
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.
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 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...
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 extended-spectrum cephalosporins, carbapenems, aminoglycosides and fluoroquinolones, and susceptibility to colistin. One of the MDR isolates was also susceptible to tigecycline.

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 of 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).
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 (vLD90) 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 vLD90 of >30 μg/ml, suggesting that the vLD90 was <20 μg/ml for more than 90% of them. In addition the virtual 99% lethal dose (vLD99) 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 (vLD99 < 10 μg/ml) while the 7 non-MDR Acinetobacter isolates demonstrated an average vLD99 > 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 vLD90 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 vLD90 was ranging between 3.25 and 4.5 μg/ml for all MDR strains while the vLD90 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 vLD90 = 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 vLD90 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 and cefepime), (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;
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).

<table>
<thead>
<tr>
<th>HBD-2</th>
<th>AMP/SULB</th>
<th>AMOX/CLAV</th>
<th>PIP/TAZO</th>
<th>AZTR</th>
<th>IMIP</th>
<th>CIPRO</th>
<th>CEPH</th>
<th>CEFAZ</th>
<th>CEFUR</th>
<th>CEFEP</th>
<th>CEFTR</th>
<th>GENT</th>
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<td>50%</td>
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<td>4</td>
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<tr>
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<td>20%</td>
<td>10%</td>
<td>20%</td>
<td>10%</td>
<td>0%</td>
<td>0%</td>
<td>13%</td>
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<td>10</td>
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<td>0%</td>
<td>6%</td>
<td>0%</td>
<td>0%</td>
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<td>6</td>
</tr>
<tr>
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<td>0%</td>
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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 keratinocytes 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 vLD₉₀ < 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 vLD₉₀ > 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 vLD₉₀ to hBD-2 < 10 µg/ml were resistant to the above antibiotics, while only a minority (15%) of the strains with vLD₉₀ 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 vLD₉₀ 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 vLD₉₀ < 4.5 µg/ml. These concentrations were significantly lower than the concentrations of hBD-2 required to kill Acinetobacter isolates susceptible to antibiotics (mean vLD₉₀ = 7.5 µg/ml) or other susceptible bacterial strains belonging to other species such as K. pneumonia, E. coli or P. mirabilis (vLD₉₀ > 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 susceptibility 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 hydrophobic 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 synergistic effect [6]. Taken together, introduction of hBD-2 to the antimicrobial therapy of multi-drug resistant bacteria could potentially have additional therapeutic benefits beside its direct bacterial 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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References


[22] Mookherjee N, Hancock RE. Cationic host defence peptides: 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 hydrophobic 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 synergistic 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 bacterial activity.

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