneumoniae* carbapenemase , metallo- β-lactamases , and OXA-48 confer carbapenem-resistance to *K. pneumoniae* [7]. In addition to carbapenem-resistance, these carbapenemases also confer resistance to most or all β-Lactam antibiotics and most are not susceptible to the effects of β-Lactamase inhibitors [7, 8]. In

addition, *K. pneumoniae* strains are able to resist many other antibiotics through the expression of efflux pumps [1]. Thus, developing new antibacterial compounds against this MDR organism is highly challenging.

Cyclic peptide antibiotics have been shown to be effective against carbapenem-resistant *K. pneumonia,* either alone or in combination with other drugs. Both polymyxin B (Minimum inhibitory concentration (MIC) of 2 μg/mL) and colistin (MIC of ≤1μg/mL) exhibit *in vitro* efficacy against carbapenem-resistant *K. pneumoniae,* and colistin is often used to treat carbapenem-resistant K. *pneumoniae* as a drug of last resort [9-11]. While effective, polymyxin B and colistin both have serious side effects including nephrotoxicity and neurotoxicity that make them potentially harmful [12]. Additionally, resistance to both of these peptides has been reported in some carbapenem-resistant *K. pneumoniae* strains [13, 14]. Thus, we sought to find a cationic antimicrobial peptide that was effective against this organism.

Cationic antimicrobial peptides (CAMPs) are positively charged peptides with antimicrobial activity. They are found in all domains of life, including bacteria and archaea [15, 16]. Theses peptides are an important part of innate immunity and can be grouped into different families based on their structural and functional properties. Canonical vertebrate CAMPs include α-defensins, β-defensins and cathelicidins [17, 18]. Cathelicidins, as a family, are grouped together based on conserved N-terminal signal and pro-regions that are present in the inactive precursor proteins [19]. While their signal and pro-regions are highly conserved, the active mature cathelicidin peptides show a high degree of divergence, with little homology between species. Similarly, cathelicidin

peptides can vary greatly in species that produce more than one cathelicidin peptide [15, 20]. We have previously studied and predicted cathelicidin peptides in the Chinese cobra (*Naja atra*) and the American alligator [16, 21-25]. We demonstrated that CAMPs from the American alligator along with peptides derived from its cathelicidin (AM-CATH) exhibited antibacterial activity against many multi-drug resistant pathogens [21, 26]. Additionally, we showed that a cathelicidin peptide from the Chinese cobra, NA-CATH, and synthetic peptide variants were antimicrobial against many medically important Gram-negative bacteria, including biothreat agents and *Klebsiella pneumonia* [21, 24, 25, 27, 28]. We also previously demonstrated that the snake cathelicidin NA-CATH was effective against carbapenem-resistant *K. pneumoniae* (MICs of 5.2 μg/mL-5.6 μg/mL) [21]. Other researchers have found that the antimicrobial peptide CM-11, a hybrid of cecropin and melittin, was effective against carbapenem-resistant *K. pneumoniae* (MIC of 8 μg/mL-16μg/mL) [29]. Additional antimicrobial peptides have been shown to be effective against *K. pneumoniae*, although their effectiveness against carbapenem-resistant *K. pneumoniae* is not currently known, including dermaseptin (MIC of 5 μg/mL), Cys-LC-LL-37 (MIC of 5μg/mL), Human beta-defensin-1 (MIC of 5 μg/mL), and Magainin 1 (MIC of 4.17 μg/mL) [30]. Thus, there have been CAMPs identified that have activity against CRKP; however, most of the peptides identified so far have MICs that are higher than the level that could be considered for therapeutic development (MIC>4 μg/mL). In our work discovering new CAMPs in reptiles, we sought to test whether we could identify a more powerful CAMP against CRKP.

We have recently identified two potential cathelicidins from the Komodo dragon, VK-CATH4.1 and VK-CATH4.2 through bioinformatics analysis of the Komodo dragon genome [31]. Additional synthetic peptides inspired by these varanid peptides were then generated to make a library to systematically explore the contribution of the N-terminal, C-terminal and helical components to antimicrobial performance. The purpose of this study was to screen this limited library of natural cathelicidin and novel synthetic antimicrobial peptides for antibacterial activity against carbapenem-resistant *K. pneumoniae* (ATCC BAA-1705).

METHODS

Bacteria strain and growth conditions

Klebsiella pneumoniae ATCC BAA-1705 was purchased from the American Type Culture Collection (Manassas, VA) and grown in Cation-Adjusted Mueller Hinton Broth. Bacteria were aliquoted and frozen at -80 °C with 20% glycerol and enumerated via serial dilution and plating prior to experimentation.

Peptide synthesis

All peptides were synthesized to order by ChinaPeptides, Inc (Shanghai, China) using Fmoc chemistry. Peptides were provided at >95 % purity, with their purity and sequences being confirmed via tandem mass spectrometry using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Peptides are provided and used as TFA salts.

CLSI protocol for MIC

The minimum inhibitory concentration (MIC) of the peptides was determined according to Clinical & Laboratory Standards Institute (CLSI) guidelines [32] Bacteria were grown overnight on Cation-adjusted Mueller Hinton Agar (BD 211438, CAMHA) at 37 °C. Assays were performed in Cation-adjusted Mueller Hinton Broth (BD 212322, CAMHB) in polypropylene 96 well plates (Corning 3879) or tissue culture treated

polystyrene 96 well plates(need catalog number). Each experiment was performed with three replicates at least three times.

DiSCS(3)5 assay

Membrane depolarization was measured using $DiSC_3(5)$ (3,3'-dipropylthiadicarbocyanine iodide) as previously described with some modifications [21]. Stocks of enumerated frozen *Klebsiella pneumonia* ATCC BAA-1705 were pelleted and washed twice in phosphate buffered saline (PBS) and then resuspended to 4×10^7 CFU/ml in PBS containing 50 μg/ml $DiSC_3(5)$. One hundred μl of this suspension was added to wells of a black 96 well polypropylene plate. The plate was incubated in a fluorescence spectrophotometer (Tecan Infinite 200) and monitored until fluorescence stopped decreasing. One hundred μl of various concentrations of peptide in PBS were added to each well. Bacteria without peptide and peptide without bacteria served as controls. Plate was immediately returned to the spectrofluorometer. Readings were taken at 20 minutes after addition of peptide (excitation = 635 nm; emission = 670 nm). The experiment was performed with three replicates twice.

EtBr assay

The ethidium bromide assay was performed as previously described with some modifications [21]. *Klebsiella pneumonia* ATCC BAA-1705 was grown overnight on cation-adjusted Mueller Hinton agar plates at 37°C. Isolated colonies were collected and suspended in PBS, centrifuged and washed with PBS, then adjusted to an OD_{600nm} of .1 in PBS. 180 μL of bacteria were added to 10 μL ethidium bromide (10mM final concentration) and 10 μL peptide in various concentrations. The Excitation and emission

wavelengths were set at 530 and 590 nm, respectively. The increase in fluorescence was then measured 20 minutes after the addition of peptides using a fluorescence spectrophotometer (Tecan Infinite 200). The experiment was performed with three replicates twice.

Hemolysis assay

Hemolytic activities of the peptides were determined using a solution of 2% sheep erythrocytes (Hemostat, LLC) in an assay adapted to a microtiter plate format as previously described [25]. Defibrinated sheep's blood was centrifuged at 2000 x g and washed three times with PBS. The wash red blood cells were then resuspended up to their original volume and then diluted to a 2% concentration. 2% erythrocytes were combined with various concentrations of peptides. Sterile deionized water was used as 100% hemolysis and PBS was used as 0% hemolysis. The plate was then incubated at 37°C for one hour. It was then centrifuged at 1000 x g for two minutes. The supernatant was then transferred to a fresh flat-bottomed plate and read at OD_{540nm}. The percent Hemolysis was calculated as the ratio of the experimental well and averaged 100% hemolysis, with the absorbance of averaged 0% hemolysis subtracted from each. The experiment was performed three times with three replicates.

Circular dichroism spectroscopy

Circular dichroism (CD) analysis of the peptides was performed using a Jasco J-1500 spectropolarimeter, as previously described [21, 24, 26]. 100µg/mL of peptide was used in each experiment. Samples were allowed to equilibrate for 5 min prior to data

collection at 25 °C in a 1mm path length cuvette. Spectra were collected from 190 to 260nm with at 20nm/min, a data integration time of 4 seconds and a 1nm bandwidth. Data shown represents the average of four spectra. The peptides were analyzed in 10mM sodium phosphate buffer (6.12mM sodium monohydrogen phosphate heptahydrate; 3.92mM monosodium phosphate anhydrous; pH 7.4), 50% (v/v) trifluoroethanol (TFE) in phosphate buffer, or 60mM sodium dodecyl sulfate (SDS) in phosphate buffer. Percent contribution to secondary structure was measured using methods described in Raussens et al [33].

Waxworm infection model

Galleria mellonella larvae were obtained from Vanderhorst Wholesale (Saint Marys, OH, USA). 10 larvae weighing between 250 and 300mg were randomly assigned to each group. Waxworms were injected with 10μL containing $5x10^5$ CFU suspended in DPBS in their rear left proleg. The waxworms were then allowed to recover for 30 minutes at 37° C. They were then injected with 10μL DPBS containing the various treatments into their rear right proleg. They were then kept at 37° C and scored for survival every 24 hours.

PEPTIDE DEVELOPMENT AND STRUCTURE

We recently reported the sequencing and assembly of the Komodo dragon genome, focusing on genes associated encoding antimicrobial host defense peptides. In this study, we identified genes that were predicted to encode Komodo dragon defensins and cathelicidin peptides [31]. Specifically, these efforts yielded genes for two potential active cathelicidin peptides, VK-CATH4.1 and VK-CATH4.2 (**Table 1**), which in the present study have been chemically synthesized and their antibacterial activity against CRKP was assessed.

Table 1: Table of Peptide sequences used in this paper and their predicted properties. PI or isoelectric point, GRAVY or "Grand Average of Hydropathy" measuring overall hydrophilicity (negative values) or hydrophobicity (positive values).

Peptide Name		Sequence	Molecular Weight (APD3)	Hydrophobic residue% (APD3)	PI	GRAVY (APD3)	Charge (APD3)
	Putative Komodo cathelicidin						
VK-CATH4.1		FRWRRFFRKAKRFLKRHGVSIAIGTVRLLRRFG	4133.019	45%	13.01	-0.3818	(+) 12
VK-CATH4.2		RRWRRFFQKAKRFVKRHGVSIAVGAYRIIG	3660.383	43%	12.51	-0.473	(+) 10
	Synthetic peptides						
DRGN-2		FRWRRFFRKAKRFLKRHAVSIAIGTVRLLRRFG	4147.046	48%	13.01	-0.315	(+) 12
DRGN-3		Ac-FRWRRFFRKAKRFLKRHAVSIAIGTVRLLRRFG-NH2	4187.095	N/T	14	N/T	(+) 12
DRGN-4		FRWRRFFRKAKRFLKRHGVSIAIGTVRLLRRFG-NH2	4174.048	N/T	14	N/T	(+) 13
DRGN-5		Ac-FRWRRFFRKAKRFLKRHGVSIAIGTVRLLRRFG-NH2	4173.068	N/T	14	N/T	(+) 12
DRGN-6		RRWRRFFQKAKRLLRRFG	2477.997	38%	12.88	-1.461	(+) 9

DRGN-7		RRWRRFFQKAKRLLRRFG-NH2	2519.026	N/T	14	N/T	(+) 10
DRGN-8		RRWRRFFRKAKRLLRRFG	2506.054	38%	12.95	-1.516	(+) 10
DRGN-9		RRWRRFFRKAKRLLRRFG-NH2	2505.074	N/T	14	N/T	(+) 11
DRGN-10		RRWRRFFRKAKRIIG	2046.501	40%	12.81	-1.313	(+) 8
DRGN-11		RRWRRFFRKAKRIIG-NH2	2045.521	N/T	14	N/T	(+) 9
	Control peptides						
NA-CATH		KRFKKFFKKLKNSVKKRAKKFFKKPKVIGVTFPF	4175.26	38%	12.34	N/T	(+) 15
LL-37		LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	4493.28	35%	11.15	N/T	(+) 6

Table 2 shows the APD3 homology and alignment of VK-CATH4.1 with known peptides in the Antimicrobial Peptide Database. For VK-CATH4.2 the predicted cathelin-domain includes the requisite four cysteines. In addition, the sequence "VTR" is present within 10 amino acids of the last cysteine. When analyzed in the Antimicrobial Peptide Database [34], the amino acid sequence of the peptide that follows the "VTR" sequence is 30 amino acids in length, would have a net +10 charge, and is predicted to be helical. This peptide demonstrates some homology to other known antimicrobial peptides in the Antimicrobial Peptide Database APD3 [34]. Thus, this candidate peptide has many of the hallmark characteristics of a potential cathelicidin peptide and is a second strong candidate for further study.

Table 2 APD3 alignment of VK-CATH4.1 and known antimicrobial peptides

Peptide Name	Alignment	Similarity
CATHPb4	-Alignment Result-: T R S R W R R F I R G A G R F A R R Y G W R I A L G + + + + L + + V G --Input Sequence---: + + F R W R R F F R K A K R F L K R H G V S I A I G T V R L L R R F G	45.71 %

Cm-CATH2	-Alignment Result-: R R S R F G R F F K K V R K Q L G R V L + R H + + S + R I + T V + G G R M R F + --Input Sequence---: F R W R + + R F F + + + R K + A K R F L K R H G V S I A I G T V R L L R + R F G	45 %
Chicken CATH-2	-Alignment Result-: + R F G R F L R K I R R F + + R P K V T I T I Q G + + + + S A R F G --Input Sequence---: F R W R R F F R K A K R F L K R H G V S I A I + G T V R L L R R F G	44.11 %
Ps-CATH4	-Alignment Result-: T R G R W G R F K R R A G R F I R R N R W Q I + I S T + G L K L + + I G --Input Sequence---: + + F R W R R F F R K A K R F L K R H G V S I A I G T V R L + L R R F G	41.66 %
cc-CATH2	-Alignment Result-: L V Q R G R F G R F L K + K V R R F I P K + + + V I I A A Q I G + + + + + S R F G --Input Sequence---: F R W R + R F + + F R K A K + + R F + L K R H G V S I + A + I G T V R L L R R F G	41.46 %

Table 3 shows the APD3 homology and alignment of VK_CATH4.2 with known peptides in the Antimicrobial Peptide Database.

Table 3 APD3 alignment of VK-CATH4.2 and known antimicrobial peptides

Peptide Name	Alignment	Similarity
CATHPb4	-Alignment Result-: T R S R W R R F I R G A G R F A R R Y G W R I A L G + + + L V G --Input Sequence---: + R + R W R R F F Q K A K R F V K R H G V S I A V G A Y R I I G	46.87 %
Cm-CATH2	-Alignment Result-: R R S R F G R F F K K V R K Q L G R + V L R H + + S R I T V G + G R M R F --Input Sequence---: R R W R + + R F F Q K + A K + + + R F V K R H G V S + I A V G A Y R I I G	45.94 %
Chicken CATH-2	-Alignment Result-: + R F G R F L R K I R R F + + R P K V T I T I Q G S A + R + F G --Input Sequence---: R R W R R F F Q K A K R F V K R H G V S I + A V G + A Y R I I G	40.62 %
cc-CATH2	-Alignment Result-: L V Q R G R F G R F L K + K V R R F I P K + + + V I I A A Q I G + S R + F G --Input Sequence---: R R W R + R F + + F Q K A K + + R F + V K R H G V S I + A + V G A Y R I I G	39.47 %
Cm-CATH3	-Alignment Result-: T R G R W K R F W R G A G R F F R R H K E K I I R A A V D I V L S --Input Sequence---: + R + R W R R F F Q K A K R F V K R H G V S I A V G A Y R I + I G	39.39 %

A rational design approach has been applied to the putative Komodo dragon cathelicidin sequences in order to generate a series of novel synthetic peptide derivatives (**Table 1**).

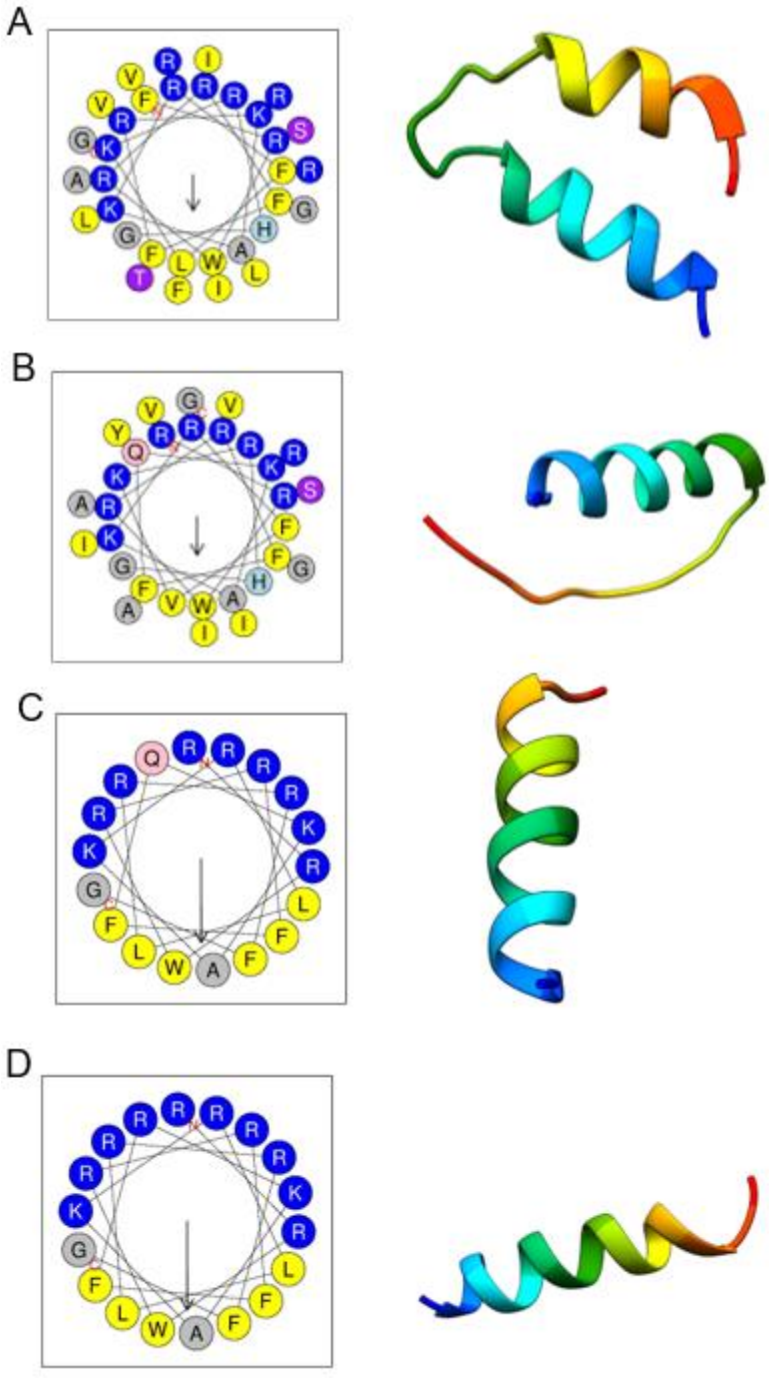
Carbapenem-resistant *Klebsiella pneumonia* (CRKP) is often highly resistant to multiple antibiotics including all beta-lactams, fluoroquinolones and aminoglycosides [35]. To investigate the ability of a library of novel, synthetic antimicrobial peptides against carbapenem-resistant *Klebsiella pneumonia,* we performed minimum inhibitory concentration (MIC) assays according to CLSI standards. Many CAMPs are unable to function in "high salt" environments [36], including LL-37, which was confirmed for CRKP. LL-37 demonstrated very little antibacterial activity against CRKP under these test conditions (**Table 4**). The proposed Komodo dragon cathelicidin peptides VK-CATH4.1 and VK-CATH4.2 along with designed synthetic peptide variants were tested for their antimicrobial activity in Cation adjusted Mueller Hinton Broth (CA-MHB), which contains a relatively high concentration of divalent cations. The predicted cathelicidin VK-CATH4.1 showed relatively low activity with a MIC of 32 µg/mL against CRKP, as did the predicted VK-CATH4.2. By contrast, the *Naja atra* cathelicidin NA-CATH showed a MIC of 8 µg/mL.

Table 4: MIC activity of the peptides against carbapenem-resistant *K. pneumoniae* strain (ATCC BAA-1705). MIC was determined following CLSI protocol in CA-MHB [32].

Peptide Name	MIC (µg/mL)	MIC(µM)
VK-CATH4.1	32	7.7
VK-CATH4.2	32	8.7
DRGN-2	32	7.7
DRGN-3	32	7.6
DRGN-4	32	7.7
DRGN-5	32	7.7
DRGN-6	4	1.6
DRGN-7	4	1.6

DRGN-8	8	3.2
DRGN-9	16	6.4
DRGN-10	>64	>31
DRGN-11	>64	>31
NA-CATH	8	1.9
LL-37	>64	>14
Colistin	4	3.46

We previously prepared chimeric NA-CATH-based peptides, which were designed to improve on the naturally occurring peptide's helical properties and antibacterial activity. NA-CATH contains two imperfect copies of the 11 amino acid motif ATRA in the first half of the peptide, which were found to be critical to its antibacterial activity [25].The first copy is termed ATRA1 with the second copy termed ATRA2 We designed a more active peptide by replacing the ATRA2 domain with another copy of the ATRA1 domain [25], suggesting that different parts (domains) of natural peptides could be combined improve their activity. We have employed a similar approach in designing the peptide DRGN-6, which combines elements from the sequences of VK-CATH4.1 and VK-CATH4.2. The N-terminal 12 residues of DRGN-6 and DRGN-7 are very similar differing only at the first and eighth residues. DRGN-6 is an eighteen-residue peptide. The N-terminal 12-residue segment of DRGN-6 comes from VK-CATH4.2, while the C-terminal six residues of DRGN-6 come from VK-CATH4.1 (amino acids 28-33). This strategy affords a shorter synthetic peptide that is predicted to be helical and much more strongly amphipathic than either of the parent peptides as shown in the helical wheel projection (**Figure 1)**.


A
B
C
D

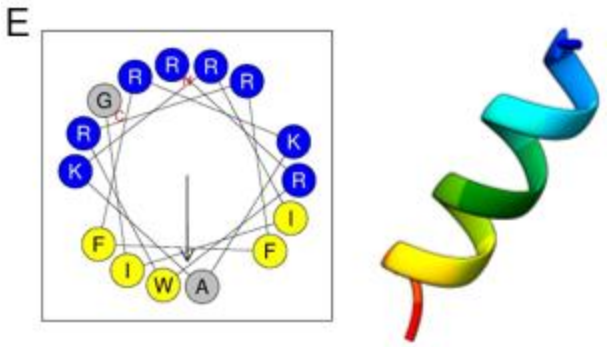


Fig. 1. Predicted secondary structures and helical wheel diagrams of **A.** VK-CATH-4.1 **B.** VK-CATH-4.2 **C.** DRGN-6, **D.** DRGN-8, and **E.** DRGN-10. Structures were predicted by I-TASSER and highest rated PDB file was visualized using Chimera [37, 38].

Three additional peptides (DRGN-7, DRGN-8 and DRGN-9) were generated based on the sequence of DRGN-6. These peptides were designed to assess how increases in net peptide positive charge would impact antibacterial activity. This change to the DRGN-6 sequence included replacing the neutral polar glutamine residue at position 8 with a cationic basic arginine residue as well as elimination of the negatively charged C-terminal carboxylate group with a carboxamide (**Table 1**). It has been suggested that the cationic character of an antimicrobial peptide influences its antimicrobial potency [39]. The sequence of the peptide DRGN-7 is identical to that of DRGN-6, however in DRGN-7 the C-terminal carboxyl group of DRGN-6 has been replaces replaced with a carboxamide. This modification effectively increases the net cationic character of the peptide without introducing new basic residues. In DRGN-8, the glutamine residue at position eight was replaced with an arginine further increasing the net charge. Assuming that DRGN-6 adopts a helical conformation, the basic residues are localized to one face of the helix with the glutamine at position eight residing on the

cationic face of the helix. Thus, replacing it with a basic residue, such as arginine, should provide a means of increasing net charge that is consistent with the existing predicted polarity of the helical peptide. Finally, DRGN-9 combines the changes introduced in DRGN-7 and -8 to increase cationic character in one peptide, resulting in a greater increase in net positive charge. When the Q in DRGN-6 was switched to an arginine in DRGN-8 and DRGN-9, the MIC values for these peptides increased 2 to 4-fold, contrary to our prediction of increased antibacterial activity with increased cationicity. However, the C-terminal amidation of DRGN-7 had no effect on MIC compared to its unmodified counterpart, DRGN-6, while this same C-terminal amidation to DRGN-9 resulted in a 2-fold increase in MIC (decreased effectiveness) compared to its unmodified counterpart, DRGN-8.

A second pair of truncated peptides, DRGN-10 and DRGN-11, were designed to incorporate the N-terminal helical region of VK-CATH4.2 (amino acids 1-12) and the three C-terminal residues of VK-CATH4.2 (amino acids 28-30). The strategy used to design the DRGN-10 and DRGN-11 peptides is analogous to that which was used to generate DRGN-8 and DRGN-9 respectively. However, in DRGN-10 and DRGN-11 we replaced the C-terminal six-residue sequence LLRRFG from VK-CATH4.1 with the three-residue sequence IIG from VK-CATH4.2. As was in DRGN-7 and DRGN-9, the C-terminal carboxylate in DRGN-11 has been amidated as opposed to DRNG-10. The DRGN-10 and DRGN-11 peptide variants were prepared in order to probe whether the specific C-terminal sequences of VK-CATH4.1, VK-CATH-4.2 and designed variants were uniquely significant in influencing antibacterial performance. As can be seen from

Table 4, DRGN-10 and DRGN-11 peptides were both ineffective against CRKP (MIC>64) suggesting that the sequence LLRRFG from VK-CATH4.1 is critical for antimicrobial activity of the DRGN-6 peptide. Furthermore, we conclude that C-terminal amidation provided no positive contribution to antibacterial activity.

Additional peptides, DRGN-2, DRGN-3, DRGN-4 and DRGN-5 were designed and tested (**Table 1**). The peptide DRGN-2 was derived from VK-CATH4.1 by replacing the glycine residue at position 18 of the wild-type sequence with alanine in order to increase the helical propensity of the peptide [40]. In DRGN-3 the N-terminal amine of DRGN-2 is acetylated and its C-terminal carboxy group is amidated in order to reduce susceptibility to proteolytic degradation [41]. These modifications, however, had no significant impact on antimicrobial activity showing little to no positive contribution from the glycine to alanine substitution nor from capping of the N- and C-termini. DRGN-4 is identical to VK-CATH-4.1 except its C-terminus is amidated in order to increase the overall charge of the peptide. In DRGN-5, the N-terminal amine is acetylated and C-terminus amidated. Both of these peptides showed no discernible increase in antimicrobial effectiveness, again suggesting these N- and C-terminal modifications do not significantly impact activity.

In summary, VK-CATH chimeric peptides demonstrated significantly improved antimicrobial activity against CRKP relative to the parental peptides. DRGN-6, -7, -8 and -9, all of which contain the N-terminal helical region of VK-CATH4.2 connected to the C-terminal helical region of VK-CATH4.1 (**Figure 1**), displayed higher activity compared to either of the parent peptides. The peptides containing the N-terminal and C-

Terminal helical regions of VK-CATH4.2 (DRGN-10 and DRGN-11) both had MICs greater than the range tested, thus were determined to be virtually inactive. Combining the N-terminal region of VK-CATH4.2 coupled to a C-terminal helical region of VK-CATH led to the most active peptide in this series. This most active peptide was analyzed for its overall helicity below.

Our results showed that peptides with higher antimicrobial activity such as DRGN-6 did not score higher in terms of GRAVY score, hydropathy index, PI or net charge suggesting that these physicochemical properties are not the only factors contributing to antimicrobial peptide activity of any one cationic, helical AMP. As is shown by the results of this study, there are sequence motif contributors to activity as well as individual residues.

We used circular dichroism spectroscopy to ascertain the general secondary structure properties of the most active peptides (DRGN-6, -7 and -8). The secondary structures of many CAMPs are disordered in the absence of anionic membranes or micelles, but adopt more defined secondary structures in their presence. To detect and measure any changes that the DRGN peptides may undergo based on environment, we studied their structural properties in 10mM phosphate buffer as a negative control, 60mM SDS in 10mM phosphate buffer and 50% 2,2,2-Trifluoroethanol (TFE) in 10mM phosphate buffer. Phosphate buffer without SDS or TFE provided an environment for studying the conformational properties of the peptides structure in the absence a micelle or membrane. Sodium dodecyl sulfate is an anionic surfactant that can form micelles in aqueous buffers mimicking bacterial membranes and simulating the effects an anionic

membrane can have on the conformations of antimicrobial peptides [42, 43]. Addition of trifluoroethanol to aqueous buffer promotes increased structure, usually helicity, in peptides with suitable sequences [44].

In these studies, the structures of both DRGN-6 and DRGN-7 were calculated to contain 70.5% and 72.6% helicity, respectively, in 50% TFE buffer. Interestingly, both showed even greater helical character in SDS buffer (84.2% and 77.0%, respectively). As expected, the CD spectra for both peptides showed little to no helicity in 10mM phosphate buffer (2.6% and 3.0%, respectively) (**Table 5**).

Table 5: Percent alpha-helicity calculated using the equation $27.58 - 14.46 * E_{193} + 1.86 * E_{193}^2 - 5.66 * E_{211} - 14.72 * E_{211}^2$ as described in Raussens et al. [33]

	TFE	SDS	10mM phosphate buffer
DRGN-6	70.5%	84.2%	2.6%
DRGN-7	72.6%	77.0%	3.0%
NACATH	63.5%	55.6%	3.4%

Sequences were submitted to I-TASSER, which predicted that the peptides would have significant helical character, consistent with the CD data (**Figure 2**).

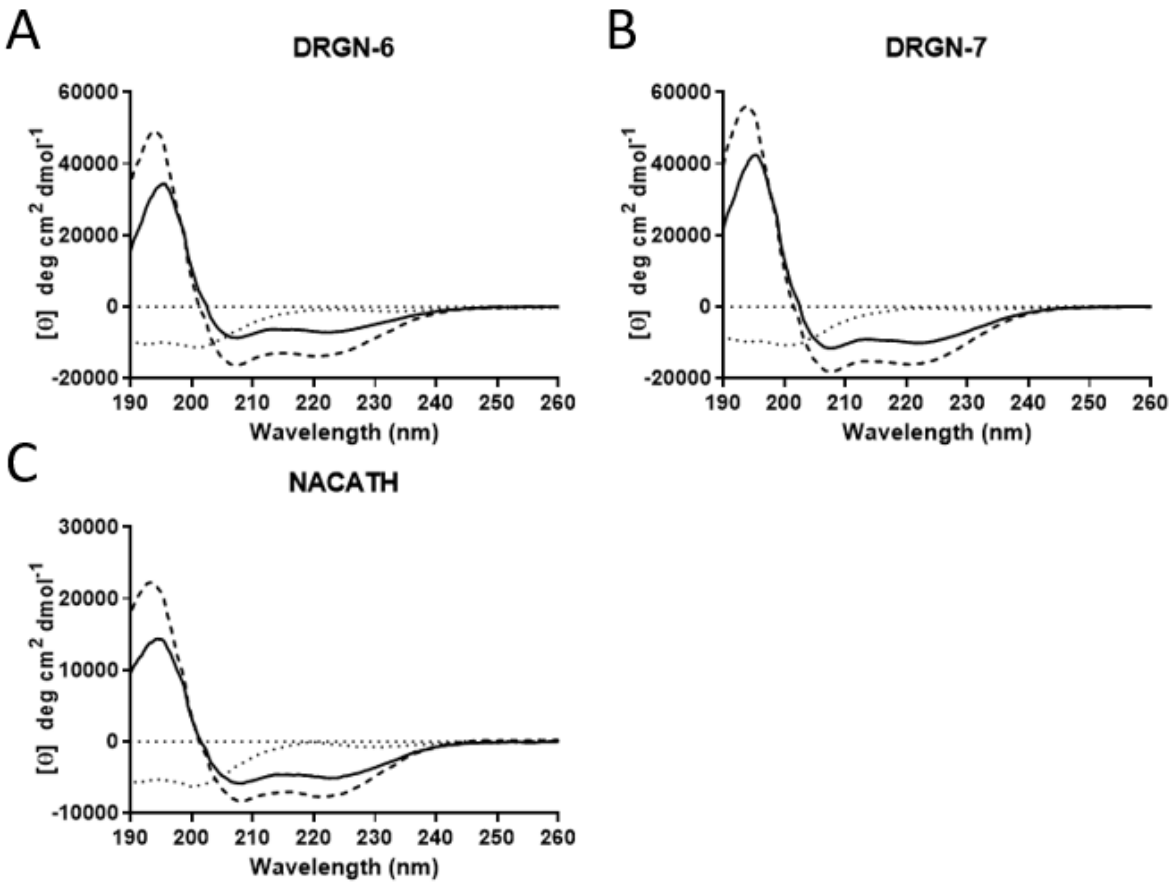


Fig. 2. Circular dichroism (CD) spectra. CD was performed on a Jasco-1500 with **A.** DRGN-6, **B.** DRGN-7, **C.** NACATH. All spectra were taken with peptide concentrations of 100 µg/ml in a 1 mm pathway cuvette. Spectra were gathered in 10 mM phosphate buffer (dots), 50% TFE (dashes), and 60 mM SDS (line).

Helical wheel projections show DRGN-6 and DRGN-8 have a well-structured helical character with a strong amphipathic face (**See Figure 1**).

PEPTIDE ACTIVITY

Section 1: Prediction of Antimicrobial activity of designed peptides using different databases.

The antimicrobial activity of peptides was then predicted using a number of prediction models (**Table 6**).

Table 6: Antimicrobial Peptide Prediction based on Sequence Analysis. Using 2 different web-based CAMP prediction applications ($CAMP_{R3}$ database and AntiBP2) [45, 46] each peptide was scored and given a prediction of whether it would have antimicrobial activity (AMP) or not (Non-AMP). The activity of peptides with modified N and C-termini was not able to be predicted.

Peptide Name	**CAMP Prediction Score from $CAMP_{R3}$**			**AntiBP2 Prediction Score**
	SVM	RF	DA	
Komodo putative cathelicidins				
VK-CATH4.1	0.998: AMP	0.9345: AMP	1.000: AMP	1.067: AMP
VK-CATH4.2	0.989: AMP	0.9955: AMP	0.999: AMP	0.833: AMP
Synthetic peptides				
DRGN-2	0.999: AMP	0.932: AMP	0.999: AMP	0.876: AMP
DRGN-3	N/P	N/P	N/P	N/P
DRGN-4	N/P	N/P	N/P	N/P
DRGN-5	N/P	N/P	N/P	N/P
DRGN-6	0.999: AMP	0.8765: AMP	0.952: AMP	0.050: AMP
DRGN-7	N/P	N/P	N/P	N/P
DRGN-8	1.000: AMP	0.902: AMP	0.990: AMP	-0.085: Non-AMP
DRGN-9	N/P	N/P	N/P	N/P
DRGN-10	0.999: AMP	0.7355: AMP	0.957: AMP	-0.009: Non-AMP
DRGN-11	N/P	N/P	N/P	N/P
Control peptides				
NA-CATH	0.991: AMP	0.9615: AMP	0.998: AMP	1.000: AMP
LL-37	0.762: AMP	0.749: AMP	0.765: AMP	1.474: AMP

All peptides were predicted to be antimicrobial by the $CAMP_{R3}$ database's Support Vector Machine, Random forest and discriminant analysis classifiers. Meanwhile, AntiBP2 predicted all but DRGN-8 and DRGN-10 to be antimicrobial. These predication methods however, were not able to analyze peptides with N- or C-terminal modifications. Given the data in **Table 4**, which showed only DRGN-6 and 8 to be even somewhat active antimicrobial peptides (MIC<16), it can be seen that these computational predictions have a very poor correlation to laboratory results.

Most active peptides

The most active peptide was DRGN-6, and the sequences the other DRGN peptide variants that exhibited significant antibacterial activity were all based on that of DRGN-6, with N-terminal modifications and/or amino acid substitutions such as DRGN-7, DRGN-8, DRGN-9, DRGN-10 and DRGN-11 (**Table 4**). This suggests that the DRGN-6 sequence is a dominant contributor to the antimicrobial activity observed for the whole suite of related peptides. The modifications that were made only reduced the antimicrobial activity of DRGN-6. In particular, DRGN-9 illustrates that the last 6 AA of DRGN-6 are important to its' antimicrobial activity. Based on these studies, a new library of peptides will be designed, starting with DRGN-6 and aiming to preserve or improve upon its helicity and amphipathicity.

We compared the sequence of DRGN-6 to known peptides that are deposited in APD3. It did share limited similarity with a number of peptides, roughly forty percent

similarity to cathelicidins from *Python bivittatus, Sarcophilus harrisii, and Chelonia mydas.* This showed that our designed peptides were distinct and original from natural peptides. **Table 7** shows APD3 homology and alignment of DRGN-6 with known peptides in the Antimicrobial Peptide Database.

Table 7 APD3 alignment of DRGN-6 and known antimicrobial peptides

Peptide Name	Alignment	Similarity
Saha-CATH5	-Alignment Result-: K R I G L I R L I G K I L R G L R R L G --Input Sequence---: + R + R W R R F F Q K A K R L L R R F G	40 %
Cm-CATH2	-Alignment Result-: R R S R F G R F F K K V R K Q L G R V L R H S R I T V G G R M R F --Input Sequence---: R R W R + + R F F Q K + A K + + + R L L R + + R + + + F G + + + +	39.39 %
Pep39	-Alignment Result-: R L F R H A F + K A + + V L + R + L --Input Sequence---: R R W R R F F Q K A K R L L R R F G	38.88 %
CATHPb4	-Alignment Result-: T R S R W R R F I R G A G R F A R R Y G W R I A L G L V G --Input Sequence---: + R + R W R R F F Q K A K R + L L R + + + R + + + + + F G	37.93 %
P1	-Alignment Result-: T H R L R R + W C R A + R G L A R + + --Input Sequence---: + R R W R R F F Q K A K R L L R R F G	36.84 %

Thus, DRGN-6 is a unique peptide when compared to the databases.

Effects on membrane permeability and hyperpolarization

To measure the effects of the most active peptides on *Klebsiella pnuemoniae's* membrane, two assays were performed. Ethidium Bromide (EtBr) was used to measure permeabilization of the membrane and Dipropylthiadicarbocyanine Iodide ($DiSC_3(5)$) was used to measure depolarization of the membrane[21, 25, 26]. EtBr uptake assays showed that DRGN-6, DRGN-7, and DRGN-8 all caused significant increases in the permeability of the cells (**Figure 3A**). ($DiSC_3(5)$ assays showed that all peptides tested caused significant depolarization at all concentrations tested (**Figure 3B).**

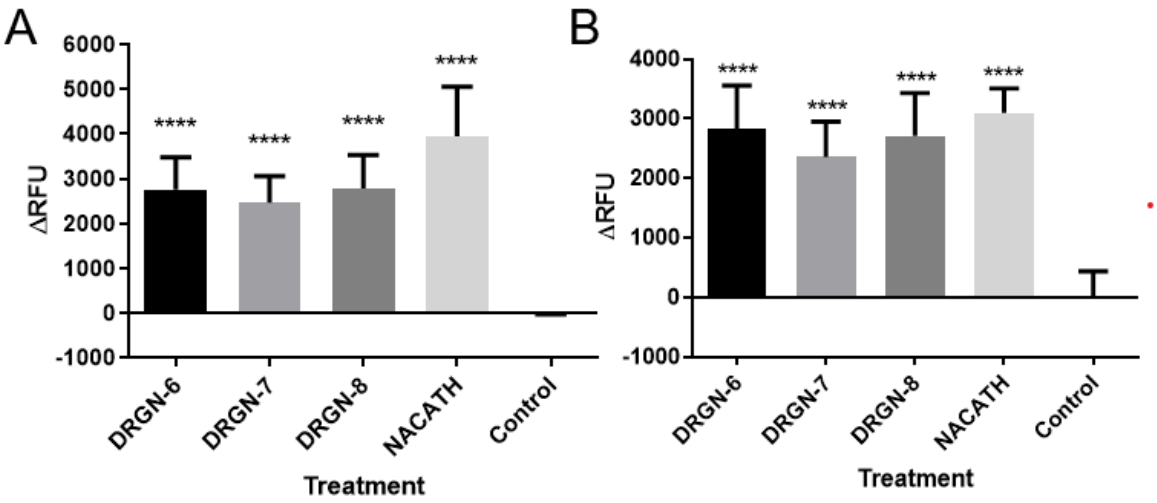


Fig. 3. (A) Membrane disruption as measured by ethidium bromide uptake against *K. pneumoniae* strain ATCC BAA-1705 in PBS at 50 µg/ml. ΔRFU shown is after 20 min incubation, done twice in triplicate. A one-way ANOVA with multiple comparisons was performed to determine statistical significance (*p 0.05, **p 0.01, ***p 0.001).(B) Membrane depolarization Membrane depolarization was measures using 3,3'-dipropylthiadicarbocyanine iodide (DiSC3(5)) against *K. pneumoniae* strain ATCC BAA-1705 in PBS at 100, 10 and 1µg/ml. ΔRFU shown is after 20 min incubation, done twice in triplicate.

Hemolysis assays

Hemolysis assays were performed against 2% sheep's blood and demonstrated that at 100µg/mL there was significant hemolysis of 20-25% in all of the synthetic peptides tested [24, 25]. However, at concentrations closer to their bactericidal concentrations, the peptides showed significantly less hemolysis. At 10µg/mL there was roughly 10% hemolysis in all and at 1µg/mL no significant hemolysis was observed (**Figure 3**). This is in comparison to LL-37, which is reported to have hemolysis of less

than 10% at greater than 250 µg/ml and NA-CATH which has hemolysis of less than 10% at 100 µg/ml [25]. This result suggests that some further modification of the sequence of DRGN-6 may be needed to reduce host-directed toxicity. Overall, these data suggest that additional development of the DRGN-6 peptide to reduce host-cell cytotoxicity will be necessary.

Figure 4

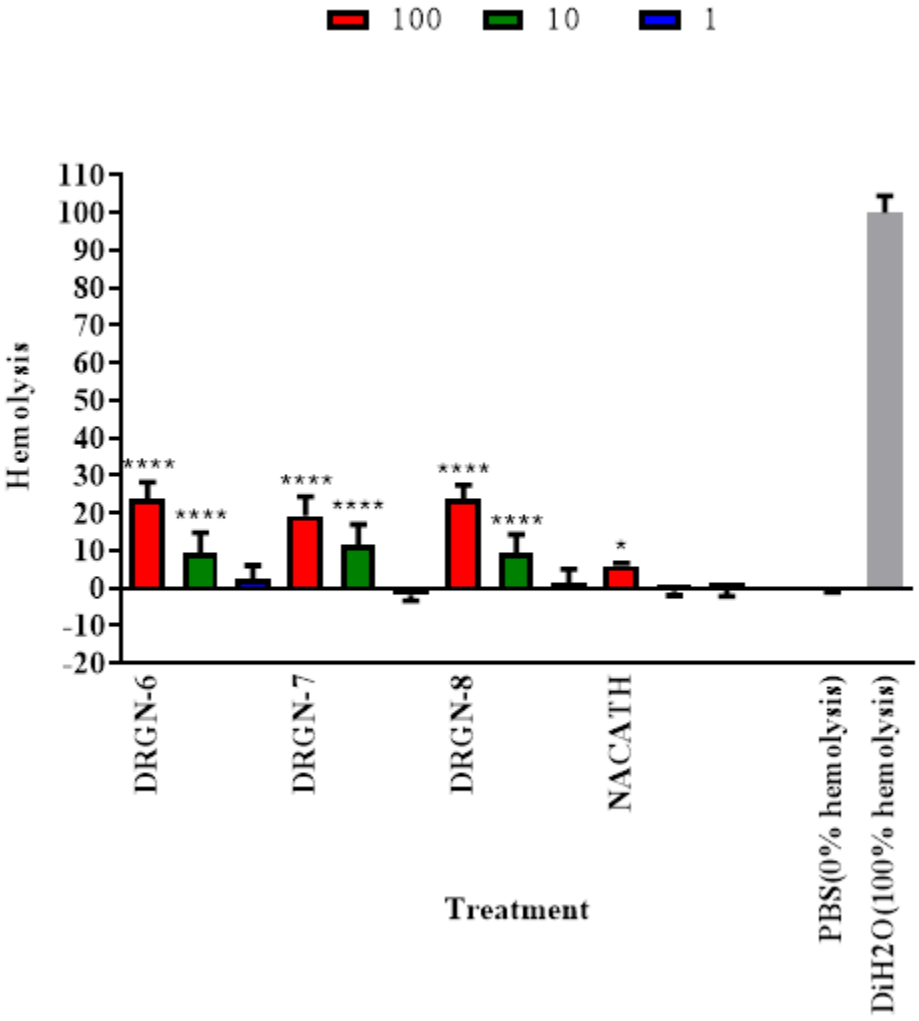


Fig 4. Hemolytic activity against sheep red blood cells. Peptides were tested at 100, 10 and 1 µg/ml. To determine 0% hemolysis, Dulbecco's PBS was added without peptide. To determine 100% hemolysis, sterile deionized water was added without peptide.

Waxworm-model studies

We tested these antimicrobial peptides in the waxworm, *G. mellonella*. When injected at 10 µg per caterpillar, none of the peptides GATR-6, -7 and -8 alone caused toxicity (data not shown). However, when injected into CRKP infected waxworms, no survival benefit was observed. This suggests that either there was not enough peptide provided in each injection to rescue the infection, or the peptide was not able to reduce the infectious load sufficiently. When the control antibiotic tigecycline was injected, 100% of the caterpillars survived, indicating that it is possible to rescue the waxworms from this infection (**Figure 5**).

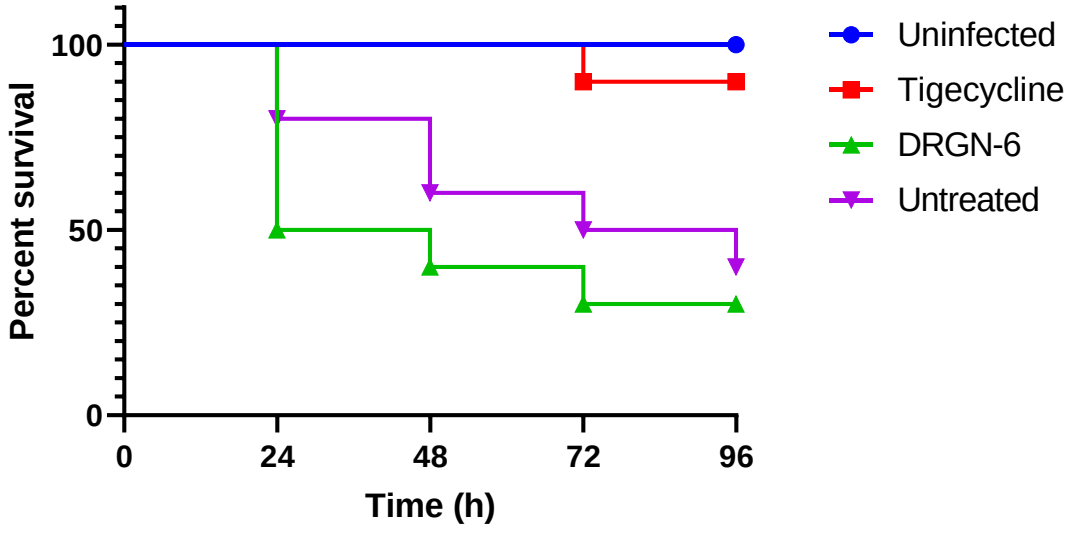


Figure 5: Waxworm survival curves. Waxworms were injected with 10µL containing $5x10^5$ CFU in their rear left proleg. The waxworms were then allowed to recover for 30 minutes at 37° C. They were then injected with 10µL containing the various treatments. They were then kept at 37° C and scored for survival every 24 hours**.**

Overall, these results suggest that the CRKP waxworm model may be oversensitive to CRKP infection, may be insensitive to rescue by this peptide, or a different model is needed that is less sensitive to CRKP so that we might observe any improvements in disease course due to DRGN-6 peptide derivative treatment in our future studies.

Synergy

We tested multiple antibiotics in checkerboard assays with DRGN-6 to identify whether any antibiotic displayed synergy with the DRGN peptide. Notably, synergy has been observed between Rifampicin as well as Meropenem with antimicrobial peptides [47-49]. We tested the following antibiotics: Rifampicin, Meropenem, Imipenem, Colistin and Tigecycline in combination with DRGn-6. None of these antibiotics exhibited synergy with the GATR-6 peptide in *in vitro* checkerboard assays, where synergy is defined as a FIC index of < 0.5 (Data not shown).

DISCUSSION

As Carbapenem-resistant *Klebsiella pneumonia* (CRKP) continues to emerge as a threat, the need for novel therapeutics against this organism increases dramatically. We sought to find and rationally design antimicrobial peptides that would be active against CRKP, despite the significant antibiotic resistance of this organism. This was a challenging goal for this multi-drug resistant gram-negative organism.

We have previously demonstrated antibacterial activity of another Komodo dragon-inspired antimicrobial peptides, DRGN-1, against both *Pseudomonas aeruginosa* and *Staphylococcus aureus in vitro* and in an infected wound in mice (*in vivo*) [50]. Given our previous successes we looked to peptides that we have recently discovered in the Komodo dragon genome [31] as inspiration for new peptides. Through testing a series of natural and synthetic antimicrobial peptides that we invented (VK-CATH4.1, VK-CATH4.2, DRGN-1-11), we found DRGN-6 to be the most active synthetic peptide with a MIC of 4 μg/ml, comparable to colistin, a cyclic peptide antibiotic used as a last-option in the treatment of CRKP (**Table 4**). As polymyxin/colistin resistance emerges in CRKP, perhaps DRGN-6 will provide an alternative avenue for treatment of this deadly infection.

As the success of DRGN-6 shows, chimeric peptides represent a powerful tool in antimicrobial peptide development and optimization. We have previously shown the

efficacy of this strategy using the peptide NA-CATH as a basis to create ATRA1-ATRA1 where we replaced the motif ATRA2 with a second ATRA1 motif, which increased the helicity of the peptide and significantly increased the activity [25]. This design approach applies rational peptide design and experimental feedback to build novel enhanced peptides based on natural antimicrobial host defense peptides that have evolved over millions of years of selective pressure. Here we combined and evaluated motifs, keeping those that have desired characteristics and enhance activity while discarding those that do not.

The differences in activity between the synthetic peptides allows us to draw conclusions about what residues or regions are important for their activity. The lack of activity in DRGN-10 and DRGN-11 compared to DRGN-6, DRGN-7 and DRGN-8 shows the importance of the C-terminal domain (LLRRFG) that was obtained from VK-CATH4.1. This may be due to the added positive charge or increased helicity in DRGN-6, DRGN-7 and DRGN-8. Alternatively, potentially the shorter C-terminal of DRGN-10 gives the peptide less structure and a weaker amphipathic face. The lower activity of DRGN-8 compared to DRGN-6 and DRGN-7 shows the importance of the glutamine residue for antibacterial activity. Glutamine residues have previously been shown to be important in the activity of other peptides [51]. The lack of difference between DRGN-6 and DRGN-7 shows that the inclusion of the C-terminal amidation does not significantly affect antimicrobial activity in this peptide. Thus, DRGN-6 is our lead peptide for further development against CRKP, and it meets the performance criteria of being at least as good as colistin.

Overall, while issues of hemolysis of the DRGN-6 remain to be solved, this unique and novel peptide did show significant antibacterial activity against Carbapenem-resistant *Klebsiella pneuomiae*, as good as colistin, a known antibiotic, suggesting that with further modification, this peptide is promising for future development.

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BIOGRAPHY

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