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### Permalink

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### Journal

Polymers, 4(4)

### ISSN

2073-4360

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### Publication Date

2012-03-01

### DOI

10.3390/polym4010747

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Article

## Antibacterial Activity of Four Human Beta-Defensins: HBD-19, HBD-23, HBD-27, and HBD-29

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Received: 1 February 2012; in revised form: 21 February 2012 / Accepted: 21 February 2012 / Published: 1 March 2012

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**Abstract:** Human  $\beta$ -defensins (HBD) are a family of small antimicrobial peptides that play important roles in the innate and adaptive immune defenses against microbial infection. In this study, we predicted the mature sequences and assessed the antibacterial properties of synthetic HBD-19, HBD-23, HBD-27, and HBD-29 against three species of clinically relevant bacteria: *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We also examined the cytotoxicity of each  $\beta$ -defensin to human cells. HBD-19 exhibited modest antibacterial effects against *E. coli* and *S. aureus* but had little effect on the growth of *P. aeruginosa*. HBD-23 exhibited substantial antibacterial effects against all three bacterial species and was particularly potent against the Gram-negative species, *E. coli* and *P. aeruginosa*. HBD-27 exerted modest antibacterial activity only towards *S. aureus* while HBD-29 had modest antibacterial activity for *E. coli* and *P. aeruginosa*. HBD-23 and HBD-27 showed little or no toxicity to human peripheral blood mononuclear cells, while HBD-19 and HBD-29 decreased cell viability by 20% at 30  $\mu$ g/mL.

**Keywords:**  $\beta$ -defensin; antibacterial; *Escherichia coli*; *Staphylococcus aureus*; *Pseudomonas aeruginosa*

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## 1. Introduction

Human  $\beta$ -defensins (HBD) are a family of cationic peptides that share a pattern of six cysteine residues forming three invariant disulfide bonds. Defensins constitute one of the first lines of innate immune defense against an array of bacteria, fungi, and viruses [1–5]. Many studies have demonstrated that human  $\beta$ -defensins, such as HBD-1, HBD-2, HBD-3, and HBD-4 are antimicrobial peptides expressed by epithelial cells initially as longer precursors [6–10]. Following processing to their mature forms, one of their mechanisms of action against bacteria is characterized by the electrostatic binding of cationic defensins with the negatively charged membrane [11,12]. This results in the disruption or perforation of the bacterial membrane, leading to cytoplasmic leakage and cell death [13,14]. Due to their broad microorganism-killing spectrum,  $\beta$ -defensins may be ideal candidates for the development of human antibiotics. Beta-defensins are also chemotactic for immature dendritic cells, memory helper T cells, monocytes, macrophages and activated neutrophils [6,15–18]. They provide a link between the innate and adaptive responses to microbial infection. The study of  $\beta$ -defensins will therefore lead to a greater understanding of innate and adaptive immunity at epithelial surfaces.

In this study, we report the antibacterial activities of four synthetic defensins whose mature sequences we predicted: HBD-19, HBD-23, HBD-27, and HBD-29. These peptides were chosen based on their common origin in the epithelial cell layer of the testis and epididymis [19–21]. Although these peptides differ in primary structure from known  $\beta$ -defensin peptides, they share a conserved arrangement of six cysteine residues with previously studied  $\beta$ -defensins and may therefore play important roles in defense against invading microbes in the male reproductive tract. The aims of this study were to characterize the antibacterial spectrum of these four human  $\beta$ -defensins, explore the effects of salt and a reducing agent on their antibacterial activities, and assess their cellular toxicity on human peripheral blood mononuclear cells (PBMC).

## 2. Experimental Section

### 2.1. Predicting Mature Defensin Amino and Carboxy Termini

SignalP, a computational tool available on the world-wide web [22] was used to predict the amino terminal signal peptide cleavage sites of HBD-19, HBD-23, HBD-27 and HBD-29. SignalP predictions were compared with known mature  $\beta$ -defensin peptide sequences to predict which amino terminal variants were most likely to yield biologically active  $\beta$ -defensins. Similarly, the mature carboxy termini of HBD-19, HBD-23, HBD-27 and HBD-29 were predicted by comparison with the known mature  $\beta$ -defensin peptide sequences.

### 2.2. Reduction and Refolding of Synthetic Defensins

HBD-23, HBD-27, and HBD-29 were chemically synthesized by Biosynthesis Inc. (Louisville, TX, USA) and HBD-19 was synthesized and kindly provided by Drs. Saskia Milton and Charles Glabe (University of California, Irvine). After reducing 4 mg of synthetic defensins with 1  $\mu$ g/mL dithiothreitol (DTT) at 55  $^{\circ}$ C for 4 h, the peptides were passed through Sep-Pak cartridges with a C-8 sorbent, washed, and eluted with acetonitrile plus 0.1% trifluoroacetic acid. Peptides were dried overnight and

subsequently dissolved in 0.1 M ammonium carbonate buffer supplemented with 1 mM cysteine and 1 mM cystine under nitrogen gas [23]. The mixtures were stirred slowly at 25 °C, allowing the peptides to refold. Peptides were examined every 24 h by C-18 reverse-phase HPLC to assay the presence of foldamers. Peptides were further examined by reducing and non-reducing SDS-PAGE to assay the presence of multimers. Each lane was loaded with 3 µg of peptide. Gels were stained with Coomassie blue dye and destained in (5% (v/v) methanol, 7% (v/v) acetic acid, 88% H<sub>2</sub>O). After seven days peptides were found to be >95% monomeric, single foldamers.

### 2.3. Antibacterial Activity Assays

The colony forming unit (CFU) reduction assay was performed using *Escherichia coli* ML35 (provided by Dr. Andre Ouellette, University of Southern California), *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 as described by Dr. Ouellette and colleagues [23]. Briefly, various concentrations of defensins were mixed with  $1 \times 10^6$  to  $5 \times 10^6$  bacteria in the logarithmic growth phase in 200 µL of 10 mM PIPES [piperazine-N,N'-bis (2-ethanesulfonic acid), pH 7.4] buffer and 1% (vol/vol) trypticase soy broth. These samples were incubated at 37 °C for 4 h. After this incubation, samples were diluted 100-fold with cold 10 mM PIPES and plated in triplicate on Luria broth agar. The plates were then incubated for 12 to 16 h at 37 °C and the number of colonies was recorded. In order to assess the effect of salt and a reducing agent on defensin activity, the PIPES buffer was supplemented with either 150 mM NaCl or 2 mM dithiothreitol (DTT). As a positive bactericidal control, 20 mM lysozyme was utilized. Statistical tests were performed with Student's two-tailed t-test.

### 2.4. Cellular Toxicity Assay

Human peripheral blood mononuclear cells (PBMCs) were purified from normal donor blood by centrifugation over Histopaque 1077 (Sigma) and  $10^5$  cells were cultured in 100 µL of media per well in 96-well plates. RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 µg/mL gentamicin and 20 units/mL IL-2 was used. PBMC were incubated for three days with various concentrations of refolded or crude defensins. Thereafter, an MTS based metabolic assay (Promega) was utilized to examine the cytotoxicity of the defensins. The assay was conducted according to the manufacturer's specifications.

## 3. Results

### 3.1. Predicting Mature Defensin Amino and Carboxy Termini

The SignalP software tool was used to predict the amino terminal signal peptide cleavage sites of HBD-19, HBD-23, HBD-27 and HBD-29. Subsequently, these predictions were compared with the known mature peptide sequences of HBD-1, HBD-2, HBD-3 and HBD-4 to predict which amino terminal variants were most likely to yield biologically active β-defensins (Figure 1). Similarly, the mature carboxy termini of HBD-19, HBD-23, HBD-27 and HBD-29 were predicted by comparison with the known mature β-defensin peptide sequences. The resulting predicted mature HBD-19, HBD-23, HBD-27 and HBD-29 peptides contain the core 6-cysteine motif preceded by four to ten

amino-terminal amino acid residues and followed by four to six carboxy terminal residues. The predicted mature amino terminal residue of HBD-19 and HBD-23 is glycine, which is also found at the amino terminus of HBD-1, HBD-2 and HBD-3. The predicted mature amino terminal residue of HBD-27 is glutamic acid like HBD-4 and the predicted mature amino terminal residue of HBD-29 is valine. All four of the predicted mature human  $\beta$ -defensins terminate with a positively charged amino acid, as do HBD-1 and HBD-3.

**Figure 1.** Sequences of known and predicted human  $\beta$ -defensins. The known sequences of HBD-1, 2, 3 and 4 are aligned with the predicted sequences of HBD-19, 23, 27 and 29. The synthetic versions of the latter four human  $\beta$ -defensins used in this study have the sequences shown. Gaps were introduced to align cysteine residues, which are shaded. Amino and carboxy terminal residues are in bold font.

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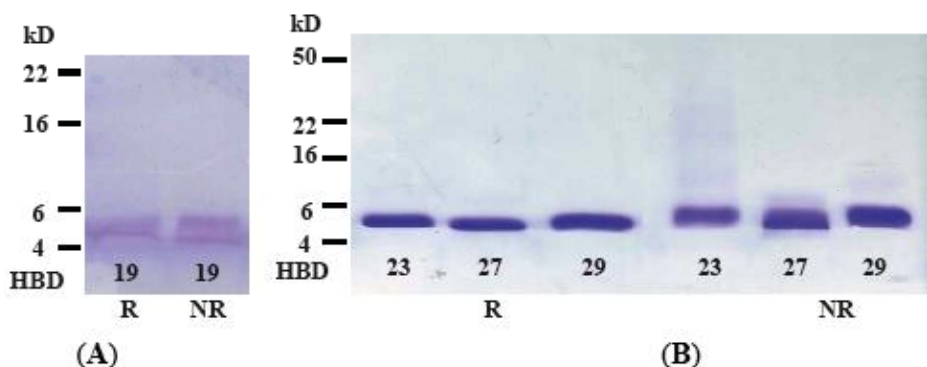
HBD-1  GNFLTGLGHRSDHYNCVSS--GGQCLYSACPIFTKIQGTCYRGKAKCCK
HBD-2          GIGDPVTCLKS--GAICHPVFCPRRYKQIGTCGLPGTKCCKKP
HBD-3  GIINTLQKYYCRVR--GGRCAVLSCLPKEEQIGKCSTRGRKCCRRK
HBD-4  EFELDRICGYG--TARCRKK--CRSQEYRIGRC--PNTYACCLRKWDESLLNRTKP
HBD-19  GKRHILRCMGN--SGICRAS--CKKNEQPYLYC--RNCQSCCLQSYMR
HBD-23          GTQRCWNL--YGKCRYR--CSKKERVYVYC--INNKMCCVKPK
HBD-27          EQLKKCWNNYVQGHCRKI--CRVNEVPEALC--ENGRYCCLNIK
HBD-29  VNTEFIGLRRCLMG--LGRCRDH--CNVDEKEIQKC--KMKKCCVGPK

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### 3.2. Refolding Synthetic Defensins

After reducing the peptides and allowing them to slowly refold, we analyzed their structural homogeneity by comparing the folded and unfolded forms on C-18 reverse-phase HPLC. We also analyzed the folded forms by SDS-PAGE under non-reducing (NR) and reducing conditions (R) to detect the presence of multimers. More than 95% of each peptide refolded to a single monomeric foldamer as indicated by the near uniform mobility of each peptide on C-18 reverse-phase HPLC (data not shown) and in SDS-PAGE under reducing and non-reducing conditions (Figure 2).

**Figure 2.** SDS-PAGE analysis of refolded synthetic human  $\beta$ -defensins. (A) HBD-19 and (B) HBD-23, HBD-27 and HBD-29 were examined in reduced (R) and non-reduced (NR) states by SDS-PAGE on 15% polyacrylamide gels followed by staining with Coomassie blue dye.

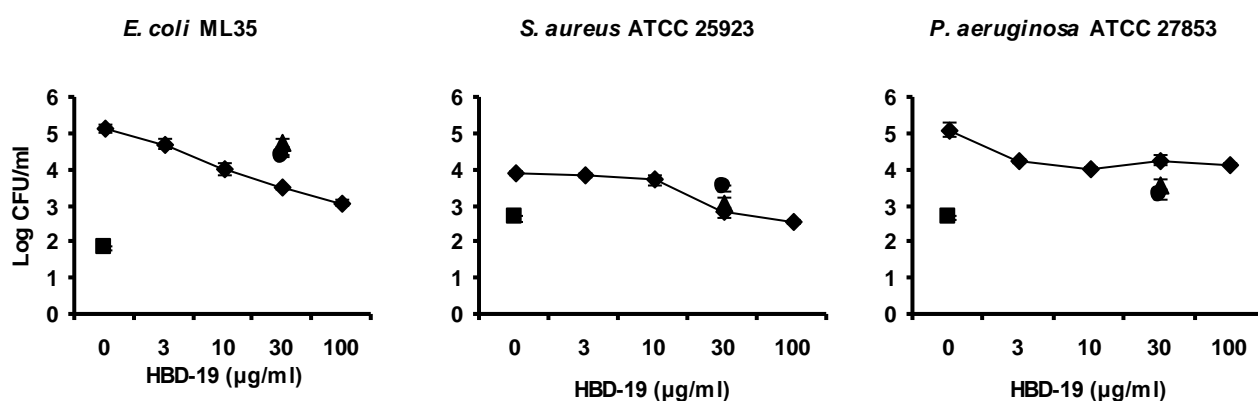


### 3.3. Antibacterial Activity Assay of Synthetic Defensins

The antibacterial activity of each peptide, for *E. coli*, *S. aureus* and *P. aeruginosa* was evaluated using the reduction of colony forming unit (CFU) assay. Each peptide was tested at concentrations ranging from 3 to 100  $\mu\text{g/mL}$  in a low ionic strength buffer. Lysozyme was used as a positive control for antibacterial activity. Moreover, each peptide was tested at one concentration in a high ionic strength solution and separately, in the presence of a reducing agent in order to probe the structural requirements of their antibacterial activities.

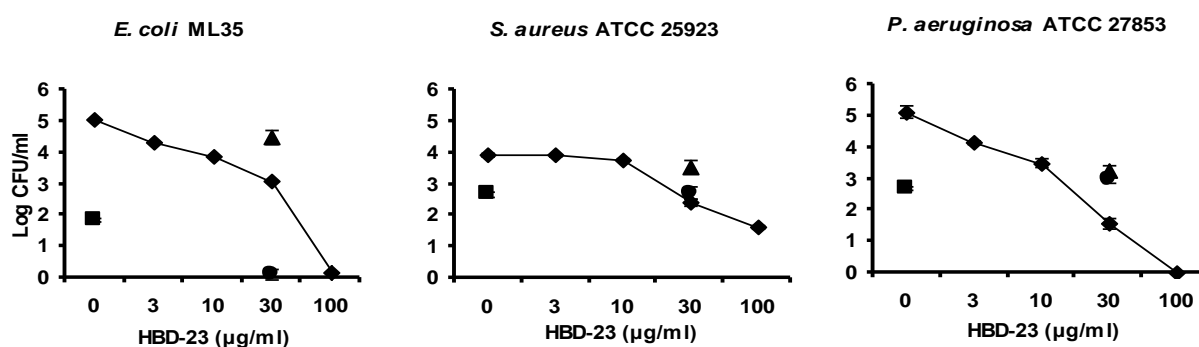
HBD-19 had antibacterial activity against *E. coli* ML35, and *S. aureus* ATCC 25923, but little against *P. aeruginosa* ATCC 27853 (Figure 3). At 100  $\mu\text{g/mL}$  HBD-19 reduced the number of *E. coli* colonies 134-fold, the number of *S. aureus* colonies 20-fold and the number of *P. aeruginosa* colonies almost 10-fold. Its activity towards *E. coli* was significantly reduced when 150 mM NaCl ( $p = 0.001$ ) or 2 mM DTT ( $p = 1.4 \times 10^{-8}$ ) was added. Only DTT significantly attenuated the anti-*S. aureus* activity of HBD-19 ( $p = 4.5 \times 10^{-5}$ ). In contrast, neither 150 mM NaCl nor 2 mM DTT had a significant effect on *E. coli* colony numbers alone. Both agents inhibited *S. aureus* colony formation significantly alone, but the inhibition was less than 2.5 fold in both cases so it did not obscure the attenuation of HBD-19 antibacterial activity against *S. aureus*. Neither 150 mM NaCl nor 2 mM DTT had a significant effect on *P. aeruginosa* colony numbers alone,  $p = 0.11$  and  $p = 0.31$  respectively, but paradoxically both agents potentiated HBD-19's activity against *P. aeruginosa* colony formation, albeit modestly, 4.7 and 8.1 fold respectively.

**Figure 3.** Antimicrobial activity of HBD-19 against three bacterial species. The antimicrobial activity of HBD-19 was evaluated by colony forming unit (CFU) assay and the results were plotted on a logarithmic scale. The experiments were carried out using four different concentrations of HBD-19 (3  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 30  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ ) with 20 mM lysozyme (square) as the positive bactericidal control. The bactericidal activity of 30  $\mu\text{g/mL}$  HBD-19 was also tested in the presence of 150 mM NaCl (triangle) and 2 mM dithiothreitol (circle). The bacteria used were *Escherichia coli* ML35, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The results shown are the average of six complete experiments with *E. coli*, four with *S. aureus* and five with *P. aeruginosa*; while some points on the graphs represent more experimental repetitions. Error bars show standard deviations.



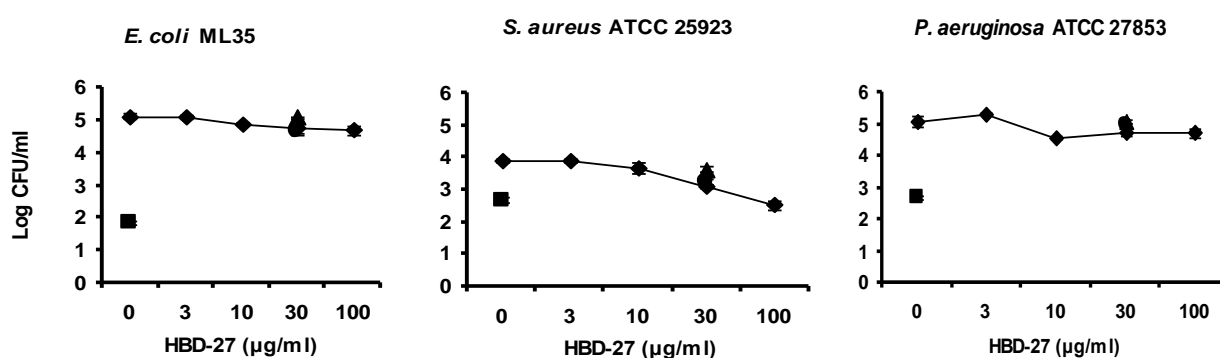
Among the four defensins tested, HBD-23 exerted the strongest antibacterial activity (Figure 4). At 100  $\mu\text{g}/\text{mL}$ , HBD-23 reduced the number of *E. coli* colonies by nearly five logs ( $5.6 \times 10^4$ -fold), the number of *S. aureus* colonies 185-fold and the number of *P. aeruginosa* colonies by more than five logs ( $1.4 \times 10^5$ -fold). The anti-*E. coli* activity of 30  $\mu\text{g}/\text{mL}$  HBD-23 was decreased under physiological salt conditions ( $p = 0.004$ ) and enhanced by 2 mM DTT ( $p = 7.7 \times 10^{-5}$ ). As stated above, neither agent affected *E. coli* colony formation alone. The addition of 2 mM DTT did not significantly affect the anti-*S. aureus* activity of HBD-23 but addition of 150 mM NaCl attenuated this activity ( $p = 7.6 \times 10^{-6}$ ). As above for HBD-19, the modest inhibition of *S. aureus* colony formation mediated by NaCl alone did not mask its ten-fold attenuation of the anti-*S. aureus* activity of HBD-23. Both NaCl and DTT significantly inhibited the anti-*P. aeruginosa* activity of HBD-23 ( $p = 0.007$  and 0.001 respectively), but as stated above neither agent significantly affected *P. aeruginosa* colony formation alone.

**Figure 4.** Antimicrobial activity of HBD-23 against three bacterial species. The antimicrobial activity of HBD-23 was evaluated by colony forming unit (CFU) assay and plotted on a logarithmic scale. The experiments were carried out using four different concentrations of HBD-23 (3  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{g}/\text{mL}$ , 30  $\mu\text{g}/\text{mL}$ , 100  $\mu\text{g}/\text{mL}$ ) with 20 mM lysozyme (square) as the positive bactericidal control. The bactericidal activity of 30  $\mu\text{g}/\text{mL}$  HBD-23 was also tested in the presence of 150 mM NaCl (triangle) and 2 mM dithiothreitol (circle). The bacteria used were *Escherichia coli* ML35, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The results shown are the average of three complete experiments with *E. coli*, five with *S. aureus* and five with *P. aeruginosa*; while some points on the graphs represent more experimental repetitions. Error bars show standard deviations.

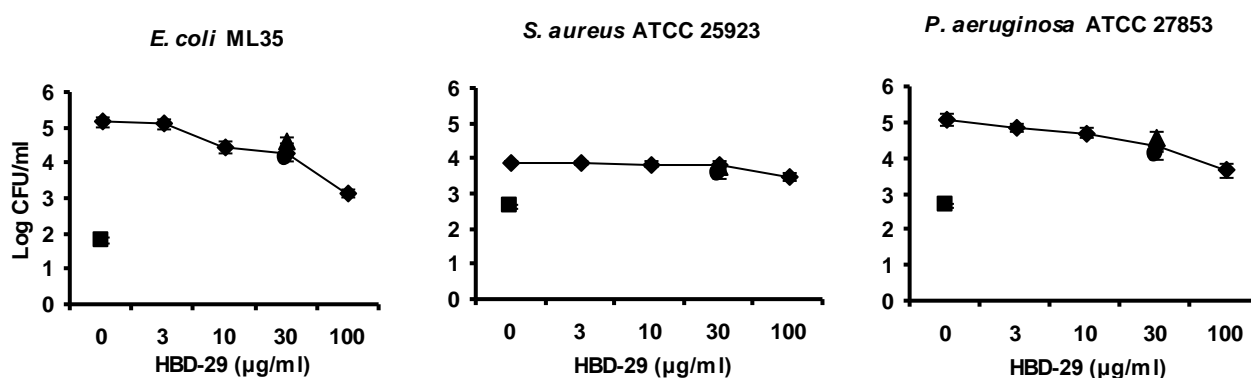


Unlike HBD-23, HBD-27 showed little antibacterial activity against *E. coli* or *P. aeruginosa* (Figure 5). HBD-27 was weakly active, however, against *S. aureus*, but only at high concentrations. At 100  $\mu\text{g}/\text{mL}$  HBD-27 reduced the number of *P. aeruginosa* colonies by nearly 22-fold. This modest antibacterial activity of HBD-27 against *P. aeruginosa* was attenuated by the addition of 150 mM NaCl ( $p = 0.001$ ) or by the addition of 2 mM DTT ( $p = 0.02$ ).

**Figure 5.** Antimicrobial activity of HBD-27 against three bacterial species. The antimicrobial activity of HBD-27 was evaluated by colony forming unit (CFU) assay and plotted on a logarithmic scale. The experiments were carried out using four different concentrations of HBD-27 (3  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 30  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ ) with 20 mM lysozyme (square) as the positive bactericidal control. The bactericidal activity of 30  $\mu\text{g/mL}$  HBD-27 was also tested in the presence of 150 mM NaCl (triangle) and 2 mM dithiothreitol (circle). The bacteria used were *Escherichia coli* ML35, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The results shown are the average of six complete experiments with *E. coli*, six with *S. aureus* and five with *P. aeruginosa*; while some points on the graphs represent more experimental repetitions. Error bars show standard deviations.



**Figure 6.** Antimicrobial activity of HBD-29 against three bacterial species. The antimicrobial activity of HBD-29 was evaluated by colony forming unit (CFU) assay and plotted on a logarithmic scale. The experiments were carried out using four different concentrations of HBD-29 (3  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 30  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ ) with 20 mM lysozyme (square) as the positive bactericidal control. The bactericidal activity of 30  $\mu\text{g/mL}$  HBD-29 was also tested in the presence of 150 mM NaCl (triangle) and 2 mM dithiothreitol (circle). The bacteria used were *Escherichia coli* ML35, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The results shown are the average of five complete experiments with *E. coli*, four with *S. aureus* and five with *P. aeruginosa*; while some points on the graphs represent more experimental repetitions. Error bars show standard deviations.



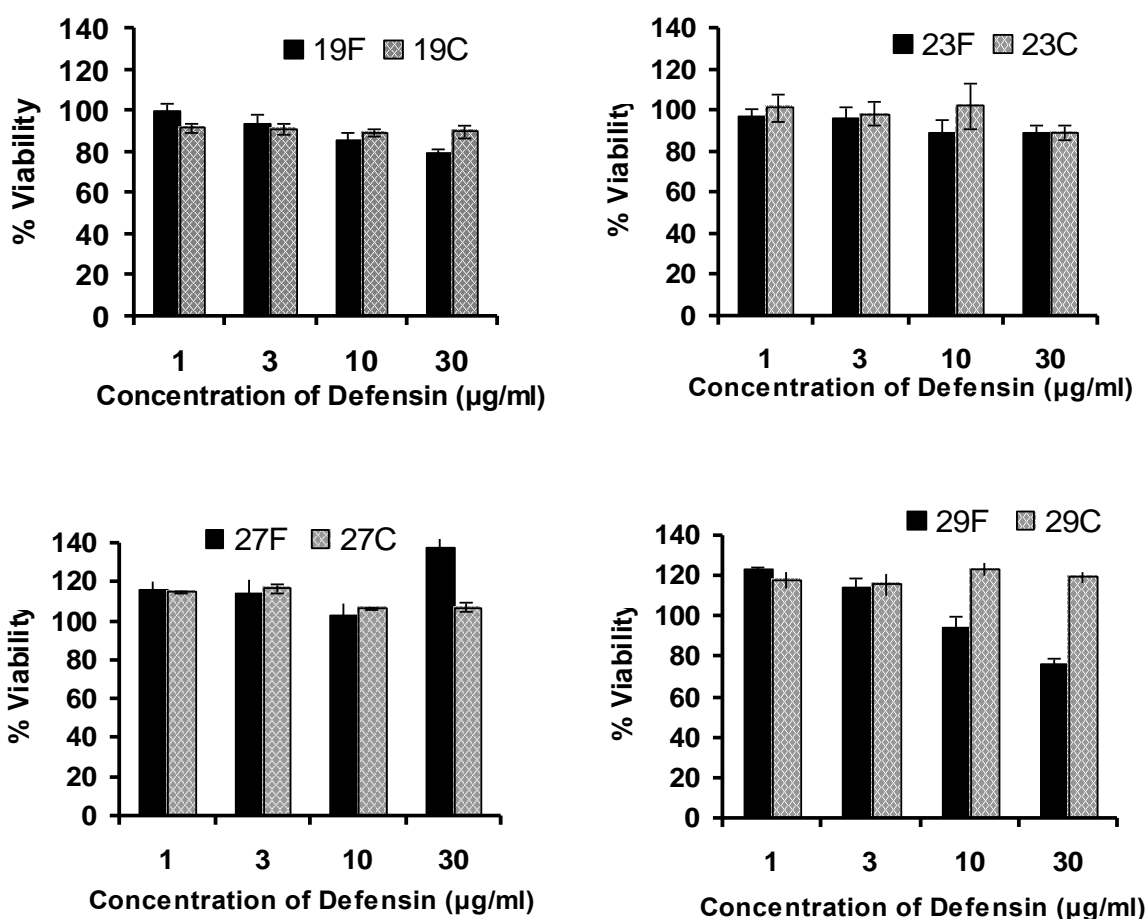


Lastly, HBD-29 exhibited antibacterial activity against *E. coli* and *P. aeruginosa*, but had little to no activity against *S. aureus* (Figure 6). At 100  $\mu\text{g}/\text{mL}$  HBD-29 reduced the number of *E. coli* colonies 112-fold and the number of *P. aeruginosa* colonies 26-fold. The antibacterial activity of HBD-29 against *E. coli* was attenuated by NaCl ( $p = 0.0006$ ), but not by DTT. In contrast, its activity against *P. aeruginosa* was blocked by DTT ( $p = 0.002$ ), but not by NaCl.

### 3.4. Cellular Toxicity in Peripheral Blood Mononuclear Cells

Previous studies reported that defensins interact electrostatically with bacterial membranes and thereby destroy their structure [3,12,14]. Thus, it is important to know whether these peptides can similarly interfere with human cellular membranes. The cellular toxicity of HBD-19, HBD-23, HBD-27, and HBD-29, both in their crude and folded states, was investigated using a colorimetric metabolic-activity assay on human peripheral blood mononuclear cells (PBMC; Figure 7).

**Figure 7.** HBD cytotoxicity on human peripheral blood mononuclear cells (PBMCs). The cellular toxicity of folded (F) and crude (C) HBD-19, HBD-23, HBD-27, and HBD-29 was analyzed by measuring the amount of the tetrazolium salt MTS reduced to a colored product by PBMCs after 3 days of incubation with various concentrations of defensins. The results shown are the average of three experiments and the error bars represent the standard deviations.



Folded HBD-19 showed a low level of cellular toxicity that was concentration dependent, reaching 20% at 30  $\mu\text{g/mL}$ . Crude HBD-19, however, had little effect on PBMC viability. Likewise, crude and folded HBD-23 and HBD-27 demonstrated little or no cytotoxicity. In fact, folded HBD-27 was somewhat stimulatory, particularly at 30  $\mu\text{g/mL}$ . In contrast, folded HBD-29 exhibited concentration dependent toxicity for PBMC; up to 23% at 30  $\mu\text{g/mL}$ , while crude HBD-29 stimulated PBMC metabolism slightly.

### 3.5. Discussion

This study examined the antibacterial properties of four human  $\beta$ -defensins—HBD-19, 23, 27, and 29—that are normally expressed, at least at the RNA level, in the male reproductive tract [19–21]. Because the epididymis is anatomically continuous with the urethra, host defense against ascending bacterial pathogens is pertinent in the protection of spermatozoa from bacterial contamination. Previous studies showed HBD-1 and HBD-4 are also expressed in the urinary tract and are known to be microbicidal [7,24].

The results of this study showed HBD-23 to be the most potent antibacterial agent among the four defensins tested. However, we found the antibacterial activity of HBD-23 was reduced in the presence of salt, an effect that has been documented by Shimoda *et al*, who observed that ultrastructural changes on the *S. aureus* surface caused by defensins were prevented at high-salt concentration [14]. This supports the hypothesis that HBD-23, like other previously studied antimicrobial defensins, permeabilizes the bacterial cell membrane by an electrostatic charge-based mechanism [12,25,26]. Antimicrobial peptides can also damage eukaryotic cells in the same manner [3]. Our data revealed that refolded HBD-19 and HBD-29 decreased cellular viability in a dose-dependent manner. However, HBD-23, and HBD-27 demonstrated low levels of cytotoxicity on human peripheral blood mononuclear cells, which is in agreement with some other defensins demonstrating low levels of hemolytic activities [16]. The presence of cholesterol and lipid-anchored proteins may interfere with defensin binding, preventing host damage [27,28].

We found that HBD-23 killed *E. coli* more efficiently *in vitro* when its disulfide bridges were reduced. This may be due to the positive charges being more exposed to anionic bacterial membrane and thereby killing at a faster rate. Nevertheless, it remains to be determined whether these disulfide bridges have a functional role *in vivo*, as one study reported that they stabilize defensins against enzymatic digestion [29].

Interestingly, Hwang *et al* showed that certain defensins are more efficient in killing bacteria when more positive charges are present within the cysteine core region [30]. This may explain why HBD-23 was the most potent as it contained the most net positive charges within the six cysteine motif, followed by HBD-29, HBD-19, and finally HBD-27, which showed the least amount of killing. These observations were consistent with the data presented by Rodríguez-Jiménez, who found that synthetic HBD-23 and HBD-29 had broad spectrums of antibacterial activity, while HBD-27 did not [20].

The human  $\beta$ -defensins studied here may also have other roles in immune defense including antiviral activity like HBD-2 and HBD-3 [4,31,32]. Moreover, multiple studies have reported that  $\beta$ -defensins recruit monocytes, T-cells, immature dendritic cells and activated neutrophils by acting as chemokines that interact with the chemokine receptors CCR2 and CCR6 [15–18,33]. Human

$\beta$ -defensins 1–4 activate keratinocytes to produce IL-18 and HBD-3 also activates macrophages and myeloid dendritic cells via the toll-like receptors (TLR) 1 and 2 [34,35]. Further investigation of HBD-19, 23, 27 and 29 may therefore shed more light on the intricate functions of human immunity.

#### 4. Conclusions

We assayed the antibacterial properties of synthetic HBD-19, HBD-23, HBD-27, and HBD-29 against three species of clinically relevant bacteria: *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. HBD-23 was by far the most potent peptide against all three species of bacteria. Moreover, HBD-23 exhibited very modest toxicity for normal human peripheral blood mononuclear cells at concentrations that inhibited the growth of the Gram-negative species *E. coli* and *P. aeruginosa* by five logs and the Gram-positive bacterium, *S. aureus* by more than two logs.

#### Acknowledgments

Maira Soto was the recipient of a California HIV/AIDS Research Program Research Training Award, and both Bryan Chow and Bonnie Lo received support from the University of California, Irvine Undergraduate Research Opportunity Program, for which we thank the funding agencies. We would also like to thank Hugh Fisher for helpful discussions and Kimberly Lam for excellent technical support.

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