Hydrophilic/hydrophobic characters of antimicrobial peptides derived from animals and their effects on multidrug resistant clinical isolates

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ABSTRACT

Multidrug resistant (MDR) pathogen infections are serious threats to hospitalized patients because of the limited therapeutic options. A novel group of antibiotic candidates, antimicrobial peptides (AMPs), have recently shown powerful activities against both Gram-negative and Gram-positive bacteria. Unfortunately, the viability of using these AMPs in clinical settings remains to be seen, since most still need to be evaluated prior to clinical trials and not all of AMPs are potent against MDR clinical isolates. To find a connection between the characteristics of several of these AMPs and their effects against MDR pathogens, we selected 14 AMPs of animal origin with typical structures and evaluated their *in vitro* activities against clinical strains of extensive drug-resistant *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus*, extended spectrum β-lactamase-producing *Pseudomonas aeruginosa* and extended spectrum β-lactamase-producing *Escherichia coli.* Our results showed that these peptides' hydrophilic/hydrophobic characteristics, rather than their secondary structures, may explain their antibacterial effects on these clinical isolates. Peptides that are amphipathic along the longitudinal direction seemed to be effective against Gram-negative pathogens, while peptides with hydrophilic terminals separated by a hydrophobic intermediate section appeared to be effective against both Gram-negative and Gram-positive pathogens. Among these, cathelicidin-BF was found to inhibit all of the Gram-negative pathogens tested at dosages of no more than 16 mg/L, killing a pandrug-resistant *A. baumannii* strain within 2 h at 4×MICs and 4 h at 2×MICs. Tachyplesin III was also found capable of inhibiting all Gram-negative and Gram-positive pathogens tested at no more than 16 mg/L, and similarly killed the same *A. baumannii* strain within 4 h at 4×MICs and 2×MICs. These results suggest that both cathelicidin-BF and tachyplesin III are likely viable targets for the development of AMPs for clinical uses.

Keywords: Hydrophilic/hydrophobic character; Multidrug resistant clinical isolate; Cathelicidin-BF; Tachyplesin III

INTRODUCTION

Multidrug resistant (MDR) pathogens continue to pose serious threats to hospitalized patients because there are limited effective therapies capable of combatting the infection. Among these pathogens, *Acinetobacter baumannii* is particularly intractable because several of its clinical isolates have gained resistance to nearly all currently available antibiotics, leading it to be described as "extensive drug resistant" (XDR) (Falagas & Karageorgopoulos, 2008). Though work on developing novel cocktails of antibiotics that can be used in tandem may help in the short-run, a long-term solution is urgently needed.

One promising candidate source of novel antibiotic treatments, antimicrobial peptides (AMPs), have gained increased attention. These AMPs, which are innate immune

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molecules that are widely distributed among animals, have previously exhibited powerful killing effects to both Gram-negative and Gram-positive bacteria. Unfortunately, clinical applications of these AMPs have made relatively little progress over the last decade and have been rarely reported (Hancock & Sahl, 2006; Lipsky et al, 2008; Vaara, 2009). The reasons for this lack of progress are multifaceted; aside from the instability of these peptides *in vivo*, three further reasons warrant some explanation. First, not all of the currently AMPs reported are potent AMPs. Traditionally, AMPs are purified directly from tissues following their activities, which may assure their antimicrobial activities. Currently, a growing number AMPs are simply predicted via bioinformatics, and then sometimes synthesized as peptides to test for antimicrobial activities. Typically, these synthetic AMPs are found to exhibit comparatively weak antimicrobial effects. Second, the structures of AMPs are highly diversified. It is nearly impossible to test the antimicrobial activities of these AMPs one by one, largely due to the intensive time and financial requirements. To get around this obstacle, it is usually necessary to select typical AMPs and then estimate the antibacterial activities of their corresponding groups, of which four predominate: helix, beta-sheet formed with 2-3 disulfide bridges, linear peptides rich in special amino acids and loop peptides formed by one disulfide bridge (Vaara, 2009). Third, the primary laboratory screening procedure of AMPs usually involves testing their antibacterial activities on several type culture strains (e.g., American Type Culture Collection, or ATCC strains) or several randomly selected clinical isolates. Though occasionally insightful, these findings are rarely translatable to combatting clinical MDR strains.

To date, more than 2 300 AMPs have been discovered, and more are constantly being reported (http://aps. unmc.edu/AP/main.php). This growing pool of usable materials is quite timely (Wang et al, 2009). In the present study, we have attempted and elucidate some relationship between the characters of several discovered AMPs and their activities against MDR clinical pathogens, so as to provide clues to the screening procedure of clinically valuable candidates. Here, we selected 14 AMPs of animal origin with typical structures to test their *in vitro* effects on XDR *A. baumannii*, methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum β-lactamase (ESBL)-producing *Pseudomonas aeruginosa,* and ESBL-producing *Escherichia coli*.

MATERIALS AND METHODS

Ethics

The protocols of this study were approved by the ethics committee of Kunming Medical University and ethics committee of Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College. Isolation of multidrug resistant clinical strains were undertaken with the informed and written consent of each patient. The study methodologies conformed to the standards set by the Declaration of Helsinki and all other relevant national and international regulations.

Bacterial strains

XDR *A. baumannii* strains Ab5753 and Ab5755 were isolated from sputum samples of two different ICU patients in the First Affiliated Hospital of Kunming Medical University. All other strains were isolated from patients at the Second Affiliated Hospital of Kunming Medical University. These strains tested in this study include the following: (I) XDR *A. baumannii* strain Ab1408 isolated from the sputum sample of one oncology department patient; (II) MRSA strain Sa1390 isolated from the sputum sample of one surgery intensive care unit (SICU) patient; (III) ESBL *P. aeruginosa* strains Pa1409 and Pa4216 isolated from the sputum samples of two different oncology department patients; and (IV) ESBL *E. coli* strain Ec513 isolated from the blood sample of one general surgery department patient. Bacterial identification was performed using a Vitek 32 system (bioMérieux, France).

Antimicrobial peptides and antibiotics

All tested AMPs were supplied by GL Biochem Ltd (Shanghai, China), and had a purity ≥95%. The tested AMPs were dissolved in water at 2 mg/mL and stored at −20 °C before use. Determining whether the AMPs are amidated on the C-terminus or not was made through the previously published literature (Table 1). Antibiotics used as references were supplied by Sigma-Aldrich, and dissolved in water at 2 mg/mL prior use, and included colistin sulfate salt (colistin), vancomycin hydrochloride hydrate (vancomycin), and tigecycline hydrate (tigecycline).

Susceptibility testing

The minimal inhibitory concentrations (MICs) of all AMPs and antibiotic references to clinical isolates were determined by broth dilution method in MHII following Clinical and Laboratory Standards Institute (CLSI) recommendations (Clinical and Laboratory Standards Institute, 2013).

Time-killing curves

Time-kill studies of tachyplesinIII, cathelicidin-BF, colistin, and tigecycline on XDR *A. baumannii* strain Ab1408 with initial inocula between 1×10^6 and 1×10^7 CFU/mL were performed using 4×MICs, 2×MICs, 1×MIC, and 0.5×MIC concentrations. Samples were taken at 0, 0.5, 2, 4, 8, and 24 h after incubation. The effects of drug carryover were addressed via three dilution steps. Only plates with between 30 and 300 colonies were counted. An antibiotic was considered bactericidal when a reduction of 3 $\log_{10}$ CFU/mL was achieved, as compared with the initial inocula (Isenberg, 2004). These tests were each performed in duplicate.

Antimicrobial peptide structure prediction

Human LL-37 and beta-defensin-2—both of which are longer than 30 amino acids—were sourced directly from NCBI PDB database PDB 2K6O and PDB 1FD3. The other 12 AMPs were predicted by the structure prediction software PEP-FOLD at http://bioserv.rpbs. univ-paris-diderot.fr/PEP-FOLD/ (Thévenet et al, 2012), and model 1 of each peptide with the best conformation was selected for further analysis.

Table 1 Peptides tested for antimicrobial effects against drug resistant bacteria

Name	Sequence	Structure	Source and references
CA(1-7)M(2-9)	KWKLFKKIGAVLKVL-NH_2	Helix	*Hyalophora cecropia* (silk moth)+*Apis mellifera* (bee venom) (Giacometti et al, 2003)
[E4K]Alyteserin-1c	GLKKIFKAGLGSLVKGIAAHVAS-NH_2	Helix	*Alytes obstetricans* (midwife toad) (Conlon et al, 2009)
[D4K]B2RP	GIWKTIKSMGKVFAGKILQNL-NH_2	Helix	*Lithobates septentrionalis* (mink frog) (Conlon et al, 2010)
Cathelicidin-BF	KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF	Helix	*Bungarus fasciatus* (banded krait) (Wang et al, 2008)
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN LVPRTES	Helix	*Homo sapiens* (Thomas-Virnig et al, 2009)
Cramp	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVP QPEQ	Helix	*Mus musculus* (house mouse) (Chromek et al, 2006)
Oncocin	VDKPPYLPRPRPPRRIYNR-NH_2	Linear, Proline/Arginine rich	*Oncopeltus fasciatus* (milkweed bug) (Knappe et al, 2010)
Indolicidin	ILPWKWPWWPWRR-NH_2	Linear, Tryptophane rich	*Bos taurus* (cattle) (Selsted et al, 1992)
Histatin	AKRHHGYKRKFH-NH_2	Linear, Histatine rich	*Homo sapiens* (Giacometti et al, 2005)
Thanatin	GSKKPVPIIY*C*NRRTGK*C*QRM[a]	Loop, one disulfide bridge	*Podisus maculiventris* (spined soldier bug) (Pagès et al, 2003)
Ranalexin-1Ca	FLGGLMKAFPALI*C*AVTKK*C*[a]	Loop, one disulfide bridge	*Rana clamitans* (green frog) (Halverson et al, 2000)
Tachyplesin III	KW*C*FRVCYRGICYRK*C*R-NH_2[a]	Beta-sheet, two disulfide bridges	*Tachypleus gigas* (Southeast Asian horseshoe crab) (Cirioni et al, 2007)
Beta-Defensin-2	GIGDPVT**C**LKSGAI*C*HPVFCPRRYKQIG-TC*G*LPGTK**C**CKKP[a]	Beta-sheet, three disulfide bridges	*Homo sapiens* (Routsias et al, 2010)
Alpha-defensin-2	LRDLV**C**YCRTRGCKRRERMNGTC*R*KGH LMYTLC**C**R[a]	Beta-sheet, three disulfide bridges	*Mus musculus* (house mouse) (Ouellette et al, 1992)

[a]: For antimicrobial peptides with disulfide bridges, cysteine (C) with the same type font or underlined formed one disulfide.

RESULTS

Minimal inhibitory concentrations (MICs)

All MICs are listed in Table 2. We found that all three *A. baumannii* strains were resistant to colistin according to CLSI standards (i.e., ≥4 mg/L). According to the susceptibility/ resistance breakpoints of tigecycline, as interpreted by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (susceptible, ≤1 mg/L; resistant, ≥4 mg/L) (The European Committee on Antimicrobial Susceptibility Testing (EUCAST) Steering Committee, 2006), Ab5753 has intermediate resistance to tigecycline (XDR) whereas Ab5755 and Ab1408 are resistant to tigecycline (Pandrug resistance, PDR). Four AMPs, including CA(1-7)M(2-9), [D4K] B2RP, cathelicidin-BF, and Tachyplesin III, showed MICs between 4 and 16 mg/L to all of the three *A. baumannii* strains. Given that the molecular weights of these peptides (from 1 770 to 3 638) are about three times to six times that of tigecycline (586), these peptides are considered as effective as tigecycline in inhibiting XDR *A. baumannii*.

ESBL *E. coli* and *P. aeruginosa* are still sensitive to colistin, but the two *P. aeruginosa* strains were resistant to tigecycline. Four AMPs, namely, CA(1-7)M(2-9), [D4K]B2RP, cathelicidin-BF, and tachyplesinIII, showed MICs of between 4 and 8 mg/L to Ec513. By contrast, only two AMPs, namely, cathelicidin-BF and tachyplesin III, showed MICs between 4 and 8 mg/L to Pa4216 and Pa1409. This phenomenon is consistent with the results on tigecycline; that is, ESBL *P. aeruginosa* is more resistant than ESBL *E. coli* is.

MRSA strain Sal1390 is sensitive to vancomycin, but intermediately resistant to tigecycline. Three AMPs, namely, CA(1-7)M(2-9), [D4K]B2RP, and tachyplesin III, showed MICs of 16 mg/L to this MRSA strain. Notably, [E4K]Alyteserin-1c and cathelicidin-BF, which showed a generally effective inhibitory activity on all MDR Gram-negative bacteria, had no effect on Gram-positive MRSA.

Time-killing kinetics

Figure 1 shows the time-killing kinetics. Cathelicidin-BF kills XDR *A. baumannii* strain Ab1408 within 4 h at 2×MICs and 2 h at 4×MICs respectively. This is similar to the action of colistin, i.e., Cathelicidin-BF was also able to rapidly kill bacteria. By comparison, tachyplesin III kills XDR *A. baumannii* after 4 h of incubation at 2×MICs and 4×MICs. Compared with colistin, tigecycline showed a markedly slower killing effect (after 8 h of incubation at 2×MICs and 4×MICs), but this effect lasted longer (24 h at 4×MICs). The optimum killing effects of cathelicidin-BF and tachyplesin III appeared to be 4 h of incubation, and afterward gradually declined.

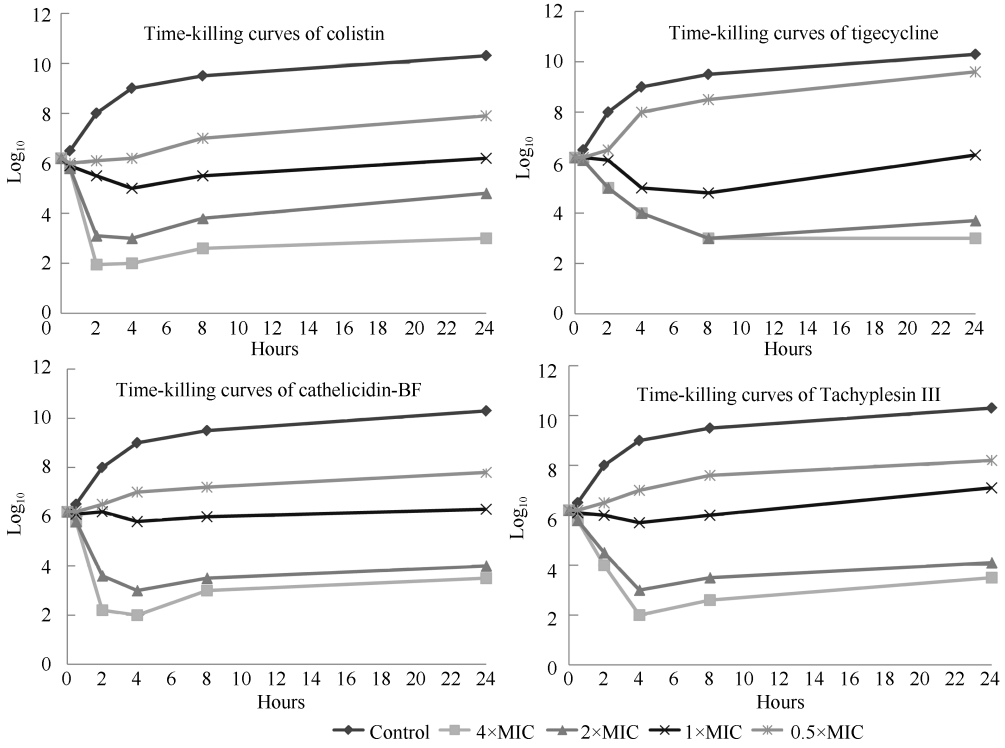

Figure 1 **Time-killing curves of colistin, tigecycline, cathelicidin-BF, and tachyplesin III on one XDR *Acinetobacter baumannii* strain (Ab1408)**

Initial inocula between 1×10^6 and 1×10^7 CFU/mL were performed using 4×MICs, 2×MICs, 1×MIC, and 0.5×MIC concentrations. Samples were obtained at 0, 0.5, 2, 4, 8, and 24 h after incubation.

Amphipathic structure distributions of AMPs

Although CA(1-7)M(2-9), [D4K]B2RP, [E4K]Alyteserin-1c, cathelicidin-BF, LL-37, and cramp are all classified as helical peptides by their secondary structures, these AMPs showed different antibacterial effects. Since the amphipathic structure distributions are generally considered crucial for AMPs to kill bacteria (Brogden, 2005), we studied the three-dimensional structures of the eight AMPs that were found to be effective on our tested MDR clinical isolates (Figure 2). [E4K]Alyteserin-1c and cathelicidin-BF, which were effective on all three Gram-negative bacteria, both have classical long linear amphipathic structures, withhydrophobic regions on one side and hydrophilic regions on the other side along the linear peptides. Similar structures exist in LL-37 and cramp, but these AMPs only showed slight activity on a portion of these Gram-negative strains. The helical peptides of CA(1-7)M(2-9) and [D4K]B2RP, which could inhibit not only Gram-negative but also Gram-positive bacteria, tend to manifest a more contractive style, with hydrophilic terminals separated by a hydrophobic intermediate section. A similar amphipathic structure distribution can be seen in tachyplesin III, which is also effective on both Gram-negative and Gram-positive clinical isolates, but is classified as a beta-sheet formed by two disulfide bridges according to its secondary structure. Interestingly, a similar structure also exists in Ranalexin-1Ca, which only showed slight activity to Gram-positive strains, but was ineffective on Gram-negative strains.

AMPs (mainly beta-sheet AMPs like beta-defensin-2 and alpha-defensin-2 that are formed with 3 disulfide bridges, and linear AMPs like oncocin, indolicidin, histatin and thanatin, which are rich in special amino acids) with neither classical long linear amphipathic structures nor hydrophilic terminals separated by hydrophobic intermediate sections, showed no effects on the tested MDR clinical isolates (Table 2, Figure 3).

DISCUSSION

In total, more than 2 300 antimicrobial peptides have been reported to date, but as we mentioned earlier, clinical applications of these AMPs are held up by a number of roadblocks. In this study, we collected a set of AMPs with different structures to evaluate their *in vitro* effects on typical MDR clinical isolates, which included XDR *A. baumannii*, MRSA, ESBL-producing *P. aeruginosa* and *E. coli.* Our results showed that helical AMPs appear to be more effective at killing MDR bacteria than other types of AMPs. By contrast, linear AMPs rich in special amino acids and AMPs with

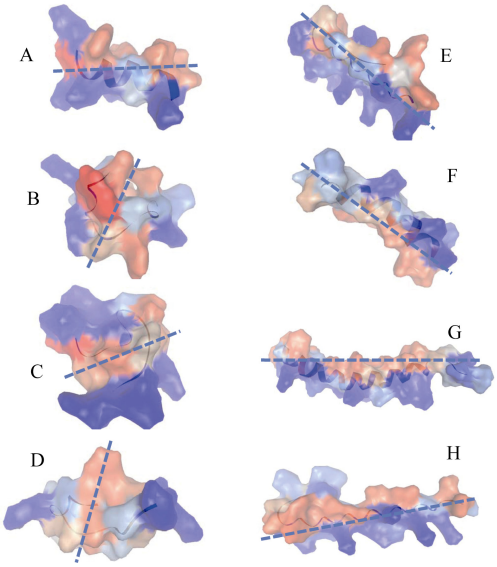

Figure 2 Structures and three-dimensional hydrophilic /hydrophobic arrangements of effective antimicrobial peptides

The backbones of these AMPs are shown in flat ribbons, and the three-dimensional surfaces of these AMPs are shown in solid. Blue: hydrophilic regions; Red: hydrophobic regions. A: CA(1-7)M(2-9); B: [D4K]B2RP; C: Tachyplesin III; D: Ranalexin-1Ca; E: cathelicidin-BF; F: [E4K]Alyteserin-1c; G: LL-37; H: cramp. Dotted lines indicate different hydrophilic/ hydrophobic region arrangement styles between A-D (both Gram-negative and Gram-positive effective, except for D, i.e., Ranalexin-1Ca, which is only Gram-positive effective) and E-H (Gram-negative effective).

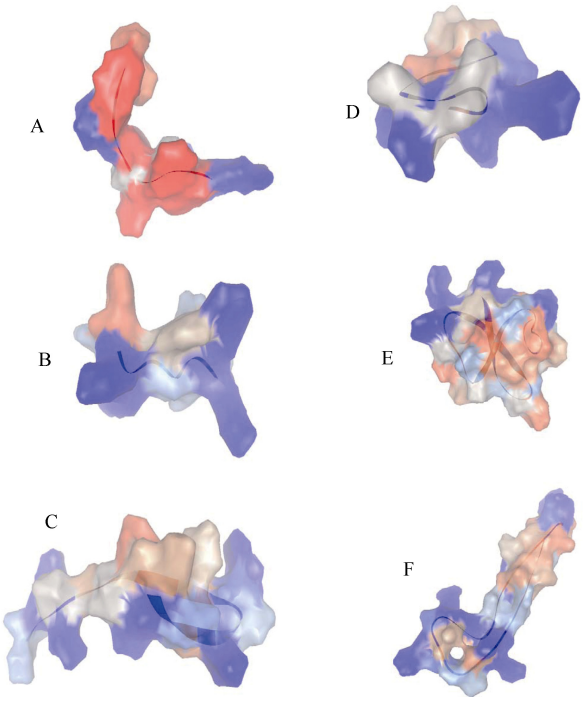

Figure 3 Structures and three-dimensional hydrophilic/ hydrophobic arrangements of ineffective antimicrobial peptides

The backbones of these AMPs are shown in flat ribbons, and the three-dimensional surfaces of these AMPs are shown in solid. Blue: hydrophilic regions; Red: hydrophobic regions. A: Indolicidin; B: Histatin; C: Thanatin; D: Oncocin; E: beta-Defensin-2; F: alpha-defensin-2.

Table 2 MICs (mg/L) of AMPs and antibiotics against drug resistant bacteria

AMPs & antibiotics	XDR *A.baumannii*			ESBLs *E.coli* & *P. aeruginosa*			MRSA
	Ab5753	Ab5755	Ab1408	Ec513	Pa1409	Pa4216	Sa1390
CA(1-7)M(2-9)	8	4	8	8	64	32	16
[D4K]B2RP	8	4	16	8	32	32	16
[E4K]Alyteserin-1c	64	8	64	32	>128	64	>128
Cathelicidin-BF	16	4	16	8	8	4	>128
LL-37	>128	32	>128	128	>128	>128	>128
Cramp	>128	128	>128	128	>128	>128	>128
Oncocin	>128	>128	>128	>128	>128	>128	>128
Indolicidin	>128	>128	>128	>128	>128	>128	>128
Histatin	>128	>128	>128	>128	>128	>128	>128
Thanatin	>128	>128	>128	>128	>128	>128	>128
Ranalexin-1Ca	>128	>128	>128	>128	>128	>128	64
Tachyplesin III	8	8	16	4	8	8	16
Beta-Defensin-2	>128	>128	>128	>128	>128	>128	>128
Alpha-defensin-2	>128	>128	>128	>128	>128	>128	>128
Colistin	>128	16	8	<1	<1	<1	>128
Vancomycin	>128	>128	>128	>128	>128	>128	2
Tigecycline	2	4	4	2	16	16	2

Abbreviations: AMP: antimicrobial peptides; MIC: minimum inhibitory concentration; XDR: extensive drug resistance; ESBLs: extended spectrum beta-lactamase producing; MRSA: methicillin-resistant *S. aureus*.

disulfide bridges (with the exception of tachyplesin III) are generally less effective in combatting MDR bacteria as compared with helical AMPs. Three-dimensional analysis showed that the hydrophilic/hydrophobic arrangements of these peptides, and not their secondary structures, seem to contribute more to their efficacy and position on the antimicrobial spectra. Peptides with long linear amphipathic structures were found to be effective only on Gram-negative pathogens, whereas peptides with more contractive styles with hydrophilic terminals separated by a hydrophobic intermediate section appeared to be effective on both Gram-negative and Gram-positive pathogens. Even though all AMPs are reported as antimicrobial peptides, only 2 of the 14 tested AMPs (tachyplesin III and cathelicidin-BF) were effective against all the tested MDR pathogens within their antimicrobial spectrum and with low MICs (≤16 mg/L). Since the net charge these AMPs carried does not correlate with their antibacterial activities (data not shown), the underlying mechanisms that lead to different MICs to kill bacteria remains to be further determined in more targeted follow-up studies.

Our results also showed that Cathelicidin-BF kills bacteria as quickly as colistin (within 4 h at 2×MICs and 2 h at 4×MICs), while Tachyplesin III is slower (after 4 h of incubation at 2×MICs and 4×MICs) than colistin, but faster than tigecycline. Considering the difference of the amphipathic structure distributions between these two peptides, the different rate of killing bacteria may imply a difference in the mechanism underlying their activities. Notably, the killing effects of these two potent peptides all declined after 4 h of incubation, which may, in part, be due to the nature of the peptides. Again, further targeted studies are needed to shed more light into the differences of these AMPs.

In conclusion, in the present study not all of the reported AMPs were effective against several tested MDR clinical isolates. Of the 14 potential AMPs, only two, Tachyplesin III and cathelicidin-BF, which differ in both their secondary structures and three-dimensional hydrophilic/ hydrophobic arrangements, showed potent activities (≤16 mg/L) against nearly all of the MDR clinical isolates within corresponding antimicrobial spectrum *in vitro*. What differentiates these two AMPs in terms of their efficacy to kill bacteria compared with other AMPs with similar hydrophilic/ hydrophobic arrangements remains to be determined.

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