Advanced Journal of Microbiology Research Vol. 2016
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Antibacterial activity and mechanism of recombinant human-defensin 5 against clinical antibiotic-resistant strains

A. P. Wang, Y. P. Su, S. Wang, M. Q. Shen, F. Chen, M. Chen, X. Z. Ran, T. M. Chen and J. P. Wang*

State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Combined Injury of PLA, Third Military Medical University, Chongqing, 400038, People's Republic of China.

Accepted 1 March, 2016

The increasing clinical bacterial strains resistant to conventional antibiotics have being a great challenge to the public's health. As a novel kind of antimicrobial agent, defensins are undoubtedly worthy of exploitation for the treatment of antibiotic-resistant bacteria. To evaluate the antibacterial potency of recombinant mature human -defensin 5 ($rmHD_5$) against clinical pathogenic strains, we examined its antibacterial kinetics and bactericidal efficacy on forty-nine bacterial strains (belonging to eleven species) with different antibiotic-resistant phenotypes, isolated from digestive and urogenital tracts of the inpatient. Meanwhile, the action mechanism of $rmHD_5$ was analyzed by transmission electron microscopy observation and membrane permeability detection. The peptide of $rmHD_5$ was found to possess high potency against all the tested isolates at concentrations of 6 - 12 g/ml for gram-negative (G^-) bacteria and 28 - 32 g/ml for gram-positive (G^+) bacteria. G^- bacteria were more susceptible to the peptide than G^+ bacteria. Abnormal morphological changes and increased permeabilization of the cytomembrane were observed in both G^- bacteria and G^+ bacteria treated with $rmHD_5$. The antibacterial activity of $rmHD_5$ may be tightly associated with the biomembrane permeabilization. Recombinant mHD_5 is a promising candidate to be developed into therapeutic agents for bacterial infections.

Key words: Antibacterial activity, mechanism, antibiotics resistant strain, human alpha defensin 5.

INTRODUCTION

Currently, the increasing morbidity and mortality caused by microbial infection are arousing the public's concern (Gastmeier and Vonberg, 2008). Notably, the rapid diffusion of drug-resistant microbial pathogens has resulted in a series of medical problems, such as an increased rate of hospitalization, elongated hospital stay and consequently increased medical cost. In light of this situation, new antibacterial drugs, especially against pathogens resistant to conventional antibiotics, are undoubtedly worthy of exploitation (Wilson, 2008).

Antimicrobial peptides possess some characteristics that make them attractive candidates as a kind of novel anti-infection agent (Hancock and Sahl, 2006). Defensins in humans are a family of Cysteine-rich cationic peptides (3 - 5 kDa). Up till now, six human -defensins and thirty human -defensins have been reported (Chen et al., 2006). The studies showed that a few defensins have distinct antimicrobial function and are involved in innate and adaptive immunity (Meyer et al., 2006; Shinwari et al., 2009). Based on the recent findings that human -defensin 5 (HD_5) is highly expressed in small intestine and urogenital tracts (Cunliffe et al., 2001; Shen et al., 2005; Svinarich et al., 1997), we speculate that HD_5 may have potential to protect digestive/urogenital tracts from pathogenic bacteria. Lately, the process of preparing a large amount of recombinant mature peptide of HD_5 ($rmHD_5$) at low cost has been successfully established in our lab (Wang et al., 2009). This facilitates us to carry out the investigation of antibacterial activity of HD_5.

*Corresponding author. E-mail: wangjunp@yahoo.com. Tel: +8602368752283. Fax: +8602368752009.

Therefore, this study was designed to observe the antibacterial kinetics and bactericidal efficacy of $rmHD_5$ on the antibiotics-resistant pathogenic strains isolated from the inpatient with digestive/urogenital tracts infection, and meanwhile to explore action mechanism of the peptide.

MATERIALS AND METHODS

Bacterial strains, peptide and reagents

A panel of bacterial strains isolated from clinical samples of digestive and urogenital tracts were obtained from the Department of Clinical Laboratory of South-west Hospital (Chongqing, China). According to National Clinical Laboratory Operation Regulations, all bacterial species were identified, and tested for antibiotics susceptibility by applying VITEK 60 system (BioMerieux, Lyon, France). Phenotypic confirmation of extended-spectrum - lactamase produced by clinical isolates was performed using E-test method (Ramalivhana et al., 2009). The peptide of $rmHD_5$ was prepared by our previously established method (Wang et al., 2009). Briefly, after high-cell density fermentation of P. pastoris GS115-HD_5, a two-step purification strategy of macroporous resin adsorption chromatography followed by cation exchange chromatography was performed to obtain $rmHD_5$. The lyophilized $rmHD_5$ with a purity of 91.7% was analyzed and identified by RP-HPLC and MALDI-TOF mass spectrometer. Calcein-AM was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibacterial assay

The antibacterial activity of $rmHD_5$ was evaluated by a series of liquid micro-dilution and colony counting assays (Liu et al., 2009; Rattanachaikunsopon and Phumkhachorn, 2009). Exponentially growing bacteria were diluted in phosphate buffer solution (PBS; pH 7.2) to reach a density of about 1×10^6 colony forming units/ml (CFU/ml). Subsequently, 10 l of each bacterial suspension was exposed to different concentrations of $rmHD_5$ diluted from a stock solution (10 mg/ml) in 100 l PBS for various times (from 5 min to 3 h) at 37°C. Then, 20 l sample was diluted in 180 l Mueller-Hinton broth (MHB), and plated onto MHB agar. CFUs were counted after 16 h of incubation at 37°C. Bactericidal activity of $rmHD_5$ was defined as a reduction in the numbers of viable bacteria of 3 log10 CFU/ml at different time intervals (Arhin et al., 2009).

Transmission electron microscopy (TEM) assay

The *Escherichia coli* and *Staphylococcus epidermidis* cells in the mid-logarithmic phase of growth were washed twice in PBS, then incubated at 37°C for another 30 min in PBS (control) or PBS containing 7 g/ml (for *E. coli*) and 32 g/ml (for *S. epidermidis*) of $rmHD_5$ respectively. The cells were centrifuged for 5 min at 5,000 *g*, and the pellets were fixed in Karnovsky's fixative, postfixed in 1% osmium tetroxide and embedded in Epon (Baechle et al., 2006). The sections were observed under transmission electron microscope (Zeiss, Oberkochen, Germany).

Calcein-AM staining and cell membrane permeability analysis

Each bacterium isolate from MHB agar was transferred into 1 ml medium and cultured at 37°C for 13 - 16 h with vigorous shaking, in order to achieve an initial inoculums of about 1×10^6 CFU/ml. The cells were stained by adding 100 l of calcein-AM solution (50 mol/l) to 1 ml bacteria suspension followed by incubation at 37°C for 30 min (Bratosin et al., 2005). Then, different concentrations of $rmHD_5$ (7, 14 g/ml for *E. coli*, and 32, 64 g/ml for *S. epidermidis*) were added to each bacterial inoculum, respectively. The cell suspension without $rmHD_5$ was used as control. All samples were incubated at 37°C for additional 3 h. Subsequently, the samples were washed three times with PBS. Then, the fluorescence intensity of calcein-AM labeled cells was assessed immediately by a flow cytometer (FACSCalibur; BD, New York, USA) according to the method described by Budde et al. (2001), with an excitation wavelength of 490 nm and an emission wavelength of 520 nm (Ikonen et al., 2003). The mean fluorescence intensity (MFI) was defined as the ratio between the bacteria and fluorescence standard beads, by using absolute values of the fluorescence intensities. Fluorescence histogram overlay of bacterial population was acquired by CellQuest Pro software (version 4.0; BD, New York, USA).

Statistics

Data are presented as the means ± SD of at least three independent experiments. The statistical significance was assessed by 2-tailed student *t* test ($P<0.05$).

RESULTS

Antibacterial activity of $rmHD_5$

In the past six months, total of forty-nine pathogenic bacterial strains belonging to eleven species were isolated from the digestive tract or urogenital tract of the patient in the university hospital. The phenotype characteristics of these strains were identified and listed in the Table 1. Recombinant mHD_5 demonstrated antibacterial activity against all the collected isolates. After 3 h incubation in PBS, the minimal concentrations of $rmHD_5$ to exert a bactericidal effect for different isolate were determined (Table 1). The results reflected that gram-negative (G^-) bacteria were more susceptible to $rmHD_5$ than gram -positive (G^+) bacteria, and different isolates belonging to the same species showed similar susceptibility to $rmHD_5$. It is noteworthy that $rmHD_5$ also displayed high antibacterial potency to some antibiotics-resistant nosocomial pathogens, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterococcus faecium*.

To evaluate the killing kinetics, a representative isolate of each bacterial species was chosen and tested for viability after incubation with $rmHD_5$ for different times. As shown in Figure 1, $rmHD_5$ inhibited the growth of bacteria in a time- and concentration- dependent manner. In particular, after incubating for about 5 min, $rmHD_5$ exhibited killing effect against *E. coli* (strain *EsC1*), *S. enterica* (strain *SaE2*), *Staphylococcus dysenteriae* (strain *SHD5*) and *A. baumannii* (strain *AcB4*) at concentrations of 7, 6, 6, 8 g/ml, respectively (Figure 1 a to d). For the isolates of *P. aeruginosa* (strain *PsA1*), *K. pneumoniae* (strain *KlP2*), *Enterobacter cloacae* (strain

Table 1. Bactericidal activity of rmHD5 against clinical isolates with special phenotypes.

Organism	Strain[a]	Source	Resistance phenotype[b]	rmHD$_5$ con.[c] (g/ml)
Gram-negative bacteria				
E. coli	*EsC1*[a]	Intestinal tract	AMC AMP CAZ GEN FEP	7
E. coli	*EsC2*[a]	Excrement	AMC AMP CAZ SXT	6
E. coli	*EsC3*	Intestinal tract	GEN SXT LVX	6
E. coli	*EsC4*[a]	Excrement	AMK CIP FEP GEN	6
E. coli	*EsC5*	Urine	AMC GEN SXT TZP LVX	6
S. enterica	*SaE1*	Excrement	GEN	6
S. enterica	*SaE2*	Excrement	AMC GEN	6
S. dysenteriae	*ShD1*[a]	Excrement	AMP LVX GEN TET MEZ	6
S. dysenteriae	*ShD2*	Excrement	AMP LVX MEZ	6
S. dysenteriae	*ShD3*	Excrement	AMP LVX TET CRO	6
S. dysenteriae	*ShD4*	Excrement	AMK AMP TET	6
S. dysenteriae	*SHD5*[a]	Excrement	AMK AMP LVX SXT CRO	6
A. baumannii	*AcB1*[a]	Urine	AMK CAZ CIP GEN NIT	7
A. baumannii	*AcB2*	Vagina secretion	AMK GEN NIT TZP	7
A. baumannii	*AcB3*	Urine	GEN IMP LVX	7
A. baumannii	*AcB4*[a]	Urine	IMP LVX NIT TZP	8
A. baumannii	*AcB5*	Vagina secretion	AMK LVX TZP	7
P. aeruginosa	*PsA1*[a]	Urine	AMK CAZ CRO GEN NIT TZP	8
P. aeruginosa	*PsA2*	Urine	AMK GEN NIT CIP IMP	8
P. aeruginosa	*PsA3*	Urine	AMK GEN NIT TZP CIP IMP	7
P. aeruginosa	*PsA4*[a]	Vagina secretion	AMK GEN FEP CAZ NIT	8
P. aeruginosa	*PsA5*	Urine	GEN CIP IMP TZP	7
K. pneumoniae	*KlP1*	Urine	GEN SXT TZP LVX	10
K. pneumoniae	*KlP2* [a]	Urine	AMK GEN SXT LVX	12
K. pneumoniae	*KlP3*	Vagina secretion	AMK GEN	10
K. pneumoniae	*KlP4*	Urine	AMK GEN LVX	10
K. pneumoniae	*KlP5* [a]	Vagina secretion	AMK GEN TZP LVX	10
E. cloacae	*EnC1*[a]	Excrement	AMK CRO NIT CIP LVX GEN SXT	10
E. cloacae	*EnC2*[a]	Excrement	CRO NIT CIP LVX GEN SXT	10
E. cloacae	*EnC3*	Intestinal tract	NIT GEN SXT	8
E. cloacae	*EnC4*	Excrement	CRO CIP LVX GEN SXT	8
E. cloacae	*EnC5*	Excrement	CIP LVX GEN	8
V. cholera	*ViC1*	Excrement	AMP SXT	8
V. cholera	*ViC2*	Excrement	AMP	10
N. gonorrhoeae	*NeG1*[a]	Vagina secretion	CIP PEN	10
N. gonorrhoeae	*NeG2*[a]	Urine	CIP GEN PEN	12
N. gonorrhoeae	*NeG3*	Urine	CIP PEN TET	10
N. gonorrhoeae	*NeG4*	Urine	CIP PEN	10
N. gonorrhoeae	*NeG5*[a]	Vagina secretion	CIP PEN TET	12
Gram-positive bacteria				
S. epidermidis	*StE1*	Urine	GEN OXA LVX TET VAN	32
S. epidermidis	*StE2*	Urine	CIP LVX OXA PEN TET VAN	32
S. epidermidis	*StE3*	Vagina secretion	GEN CIP PEN VAN	28
S. epidermidis	*StE4*	Urine	CIP ERY LVX OXA VAN	28
S. epidermidis	*StE5*	Urine	PEN TET VAN	28
E. faecium	*EnF1*	Urine	CIP GEN PEN VAN SAM IMP TEC	28
E. faecium	*EnF2*	Excrement	CIP VAN TET IMP PEN ERY	28
E. faecium	*EnF3*	Excrement	AMK VAN GEN SAM IMP TEC	28

Table 1. Contd.

E. faecium	*EnF4*	Urine	AMC AMK ERY SAM TET TEC VAN	28
E. faecium	*EnF5*	Vagina secretion	GEN VAN TET TEC	28

[a] positive to Extended-Spectrum -lactamase

[b] AMC, amoxycillin; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; ERY, erythromycin; FEP, cefepime; GEN, gentamicin; IMP, imipenem; LVX, levofloxacin; NIT, nitrofurantoin; OXA, oxacillin; MEZ, mezlocillin; PEN, penicillin; RIF, rifampin; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TEC, teicoplanin; TZP, piperacillin-tazobactam; VAN, vancomycin.

[c] Minimum concentration of rmHD5 to produce bactericidal effect after 3 h of incubation in PBS. The values are for geometric means of three replicates of each experiment.

EnC1), *Vibrio cholera* (strain *ViC2*) and *Neisseria gonorrhoeae* (strain *NeG2*), $rmHD_5$ showed a bactericidal effect at concentrations of 8, 12, 10, 10, 12 g/ml, respectively, after incubating for 10 min (Figure 1 e to i), while it needed more than 15 min for the peptide to produce a bactericidal effect against *S. epidermidis* (strain *StE1*) and *E. faecium* (strain *EnF4*), at comparatively higher concentrations of 64 and 56 g/ml (Figure 1 j and k).

Effect of $rmHD_5$ on morphological changes of bacteria

In order to investigate how $rmHD_5$ kills bacteria, we observed the morphological changes of *E. coli* and *S. epidermidis,* which were taken as the representatives of G^- bacteria and G^+ bacteria respectively, after interaction with $rmHD_5$ for 30 min. Interestingly, the *E. coli* cells treated with $rmHD_5$ showed remarkable changes in their microscopic appearance, characterized by rough surfaces containing much electron-dense material in the periplasmic space and on the external face of the outer membranes (Figure 2b). While the control cells of *E. coli* presented smooth and complete profiles (Figure 2a). Meanwhile, membranous blebs were found in the *S. epidermidis* cells treated with $rmHD_5$, which subsequently led to an efflux of cytoplasm materials (Figure 2d). In contrast, there were no visibly morphological changes in the control cells of *S. epidermidis* (Figure 2c).

Effect of rmHD5 on membrane permeability of bacteria

To further explore the antibacterial mechanism of $rmHD_5$, *E. coli* (strain *EsC1*) and *S. epidermidis* (strain *StE1*) treated with different concentrations of $rmHD_5$ were stained with calcein-AM, and subsequently subjected to flow cytometric analysis. Compared with the control, fluorescence histograms of both *E. coli* and *S. epidermidis* treated with $rmHD_5$ shifted to the left (Figure 3 a1 and b1), which means that MFI of cell population was decreased. As shown in Figures 3, a2, after being treated with $rmHD_5$ at the concentrations of 7 and 14 g/ml, the MFIs of *E. coli* cells were significantly reduced from 34.64 ± 1.64 (control) to 25.18 ± 1.25 (7 g/ml) and 18.53 ± 0.90 (14 g/ml), respectively. Meanwhile, the MFIs of *S. epidermidis* cells treated with 32 and 64 g/ml of $rmHD_5$ were 23.95 ± 3.54 and 18.40 ± 2.31, respectively, which were remarkably lower than the control (38.66 ± 2.55) ($P<0.05$) (Figure 3 b2). Normally, calcein will be trapped within the subcellular compartments unless the cytomembranes are damaged (Bratosin et al., 2005). Therefore, the decrease of calcein in the bacterial cells indicated that $rmHD_5$ could significantly increase the membrane permeability of bacteria, which might be attributed to the cytomembrane damage induced by $rmHD_5$.

DISCUSSION

Nowadays, increasing pathogenic bacterial strains resistant to traditional antibiotics have been reported in clinical practice. It is imperatively needed to search and develop new antibiotics (Wilson, 2008). HD_5 is a promising candidate to be developed into a novel antibiotic, because it has remarkable antimicrobial activity against a panel of standard strains (Szyk et al., 2006). To evaluate the application potential of $rmHD_5$, this study is focused on the antimicrobial activity of this peptide against clinical drug-resistant isolates and its action mechanism.

HD_5 was primarily expressed in mucosa epithelium of digestive/urogenital tracts *in vivo* (Shen et al., 2005; Svinarich et al., 1997), the bacterial strains isolated from these tissues of the inpatient were tested for susceptibility to $rmHD_5$ in this study. Inspiringly, the results showed that $rmHD_5$ exhibited strong bactericidal potency against all tested clinical isolates at relatively low concentrations. Unexpectedly, different isolates belonging to the same species displayed identical susceptibility to $rmHD_5$, although they were characteristics of different antibiotics-resistant phenotypes. This result suggests that the antibacterial mechanism of HD_5 differs from that of conventional antibiotics. In the present study, whatever in terms of bactericidal concentration or bactericidal speed, $rmHD_5$ showed quite more efficient in killing G^- bacteria than G^+ bacteria. As it happens, the specificity and selectivity of HD_5 against reference bacterial strains was previously reported by Ericksen et al. (2005). This phenomenon is understandable because HD_5 is

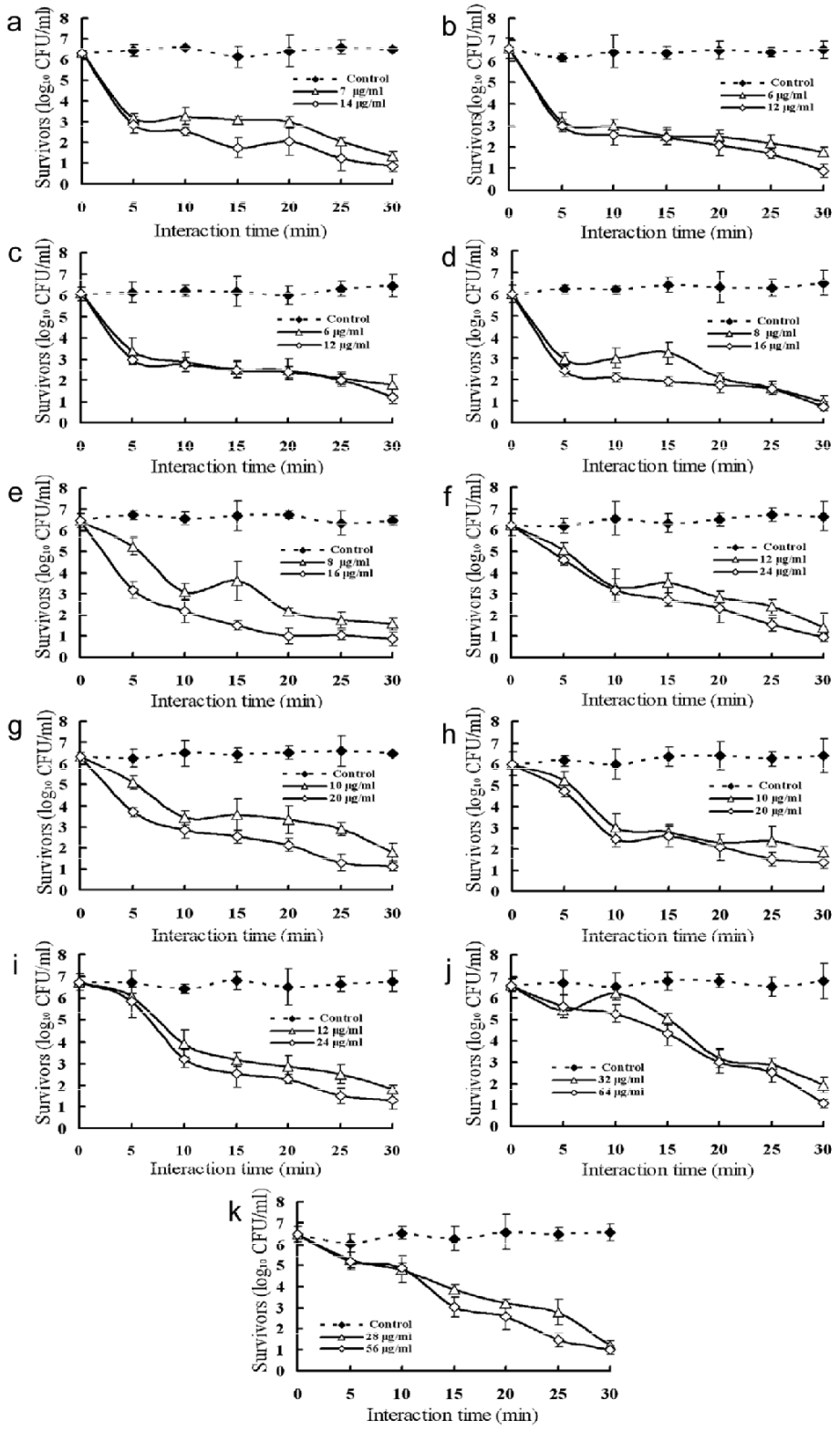

Figure 1. Time- and concentration-dependent antibacterial potency of rmHD5 against clinical isolates, *E. coli* (strain *EsC1*) (a), *S. enterica* (strain *SaE2*) (b), *S. dysenteriae* (strain *SHD5*) (c), *A. baumannii* (strain *AcB5*) (d), *P. aeruginosa* (strain *PsA1*) (e), *K. pneumoniae* (strain *KlP2*) (f), *E. cloacae* (strain *EnC1*) (g), *V. cholera* (strain *ViC2*) (h), *N. gonorrhoeae* (strain *NeG2*) (i), *S. epidermidis* (strain *StE1*) (j) and *E. faecium* (strain *EnF1*) (k). The control refers to the bacteria incubated in PBS without rmHD5.

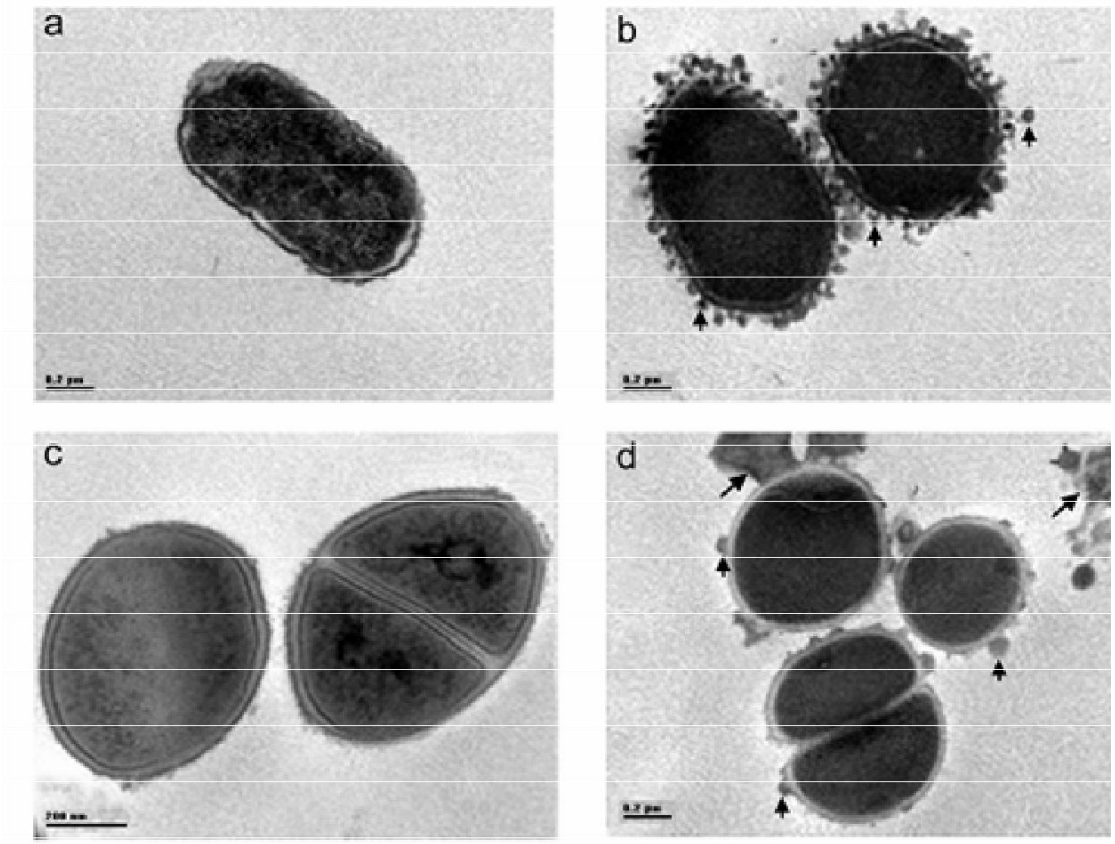

Figure 2. Transmission electron micrographs of bacteria cells. Incubated in PBS, the cells of *E. coli* (strain *EsC1*) showed normal morphology with smooth and complete membrane (a). After incubated with 7 g/ml rmHD5 for 30 min, most cells of the *E. coli* (strain *EsC1*) presented rough surface by the characteristics of much electron-dense material (arrow) in the periplasmic space and on the external face of the cytomembrane (b). The intact cell profile of *S. epidermidis* (strain *StE1*) untreated by rmHD5 was seen (c). *S. epidermidis* (strain *StE1*) was incubated with 32 g/ml of rmHD5 for 30 min, many membranous blebs (short arrow) and much solute efflux (long arrow) of cytoplasm material could be seen (d).

putatively committed to struggle with G^- pathogenic microorganism, due to the fact that it was formed and evolved under the pressure of microenvironment where G^- bacteria is predominant (Ouellette, 2006).

In order to further understand the bacteria-killing mode of HD_5, we examined the changes in membrane integrity and permeability of bacteria treated with $rmHD_5$, since it was reported that most of antibacterial peptides exert their activities by perturbing cytomembranes (Yeaman and Yount, 2003). It's known that all G^- bacteria and G^+ bacteria have their distinct characteristics of basic components and structure in cytomembranes. In this study, we chose the strain *EsC1* of *E. coli* and the strain *SeP1* of *S. epidermidis* as representative species of G^- bacteria and G^+ bacteria respectively. Interestingly, abnormal morphologic changes was observed in the cytomembranes of both *E. coli* and *S. epidermidis* cells treated with $rmHD_5$. Similar phenomena caused by $HNP_{1/2}$ were once reported in previous studies (Steffen et al., 2006), suggesting that HD_5 and $HNP_{1/2}$ may exert their antibacterial activity in a same way, which can be explained by the fact that these defensins share resemble structural properties (Szyk et al., 2006). Furthermore, the membrane permeability assays confirmed that by acting on bacterial cell directly, $rmHD_5$ could result in massive permeabilization of bacterial membranes, and subsequently impair the cell viability (Figure 3). Overall, these studies showed that HD_5-induced antibacterial activity against target bacteria is tightly associated with cytoplasmic membrane permeabili-zation. These deduction may reasonably interpret that G^-bacteria are more sensitive to $rmHD_5$ than G^+ bacteria, for the membranes of the former are more easily subjected to disturbance and permeabilization by cationic antimicrobial peptides, due to the fact that there are much negative charges in the surface of G^- bacteria cells (Brogden, 2005).

In conclusion, the present study firstly confirmed the bactericidal activity of $rmHD_5$ against clinical drug-resistant strains, through mechanism of disruption and

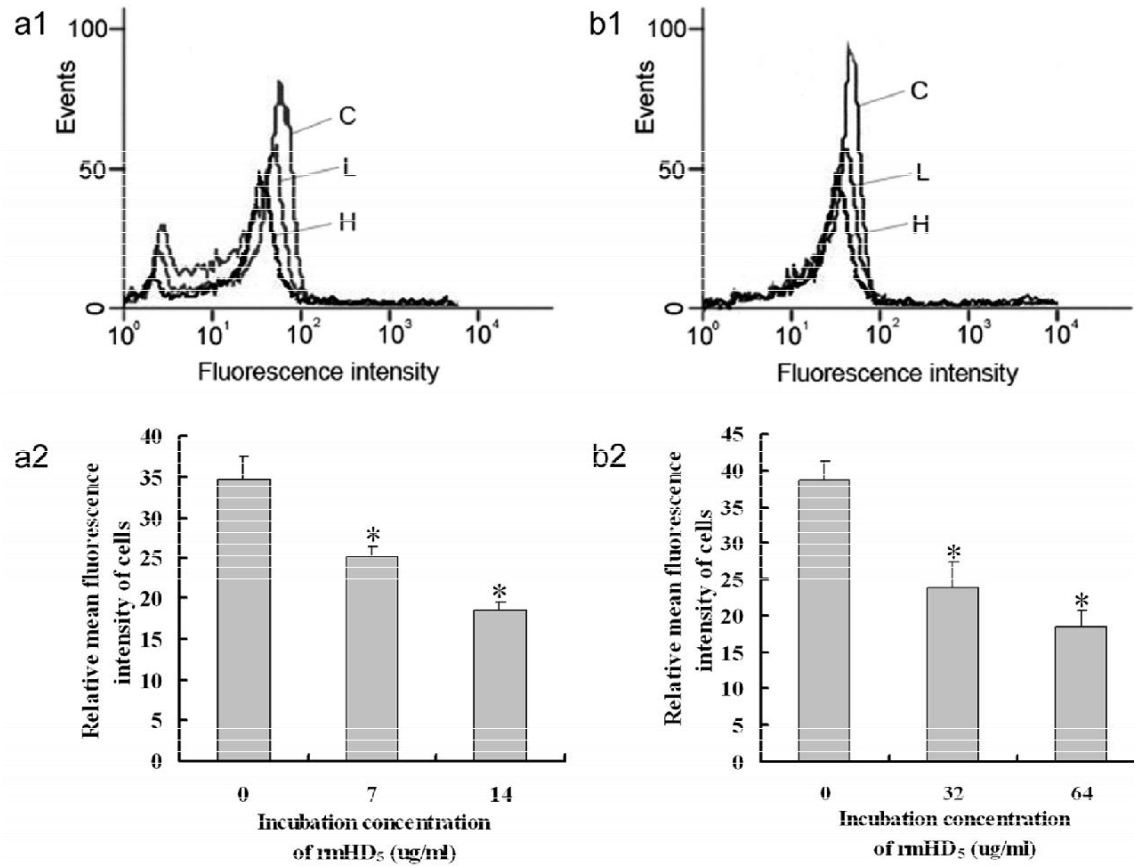

Figure 3. Fluorescence histogram overlay and relative mean fluorescence intensity of bacterial cells stained with calcein-AM prior to exposure to different concentrations of rmHD5, *E. coli* (strain *EsC1*) (a1, a2) and *S. epidermidis* (strain *StE1*) (b1, b2). The concentrations of rmHD5 used in the experiments were 0 (C), 7 (L) and 14 (H) g/ml for *E. coli*, and 0 (C), 32 (L) and 64 (H) g/ml for *S. epidermidis.* * indicates significant difference vs. the control ($P < 0.05$).

permeation of cytoplasmic membrane. This study paved substantial base for the potential clinical application of rmHD $_5$ as an antibacterial agent for digestive/urogenital tracts infection.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (No. 30771892), Academician Fund of Chongqing City (No. CSTC, 2007AB5022), Special Fund of National Key Laboratory of Trauma, Burn and Combined Injury (No. SKLZZ200821) and Natural Science Foundation of Chongqing City (No. CSTC, 2008BB5137).

REFERENCES

Arhin FF, McKay GA, Beaulieu S, Sarmiento I, Parr TR, Moeck G (2009). Time-kill kinetics of oritavancin and comparator agents against Streptococcus pyogenes. Int. J. Antimicrob. Agents 34: 550-554.

Baechle D, Flad T, Cansier A, Steffen H, Schittek B, Tolson J, Herrmann T, Dihazi H, Beck A, Mueller GA, Mueller M, Stevanovic S, Garbe C, Mueller CA, Kalbacher H (2006). Cathepsin D is present in human eccrine sweat and involved in the postsecretory processing of the antimicrobial peptide DCD-1L. J. Biol. Chem. 281: 5406-5415.

Bratosin D, Mitrofan L, Palii C, Estaquier J, Montreuil J (2005). Novel fluorescence assay using calcein-AM for the determination of human erythrocyte viability and aging. Cytometry A. 66: 78-84.

Brogden KA (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. Nat. Rev. Microbiol. 3: 238-250.

Budde BB, Rasch M (2001). A comparative study on the use of flow cytometry and colony forming units for assessment of the antibacterial effect of bacteriocins. Int. J. Food Microbiol. 63: 65-72.

Chen H, Xu Z, Peng L, Fang X, Yin X, Xu N, Cen P (2006). Recent advances in the research and development of human defensins. Peptides 27: 931-940.

Cunliffe RN, Rose FR, Keyte J, Abberley L, Chan WC, Mahida YR (2001). Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. Gut 48: 176-185.

Ericksen B, Wu Z, Lu W, Lehrer RI (2005). Antibacterial activity and specificity of the six human alpha-defensins. Antimicrob. Agents Chemother. 49: 269-275.

Gastmeier P, Vonberg RP (2008). Outbreaks of nosocomial infections:

lessons learned and perspectives. Curr. Opin. Infect. Dis. 21: 357-361.
Hancock RE, Sahl HG (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. 24: 1551-1557.
Ikonen M, Liu B, Hashimoto Y, Ma L, Lee KW, Niikura T, Nishimoto I, Cohen P (2003). Interaction between the Alzheimer's survival peptide humanin and insulin-like growth factor-binding protein 3 regulates cell survival and apoptosis. Proc. Natl. Acad. Sci. USA 100: 13042-13047.
Liu CY, Lu CL, Huang YT, Liao CH, Hsueh PR (2009). *In vitro* activities of moxifloxacin and tigecycline against bacterial isolates associated with intraabdominal infections at a medical center in Taiwan, 2001-2006. Eur. J. Clin. Microbiol. Infect. Dis. 28: 1437-1422.
Meyer JE, Schwaab M, Beier UH, Gorogh T, Buchelt T, Frese K, Maune S (2006). Association between human beta defensin expression and cholesteatoma formation. Auris. Nasus. Larynx. 33: 159-165.
Ouellette AJ (2006). Paneth cell alpha-defensin synthesis and function. Curr. Top. Microbiol. Immunol. 306: 1-25.
Ramalivhana JN, Obi CL, Moyo1 SR (2009). Antimicrobial susceptibility testing of Aeromonas hydrophila isolated from Limpopo Province, South Africa using VITEK 2 system, Micro Scan WalkAway, disk diffusion and E-test method. Afr. J. Microbiol. Res. 3(12): 870-876.
Rattanachaikunsopon P, Phumkhachorn P (2009). *In vitro* study of synergistic antimicrobial effect of carvacrol and cymene on drug resistant Salmonella typhi. Afr. J. Microbiol. Res. 3(12): 978-980.
Shen B, Porter EM, Reynoso E, Shen C, Ghosh D, Connor JT, Drazba J, Rho HK, Gramlich TL, Li R, Ormsby AH, Sy MS, Ganz T, Bevins CL (2005). Human defensin 5 expression in intestinal metaplasia of the upper gastrointestinal tract. J. Clin. Pathol. 58: 687-694.
Shinwari ZK, Khan I, Naz S, Hussain A (2009). Assessment of antibacterial activity of three plants used in Pakistan to cure respiratory diseases. Afr. J. Biotechnol. 8(24): 7082-7086.
Steffen H, Rieg S, Wiedemann I, Kalbacher H, Deeg M, Sahl HG, Peschel A, Gotz F, Garbe C, Schittek B (2006). Naturally processed dermcidin-derived peptides do not permeabilize bacterial membranes and kill microorganisms irrespective of their charge. Antimicrob. Agents Chemother. 50: 2608-2620.
Svinarich DM, Wolf NA, Gomez R, Gonik B, Romero R (1997). Detection of human defensin 5 in reproductive tissues. Am. J. Obstet. Gynecol. 176: 470-475.
Szyk A, Wu Z, Tucker K, Yang D, Lu W, Lubkowski J (2006). Crystal structures of human alpha-defensins HNP4, HD5, and HD6. Protein Sci. 15: 2749-2760.
Wang A, Wang S, Shen M, Chen F, Zou Z, Ran X, Cheng T, Su Y, Wang J (2009). High level expression and purification of bioactive human alpha-defensin 5 mature peptide in Pichia pastoris. Appl. Microbiol. Biotechnol. 84: 877-884.
Wilson P (2008). New antibiotics are needed as resistance grows. Bmj. 337: 1660.
Yeaman MR, Yount NY (2003). Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. 55: 27-55.