NET CHARGE, HYDROPHOBICITY AND SPECIFIC AMINO ACIDS CONTRIBUTE TO THE ACTIVITY OF ANTIMICROBIAL PEPTIDES

Jindal MH, Le CF, Mohd Yusof MY, Sekaran SD

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur.

Correspondence:
Prof. Dr. Shamala Devi Sekaran
Department of Medical Microbiology,
Faculty of Medicine,
University of Malaya, 50603 Kuala Lumpur.
Email: shamalamy@yahoo.com

ABSTRACT

Antimicrobial peptides (AMPs) have gained increasing attention as a potential candidate in the development of novel antimicrobial agent. Designing AMPs with enhanced antimicrobial activity while reducing the cell toxicity level is desired especially against the antibiotic-resistant microbes. Various approaches towards the design of AMPs have been described and physicochemical properties of AMPs represent the primary factors determining the antimicrobial potency of AMPs. The most common parameters include net charge and hydrophobicity, which greatly influence the antimicrobial activity of AMPs. Moreover, certain amino acids would have critical importance in affecting the antimicrobial activity as well as cell cytotoxicity of AMPS. In this review, net charge, hydrophobicity, and specific amino acid residues were discussed as factors contributing to the antimicrobial activity of AMPs.

Keywords: *Antimicrobial peptides, antibiotic-resistant, net charge, hydrophobicity*

Introduction

Living organisms are constantly interacting with other life forms in their surrounding complex environments, which include potential harmful challenges by other pathogenic life forms. The host's first line of immune defense must be able to recognize and destroy any potential invasion efficiently. It is desirable to have the pathogens eliminated without the unnecessary activation of the secondary immune mechanism to prevent overwhelming immune responses in the host (1).

Antimicrobial peptides (AMPs) are an essential innate immune component produced by a wide range of multicellular organisms (2). AMPs are relatively small in size (generally 12–50 amino acid residues long), possess multiple cationic amino acids with an overall net charge ranging from +2 to +9, and are amphipathic (containing ~50% hydrophobic residues) (3). AMPs can be classified into four major classes, namely, β-sheet structures stabilized by two or three disulphide bridges, α-helices, extended helices (polyprohelices) with a predominance of one or more amino acids, and loop structures (4). These molecules act as natural antibiotics against a wide variety of microorganisms (bacteria, fungi, parasites and virus) and they induce killing in a short contact time (5). Despite diversed sequence and secondary structures , the biological activity of AMPs have been characterized with sets of physicochemical traits and universal structural signatures (6).

The rising problem of antibiotic-resistance microorganisms to conventional antibiotics has prompted many researchers to develop AMPs as candidates of novel antibiotics (7-10). Microbial agents showed less efficiency in developing effective resistance mechanisms against AMPs than against classical antibiotics (11). Moreover, AMPs can act synergistically with classical antibiotics to improve their therapeutic activity (12). Large collections of AMPs both extracted from the natural sources and the engineered peptide variants have been documented to date and the number is expected to expand continuously in the near future.

Three major theories describing the killing of target microbes by AMPs have been postulated: 1) The loss of microbial viability might be due to the snowballing effects of energy exhaustion following equilibration of intracellular and extracellular ion concentrations through the disrupted membrane (10); 2) AMPs might create pores that admit water but do not allow osmotically active substances

to pass. The entry of water causes buildup of excessive osmotic pressure that eventually stretches and breaks the microbial membrane (7);3) AMPs could also penetrate the target cell through the disrupted membrane, bind to the intracellular molecules and disrupt their metabolic function (13). These unique mechanisms of action enable AMPs to avoid the common resistance mechanisms observed for conventional antibiotics (14). The mechanism of actions and selectivity of AMPs are suggested to be influenced by several physicochemical properties which includes charge net charge, hydrophobicity, and specific amino acid present in the peptide sequence. In this review, the influences of these three factors on the antimicrobial activity of AMPs will be discussed.

Net Charge

The most widely accepted model of antimicrobial by AMPs is the non-receptor mediated membrane lytic mechanism (15-16). The differences between prokaryotic and eukaryotic membranes enable selective targeting of AMPs against the microbes but not the host cells (17). In most cases, electrostatic interaction represents the main attraction force motivates the first contact between AMPs and microbes (4,18-20). All biological membranes are composed primarily of proteins and phospholipids. Unlike cell membranes of animals, microbial membranes are rich in anionic phospholipids, a characteristic property favored by AMPs. For instance, the presence of anionic teichoic acids (TAs) and LTAs in Gram-positive bacterial cell wall and lipopolysaccharides in Gram-negative bacterial cell wall attract the positively charged antimicrobial peptides to bind to the microbial cells membranes and make them preferred by AMPs over the mammalian cells membranes (17). On the contrary, the cell membranes of animals are rich in neutral phospholipids and cholesterol, substances that inhibit the integration of these peptides into membranes and the formation of pores. The difference in membrane composition between prokaryotic and eukaryotic cells represent the main contributor to AMPs cell selectivity against microbial pathogens while cytotoxicity against eukaryotic cells usually occur at higher concentrations of peptide (21).

Cationicity as the main factor describing the antimicrobial activity of AMPs have been documented in a number of studies (Table 1). Ringstad *et al*. found that the ability of peptides to disrupt microbial membranes increased with increasing net charge and hydrophobicity, and *vice versa* (22). Matsuzaki *et al*. studied the effect of net charge on the antimicrobial activity of Magainin 2, an AMP isolated from the amphibian *Xenopuslaevis*skin. Based on the parent peptide four analogs (MG0, MG2+, MG4+, and MG6+) have been designed with net charges ranging from 0 to +6. Peptides with higher positive charges were correlated with enhanced binding affinity against the negatively charged artificial membranes, suggesting that net charge

Table 1. *Antimicrobial activity of AMP analogues designed with variable net charge property.*

Peptide name	Peptide sequence	Net charge	Antimicrobial activity						Reference
			Radial diffusion assay at 100 μM (estimated to the nearest zone, mm)						
			P. aeruginosa		*B. subtilis*				
CNY21	CNYITELRRQHARASHLGLAR	+3	4.5		6.2				Ringstad *et al.*, 2007 (22)
CNY21L	CNYITELRRQLARASLLGLAR	+3	4.6		4.7				
CNY21K	CNYITELRRQKARASKLGLAR	+5	5.3		7.2				
CNY21R-S	CNYITELSSQHASASHLGLAS	-1	Not determined		Not determined				
MG0	GIGKFLHSAEEWGKAFVGEIMNS	0	No MIC data, based on artificial membrane analysis						Matsuzaki *et al.*, 1997 (23)
MG2+	GIGKFLHSAEKWGKAFVGEIMNS	+2							
MG4+	GIGKFLHSAKKWGKAFVGEIMNS	+4							
MG6+	GIGKFLHSAKKWGKAFVGQIMNSamide	+6							
			MIC (μM)						
			E. coli O111:B4	E. coli D21	E. coli O26:B6	A. baumannii	P. aeruginosa	S. aureus	
K5L7	KKLLKLLLKLLK-NH2	+6	25	33	25	50	37.5	37.5	Rosenfeld, Lev, & Shai, 2010 (24)
C6-K5L7	CH3(CH2)4CO-KKLLKLLLKLLK- NH2	+5	12.5	6.25	12.5	10	10	6.25	
C8-K5L7	CH3(CH2)6CO-KKLLKLLLKLLK- NH2	+5	6.25	4.5	6.25	4.5	3	3.12	
K7L5	KKLLKKLKKLLK- NH2	+8	50	5.	50	>100	>100	>100	
C6-K7L5	CH3(CH2)4CO-KKLLKKLKKLLK- NH2	+7	25	25	12.5	37.5	37.5	50	
C8-K7L5	CH3(CH2)6CO-KKLLKKLKKLLK- NH2	+7	12.5	10	6.25	19	15	20	
K9L3	KKKLKKLKKKLK- NH2	+10	50	50	50	>100	>100	>100	
C6-K9L3	CH3(CH2)4CO-KKKLKKLKKKLK- NH2	+9	50	50	50	100	100	100	
C8-K9L3	CH3(CH2)6CO-KKKLKKLKKKLK- NH2	+9	50	50	50	50	50	100	
			MIC (μg/ml)						
			P.aeruginosa	***E.coli***	***S.typhimurium***	***S.aureus***	***S.epidermidis***	***B.subtilus***	
+1	Ac–KWKEFLKEFKEAKKEVLHEALKAISEamide	+1	>64	32	>64	>64	64	32	Jiang *et al.*, 2009 (25)
+4E	AcKWKEFLKTFKEAKKEVLHTALKAISSamide	+4	8	8	4	>64	16	32	
V13K	AcKWKSFLKTFKSAKKTVLHTALKAISSamide	+7	4	8	4	32	8	32	
+8	AcKWKSFLKTFKSAKKKVLHTALKAISSamide	+8	8	8	4	>64	8	32	
+9	AcKWKSFLKTFKSAKKKVLHKALKAISSamide	+9	4	8	4	>64	8	16	

Abbreviation: MIC, minimum inhibitory concentration.

plays an important role in the binding process (23). In addition, Rosenfeld *et al*. demonstrated that increasing the peptide's net charge enhanced both antimicrobial and LPS neutralization activities of synthetic AMPs (24). Moreover, Jiang *et al*. showed that net charge has an imperative effect on the antimicrobial activity of L-V13K peptides (25). Decreasing the net charge of L-V13K analogs to levels below +4 reduce antimicrobial and hemolytic activity, and increasing the net charge from +4 to +8 can increase the antimicrobial activity (25). However, increasing the net charge to above +9 improved the antimicrobial activity but dramatically increased the undesired hemolytic activity (25).

Although cationicity of AMPs can be elevated by inserting/substituting the native residues with positively charged amino acids, this can also be achieved by reducing the proportion of negatively charged residues. Ueno *et al*. designed the three synthetic analogues NP1P, NP2P, and NP3P by using the acid-amide substitution approach, which replaced the acidic Asp and Glu to the neutral amidated residues (Asn, Gln) (26). This method prevents the potential dramatic structural changes to the peptides following substitution of unrelated amino acids. A gain in cationicity was thus achieved with indirect reduction of peptide's total negative charge. Interestingly, the newly generated peptides displayed substantial increase in antibacterial activity against both Gram-positive and Gram-negative bacteria *Bacillus subtilis*, *S. aureus*, *S. typhimurium*, *P. aeruginosa, E. coli*, and *Serratia marcescens*.

Hydrophobicity

Due to the presence of non-polar amino acid residues in the sequence, AMPs are commonly characterized by the hydrophobicid properties. Hydrophobicity rules the ability of AMPs to partition into the lipid bilayer of microbial membrane. However, increasing levels of hydrophobicity are strongly associated with mammalian cell toxicity and loss of antimicrobial specificity (17). Thus, careful consideration is necessary when altering the peptide hydrophobicity.

To investigate hydrophobicity as a function of antimicrobial activity, Wieprecht *et al*. generated a series of magainins analogues to which charge, helicity, and hydrophobic moment parameters were kept invariable while only the hydrophobicity of the peptides was systematically altered (27) (Table 2). They revealed that increase the peptide hydrophobicity can enhance antimicrobial activity although it was also correlated with hemolytic activity. Furthermore, peptides differing in hydrophobicity were found to display different spectrum of antibacterial activity (27). As for *P. aeruginosa*, the specificity of this class of

Table 2. *Antimicrobial activity of AMP analogues designed with variable hydrophobicity.*

Peptide name	Peptide sequence	Hydrophobicity[a]	Antimicrobial activity			Reference
			E. coli	*P. aeruginosa*		
$L^2R^{11}A^{20}$-M2a	GLGKFLHSAKRFGKAFVGEAMNS	-0.091	75	>75		Wieprecht *et al.*, 1997 (27)
M2a	GIGKFLHSAKKFGKAFVGEIMNS	-0.091	38	76		
I^6L^{15}-M2a	GIGKFIHSAKKFGKLFVGEIMNS	0.200	19-38	76		
$I^6A^8L^{15}I^{17}$-M2a	GIGKFIHAAKKFGKLFIGEIMNS	0.326	2.4	19-38		
			MIC (μM)			
			P. aeruginosa			
6K-F17	KKKKKKAAFAAWAAFAA-NH2	-0.253	4			Yin *et al.*, 2012 (29)
6K-F17-4L	KKKKKKALFALWLAFLA-NH2	0.218	16			
3K-F17-3K	KKKAAFAAWAAFAAKKK-NH2	-0.253	32			
3K-F17-4L-3K	KKKALFALWLAFLAKKK-NH2	0.218	8			
			MIC (μg/ml)			
			S. aureus	B. subtilis	E. coli	
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH2	0.273	0.72	0.18	11.4	Yan *et al.*, 2003 (30)
Mel(12-26)	GLPALISWIKRKRQQ-NH2	-0.607	5.6	5.6	88.9	
Mel(12-26, L^1)	LLPALISWIKRKRQQ-NH2	-0.327	1.1	2.2	70.8	
Mel(12-26, S^2)	GSPALISWIKRKRQQ-NH2	-0.913	90	67.5	>270	
Mel(12-26, L^3)	GLLALISWIKRKRQQ-NH2	-0.247	1.2	2.2	70.8	
Mel(12-26, S^3)	GLSALISWIKRKRQQ-NH2	-0.553	3.1	1.6	6.25	
Mel(12-26, L^4)	GLPLLISWIKRKRQQ-NH2	-0.473	1.3	2.4	76.7	
Mel(12-26, S^5)	GLPASISWIKRKRQQ-NH2	-0.913	250	31.2	>250	
Mel(12-26, S^6)	GLPALSSWIKRKRQQ-NH2	-0.960	14.6	39	311	
Mel(12-26, L^7)	GLPALILWIKRKRQQ-NH2	-0.300	1.4	0.64	81.7	
Mel(12-26, L^8)	GLPALISLIKRKRQQ-NH2	-0.293	13.5	10.0	161	
Mel(12-26, S^8)	GLPALISSIKRKRQQ-NH2	-0.600	38.2	76.4	>305	
Mel(12-26, S^9)	GLPALISWSKRKRQQ-NH2	-0.960	94.2	70.8	>94.4	

[a]Calculated based on Grand Average of Hydrophobicity (GRAVY) using ExPASyProtparam tool (http://web.expasy.org/protparam/).
Abbreviation: MIC, minimum inhibitory concentration.

magainins analogues appeared to reduce with increasing hydrophobicity, but this was not the case for *E. coli*.

Chen *et al*. investigated the role of hydrophobicity in the antimicrobial activity of the α-helical AMPs by systematically decreasing or increasing the hydrophobicity of a synthetic V13KL peptide (28). They noted that, decreasing peptides' hydrophobicity was associated with reduced antimicrobial activity. Improving the antimicrobial activity of the peptides by enhancing the hydrophobicity was achievable, however, only up to a certain level. Deviation from the specific hydrophobicity window would result in the significant loss of antimicrobial activity with the peptides (28). This occurs most likely due to increased peptide dimerization, which prevent the access of peptide monomers on microbial cell membranes. The lower hydrophobic variant 6K-F17 designed by Yin *et al*. was found to possess a four-fold higher antipseudomonal activity as compared to the Ala-substituted analogue 6K-F17-4L (29). Furthermore, 6K-F17 displayed minimal hemolytic activity up to the concentration of 320 μM as compared to 6K-F17-4L which showed 40 – 80% hemolysis at the same level. In addition, their data suggested that peptides with higher hydrophobicity have stronger self-association and aggregation tendencies than those with lower hydrophobicity level (29).

Yan *et al*. extracted 15 residues from the C-terminal segment of melittin and systematically altered the hydrophobicity of the peptides via individual residual substitution. The investigators found that increased in hydrophobicity but not amphipathicity enhanced antimicrobial activity (30). Moreover, they noticed that the effect of changing the individual residues was stronger at specific positions in the sequence (30). Interestingly, the antimicrobial and hemolytic activities were suggested to favor the opposite faces and this indicates that antimicrobial activity could be dissociated from the side effects with careful consideration on region/face of the peptides (30). Cornut *et al*. found that membrane lysis increased with increasing of peptide hydrophobicity (31). Notably, our preliminary results also underlined the antipneumococcal-enhancing effect by increasing the hydrophobicity of a series of peptides with fixed sequence length. We also emphasized the importance of optimum hydrophobicity window to which higher or lower hydrophobicity levels would impair the antimicrobial activity of the peptides. Hydrophobicity has also been found to be a key parameter governing the antimicrobial activity of AMPs in a number of quantitative structure–activity-relationship investigations. However, this physicochemical property shall be manipulated carefully to avoid increase of the unwanted hydrophobicity-associated peptides' toxicity on human cells(32).

Presence of specific amino acids residues

Many antimicrobial peptides encompass an unusual composition of amino acids. One group of particular interest is peptides with high content of Arginine (Arg) and Tryptophan (Trp) (33) (Table 3). Beside their importance in antimicrobial peptides, Trp play a crucial role in membrane spanning proteins, as Trp has a strong preference for the interfacial regions of lipid bilayers(34). Additionally, antimicrobial peptides with high content of Arg and Trp were found to possess the highest antimicrobial activities (35).The Arg side chain is capable of forming almost as many hydrogen bonds with the surrounding water molecules as when it is not involved in any cation–π interactions. This is in contrast to Lysine (Lys), which cannot form hydrogen bonds while engaged in cation–π interactions with an aromatic residue (36). This difference is responsible for the increased activity of Arg containing peptides over Lys substituted peptides. Many studies well documented that Trp residues have a preference for the interfacial region of lipid bilayers.

Lawyer and his fellow workers (37) tested the antimicrobial activity of 13 amino acid Trp-rich peptide and found that the peptide has strong antimicrobial activities against various Gram-positive and negative bacteria and fungi. In addition, Torcato *et al*. (12) studied the effect of Trp and Arg on the antimicrobial activity of peptides against Gram-positive and Gram-negative bacteria. In their study, they designed two new peptides RW-BP100 and R-BP100 based on previously designed peptide BP100 by the same group of researchers. In these two analogues they replaced Tyrosine (Tyr) with Trp, and Lys with Arg. Their results showed that the new peptides have stronger activity against both Gram-negative and Gram-positive bacteria. In general, the cationic nature and unique hydrogen bonding geometry of Arg and the complex properties of Trp enhance the ability of AMPs to interact and destroy the microbial membrane. The positive charge of Arg effectively increases attracting ability of the peptide to the target membranes, and hydrogen bonding assists its interaction with negatively charged surfaces. Trp represents the most suitable amino acid to enable the peptide to associate with the target membrane (33).

Functional group alteration also represents a potential strategy to enhance the antibacterial potency of peptides and to increase the proteolytic resistibility of the peptides (38). The strong antibacterial and fungicidal activities of Thanatin can be attributed to the four specific peptide regions: the C-terminal loop, the three residue extension at C-terminus, presence of seven hydrophobic residues at the N-terminus, and three N-terminal residues which is indispensable for antifungal activity (39). A C-terminally amidated thanatin which is an insect-derived *Podisusmaculiventris* AMP showed strong antibacterial effect against Gram-negative bacteria, in particular, the extended-spectrum B-lactamase-producing *E. coli*. (38-39). This peptide also conferred dose-dependent therapeutic efficacy with survival rates of 50.0%, 66.7%, 91.7% following low-, medium-, and high-dose A-thanatin treatment in a mice septicaemic model as compared to 0% survival at day 2 post treatment using ampicillin (38).

Table 3. *AMP analogues designed to investigate the role ofspecific amino acids in relation to antimicrobial activity.*

Peptide name	Peptide sequence	Antimicrobial activity								Reference
		MIC (μM)								Torcato *et al.*, 2013 (12)
		E. coli	*K. pneumoniae*	*P. aeruginosa*		*S. aureus*	*S. pneumoniae*	*E. faecium*		
RW-BP100	RRLFRRILRWL-NH2	0.2±0.1	0.3±0.0	2.5±0.5		0.3±0.1	0.4±0.1	1.0±0.0		
R-BP100	RRLFRRILRYL-NH2	0.4±0.1	0.6±0.3	2.0±0.0		3.0±0.6	4.0±0.0	2.0±0.0		
BP100	KKLFKKILKYL-NH2	0.7±0.2	4.0±0.0	8.0±0.0		16.0±0	16.0±0.0	16.0±0.0		
		Aspergillusfumigatus			*Candida albicans*					Lawyer *et al.*, 1996 (37)
1	VRRFPWWWPFLRR	250			1000					
2	RRRFPWVCWPFLRRR	250			1000					
3	RFPWWWPFLR	500			>1000					
4	FPWWWPF	500			>1000					
		MIC (μg/ml)								Hou *et al.*, 2011 (38)
		S. aureus	*E. coli* ATCC25922	*E. coli* ATCC35218	*E.coli* HN10349	*E.coli* HN10318	*E.coli* SX49660	*E.coli* XJ74283	*E.coli* JT11092	*K. pneumoniae*
A-thanatin	GSKKPVPIIYCNRRTGKCQRM	256	16	4	2	2	4	8	2	4

Abbreviation: MIC, minimum inhibitory concentration.

Discussion

In the face of increasingly common antibiotic-resistant microbes, novel antimicrobial agents are urgently needed to serve as standalone therapeutics or in combination to support the use of conventional antibiotics. AMPs possess multiple advantages in term of its potent and rapid bactericidal activity while also being broad spectrum to serve as the candidate for alternative drug development. The design of new synthetic analogues of AMPs mainly focus on one common goal – better therapeutic index with higher antimicrobial activity and lower cell toxicity levels. This will require careful and systematic design strategy. The physicochemical properties of AMPs represent important factors to be considered as net charge, hydrophobicity, and the specific amino acids presence would affect the antimicrobial activity of the peptides. More studies have been documented in support of the notion that increasing net charge (to a certain limit) shall be followed. The same principle goes to the hydrophobicity of the peptides. Moreover, certain amino acids are responsible for the antimicrobial activity but not cytotoxicity of the peptides and vice versa. Although there are many factors to be considered in designing AMPs, one should not interpret these factors independently, but to consider all factors within the same formulation.

Although thousands of peptides have been discovered and synthesized in the past decades, only limited fraction of them have been studied and tested for potential development. Several AMPs possess excellent antimicrobial activity *in vitro*. However, like any new class of drugs, AMPs need to gain approval through multiple clinical assessments and trials before being brought into the actual clinical uses by the pharmaceutical companies. These challenges include drug stability *in vivo*, associated toxicity to human cells, demonstration of good antimicrobial activity, and the relatively high costs in peptide-antibiotic development and manufacturing.

Up to date, only limited success has been achieved with those AMPs that have entered into clinical trials and to the best of our knowledge, none have obtained FDA agreement for clinical use. Despite the fact that AMPs are essential components of the host innate immune system against microbial pathogens, their possibilities as a new class of therapeutic agents still remain to be proven. Nevertheless, the eventual goal to develop AMPs into clinically useful drug should not have just been hampered by the obstacles and further exploration in the field should continue.

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