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

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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

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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 $1.5 \times 10^8$ 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.

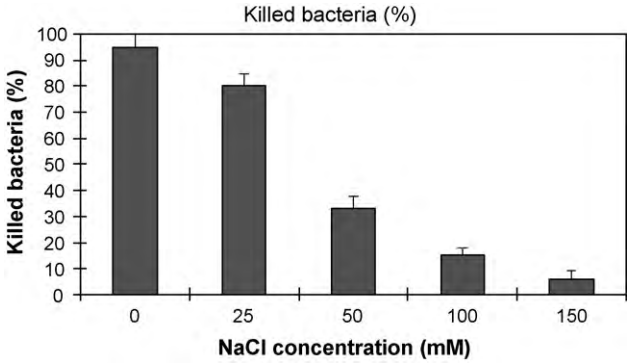


**Fig. 1.** Effect of NaCl concentration on the antimicrobial activity of hBD-2. The data presented regard the mean values of 5 individual experiments. Enhanced bactericidal activity of hBD-2 was observed in low salt concentrations.

### 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).