CHEMBIOCHEM

DOI: 10.1002/cbic.200700560

Linear Analogues of Human β-Defensin 3: Concepts for Design of Antimicrobial Peptides with Reduced Cytotoxicity to Mammalian Cells

Shouping Liu,[a] Lei Zhou,[a, b] Jing Li,[a, b] Anita Suresh,[c] Chandra Verma,[c] Yong Hwee Foo,[a] Eric P. H. Yap,[d] Donald T. H. Tan,[a, b, e] and Roger W. Beuerman*[a, b]

A series of engineered linear analogues [coded as F6, W6, Y6, A6, S6 and C(Acm)6] were modeled, designed, synthesized and structurally characterized by mass spectra, circular dichroism, hydrophobicity analysis and molecular modeling. We have screened antimicrobial activity, hemolysis to rabbit erythrocytes, and cytotoxicity to human conjunctival epithelial cells. No significant hemolytic effect was observed for hBD3 or from five of the six analogues [F6, Y6, A6, S6 and C(Acm)6] over the range of 3–100 μg mL^{-1}. The six linear analogues have reduced cytotoxicity to human conjunctival epithelial cells over the range of 6–100 μg mL^{-1} compared to hBD3. By tuning the overall hydrophobicity of linear hBD3 analogues, reduced cytotoxicity and hemolysis were obtained while preserving the antimicrobial properties. The decreased cytotoxicity of the linear analogues is suggested to be structurally related to the removal of disulfide bridges, and the flexible structure of the linear forms, which seem to be associated with loss of secondary structure. These results suggest a new approach for guiding the design of new linear analogues of defensin peptides with strong antibiotic properties and reduced cytotoxicity to mammalian cells.

Introduction

The defensins are small cationic and cysteine-rich molecules with molecular weights between 3–6 kDa, and are about 45 amino acids in length. Based on the spatial distribution of the three cysteine intramolecular bonds, mammalian defensins can be divided into two major groups termed α and β-defensins. θ-Defensins make up a third group, but to-date are found only in nonhuman primates, and have only 18 amino acids.[1–3] Defensins are an important component of the innate immune system and provide an initial antimicrobial barrier for mucosal surfaces such as the surface of the eye, oral tissues, the airways and lungs, and also the skin.[4–8] As antimicrobial agents they show broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi and enveloped viruses, and also against bacteria that have demonstrated resistance to the currently used antibiotics.[9] Because of this ability, they have come to be known as "natural antibiotics". Interest in the defensins has also been aided by an increase in bacterial strains with resistance to the standard antibiotics, an increase in the population with reduced immunity, and the water solubility of defensins, which makes them potentially rapidly deployable in response to a pathogen release into the environment.[10] Current efforts are focused toward designing analogues of defensins that have properties that are appropriate for therapeutic application. However, as improvements to the structure–activity relationship are attempted, issues such as charge density and hydrophobicity emerge because analogues can interact with both the membrane of a pathogen as well as the membrane of human cells. Toxicity to human cells, of both native forms and of analogues, has been a concern.[11–12] Peptide analogues have been developed with diverse capabilities, and it appears that the disulfide bonds can be rearranged or removed with preservation of antimicrobial activity.[13–15]

The β-defensins have been discovered more recently than α-defensins, and have been given special attention because they are found in skin and epithelial cells that line mucosal surfaces.[7,16] In β-defensins, the disulfide bridges that likely stabilize these molecules are Cys1–Cys5, Cys2–Cys4 and Cys3–Cys6 while the α-defensins, which are largely produced by the Paneth cells of the intestine and neutrophils, have bridges at

[a] *Dr. S. Liu,[+] Dr. L. Zhou,[+] Dr. J. Li,[+] Y. H. Foo, Prof. D. T. H. Tan, Prof. Dr. R. W. Beuerman*
Singapore Eye Research Institute
11 Third Hospital Avenue, #06-00, Singapore 16875 (Singapore)
Fax: (+65) 6322-4599
E-mail: rbeuer@pacific.net.sg

[b] *Dr. L. Zhou,[+] Dr. J. Li,[+] Prof. D. T. H. Tan, Prof. Dr. R. W. Beuerman*
Department of Ophthalmology, Yong Loo Lin School of Medicine
National University of Singapore
Singapore 16875 (Singapore)

[c] *A. Suresh, Dr. C. Verma*
Bioinformatics Institute
Singapore 16875 (Singapore)

[d] *Dr. E. P. H. Yap*
Defence Medical & Environmental Research Institute
Singapore 16875 (Singapore)

[e] *Prof. D. T. H. Tan*
Singapore National Eye Center
Singapore 16875 (Singapore)

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: RP-HPLC-UV chromatograms and MS spectra of the six linear analogues of hBD3. (NMRC/CPG/007/2004)

Cys1–Cys6, Cys2–Cys4 and Cys3–Cys5. However, in the eye, both of these are active in response to injury, as has been found in tears.[6,17] Within the family of β-defensins, hBD1, hBD2 and hBD3 have been the most extensively studied; additionally hBD4–6 have been recently reported. Further, genome bioinformatics studies have pointed to the existence of an additional 28 β-defensins that have yet to be found by proteomic studies.[18–20] Studies of the tissue distribution of hBD3 by using RNA levels found only low levels in most tissues, except in oral mucosa and more recently in epithelial cells from the ocular surface.[7,21] Of the β-defensins, hBD3 has been of particular interest as it appears to possess better broad-spectrum antimicrobial activity than either hBD1 or hBD2.[22]

The goal of this study was to design analogues of hBD3 that exhibit good antimicrobial properties and display reduced toxicity to human cells. We designed and synthesized linear analogues of hBD3 by retaining the same net positive charge (+11), and by replacing the six bridging cysteine residues with residues of varying hydrophobicities. Factors including linearity, hydrophobicity, antimicrobial activity, hemolysis and epithelial cell cytotoxicity were determined. Within this group of linear analogues, the overall hydrophobicity was generally but not specifically related to decreased cytotoxicity to human ocular surface cells; however, antimicrobial capability remained at the same level as native hBD3.

Table 1. Physicochemical properties of the peptides.

Variant	Number of residues total	Number of residues hydrophobic (aromatic)	Net positive charge	Relative hydrophobicity[a]
C(Acm)6	45	14 (2)	11	n.c.
S6	45	14 (2)	11	28.3
A6	45	14 (2)	11	23.5
wt-hBD3	45	14 (2)	11	20.5
Y6	45	14 (8)	11	12.7
F6	45	14 (8)	11	11.5
W6	45	14 (8)	11	6.1

[a] The overall hydrophobicity was calculated based on the hydrophobicity scale of Hopp–Woods hydrophilicity scale.[23] The lower value corresponds to lower hydrophilicity or higher hydrophobicity; n.c., not calculated.

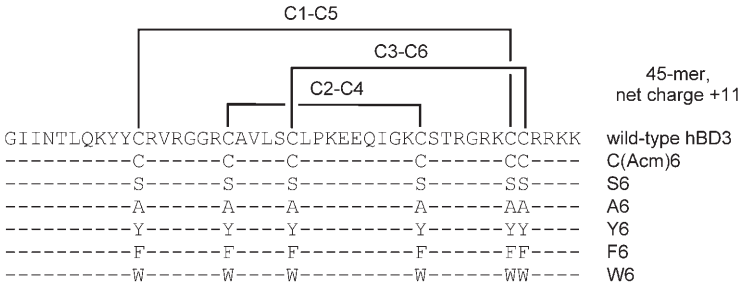
Table 2. The overall hydrophobicity of the peptides in terms of retention time (t_R) in RP-HPLC–MS.

Variant	t_R [min] 500 μg mL^{-1}	t_R [min] 100 μg mL^{-1}
C(Acm)6	20.15 ± 0.19	20.94 ± 0.21
S6	20.33 ± 0.25	20.44 ± 0.15
A6	22.36 ± 0.07	22.36 ± 0.28
wt-hBD3	23.24 ± 0.03	24.28 ± 0.13
Y6	23.85 ± 0.16	24.40 ± 0.05
F6	27.65 ± 0.13	28.25 ± 0.12
W6	29.29 ± 0.03	29.66 ± 0.06

Results and Discussion

Synthesis of analogues of hBD3

The solid-phase synthesis of six linear analogues of hBD3 with 45 residues was performed by using Fmoc chemistry. The peptide sequences are illustrated in Scheme 1. In the analogues, all cysteine residues were uniformly replaced by one of the following amino acids: alanine (A), serine (S), cysteine that was protected by Acm [C(Acm)], tryptophan (W), tyrosine (Y), or phenylalanine (F). The six analogues were coded as follows: A6, S6, C(Acm)6, W6, Y6 and F6, respectively. Physicochemical properties of the peptides are shown in Table 1 (sequence length, numbers of hydrophobic and aromatic residues, net charge, relative hydrophobicity) and Table 2 (retention time in HPLC). The UV spectroscopy and mass spectrometry (MS) of these peptides are shown in Figure S1 in the Supporting Information.



Scheme 1. Sequence of wild-type hBD3 and its linear analogues.

Compared with native hBD3, the six linear analogues of hBD3 are of the same length and net positive charge (+11) because they are designed and synthesized by uniform replacement of the six bridging cysteine residues in hBD3 with six residues of varying hydrophobicities (viz., F, W, Y, A, S and C(Acm)). Therefore, these linear analogues provide a well-defined model to study the effect of overall hydrophobicity, secondary structure conformation, removal of the native disulfide bridges on antimicrobial, hemolytic and cytotoxic activities.

Molecular hydrophobicity

We have measured the relative molecular hydrophobicity by RP-HPLC–MS in terms of retention time at 500 μg mL^{-1} and 100 μg mL^{-1} (Table 2). RP-HPLC is an approach that is commonly employed for comparisons of peptides or amino acid side chains on antibacterial peptides.[31–35] Because the stationary phase of C18-modified silica is hydrophobic and the mobile phase (water/acetonitrile) is hydrophilic, a longer retention time is a measure of greater hydrophobicity. The measured order for the relative molecular hydrophobicity of the peptides was as follows: W6 > F6 > Y6 > native hBD3 > A6 > S6 > C(Acm)6. The relative hydrophobicities of the peptides were also calculated based on the Hopp–Woods hydrophilicity scale[23] (Table 1). The scale is a hydrophilic index in which apolar residues have been assigned negative values, and is typically

used to identify antigenic regions based on hydrophilic patches. For each peptide, the values that correspond to each residue were summed to give an overall measure. The general trend in computed hydrophobicity of the peptides by using this scale matched that of the experimental HPLC retention time data. C(Acm)6, was excluded because Acm has not been parameterized in the Hopp–Woods scale.[23]

CD spectroscopy

Previous X-ray and NMR spectroscopic studies of human β-defensins (hBD1–3)[27,36–37] have shown that the tertiary structures are similar, and have a short α-helical segment before a triple-stranded antiparallel β-sheet, all rigidly held together by three disulfide bonds. It follows that in the absence of the constraints that are imposed by these disulfide bonds, the linear analogues of hBD3 are likely to be flexible and random in aqueous solution, and they could adopt conformations that are dictated by the environment.[13,15] Therefore, it was of interest to examine the solution conformations of the linear analogues in different media. CD spectroscopy of the hBD3 derivatives was carried out in aqueous solution in the presence of 50% organic modifier trifluoroethanol (TFE), and in the presence of 20 mM SDS micelles, which is a membrane-mimetic (Figures 1, 2, and 3). TFE has been widely used in protein structure studies because it can induce stable, structured conformations from otherwise unstructured/random peptides in aqueous solution.[38–41] The membrane-mimicking SDS was chosen because it is most similar to the prokaryotic membrane, and is therefore a good model for the natural target of an antibiotic.[42] While all of the tested peptides appeared unstructured in aqueous solution, they displayed an observable level of structuring in the presence of 50% TFE or 20 mM SDS micelles, as was determined by CD spectroscopy.

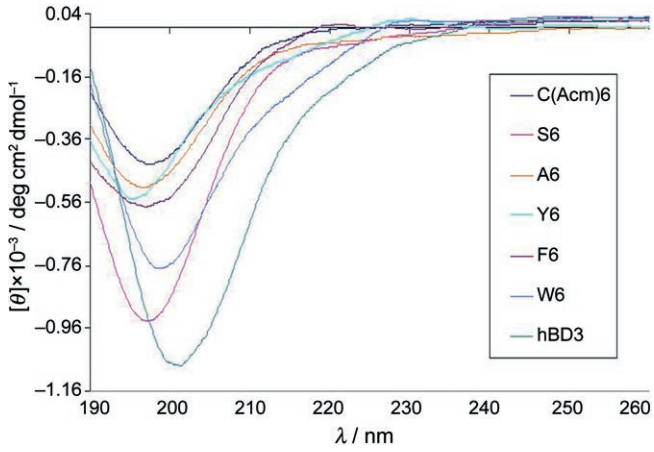


Figure 1. CD spectra of hBD3 and its linear analogues in water.

The CD spectrum of wild-type hBD3 agrees very well with that of native hBD3, which was reported elsewhere.[13,42] The spectra of the six linear analogues and wild-type hBD3 in aqueous solution were similar, and suggested a mix of random and

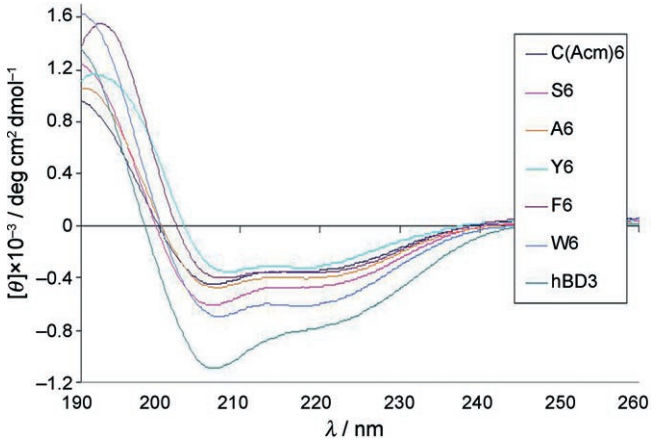


Figure 2. CD spectra of hBD3 and its linear analogues in 50% TFE solution.

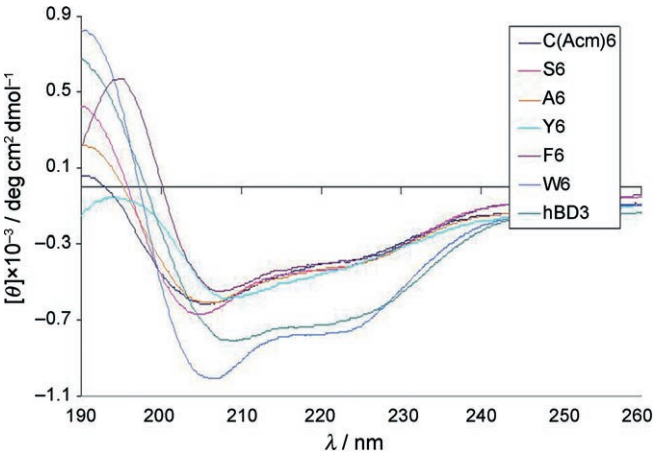


Figure 3. CD spectra of hBD3 and its linear analogues in 20 mM SDS micelles.

β-hairpin conformations, although to different extents because the intensities at the negative extrema varied (Figure 1). The absence of prominent crossover at wavelengths of 195 nm was indicative of an absence of significant amounts of structurally distinct conformers, while the minima at ~200 nm was suggestive of transient β-hairpin or turn-like conformer. This suggests that the existence of disulfide bonds and the type of Cys-replacing residues impose no significant effect on the secondary structure of hBD3 derivatives. The minima at ~195–200 nm for native defensin has been observed previously in the case of native HNP-1[43] and BNBD-12,[44] and is characteristic of peptides that contain β-sheet or β-turn secondary structure with some amount of random coil.[42] Thus, the secondary structure seems to be preformed in the peptides independent of the presence of multiple disulfide bridges or the Cys-replacing residues. This observation is in accordance with the result that was reported by Kluver et al.,[13] and is further supported by molecular dynamics simulations (Figure 4) that show that the linear variants adopt a variety of conformations in aqueous conditions.

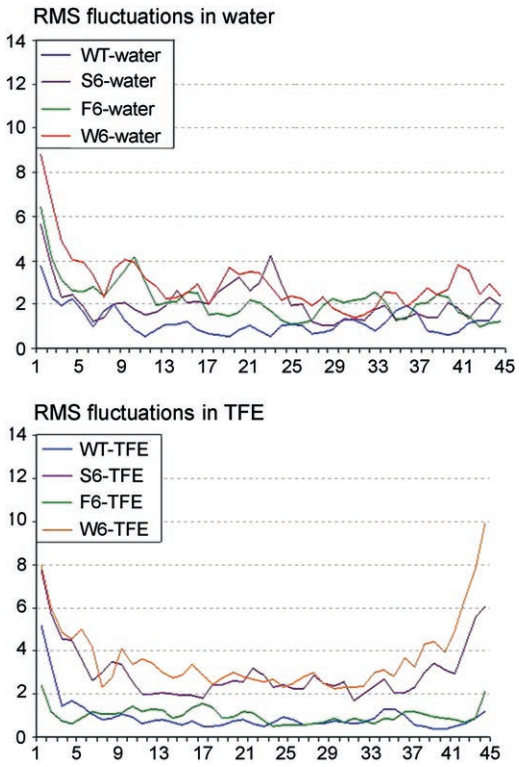


Figure 4. Mobility of main-chain atoms of hBD3 and linear analogues S6, F6 and W6 in water and TFE environments.

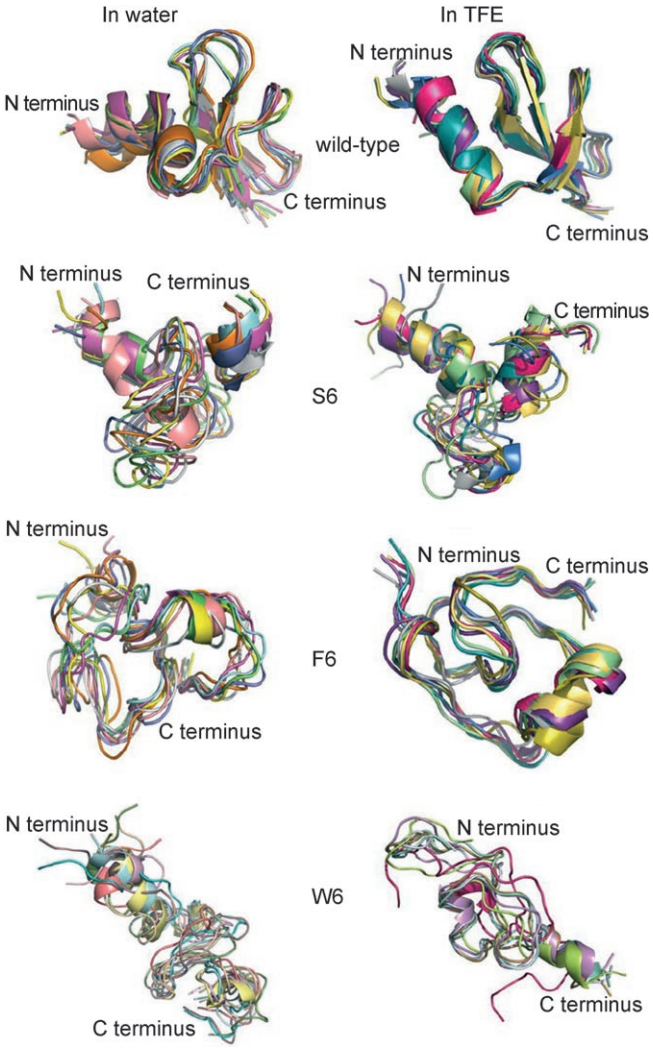


Figure 5. Snapshots of folded conformations of hBD3 and linear analogues S6, F6 and W6 in water and TFE environments.

In the presence of 50% TFE in water, all linear analogues as well as native hBD3 underwent a marked conformational transition (Figure 2). Their CD spectra became red-shifted with clearly observable double minima at wavelengths of 209 and 222 nm, and strong positive peaks at 190–193 nm. Such a spectrum is characteristic of α-helical structures.[41] Because the N terminus of native hBD3 can assume a helical conformation, it might be reasonable to conclude that the presence of TFE increased the proportion of helical conformation in these peptides.[42] The α-helical content of the six analogues and wild-type hBD3 perceptibly increased in the presence of TFE, which is known to stabilize this type of conformation;[43] this is also seen in our molecular dynamics simulations (Figure 5). The negative ellipticity near 205 nm and crossover at 200 nm for Y6, F6, C(Acm)6 and A6 spectra in TFE suggest populations with β-hairpin conformations. A pronounced positive peak at ~195 nm for F6 indicates the presence of a β-sheet conformation. The double minima at ~205 nm and ~223 nm, with a crossover at ~200 nm indicates the presence of populations with both β-hairpin and helical conformations.

Examination of the populations of hydrogen bonds from our molecular dynamics (MD) simulations show that in the wild-type, the induction of structure in the presence of TFE (in contrast to water) is brought about by an increase in the number of side-chain–side-chain interactions, whereas in S6 this is brought about by an increase in hydrogen bonds that involve the backbone atoms. In both of these systems, an increase in order is seen in the N-terminal helical regions. In contrast, the bulky side-chains of Phe in F6 and Trp in W6 lead to a larger increase in interactions that involve the side-chain atoms. The increase in order is seen in the middle and the C-terminal ends of these two analogues. The larger increase in order in S6 is understandable because upon the reduction in the dielectric (TFE), it is easier for the smaller side chain of Ser to bridge polar atoms in the backbone than it is for the bulkier Trp. This is also seen in the fluctuations of the systems (Figure 4), where we see that in TFE, the larger increase in order in wild-type and S6 results in a clear decrease in their mobility relative to that in F6 and W6.

The CD transition also occurs in 20 mM SDS micelles, which simulates the anisotropic lipid environment of bacterial membranes. The six analogues and wild-type hBD3 increased their α-helix content in the SDS environment. The negative minima at ~205 nm and shoulder at 222 nm for W6 indicates a propensity for a helical conformation in SDS micelles. While having a greater proportion of the molecule that develops an α-helix structure has been correlated with strong hemolytic properties, no obvious correlation has been reported to date between the degree of α-helix formation and antibacterial

activity.[47] The broad minimum for hBD3 with a slight dip at 208 nm could arise as a result of the presence of both helical and β-sheet structure. In the case of BNBD12, CD spectra suggested that the presence of disulfide bridges did not "constrain" the peptides into adopting ordered conformations in aqueous medium.[44] If the molecule is rigid, it would be expected to resist any major solvent-induced conformational changes. However, in the presence of TFE and SDS, the CD spectra of linear analogues and wild-type hBD3 change dramatically; this indicates possible interactions between the peptides and the membrane-like environment. Thus, disulfide bridges do not necessarily dictate the presence of rigid secondary structures. Kluver et al. carried out only a minimal study of CD spectra in water and their results were essentially indifferent.[13] Our CD values in two of the three media that we used were informative, and our simulations corroborated those findings.

Antibacterial activity

The LD_{50} and LD_{99} of wild-type hBD3 and its full-length linear analogues were determined against two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and two Gram-positive bacteria (*B. cereus* and *S. aureus*) (Table 3). Our results for full-length linear hBD3 analogues showed that the replacement of the six cysteine residues with other residues had a measurable effect on the antibacterial activity. W6, S6 and F6 have antibacterial activities that are comparable to hBD3, while Y6, C(Acm)6 and A6 are less potent. W6 has the lowest LD_{50} against *E. coli* and *S. aureus* (lower than 5 $\mu g\,mL^{-1}$), and a comparable LD_{50} to hBD3 against *P. aeruginosa* and *B. cereus* (lower than 8 $\mu g\,mL^{-1}$). S6 and F6 also have low LD_{50} values (less than 10 $\mu g\,mL^{-1}$) against the four pathogens. For C6, Y6 and A6, the LD_{50} values against pathogens are in the range of 10–20 $\mu g\,mL^{-1}$. The results indicate that the series of synthesized linear analogues, especially W6, S6 and F6 have comparable killing activity against *E. coli, P. aeruginosa* and *S. aureus* compared to the cyclic or noncyclic 40-residue hBD3 derivatives,[13] which were measured in terms of the minimum inhibition concentrations (MIC). The LD_{90} concentrations for S6, W6 and F6 are in the range of 5–20 $\mu g\,mL^{-1}$, which is comparable to that of wild-type hBD3 and other cyclic or linear hBD3 analogues.[45]

The fact that the 45-residue full-length linear variants of hBD3 are as active as their wild-type counterpart with native multi-disulfide bridges suggests that the presence of the three disulfide bonds is not essential for antimicrobial activity. These observations and results are consistent with other studies.[13,45,46] However, reduced hBD2 (linear hBD2) was reported earlier to be inactive against bacteria; this indicates that both the native structure of hBD2 and its charge-based interaction with the membrane are critical for the activity of this peptide.[48] The reason for the loss of antimicrobial activity of linear hBD2 might be due to its low net positive charge of +6.

W6 is the most potent antimicrobial peptide in our series. The high activity is likely due to the presence of six tryptophan residues. This tryptophan-rich variant can be compared with other tryptophan-containing antimicrobial peptides, for example, tritrpticin,[49] indolicidin[50–51] and lactoferricin,[52–54] in which the tryptophan has been reported to be an essential constituent.[52–53] The aromatic indolyl side chain of tryptophan is capable of π–π stacking interactions, and can participate in hydrogen bonding, particularly in an interfacial environment.[55–56] Structurally, this could possibly arise from the fact that during our MD simulations, there is a propensity for W6 to be the most extended. This could possibly enhance its ability to rapidly aggregate into ordered helices and insert into membranes in a cooperative manner, as has been shown elsewhere for helical peptides.[57]

From the results of the pathogen-killing ability of the full-length linear peptides (Table 3), the antimicrobial activity appears to be related to the replacement residues of six cysteines in hBD3. For example, W6, F6 and S6 show potent antimicrobial activity against Gram-positive and Gram-negative bacteria; however, Y6, A6 and C6 display less potency against Gram-positive bacteria. As was found by others, we also conclude that the replacement of cysteine by alanine (A), tryptophan (W) or carboxamidomethylated cysteine [C(Cam)] did not significantly change the antibacterial activity compared to the fully disulfide-bridged hBD3 peptides;[13] however, interestingly, the MIC of variants of the N and C-terminal fragment peptides of hBD3 were dependant on the type of amino acid (A or W) by which the cysteine residues were replaced.[13]

It was observed that the overall hydrophobicity of this series of full-length analogues of hBD3, which is represented as their RP-HPLC retention time has no correlation with their antimicrobial activity. However, the activity of hBD3 derivatives was shown to be dependent on overall hydrophobicity, and not on the organization of secondary structure elements.[13] Increasing the hydrophobicity of some antimicrobial peptides above a certain level led to the loss of recognition selectivity between

Table 3. Pathogen killing ability of wild-type hBD3 and its linear analogues.

Peptide	*P. aeruginosa*		*E. coli*		*S. aureus*		*B. cereus*	
	LD_{50} [$\mu g\,mL^{-1}$]	LD_{99} [$\mu g\,mL^{-1}$]	LD_{50} [$\mu g\,mL^{-1}$]	LD_{99} [$\mu g\,mL^{-1}$]	LD_{50} [$\mu g\,mL^{-1}$]	LD_{99} [$\mu g\,mL^{-1}$]	LD_{50} [$\mu g\,mL^{-1}$]	LD_{99} [$\mu g\,mL^{-1}$]
hBD3	2.7	11.4	9.3	12.4	5.5	12.2	3.9	6.2
C(Acm)6	11.2	24.7	18.7	24.9	25	91	19.6	47.8
S6	4.7	6.2	9.3	12.4	9.8	24.5	11.3	24.75
A6	10.3	24.5	18.7	24.9	20.8	50	19.6	47.9
Y6	9.4	21.5	13	24.8	17.7	24.9	18.7	24.8
F6	9.2	12.4	9.4	17.7	10.1	24.4	9.4	12.5
W6	5.1	12.2	4.8	11.9	1.5	10.4	7.4	12.4

prokaryotic and eukaryotic membranes, thereby causing an increase in cytotoxicity.[58] The observed hydrophobicity of the 13-amino acid α-helical PTP7 derivatives by RP-HPLC retention time correlated well with the activity against Gram-positive bacteria; however, antimicrobial activity against Gram-negative bacteria, such as *E. coli*, did not correlate with RP-HPLC retention time.[34]

The exact mechanism of the hBD3-mediated antimicrobial activity is not clear. Previous studies suggest that the antimicrobial activity of hBD3 derivatives depends on their ability to contact the anionic pathogen cell membrane via electrostatic interactions that are mediated by the cationic peptide residues, and subsequently, to infiltrate the membrane by hydrophobic interactions. The key determinants of this action are the net positive charge and the overall hydrophobicity of the peptide. In our study, the overall hydrophobicity of the peptides was calculated based on an empirical scale (Table 1), and also measured by an experimental HPLC approach (Table 2). Among the linear analogues, the most hydrophobic, W6, and the most hydrophilic, S6 are potent antimicrobials; this suggests that the other structural determinants for the maintenance of high antimicrobial activity should include positive charge density, which is associated with folding and hydrophilic surface area, and a delicate balance between the hydrophilic surface area and hydrophobic surface. MD simulations suggest that both W6 and S6 tend to have the most "extended" conformations, and their propensity to have helical regions might be enhanced cooperatively. The structural feature of the facially amphiphilic conformations is thought to be responsible for their ability to kill cells by disrupting phospholipid membranes.[59–61] In addition, by controlling the hydrophobic/hydrophilic balance of amphiphilic macromolecules, it is possible to obtain high selectivity between antimicrobial activity and hemolytic activity/cytotoxicity against host cells.[62]

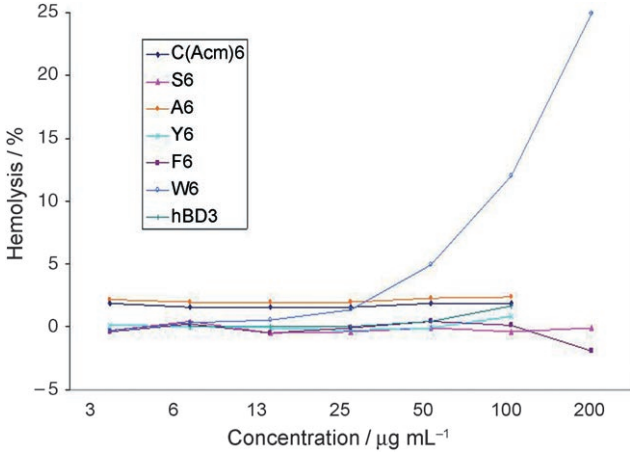


Figure 6. Hemolytic effects of hBD3 and its linear analogues on rabbit erythrocytes. Freshly isolated rabbit red blood cells (100 μL) were diluted to 8% (*v/v*) in sterile and mixed with equal volume of defensin analogues to achieve the targeted concentrations in 96-well plates in triplicate. The plate was incubated at 37 °C for 60 min with occasional gentle agitation. The supernatant was transfered to a fresh 96-well plate at the end of the incubation after brief centrifugation. The hemoglobin protein that was released in the supernatant was determined by measuring the absorbance at 414 nm. The averaged readings from the positive control (0.1% Triton X-100) were set as 100% and the hemolytic activities of samples were calculated as a percentage of the positive control. The experiment was repeated 3 times with triplicate wells for each sample.

Hemolytic activity and cytotoxicity to mammalian cells

It is well established that cationic peptides not only interact with pathogens, but can also be toxic to mammalian cells. Bacterial cell membranes consist of anionic phosphatidylglycerol as a major component, while eukaryotic cell membranes mainly contain zwitterionic phosphatidylcholine and phosphatidylethanolamine, which are susceptible to hydrophobic interactions.[63] It has been proposed that the hemolytic effects of cationic antimicrobial peptides are directly linked to the hydrophobicity of these peptides.[64] High levels of hydrophobicity are known to decrease the selectivity between the desired bacterial target membranes and mammalian host cell membranes.[65] Therefore, the linear analogues and wild-type hBD3 were tested for their ability to induce hemolysis in fresh rabbit red blood cells (RBC; Figure 6). W6, the most hydrophobic of the analogues, also shows the most potent hemolytic effect; it gives to 4.9–24.9% hemolysis over the concentration range of 50–200 μg mL^{-1}. The results show that W6 has potent antimicrobial activity, but displays low selectivity between antimicrobial activity and hemolytic activity, the high hemolytic activity is likely due to the hydrophobicity,[64–65] or the possibility that it lacks hydrophobic/hydrophilic balance.[62] Analogues C(Acm)6 and A6, which had low hydrophobicity displayed less hemolysis of 1.5–2.3% in the concentration range of 3–100 μg mL^{-1}, while F6, Y6, S6 and wild-type hBD3 showed the least amount of hemolysis of less than 1% in the concentration range of 3–100 μg mL^{-1}. While no significant hemolytic effect was observed in wild-type hBD3 or from five of the six analogues [F6, Y6, S6, A6 and C(Acm)6] in the concentration range of 3–100 μg mL^{-1}, W6 showed a significantly increased hemolytic effect on rabbit erythrocytes in the same concentration range.

Hemolytic activity is conventionally used as a measure of cytotoxicity and a model for mammalian cells because red blood cells are, in general, extremely fragile.[62,66–67] The hemolysis analysis reflects the systemic effect of the drug administration. As part of the innate immune system, which is associated with moist mucosal surfaces, and topical applications for eye, mouth or lung, the toxicity of the synthetic molecules to surface epithelial cells is also relevant. In the present study, the potential toxicity of the analogues to epithelial cells was analyzed by using primary cultured human conjunctival epithelial cells. Surprisingly, all six analogues showed improved cytotoxicity to epithelial cells at the tested concentration compared to wild-type hBD3 (Figure 7). It was also clear that the six analogues fell into two groups, one group included C(Acm)6, A6 and S6 and showed negligible cytotoxicity to epithelial cells at concentrations up to 200 μg mL^{-1}, the other group included F6, W6 and Y6, and showed IC_{50} values at concentrations of 30–50 μg mL^{-1} compared to an IC_{50} of around 12.5 μg mL^{-1} for wild-type hBD3.

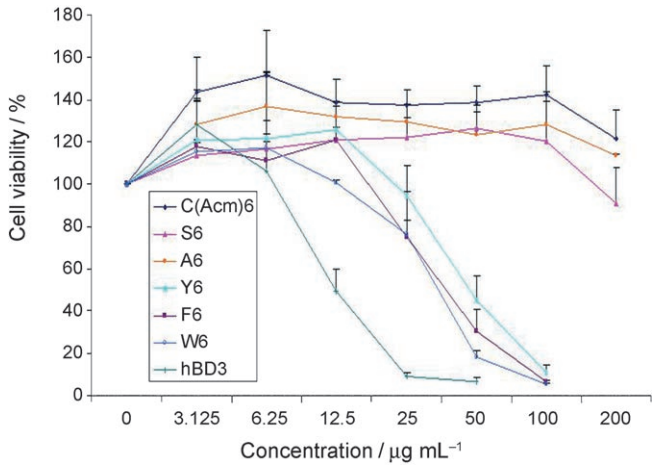


Figure 7. Cytoxicity of hBD3 and its linear analogues on human conjuctiva epithial cells. Primary normal human conjunctival epithelial cells were prepared as described in the Experimental Section; 2×10^4 cells per well per 100 μL were incubated with defensin analogues at the desired concentrations for 24 h before the cell death was determined by the reading of reduced resazurin (resorufin) at $579_{Ex}/584_{Em}$. The readings from wells without analogues were set as 100% and used to calibrate the readings that were obtained from treated wells. Each experiment was repeated three times.

It has been previously suggested that the cytotoxicity of antimicrobial peptides is related to greater hydrophobicity.[58,65,68] The cytotoxicity levels of linear analogues show general correlation with hydrophobicity values, but native hBD3 with three disulfide bonds does not fit the pattern. The order for the overall hydrophobicity is as follows: W6 > F6 > Y6 > wild-type hBD3 > A6 > C(Acm)6 > S6 (Tables 1 and 2). However, regardless of the overall hydrophobicity of the linear analogues, they showed reduced cytotoxicity compared with wild-type hBD3 in the concentration range of 6.25–200 μg mL^{-1}, with the overall order of epithelial cell cytotoxicity as follows: wild-type hBD3 > W6 > F6 > Y6 > S6 > A6 > C(Acm)6. Moreover, they can be put into three clusters based on cytotoxicity values: one cluster consists of A6, S6 and C(Acm)6 with low hydrophobicity and the lowest cytotoxicity over the concentration range of 6.25–200 μg mL^{-1}, a second cluster is made up of W6, F6 and Y6 and had the highest hydrophobicity and mid-level cytotoxicity, and a third cluster is represented by the single wild-type hBD3 with medium hydrophobicity and the highest cytotoxicity among the series.

To understand the discontinuity in the hydrophobicity–cytotoxicity relationship between the linear analogues and wild-type hBD3, we compared the critical physicochemical parameters of these peptides. First, net charge could be a factor; however, the six linear analogues have the same length and the same net positive charge of +11, as does hBD3. Therefore, at the sequence level, the differences in physicochemical properties of the cysteine-replacing residues should determine most of the differences between the peptides. Second, the linear analogues and wild-type hBD3 appear to have similar 2D conformations in aqueous solution, as well as in 50% TFE solution and 20 mM SDS micelles. All the peptides are predominantly "random-coil" in water and acquire detectably distinct populations of helical conformations upon exposure to membrane-mimetic environments. Third, the primary three-dimensional structural difference between the linear analogues and wild-type hBD3 lies in the linearity of the molecular backbone of the analogues due to the replacement of six cysteine residues of hBD3 with other residues compared to the pseudo-cyclic disulfide-bridge backbone of native hBD3. The overall folding of wild-type hBD3 is stabilized by the native disulfide bridges, whereas the folded structures of all linear analogues are likely to be more flexible and less restrained owing to the lack of strong disulfide bridges; they are primarily stabilized by weak hydrogen bonds and hydrophobic interactions. These peptides have different overall hydrophobicities (estimated by the change in free energy) and relative hydrophobicity, which are associated with the three-dimensional folding. By tuning the overall hydrophobicity of linear hBD3 analogues, reduced cytotoxicity, reduced hemolysis and antimicrobial competency were obtained.

Kluver et al. reported a series of analogues of hBD3 that had 40, 27, and 17 amino acid residues with or without disulfide bonds. In comparing structural parameters between the 45-residue wt-hBD3 and 40-residue analogues, the effect of the 5-amino-acid (GIINT) N-terminal fragment of hBD3 on cytotoxicity has been ignored or has not been considered.[13] Compared to the work by Kluver et al., we have successfully created a series of new model peptides of well-defined structurally homogenous analogues, which are used to examine the effects of structural variation of hBD3 on antibacterial activity, cytotoxicity, and conformational preferences; all analogues are full-length linear analogues, which include the N-terminal sequence and have the same positive charge and the same length (full length) as wt-hBD3 does, and have broader physiochemical properties, for example, six different cysteine-substituted residues [S, A, C(Acm), F, Y and W] and a bigger range of overall hydrophobicity.

We have proposed and elucidated the structural parameters for the reduced cytotoxicity to mammalian cells. The decreased cytotoxicity of the linear analogues is first suggested to be structurally related to the removal of disulfide bridges, and the flexible structure of the linear forms, which seem to be associated with loss of secondary structure. Moreover, by tuning the overall hydrophobicity of linear hBD3 analogues, reduced cytotoxicity and hemolysis were obtained, and were controllable while preserving antimicrobial properties. Therefore, based on previous reports (ref. [13] and others), our work provides additional evidence for the suggestion that linear analogues of hBD3 have reduced cytotoxicity to host cells, but comparable antimicrobial activity to wt-hBD3.

Based on the analysis of these structural parameters, we can hypothesize how the structural determinants govern and control the cytotoxicity of this group of defensin peptides. The native cyclic structure of wild-type hBD3, as stabilized by the disulfide bridges, is one of the main structural determinants of its high cytotoxicity. In other words, the linearity of analogues and the resultant flexible conformation of these analogues in the absence of structural restraints that are imposed by bridging disulfide bonds are key structural parameters that are asso-

ciated with their reduced cytotoxicity. A study that used α-helical peptides, showed that partial disruption of secondary structure can lead to a decrease in cytotoxicity to host cells.[68] Our observations of the disruption of secondary structures and decrease in host-cell cytotoxicity in systems that are more complex than α-helical peptides extend the suggestion that the loss of secondary structure is a possible general mechanism which might be useful for a more efficient design of defensins analogues.

Conclusions

While human β-defensin 3 (hBD3), like other defensins, is structurally characterized by high cationic capability and amphiphilicity, there are a variety of structural determinants (high net positive charge, sequence and residue distribution, amphiphilic conformation, hydrophobicity and folding) that might play a part in antimicrobial activity, hemolysis and cytotoxicity. We have designed and synthesized a series of well-defined structurally homogenous full-length linear analogues of hBD3 that provide a good model for further investigation into the effects of the following structural factors on antimicrobial activity, hemolysis and cytotoxicity of the peptides: native disulfide bridges or native cyclic structure of wild-type hBD3, the linearity and flexibility of analogues in the absence of native disulfide bridges, the physicochemical properties of the Cys-replacing residues, and the overall hydrophobicity of peptides.

We have observed that all linear hBD3 derivatives, especially W6, S6 and F6 have potent antimicrobial activity. We have discovered that a series of six linear analogues exhibit decreased cytotoxicity to human conjunctival epithelial cells compared to wild-type hBD3. It is suggested that the presence of the three disulfide bridges that restrain the native hBD3 into a pseudocyclic form is one of the main structural determinants of its high cytotoxicity. Conversely, the linearity and the flexible nature of the Cys-replaced analogues due to the absence of the stabilizing forces of native disulfide bridges are key structural factors that underlie their reduced cytotoxicity. Our observations provide additional evidence that partial disruption of secondary structure can lead to a decrease in cytotoxicity to host cells. Additionally, our finding suggests that the cytotoxicity can be controlled by modulating the overall hydrophobicity of the defensin molecules.

Linear constructs of hBD3-based peptides provide a simple architecture for design compared to the cyclic structure of wild-type hBD3, which contains specific disulfide bridges. From a chemical synthesis point of view, the incorporation of specific patterns of disulfide bonds and subsequent purification and identification is time and resource consuming. Compared to the synthesis, identification and purification of wt-hBD3 and certain other cyclic peptides with disulfide bonds, it is much easier and quicker to synthesize, identify and purify linear analogues. The development of potent defensin-based antibiotics with low-to-no toxic effects on host cells, high microbial selectivity and lower costs of production could be designed and developed with the guide of novel concepts from the present discovery.

Experimental Section

Solid-phase peptide synthesis (SPPS): Recombinant wild-type hBD3 was purchased from CytoLab Ltd (Rehovot, Israel). Fluorenylmethoxycarbonyl (Fmoc)-protected L-amino acids and resin were purchased from Advanced ChemTech (now Advanced Automated Peptide Protein TECHNOLOGIES, AAPPTEC) (Louisville, KY, USA) and used with the following side-chain protective groups: Arg(pbf), Lys(Boc), Tyr(But), Trp(Boc), Thr(But), Ser(But), Gln(Trt), Glu(OBut), Asn(Trt), Cys(Acm), and Fmoc-Lys(Boc)-Wang resin (substitution 0.72 mmol g^{-1}). Syntheses of the six linear analogues of hBD3 were carried out on Apex 396 (Advanced ChemTech) by Fmoc chemistry. Acylation (coupling reaction) was carried out with HBTU–HOBT in DMF at a synthesis scale of 0.04 mmol. Fmoc deprotection was carried out with 20% piperidine in DMF. The resulting peptidyl resins were treated with a freshly prepared mixture of TFA/TIS/phenol/thionisole/water (90:1:2.5:5:1.5, the ratio of volume percent) for 2–3 h at room temperature. The crude peptides were precipitated by filtration into ice-cold diethyl ether, separated by centrifugation, washed three times with ice-cold diethyl ether and dried by automated evaporation or under vacuum at room temperature. For further purification, the crude products were dissolved in a solvent that contained 5% acetonitrile and 0.1% TFA in H_2O and loaded onto a semipreparative HPLC column (Delta PAK C18, 300×7.8 mm I.D., 15 μm, 100 Å, Waters Associates, Milford, MA, USA) at a flow rate of 3 mL min^{-1}. The Waters HPLC system was equipped with a 2690 separation module, an auto-sampler and a 996 photodiode array detector (PDA; Waters Associates, Milford, MA). Gradient elution was started at 80% A/20% B (eluent A: 0.01% TFA in water; eluent B: 0.01% TFA in acetonitrile) and linearly changed to 65% A/35% B in 20 min. The chromatogram was monitored by PDA at 210 nm. Final products were characterized by analytical LC–MS (Micromass Platform LCZ, Manchester, UK) by using a Delta PAK C18, 150×3.9 mm I.D.: 5 μm, 100 Å (Waters Associates, Milford, MA, USA) at a flow rate of 0.2 mL min^{-1}. A linear gradient of eluent B, which was increased from 18% to 38% over 32 min was used. For RP-HPLC–UV chromatogram and MS spectrum of the six linear analogues of hBD3, see the Supporting Information.

Molecular hydrophobicity analysis: The overall molecular hydrophobicity of the peptides was measured by reverse phase (RP)-HPLC–ESI-MS under the same experimental conditions, that is, peptide concentration (measured at 500 or 100 μg mL^{-1}, respectively), injection volume (5 μL) and the flow rate of 0.2 mL min^{-1} in the gradient 18–38% of eluent B in 32 min, and was compared in terms of the retention time of the peptide peak.

The relative overall hydrophobicities of hBD3 analogues were calculated and determined by using the amino acid hydrophilicity scale of Hopp–Woods.[23] The Hopp–Woods scale was derived for predicting potential antigenic sites of globular proteins that are likely to be rich in charged and polar residues.

CD spectroscopy: The linear analogues and wild-type hBD3 were analyzed by circular dichroism spectroscopy by using a Jasco J-810 spectropolarimeter in dilute aqueous solution (pH 5.0–5.5) or in 50% (*v/v*) trifluoroethanol (TFE) in deionized H_2O or 20 mM sodium dodecyl sulfate (SDS). The six linear analogues were examined at concentrations of 125 μg mL^{-1}, while the wild-type hBD3 was at 100 μg mL^{-1}. CD measurements were performed at 27 °C within a wavelength range of 190–260 nm. Every sample and solvent was scanned three times, and the solvent CD was subtracted from that of the sample solution.

Molecular modeling: To obtain structural insights into the nature of the folded conformations of the analogues, molecular dynamics

(MD) simulations were carried out for four peptides (wild-type hBD3, S6, F6, W6) in two different environments (water and membrane-like TFE); a continuum model of the solvent was used in the form of the Generalized Born solvation model.[24] Simulations were performed by using the AMBER 8.0 package and the parm99 force field,[25] by following the protocol that was outlined by others.[26a] These methods are increasingly being used to study the folding of peptides and indeed small proteins.[26b] Briefly, the initial structures that were used for the simulations were prepared as follows: for the wild type, the known NMR spectroscopic structure (PDB code: 1KJ6) was used;[27] for the analogues, extended conformations were created with the LEaP module of AMBER. Each peptide was subject to "folding" simulations in the two different solvents. The solvents were mimicked by altering the dielectric parameters. The systems were first energy minimized and then heated to 325 K[26a] over 50 ps. This was followed by fully unrestrained production runs of 5 ns duration for each simulation, which was carried out by using the *sander* module of AMBER. Structures were saved every 1 ps for analysis, analyzed by using the *ptraj* module of AMBER, and visualized by using VMD[28] and Pymol.[29] Analysis was carried out over the last 3.5 ns of the simulations (this was done because this was the period over which the peptides were found to have folded states in equilibrium, as judged by various parameters).

Antimicrobial analysis: Antibacterial activities of the wild-type hBD3 and analogues were determined for *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus cereus* (NCTC 2599) by measuring bacterial growth in liquid broth in the presence of the serially diluted peptides. Each bacteria strain was grown to mid-logarithmic phase in Mueller–Hinton Broth (MHB) and diluted to OD 0.1 at 600 nm in a mixture of equal volumes of 10 mM potassium phosphate buffer and MHB. This bacteria suspension gave a concentration of 10^7 CFU mL^{-1}. The suspension was then mixed with an equal volume, 50 μL, of a defensin analogue at a particular concentration in 10 mM potassium phosphate buffer in sterile 96-well plates. The inner surface of the microplate cover was pre-treated with 0.05% Triton X-100 in 20% ethanol to prevent the condensation of water droplets. The mixture was cultured for 3 h in a microplate reader (Tecan Genios Plus, Zürich, Switzerland) at 37 °C for 3 h with 10 s orbital shaking and 10 s settling time. The incubation facilitates the reaction between the bacteria and the defensins. After 3 h, the 96-well plate was taken out of the Tecan Genios Plus and 2X MHB (100 μL) was added into each well. This part of the assay allowed the surviving bacteria to grow so that the bacterial concentration could be determined by using Brewster's procedure.[30] The growth kinetics of the bacteria in the 96-well plate was monitored over 18 h as OD620 in 15 min intervals with the computer-controlled microplate reader (Tecan Genios Plus) that was running Magillan v5.03 software, and a 620 nm filter at 37 °C. The ODs were then plotted out, and the LD_{50} was determined as the concentration of peptide at which 50% of the viable cells were killed. For an accurate determination of the bacterial concentrations, the OD readings and bacteria numbers were calibrated by using a tenfold serial dilution of cell suspension by starting at 10^7 CFU mL^{-1} that was made in 200 μL of an equal volume mixture of potassium phosphate buffer and MHB.

Cytotoxicity analysis: Cytotoxicity of the analogues was analyzed on primary cultured human normal conjunctival epithelial cells (HCEs) by measuring cell viability by using CellTiter-blue (Promega, Singapore). Wild-type hBD3 was used as a control in all analyses. The HCEs were isolated from cadaver conjunctiva tissue by dispase II digestion and cultured in serum-free Keratinocyte Growth Medium (KGM, Cambrex, Walkersville, MD, USA) according to protocols that were described in detail.[7] The protocol was approved by the Institutional Review Board of the Singapore Eye Research Institute. Passage 2–3 HCEs were used.

To evaluate the effect of the defensin analogues on cell survival, HCEs were seeded at a density of 2×10^4 cells/well in KGM in black 96-well plates (Corning Life Sciences, Acton, MA, USA) with a transparent bottom in the presence of different concentrations of defensin analogues, and the cells were incubated for 24 h. Resazurin was added 4 h before the end of the incubation. The amount of reduced resazurin (resorufin) that was generated by live cells was measured at 560/590 nm by using a microplate reader (Tecan GeniosPro, Tecan Asia, Singapore). A cell/fluorescence standard curve was generated at each time to ensure the linearity of fluorescence in the range used with the cells.

Hemolysis assay: The hemolytic activity of defensin analogues was determined by the amount of hemoglobin that was released from rabbit erythrocytes. Fresh rabbit red blood cells (RBCs) were isolated from the whole blood of New Zealand white rabbits by centrifugation at 250 *g* for 10 min. RBCs were further washed (4×) and diluted to 8% (*v*/*v*) in sterile PBS. Defensin analogues were diluted in sterile PBS to 2× the desired concentrations. Diluted RBCs (100 μL) and an equal volume of defensin analogues in PBS were added to 96-well plates in triplicates. The plate was incubated at 37 °C for 60 min with occasional gentle agitation. At the end of incubation, the plate was spun at 150 *g* for 5 min and the supernatant (100 μL) was transferred to a clean 96-well plate. The amount of hemoglobin in each well was determined by measuring the absorbance at 414 nm with a microplate reader. Rabbit procedures were approved by the IACUC of SingHealth and were according to the standards of the Association for Research in Vision and Ophthalmology.

Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; DMSO, dimethyl sulfoxide; EDT, ethane dithiol; ESI-MS, electrospray ionization mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBT, 1-hydroxylbenzotriazole; LD_{50}: the concentration of peptide solution at which 50% of the viable cells are killed; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl; TFA, trifluoroacetic acid; TIS, triisipropyl silane; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; Trt, trityl.

Acknowledgement

The authors appreciate the support of the following grants from the National Medical Research Council of Singapore: NMRC/0808/2003, NMRC/CPG/007/2004, CPG 002/2003 and the NMRC-IBG.

Keywords: antibiotics · cytotoxicity · epithelial cells · human beta-defensin 3 (hBD3) · molecular modeling

[1] R. E. W. Hancock, R. Lehrer, *Trends Biotechnol.* **1998**, *16*, 82–88.
[2] T. X. Nguyen, A. M. Cole, R. I. Lehrer, *Peptides* **2003**, *24*, 1647–1654.
[3] R. E. W. Hancock, G. Diamond, *Trends Microbiol.* **2000**, *8*, 402–409.
[4] J. S. Cullor, M. J. Mannis, C. J. Murphy, W. L. Smith, M. E. Selsted, T. W. Reid, *Arch Ophthalmol.* **1990**, *108*, 861–864.
[5] A. M. McDermott, R. L. Redfern, B. Zhang, Y. Pei, L. Huang, R. J. Proske, *Invest. Ophthalmol. Visual Sci.* **2003**, *44*, 1859–1865.
[6] L. Zhou, L. Q. Huang, R. W. Beuerman, M. E. Grigg, S. F. Li, F. T. Chew, L. Ang, M. E. Stern, D. Tan, *J. Proteome Res.* **2004**, *3*, 410–416.

[7] J. Li, M. Raghunath, D. Tan, R. R. Lareu, Z. Chen, R. W. Beuerman, *Invest. Ophthalmol. Visual Sci.* **2006**, *47*, 3811–3819.
[8] A. Dunsche, Y. Acil, H. Dommisch, R. Siebert, J. M. Schroder, S. Jepsen, *Eur. J. Oral Sci.* **2002**, *110*, 121–124.
[9] J. P. Powers, R. E. W. Hancock, *Peptides* **2003**, *24*, 1681–1691.
[10] G. Maisetta, G. Batoni, S. Esin, W. Florio, D. Bottai, F. Favilli, M. Campa, *Antimicrob. Agents Chemother.* **2006**, *50*, 806–809.
[11] Y. J. Gordon, E. G. Romanowski, A. M. McDermott, *Curr. Eye Res.* **2005**, *30*, 505–515.
[12] J. B. McPhee, M. G. Scott, R. E. Hancock, *Comb. Chem. High Throughput Screening* **2005**, *8*, 257–272.
[13] E. Kluver, S. Schulz-Maronde, S. Scheid, B. Meyer, W. G. Forssmann, K. Adermann, *Biochemistry* **2005**, *44*, 9804–9816.
[14] M. Mandal, R. Nagaraj, *J. Pept. Res.* **2002**, *59*, 95–104.
[15] Z. Wu, D. M. Hoover, D. Yang, C. Boulegue, F. Santamaria, J. J. Oppenheim, J. Lubkowski, W. Lu, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8880–8885.
[16] R. I. Lehrer, *Nat. Rev. Microbiol.* **2004**, *2*, 727–738.
[17] L. Zhou, R. W. Beuerman, L. Q. Huang, A. Barathi, Y. H. Foo, S. F. Li, F. T. Chew, D. Tan, *Proteomics* **2007**, *7*, 3194–3206.
[18] J. R. Garcia, A. Krause, S. Schulz, F. J. Rodriguez-Jimenez, E. Kluver, K. Adermann, U. Forssmann, A. Frimpong-Boateng, R. Bals, W. G. Forssmann, *FASEB J.* **2001**, *15*, 1819–1821.
[19] Y. Yamaguchi, T. Nagase, R. Makita, S. Fukuhara, T. Tomita, T. Tominaga, H. Kurihara, Y. Ouchi, *J. Immunol.* **2002**, *169*, 2516–2523.
[20] B. C. Schutte, J. P. Mitros, J. A. Bartlett, J. D. Walters, H. P. Jia, M. J. Welsh, T. L. Casavant, P. B. McCray Jr, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2129–2133.
[21] J. Harder, J. Bartels, J. Christophers, J. M. Schroder, *J. Biol. Chem.* **2001**, *276*, 5707–5713.
[22] G. Batoni, G. Maisetta, S. Esin, M. Campa, *Mini-Rev. Med. Chem.* **2006**, *6*, 1063–1073.
[23] T. P. Hopp, K. R. Woods, *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 3824–3828.
[24] W. C. Still, A. Tempczyk, R. C. Hawley, T. Hendrickson, *J. Am. Chem. Soc.* **1990**, *112*, 6127–6129.
[25] D. A. Case, T. E. 3rd Cheatham, T. Darden, H. Gohlke, R. Luo, K. M. Merz Jr. , A. Onufriev, C. Simmerling, B. Wang, R. J. Woods, *J. Comput. Chem.* **2005**, *26*, 1668–1688.
[26] a) C. Simmerling, B. Strockbine, A. E. Roitberg, *J. Am. Chem. Soc.* **2002**, *124*, 11258–11259; b) H. X. Lei, C. Wu, H. G. Liu, Y. Duan, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 4925–4930.
[27] D. J. Schibli, H. N. Hunter, V. Aseyev, T. D. Starner, J. M. Wiencek, P. B. McCray Jr. , B. F. Tack, H. J. Vogel, *J. Biol. Chem.* **2002**, *277*, 8279–8289.
[28] W. Humphrey, A. Dalke, K. Schulten, *J. Mol. Graphics* **1996**, *14*, 33–38.
[29] W. L. DeLano, *The PyMOL Molecular Graphics System* **2002**, DeLano Scientific, San Carlos, CA, USA. http://www.pymol.org.
[30] J. D. Brewster, *J. Microbiol. Methods* **2003**, *53*, 77–86.
[31] S. E. Blondelle, R. A. Houghten, *Biochemistry* **1992**, *31*, 12688–12694.
[32] T. L. Raguse, E. A. Porter, B. Weisblum, S. H. Gellman, *J. Am. Chem. Soc.* **2002**, *124*, 12774–12785.
[33] M. A. Schmitt, B. Weisblum, S. H. Gellman, *J. Am. Chem. Soc.* **2007**, *129*, 417–428.
[34] S. Kim, S. S. Kim, B. J. Lee, *Peptides* **2005**, *26*, 2050–2056.
[35] R. S. Hodges, Y. Chen, E. Kopecky, C. T. Mant, *J. Chromatogr. A* **2004**, *1053*, 161–172.
[36] D. M. Hoover, O. Chertov, J. Lubkowski, *J. Biol. Chem.* **2001**, *276*, 39021–39026.
[37] M. V. Sawai, H. P. Jia, L. D. Liu, V. Aseyev, J. M. Wiencek, P. B. McCray Jr., T. Ganz, W. R. Kearney, B. F. Tack, *Biochemistry* **2001**, *40*, 3810–3816.
[38] P. S. Kim, A. Bierzynski, R. L. Baldwin, *J. Mol. Biol.* **1982**, *162*, 187–199.
[39] D. Marion, M. Zasolff, A. Bax, *FEBS Lett.* **1988**, *227*, 21–26.
[40] Y. Yamamoto, T. Ohkubo, A. Kohara, T. Tanaka, M. Kikuchi, *Biochemistry* **1990**, *29*, 8998–9006.
[41] A. G. Rao, *Arch. Biochem. Biophys.* **1999**, *361*, 127–134.
[42] M. Boniotto, N. Antcheva, I. Zelezetsky, A. Tossi, V. Palumbo, M. V. V. Falzacappa, S. Sgubin, L. Braida, A. Amoroso, S. Crovella, *Biochem. J.* **2003**, *374*, 707–714.
[43] D. Roccatano, G. Colombo, M. Fioroni, A. E. Mark, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12179–12184.
[44] M. Mandal, M. V. Jagannadham, R. Nagaraj, *Peptides* **2002**, *23*, 413–418.
[45] D. M. Hoover, Z. Wu, K. Tucker, W. Lu, J. Lubkowski, *Antimicrob. Agents Chemother.* **2003**, *47*, 2804–2809.
[46] V. Krishnakumari, A. Sharadadevi, S. Singh, R. Nagaraj, *Biochemistry* **2003**, *42*, 9307–9315.
[47] Z. Oren, Y. Shai, *Biochemistry* **1997**, *36*, 1826–1835.
[48] D. M. Hoover, K. R. Rajashankar, K. R. Blumenthal, A. Puri, J. J. Oppenheim, O. Chertov, J. Lubkowski, *J. Biol. Chem.* **2000**, *275*, 32911–32918.
[49] C. Lawyer, S. Pai, M. Watabe, P. Borgia, T. Mashimo, L. Eagleton, K. Watabe, *FEBS Lett.* **1996**, *390*, 95–98.
[50] P. Staubitz, A. Peschel, W. F. Nieuwenhuizen, M. Otto, F. Gotz, G. Jung, R. W. Jack, *J. Pept. Sci.* **2001**, *7*, 552–564.
[51] C. Subbalakshmi, V. Krishnakumari, R. Nagaraji, N. Sitaram, *FEBS Lett.* **1996**, *395*, 48–52.
[52] M. B. Strom, O. Rekdal, J. S. Svendsen, *J. Pept. Res.* **2000**, *56*, 265–274.
[53] M. B. Strøm, B. E. Haug, Ø. Rekdal, M. L. Skar, W. Stensen, J. S. Svendsen, *Biochem. Cell Biol.* **2002**, *80*, 65–74.
[54] H. J. Vogel, D. J. Schibli, W. Jing, E. M. Lohmeier-Vogel, R. F. Epand, R. M. Epand, *Biochem. Cell Biol.* **2002**, *80*, 49–63.
[55] D. J. Schibli, R. F. Epand, H. L. Vogel, R. M. Epand, *Biochem. Cell Biol.* **2002**, *80*, 667–677.
[56] M. Schiffer, C. H. Chang, F. J. Stevens, *Protein Eng.* **1992**, *5*, 213–214.
[57] H. Leontiadou, A. E. Mark, S. J. Marrink, *J. Am. Chem. Soc.* **2006**, *128*, 12156–12161.
[58] M. Dathe, J. Meyer, M. Beyermann, B. Maul, C. Hoischen, M. Bienert, *Biochim. Biophys. Acta Biomembr.* **2002**, *1558*, 171–186.
[59] W. F. DeGrado, *Adv. Protein Chem.* **1988**, *39*, 51–124.
[60] A. Tossi, L. Sandri, A. Giangaspero, *Biopolymers* **2000**, *55*, 4–30.
[61] G. N. Tew, D. H. Liu, B. Chen, R. J. Doerksen, J. Kaplan, P. J. Carroll, M. L. Klein, W. F. DeGrado, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5110–5114.
[62] M. F. Ilker, K. Nüsslein, G. N. Tew, E. B. Coughlin, *J. Am. Chem. Soc.* **2004**, *126*, 15870–15875.
[63] M. R. Yeaman, N. Y. Yount, *Pharmacol. Rev.* **2003**, *55*, 27–55.
[64] P. M. Hwang, H. J. Vogel, *Biochem. Cell Biol.* **1998**, *76*, 235–246.
[65] I. Zelezetsky, U. Pag, H. G. Sahl, A. Tossi, *Peptides* **2005**, *26*, 2368–2376.
[66] W. van't Hof, E. C. I. Veerman, E. J. Helmerhorst, A. V. N. Amerongen, *Biol. Chem.* **2001**, *382*, 597–619.
[67] Z. Oren, Y. Shai, *Biopolymers* **1998**, *47*, 451–463.
[68] I. Kustanovich, D. E. Shalev, M. Mikhlin, L. Gaidukov, A. Mor, *J. Biol. Chem.* **2002**, *277*, 16941–16951.

Received: September 19, 2007
Published online on March 18, 2008

