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Human beta-defensin 2 (hBD-2) is a 41-amino acid cationic peptide of the innate immune system that serves as antimicrobial molecule. We determined the bactericidal activity of synthetic hBD-2 against nosocomial strains belonging to eight different bacterial species and exhibiting various antimicrobial resistance phenotypes. The native disulfide connectivity was found essential for the bactericidal activity of hBD-2, while sodium chloride concentration was reversely associated with its potency. hBD-2 exhibited high bactericidal activity against Acinetobacter baumannii, Pseudomonas aeruginosa, Enterococcus faecalis, Enterococcus faecium and Staphylococcus aureus clinical strains. Characteristically, A. baumannii strains that exhibited multi-drug resistant (MDR) phenotypes were susceptible to lower concentrations of hBD-2 (vLD(90)=3.25-4.5 microg/ml) in comparison with non-MDR (wild-type) A. baumannii strains (vLD(90)=3.90-9.35 microg/ml). Bactericidal activity of hBD-2 was less pronounced against Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis strains but was significantly enhanced against strains of these species that exhibited resistance to several beta-lactam antibiotics. These observations give indications that the natural hBD-2 has a potential therapeutic role against bacterial pathogens and particularly against those exhibiting MDR phenotypes.

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In vitro bactericidal activity of human β-defensin 2 against nosocomial strains

John G. Routsias*, Panagiotis Karagounis, Georgeta Parvulesku, Nikolaos J. Legakis, Athanassios Tsakris

Department of Microbiology, School of Medicine, National and Kapodistrian University of Athens, 75 M.Asias St., 11527 Athens, Greece

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ABSTRACT

Human β-defensin 2 (hBD-2) is a 41-amino acid cationic peptide of the innate immune system that serves as antimicrobial molecule. We determined the bactericidal activity of synthetic hBD-2 against nosocomial strains belonging to eight different bacterial species and exhibiting various antimicrobial resistance phenotypes. The native disulfide connectivity was found essential for the bactericidal activity of hBD-2, while sodium chloride concentration was reversely associated with its potency. hBD-2 exhibited high bactericidal activity against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterococcus faecium* and *Staphylococcus aureus* clinical strains. Characteristically, *A. baumannii* strains that exhibited multi-drug resistant (MDR) phenotypes were susceptible to lower concentrations of hBD-2 (vLD_{90} = 3.25–4.5 μg/ml) in comparison with non-MDR (wild-type) *A. baumannii* strains (vLD_{90} = 3.90–9.35 μg/ml). Bactericidal activity of hBD-2 was less pronounced against *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* strains but was significantly enhanced against strains of these species that exhibited resistance to several β-lactam antibiotics. These observations give indications that the natural hBD-2 has a potential therapeutic role against bacterial pathogens and particularly against those exhibiting MDR phenotypes.

1. Introduction

The prevalence of bacteria with resistance to most clinically useful antibiotics is growing in many parts worldwide, leaving very few alternative antimicrobial options for treatment [37]. Characteristically, *Acinetobacter baumannii* strains resistant to all potentially active antibiotic classes [26] are increasingly isolated among hospital-acquired infections [16] and the only available antimicrobials are the inconvenient peptide antibiotics polymyxin B and polymyxin E (colistin). Therefore, there is an ever-growing need for new antimicrobial agents in order to fight these infections [27].

Antimicrobial peptides (AMPs) are attractive candidates as alternative therapeutic agents for bacterial infections because of their selectivity, speed of action and inherent immunological compatibility [22]. One important subclass of AMPs is represented in humans by defensins [30]. This category consists of a group of β-sheet-rich, cationic and amphipathic peptides, forming characteristic networks of disulfide bridges that assume a conserved structural fold [21]. Based primarily on the spacing between the cysteine residues and the topology of the disulfide bridges, human defensins are classified into α-, β- and the most recently discovered θ-defensins [30].

The antimicrobial effect of defensins is believed to be achieved by creating pores or otherwise disrupting the cell membrane of target organisms, leading to the release of their cellular contents [25]. α-Defensins have broad antimicrobial activity against bacterial pathogens, fungi and enveloped viruses, while β-defensins have generally a more narrow antimicrobial spectrum being active mainly against gram-negative bacteria and yeasts [31]. Four major β-defensins, termed β-defensin-1 (hBD-1), β-defensin-2 (hBD-2), β-defensin-3 (hBD-3) and β-defensin-4 (hBD-4) have been characterized in detail in humans. hBD-1 and hBD-2 are primarily expressed in the epithelial lining of the urinary and respiratory tracts [1,25]. HBD-3, in addition to the epithelia, was also expressed at lower levels in different non-epithelial cells of organs such as the heart, liver and placenta while hBD-4 is primarily expressed in the testis and epididymis [25]. hBD-2 through hBD-4 levels are up-regulated in response to bacterial infection or proinflammatory stimuli [25,32], whereas hBD-1, is constitutively expressed, serving as a basal defence in the absence of inflammation.

Each of the β-defensins characterized to date has the capacity to kill or inhibit *in vitro* a variety of bacteria, particularly at low concentrations of salt and plasma proteins [8,25]. A number of studies have demonstrated the *in vitro* antimicrobial activity of β-defensins against a limited number of referral bacterial strains [2,15,17,35]. In a recent report the bactericidal activity of hBD-3 against 30 multi-drug resistant nosocomial strains has been also evaluated [20]. However, the antimicrobial properties of the hBD-2 have not been studied in detail and its antimicrobial activity against

* Corresponding author. Tel.: +30 2107462133; fax: +30 2107462210.
E-mail address: jroutsias@med.uoa.gr (J.G. Routsias).

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nosocomial strains has not been determined. Therefore, we sought to examine the bactericidal activity of hBD-2 against epidemiologically unrelated hospital pathogens belonging to several bacterial species. We attempted also to evaluate the activity of hBD-2 in relation with the antibiotic resistance phenotypes.

2. Materials and methods

2.1. Bacterial strains and bacterial growth

Antimicrobial activity of hBD-2 was tested against clinical strains of *Escherichia coli* (18 strains), *Klebsiella pneumoniae* (12 strains), *Proteus mirabilis* (13 strains), *A. baumannii* (21 strains), *Pseudomonas aeruginosa* (9 strains), *Staphylococcus aureus* (9 strains), *Enterococcus faecalis* (8 strains), and *Enterococcus faecium* (2 strains). The isolates were non-repetitive and selected randomly from epidemiologically unrelated patients hospitalized during 2005–2007 in three of the largest general hospitals in the region of Athens. Multi-drug resistant (MDR) *A. baumannii* isolates (14 strains) exhibited resistance to 3 or more classes of antimicrobials, including expanded-spectrum cephalosporins, carbapenems, aminoglycosides and fluoroquinolones, and susceptibility to colistin. One of the MDR isolates was also susceptible to tigecycline and one to tetracycline. The identification, as well as the antimicrobial susceptibility testing of the organisms, was determined by using the MicroScan Autoscan-4 system (AutoSCAN-4, DADE International, West Sacramento, CA). Identification was confirmed by standard conventional biochemical assays and for *A. baumannii* with the detection of the endogenous $bla_{oxa\text{-}51}$ gene. Susceptibility results were confirmed by using *E*-test.

2.2. Synthesis and oxidation of hBD-2

Based on the sequence deduced from DEFB2 cDNA, hBD-2 (GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP) peptide was synthesized using automated Fmoc [N-(9-fluorenyl)methoxycarbonyl] solid-phase synthesis (Biosynthesis Inc., San Antonio, TX). For the formation of the disulfide bonds, required for the correct peptide folding, the linear hBD-2 (possessing free thiol groups) was air oxidized according to a published protocol that ensures the formation of the natural peptide folding [34]. In this regard, the peptide solution was diluted to 100 μg/ml (to avoid the formation of undesired intermolecular disulfide bridges) in 17.4 mM ammonium acetate, pH 8.0 (volatile buffer), and stirred vigorously in an open container for 24 h at 22 °C. Acetic acid (5% final concentration) was then added to the aqueous solution and the peptide was lyophilized. After oxidative folding, the peptide was purified to homogeneity by RP-HPLC. The reduced and oxidized peptides both showed a single peak by RP-HPLC and the correct molecular weight (*M*r = 3928) by mass spectroscopy.

2.3. Antimicrobial assay

Exponentially growing bacteria were resuspended in 10 mM sodium phosphate buffer (SPB; pH 7.4) to reach a density of 1.5×10^8 CFU/ml (0.5 McFarland). The bacterial suspension was exposed at 37 °C for 2 h to different concentrations of hBD-2. Following incubation, the samples were diluted 6000-fold in tryptone soy broth (TSB), and 10 μl of each dilution was plated onto MacConkey agar (for Gram-negative bacteria) and Columbia agar with 5% sheep blood agar (for Gram positive cocci). After incubation for 24 h at 37 °C the microbial colonies were counted. Virtual lethal doses, vLD_{50}, vLD_{90} and vLD_{99} were obtained after analysis of colony counts and reported as the concentration of hBD-2 resulting in the killing of 50, 90 and 99% of bacteria, respectively.

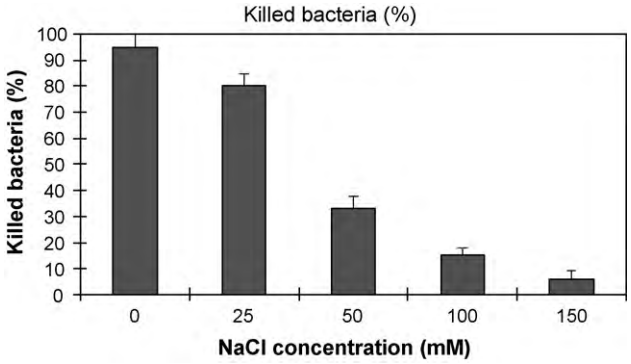

Fig. 1. Effect of NaCl concentration on the antimicrobial activity of hBD-2. The data presented regard the mean values of 5 individual experiments. Enhanced bactericidal activity of hBD-2 was observed in low salt concentrations.

2.4. Statistical analysis

Thirty-six strains, belonging to the *E. coli*, *K. pneumoniae* and *P. mirabilis species*, were categorized in four groups, according to their susceptibility in hBD-2 (vLD_{90}: 1–10, 10–20, 20–30, 30–50 μg/ml). The number of strains in the various groups, which are resistant to each antibiotic, were compared by using the Freeman–Halton generalization of the Fisher's exact probability test for the analysis of two-by-two contingency tables to those with multiple rows and columns [7]. The virtual lethal doses to hBD-2 of multi-drug resistant and sensitive *Acinetobacter* isolates were compared using two-tailed Student's *t*-test.

3. Results

3.1. Effect of incubation time on the bactericidal activity of hBD-2

In a preliminary experiment, the survival rates of three strains, randomly selected among those belonging to the most common bacterial species, were evaluated after their incubation with 10 or 30 μg/ml of hBD-2 for a period of 2 or 5 h. Incubation with 10 μg/ml of hBD-2 for 2 h was able to kill all bacterial population of *A. baumannii*. Therefore, further increase on the concentration of hBD-2 or its incubation time had no additional effect on the bactericidal activity. In contrast, 60% of *P. mirabilis* bacterial cells survived after incubation for 2 h with 10 μg/ml hBD-2. Increase of the incubation time to 5 h reduced the survival rate of *P. mirabilis* bacterial cells to 10%. Similarly, an increase of hBD-2 concentration from 10 μg/ml to 30 μg/ml reduced the survival rate of *P. mirabilis* to 16%. *E. coli* was more susceptible than *P. mirabilis* to hBD-2. After 2 and 5 h incubation with 10 μg/ml of hBD-2, only 6.4 and 2.4% of *E. coli* bacterial cells, respectively, survived. The concentration of 30 μg/ml was found to be lethal for all *E. coli* bacterial cells using both incubation times. Taking into account the above data, 2 h was the selected incubation period for the following experiments.

3.2. Effect of salt concentration on the bactericidal activity of hBD-2

It has been previously reported that the bactericidal activity of hBD-2 is reversely dependent on the NaCl concentration [10]. To confirm this observation we incubated *E. coli* bacteria with 10 μg/ml of hBD-2 for 2 h in increasing salt concentrations, ranging from 0 to 150 mM. It was found that the rate of killed bacteria was gradually decreased from 95 to 6%, as the NaCl concentration increased, from 0 to 150 mM, respectively (Fig. 1).

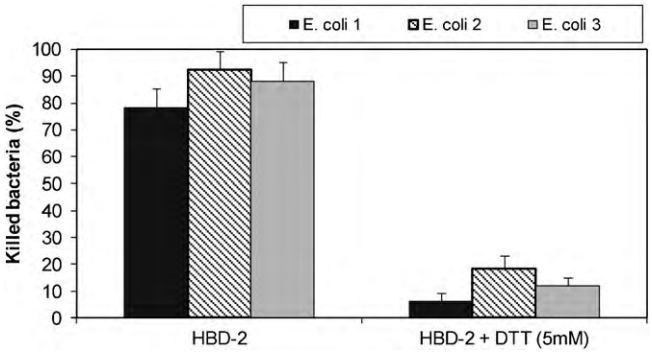

Fig. 2. Bactericidal activity of (i) oxidized hBD-2 and (ii) reduced hBD-2 (after incubation with the reducing agent dithiothreitol) against three *Escherichia coli* clinical isolates. The data presented regard the mean values of three individual experiments.

3.3. Effect of reduction of hBD-2 disulfide bridges on its bactericidal activity

In order to confirm that our synthetic hBD-2 is properly oxidised and to compare its bactericidal activity with the activity of the reduced hBD-2, experiments with three different *E. coli* isolates in the presence of DTT were performed. The presence of this reducing agent (at 5 mM concentration) significantly diminished the bactericidal activity of hBD-2 (at 10 μg/ml for 2 h incubation), decreasing the percentage of killed bacteria, approximately by 7-fold (Fig. 2). DDT alone (without hBD-2) exhibited no significant effect in the survival rate of bacteria (reduction of survival rate <7%).

3.4. Antimicrobial activity of hBD-2 against 92 bacterial strains

hBD-2 exhibited a broad-spectrum of antimicrobial activity against the 92 clinical strains, which belonged to eight different bacterial species. For *A. baumannii* (14 strains), *P. aeruginosa* (9 strains), *E. faecalis* (8 strains), *E. faecium* (2 strains), *S. aureus* (9 strains) the concentration of 10 μg/ml of hBD-2 was lethal for >99% of the bacterial cells. In contrast, *E. coli, K. pneumoniae* and *P. mirabilis* strains (43 in total) were less susceptible towards the same concentration of hBD-2 and the mean survival rate of hBD-2 treated cells ranged from 24 to 53% (Fig. 3). The virtual 90% lethal dose (vLD_{90}) varied from 5 to 50 μg/ml of hBD-2. However, only 7 (2 *E. coli*, 2 *K. pneumoniae* and 3 *P. mirabilis*) of the 92 tested strains exhibited a vLD_{90} of ≥30 μg/ml, suggesting that the vLD_{90} was ≤20 μg/ml for more than 90% of them. In addition the virtual 99% lethal dose (vLD_{99}) was ≤10 μg/ml for approximately half of the tested bacterial strains. Interestingly, the 14 *Acinetobacter* isolates that exhibited MDR phenotypes, were all (100%) sensitive to low concentrations of hBD-2 (vLD_{99} < 10 μg/ml) while the 7 non-MDR *Acinetobacter* isolates demonstrated an average vLD_{99} > 10 μg/ml.

3.5. MDR Acinetobacter isolates are susceptible to lower concentrations of hBD-2 than non-MDR Acinetobacter strains

The assay setup (using hBD-2 concentrations of 10, 20, 30 and 50 μg/ml) for determination of hBD-2 antimicrobial activity did not allow the precise estimation of vLD_{90} of *Acinetobacter* strains, since it was less than 10 μg/ml for all the MDR isolates and 2/7 of the non-MDR strains. Therefore, we incubated nine multi-resistant and seven sensitive *Acinetobacter* isolates (their antibiotic resistance profiles are presented in Table 1) with lower hBD-2 concentrations (10, 5, 2.5, 1.25, 0.63, and 0.32 μg/ml). It was found that the vLD_{90} was ranging between 3.25 and 4.5 μg/ml for all MDR strains while the vLD_{90} was significantly higher (ranging from 3.90 to 9.35) for non-MDR strains ($t = -3.74$, $p < 0.01$) (Fig. 4). In this regard, 2.1 μg/ml of hBD-2 was enough to kill approximately 50% of bacterial cells of all MDR *Acinetobacter* strains (mean vLD_{50} = 2.1 μg/ml) and this concentration was 1.5-fold lower than the concentration required for killing the 50% of non-MDR *Acinetobacter* strains and 5-fold lower than the concentration required for killing the 50% of *K. pneumoniae* strains. Therefore hBD-2 was more potent against MDR *Acinetobacter* strains than non-MDR *Acinetobacter* or other bacterial strains.

3.6. Antibiotic resistance is related to susceptibility of different bacterial strains to hBD-2

In order to examine if the bactericidal activity of hBD-2 is related with resistance to certain antibiotics, clinical isolates of *E. coli*, *K. pneumoniae* and *P. mirabilis* were divided in four groups according to their vLD_{90} to hBD-2. The susceptibility of the bacterial strains to antibiotics of different antimicrobial classes was also defined. They included (i) penicillin/β-lactamase inhibitors (ampicillin/sulbactam, amoxicillin/clavulanate, piperacillin/tazobactam), (ii) cephalosporins (cephalothin, cefazolin, cefuroxime, ceftriaxone, ceftazidime, cefotaxime andcefepime), (iii) aztreonam, (iv) imipenem, (v) gentamicin and (vi) ciprofloxacin. The bactericidal activity of hBD-2 was enhanced against strains that exhibited resistance to amoxicillin/clavulanate, aztreonam and all cephalosporins ($P < 0.01$;

Table 1
Antibiotic resistance profiles of MDR (AC1–AC9) and non-MDR (AC10–AC16) *Acinetobacter* isolates.

Isolate	AMP	SAM	TIM	ATM	FEP	CAZ	CIP	SXT	AMK	GEN	TOB	TET	COL	HBD-2
AC1	R	R	R	R	R	R	R	R	R	R	R	R	S	2.0
AC2	R	R	R	R	R	R	R	R	S	R	R	R	S	2.3
AC3	R	R	R	R	R	R	R	R	R	R	R	R	S	2.2
AC4	R	R	R	R	R	R	R	R	R	R	R	R	S	1.6
AC5	R	R	R	R	R	R	R	R	R	R	R	S	S	2.9
AC6	R	R	R	R	R	R	R	R	R	R	R	R	S	2.7
AC7	R	R	R	R	R	R	R	R	R	R	R	R	S	2.1
AC8	R	R	R	R	R	R	R	R	R	R	R	R	S	1.6
AC9	R	R	R	R	R	R	R	R	R	R	R	R	S	1.8
AC10	R	S	S	R	S	S	S	S	S	S	S	S	S	4.2
AC11	R	S	S	S	S	S	S	S	S	S	S	S	S	2.0
AC12	R	S	S	R	S	S	S	S	S	S	S	S	S	3.8
AC13	S	S	S	S	S	S	S	S	S	S	S	S	S	4.7
AC14	R	S	S	R	S	S	S	S	S	S	S	S	S	4.2
AC15	R	S	S	S	S	S	S	S	S	S	S	S	S	3.9
AC16	S	S	S	S	S	S	S	S	S	S	S	S	S	2.8

Antibiotic abbreviations are as follows: AMP, ampicillin; SAM, ampicillin–sulbactam; PIP, piperacillin;TZP, piperacillin-tazobactam; TIM, ticarcillin/clavulanic acid; ATM, aztreonam; TIC, ticarcillin; FEP, cefepime; CAZ, ceftazidime; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; TIG, tigecyclin IPM, imipenem; COL, colistin; HBD-2, vLD_{50} (μg/ml) of human beta-defensin 2.

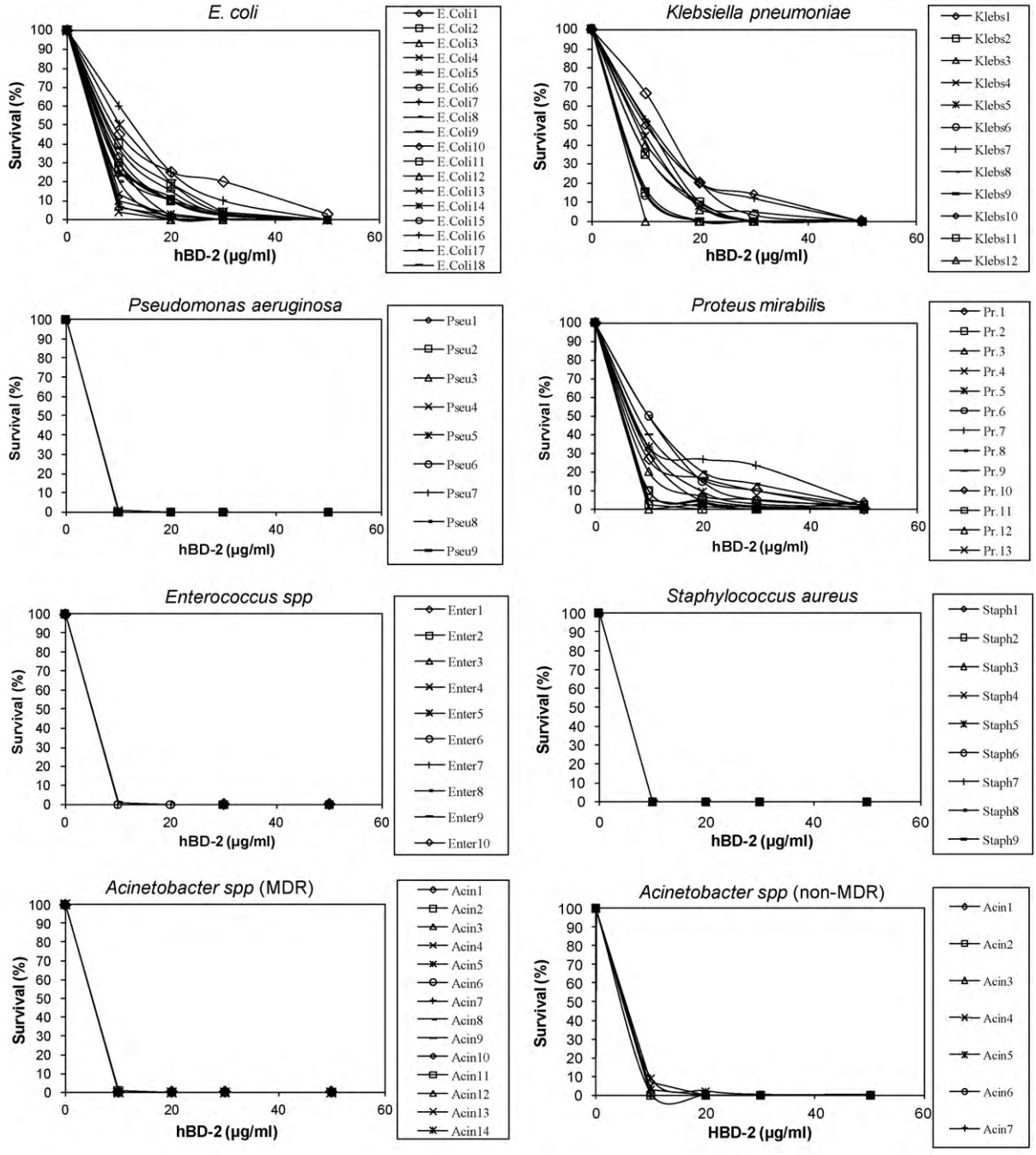

Fig. 3. Survival curves of 92 different strains belonging to eight different bacterial species exposed to hBD-2 concentrations varying from 10 to 50 μg/ml. Each square contains survival plots of a different bacterial species. The incubation time with hBD-2 peptide was 2 h.

Table 2
Resistance (%) of *Klebsiella*, *Proteus* and *Escherichia coli* species in various antibacterial agents in relation with susceptibility to different concentrations of HBD-2 ($n = 36$).

HBD-2	AMP/SULB	AMOX/CLAV	PIP/TAZO	AZTR	IMIP	CIPRO	CEPH	CEFAZ	CEFUR	CEFEP	CEFTR	GENT	*n*
<=10	75%	50%	25%	50%	25%	25%	75%	67%	50%	50%	50%	0%	4
>10, <=20	20%	10%	20%	10%	0%	0%	13%	0%	0%	0%	0%	0%	10
>20, <=30	19%	0%	0%	0%	0%	6%	0%	0%	0%	0%	0%	6%	16
>30, <=50	33%	0%	0%	0%	0%	0%	20%	0%	0%	0%	17%	0%	6
P	NS	0.01	NS	0.01	NS	NS	<0.01	<0.01	<0.01	<0.01	<0.01	NS	

AMP/SULB: ampicillin–sulbactam; AMOX/CLAV: amoxicillin–clavulanate; PIP/TAZO: piperacillin–tazobactam; AZTR: aztreonam; IMIP: imipenem; CIPRO: ciprofloxacin; CEPH: cephalothin; CEFAZ: cefazolin; CEFUR: cefuroxim; CEFEP: cefepime, ceftazidime, cefotaxim; CEFTR: ceftriaxone; GENT: gentamicin.

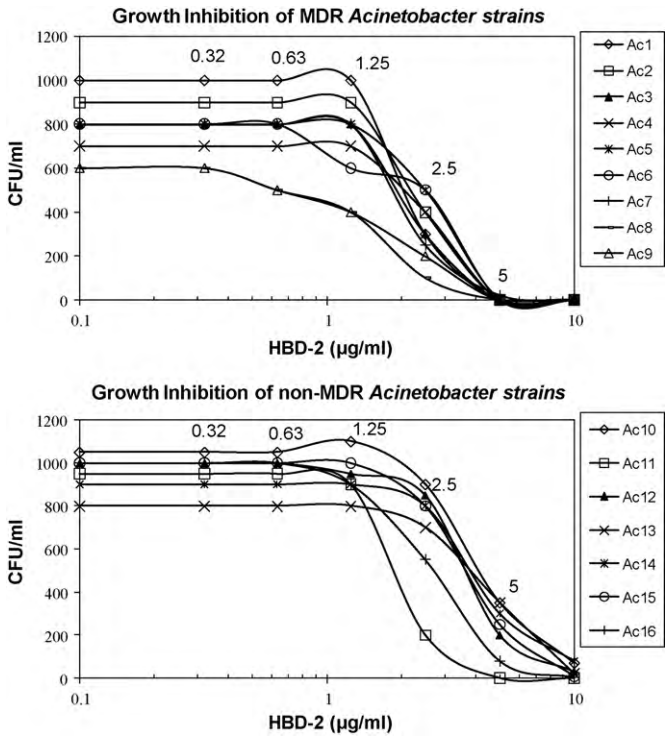

Fig. 4. Survival curves of nine MDR and seven non-MDR *Acinetobacter baumanii* strains exposed to hBD-2 concentrations varying from 0.32 to 10 μg/ml.

Table 2). Strains that were resistant to these antibiotics, exhibited a vLD_{90} to hBD-2 ≤10 μg/ml, demonstrating an increased susceptibility to hBD-2. However, resistance to other β-lactam antibiotics as well as to gentamicin and ciprofloxacin was not related with increased susceptibility to hBD-2.

4. Discussion

hBD-2 belongs to human β-defensins, which are endogenous antibiotic peptides of the innate immune system. Structurally, β-defensins are β-sheet-rich, cationic and amphipathic peptides, possessing six cysteine residues that form a characteristic pattern of disulfide bridges leading to a conserved structural fold [28]. The basal expression of hBD-2 has been documented in various tissues including freshly isolated foreskin, lung and trachea [29] and induction of hBD-2 synthesis has been observed in gastric and respiratory epithelium, peripheral blood, dendritic cells and keranatinocytes upon stimulation with inflammatory stimuli such as IL-1, TNF-α, IFN-γ or LPS [25]. hBD-2 similarly with all defensins exhibits antimicrobial activities against gram-negative and gram-positive bacterial strains and fungi [29,36]. However, the antimicrobial activity of this peptide has been tested against only a very limited number of strains, such as referral ATCC isolates and its antimicrobial potency has not been yet explored in detail against a large number of clinical isolates.

Our study shows that the disulfide connectivity is essential for the bactericidal effect of hBD-2, since incubation in the presence of the reducing agent DTT, decreased the number of killed bacteria approximately by 7-fold, which is inline with previous reports [24]. However, some studies in the literature suggest that disulfide connectivity do not significantly affect the antimicrobial activity of certain defensins, such as hBD-3 [13]. Other factors affecting the bactericidal effect of hBD-2 were also explored, including the salt concentration, the incubation time and the hBD-2 concentration. It was demonstrated that even 150 mM of sodium chloride was enough to decrease the bactericidal effect of hBD-2 at least 15-times, indicating that the maximum activity of hBD-2 can be found in sites of the human body with low salt concentration [10]. In this regard, the airway surface fluid of normal lung exhibit low salt concentration (about 80 mM of NaCl), allowing the bactericidal action of hBD-2. On the other hand, in patients with cystic fibrosis the NaCl concentrations raises up to 170 mM, diminishing the antimicrobial effect of hBD-2 [9]. It was also shown that an increase of the incubation time was capable to counterbalance, at least partially, a decrease of the concentration of hBD-2. Therefore, 5 h incubation with 30 μg/ml of hBD-2 was found to have similar bactericidal effect with 2 h incubation with 50 μg/ml of hBD-2 for *P. mirabilis* species. However, from the same experiments it was clearly suggested that the antimicrobial activity of hBD-2 is largely dependent on the type of the organism (e.g. hBD-2 was more potent against *A. baumannii* than against *P. mirabilis* strains).

The bactericidal activity of hBD-2 against different organisms was studied using 92 clinical isolates. hBD-2 was most potent against *A. baumannii*, *P. aeruginosa*, *E. faecalis*, *E. faecium* and *S. aureus* isolates, exhibiting a vLD_{90} < 10 μg/ml. On the other hand *E. coli*, *K. pneumoniae* and *P. mirabilis* strains were less susceptible towards hBD-2, with most of the strains tested having vLD_{90} >10 μg/ml. Interestingly, the susceptibility towards hBD-2 of 36 *E. coli*, *K. pneumoniae* and *P. mirabilis* strains was found to be reversibly correlated with resistance to cephalosporins, aztreonam and amoxicillin/clavulanate. More specifically, the majority of the strains with a vLD_{90} to hBD-2 ≤10 μg/ml were resistant to the above antibiotics, while only a minority (15%) of the strains with vLD_{90} to hBD-2 >10 μg/ml were resistant to these antibiotics. Since cephalosporins, aztreonam and amoxicillin/clavulanate are β-lactams sharing the same mechanism of action, the inhibition of cell wall synthesis, a common mechanism could be involved in the increased susceptibility to hBD-2 of the antibiotic-resistant strains. The antimicrobial activity of the hBD-2 includes the binding of the cationic peptide to the negatively charged membranes of bacteria, the disruption of the membranes and the leakage of cellular contents that ultimately leads to the destruction of the cell by osmolysis [25]. Therefore, any factor that alters the composition of the cell wall of gram-negative bacteria such as diminished expression of outer membrane proteins or modification of peptidoglycan structure in the periplasmic space could potentially disrupt the wall cohesion, making them more susceptible to the action of β-defensins. In fact, such changes in the membrane composition have been previously described in β-lactam resistant bacterial strains [3]. In this regard, changes that render the resistance to one class of antibiotics, may result in susceptibility to another.

Taking into account the above observations we determined the precise vLD_{90} of hBD-2 against nine multi-drug resistant *A. baumannii* strains. They were all found to be susceptible to low concentrations of hBD-2 demonstrating vLD_{90} < 4.5 μg/ml. These concentrations were significantly lower than the concentrations of hBD-2 required to kill *Acinetobacter* isolates susceptible to antibiotics (mean vLD_{90} = 7.5 μg/ml) or other susceptible bacterial strains belonging to other species such as *K. pneumonia*, *E. coli* or *P. mirabilis* (vLD_{90} > 10 μg/ml). The examination of the antibiotic resistance phenotype of MDR *Acinetobacter* isolates showed that they were susceptible only to colistin. Colistin, also known as polymyxin E, is a peptide with significant *in vitro* activity against gram-negative pathogens [4]. However, its use has been limited because of concerns about poor pharmacokinetics and nephrotoxicity. Chemically, colistin is a cationic cyclic decapeptide linked to a fatty acid chain with a similar mechanism of action to defensins [4]. More specifically, this cationic peptide interacts with the negatively charged membrane of bacteria, leading to permeability changes in the cell envelope, leakage of cell contents, and cell death [4,18]. Interestingly, increased susceptibility to colistin has been recently

documented in hypermutable bacterial strains (*P. aeruginosa*) [19], an observation that could extrapolated to our findings regarding the increased susceptibility to hBD-2 of MDR bacterial isolates. In the case of colistin, the supersusceptibility of hypermutable bacterial strains has been attributed to the acquisition of particular adaptive mutations, such as those leading to the frequently modified lipopolysaccharide structure, or to the accumulation of deleterious mutations that accompanied by the loss of function of multiple genes [19]. Such genes include the PsrA transcriptional regulator that affects energy generation and outer membrane permeability. Mutations of psrA have been recently associated with supersusceptibility of *P. aeruginosa* to polymyxin B [11]. From this point of view is not unlikely that multi-drug resistant strains besides mutations that confer resistance to certain antibiotics possess also mutations that affect energy generation that required for maintenance of membrane integrity or affect membrane structure itself, leading to supersusceptibility to the action of colistin and hBD-2.

In our days, the increasing prevalence of multi-drug resistant gram-negative organisms has rekindled interest in colistin. Thus, it has been used as a salvage therapy in patients with serious infections from multi-drug resistant bacteria with acceptable efficacy, but also with some serious side effects such as nephrotoxicity [5,12]. Our data suggest that apart from colistin, hBD-2 could also be used in the fight against MDR *Acinetobacter baumannii* strains. hBD-2, as an endogenous antibiotic peptide of the innate immune system could be less toxic than polymyxins. Studies with the porcine defensin, protegrin-1 and beetle defensin peptides, indicate that defensin peptides are less toxic than other antibiotics in experimental animals [14,33]. Moreover, in addition to their direct antibacterial activity, defensins have the potential to neutralize bacterial endotoxin by direct binding and inactivation of lipopolysaccharide (LPS) [23]. Therefore, defensins have been found to prevent the endotoxin's ability to induce shock through the release of cytokines and nitric oxide [38] and also to prevent LPS-induced mortality in C57BL/6 mice in a therapeutic approach [23]. Another, beneficial action defensins is related with their ability to form channels in lipid membranes that contain bacterial lipopolysaccharide. In one report, the human hBD-1 and the porcine defensin, protegrin, were found to be capable to disrupt cell wall of *Mycobacterium tuberculosis*, facilitating the access of the hydrophilic drug, isoniazid, inside the cells. In this regard, *M. tuberculosis* (even a strain resistant to isoniazid) was found to be susceptible to the combined action of defensin and isoniazid, which exhibited a strong synergestic effect [6]. Taken together, introduction of hBD-2 to the antimicrobial therapy of multi-drug resistant bacteria could potentially have additional therapeutic benefits besides its direct bactericidal activity.

In conclusion, the natural peptide antibiotic hBD-2 has a broad-spectrum of antimicrobial activity and, in addition, it can be potentially used as a novel therapeutic agent in the fight against MDR bacteria.

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