Structure−Activity Relation of Human β-Defensin 3: Influence of Disulfide Bonds and Cysteine Substitution on Antimicrobial Activity and Cytotoxicity†

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ABSTRACT: Human β-defensins form a group of cysteine-rich antimicrobial peptides which have been found in epithelial tissue and, more recently, in the male genital tract. They play a role in the defense against microbial pathogens in innate immunity and display additional chemotactic functions in the adaptive immune system. An important characteristic of antimicrobial peptides is that they also exhibit toxic potential on eukaryotic cells. Very little is known about the structure dependence of antimicrobial and cytotoxic effects. We investigated human β-defensin 3 (hBD-3), a potent broad-spectrum antimicrobial effector peptide, regarding the influence of structural parameters on the antimicrobial and cytotoxic activity. We have established a structure−activity relation of the hBD-3 using synthetic derivatives differing in length, charge, disulfide connectivity, and overall hydrophobicity. The antimicrobial activity of the peptides was compared to the cyctotoxic effects on monocytic THP-1 cells and the hemolytic activity on human erythrocytes. We found that it is not important for antimicrobial and cytotoxic activity whether and how cysteine residues are arranged to form disulfide bonds. Substitution of half-cystinyl residues by tryptophan resulted in increased activities, while other substitutions did not change activity. Correlation of activities with the structural changes demonstrates that the activity on eukaryotic cells appears to depend strongly on the overall hydrophobicity. In contrast, the antimicrobial potency of hBD-3 peptides is determined by the distribution of positively charged amino acid residues and hydrophobic side chains. The results facilitate the understanding of β-defensin interaction with different cell types and guide the design of antimicrobially active peptides.

β-Defensins are a mammalian and avian subgroup of the defensin peptide family, whose members play an important role in the innate immune system of most species of the animal and plant kingdom (*1*−*6*). β-Defensins are cationic and cysteine-rich peptides of about 35−50 amino acid residues and contain three disulfide bridges in the arrangement Cys^1−Cys^5, Cys^2−Cys^4, Cys^3−Cys^6 (*7*). They share a similar three-dimensional structure containing a triple-stranded, antiparallel β-sheet (*8*−*13*). In humans, three β-defensins have been isolated so far (*14*, *15*, *17*−*20*), but recent research using bioinformatic approaches has revealed the existence of a large number of genes encoding putative novel β-defensins located on chromosomes 8 and 20 (*21*−*24*). While the human β-defensins 1, 2, and 3 (hBD-1−3) are mainly expressed in epithelial tissues of various organs, such as skin, respiratory, and urogenital tract, the newly identified genes show almost exclusively specific expression in testis or epididymis (*21*, *23*, *24*).

Human β-defensins exhibit broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and yeasts (*15*−*20*) and are thus considered as a part of the early immune response to invading pathogens. The corresponding mechanism of action is thought to be based on the disruption of the microbial membrane by interaction with membrane components (*1*, *4*, *25*, *26*). β-Defensins have been demonstrated to exhibit biological activities beyond the inhibition of microbes. These include contributions to the adaptive immune response by exhibiting chemotactic activity on dendritic and T-cells via the chemokine receptor CCR6 (hBD-1, hBD-2, hBD-3) (*27*, *28*) and on monocytes, macrophages (hBD-3) (*20*), and mast cells (hBD-2) (*29*). Murine β-defensin-2 has been shown to activate dendritic cell maturation via Toll-like receptor 4 (*30*). A further significant effect of hBD-2 and hBD-3 is the inhibition of HIV-1 replication in vitro (*31*). The different effects have not yet been assigned to particular structural elements of β-defensins.

The recently discovered human β-defensin 3 (hBD-3) is a particularly interesting antimicrobial peptide due to its exceptionally high net charge (+11) and its broad spectrum of antimicrobial activity even under high-salt conditions (*19*,

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20, *32*). In contrast to other antimicrobial peptides such as indolicidin or bactenecin (*3*), hBD-3 does not exhibit a significant lytic activity on human erythrocytes (*19*) and no cytotoxicity against various human cells (*31*).

It is generally assumed that the antimicrobial activity of β-defensins is determined by the existence of an amphiphilic structure and the extent and distribution of cationic and hydrophobic regions on the peptide surface (*26*, *33*). The presence and position of disulfide bridges and N-terminal sequence variations seem to have a marginal influence on the antibacterial effect of β-defensins (*15*, *34*−*38*). For the bovine β-defensins BNBD-2 and BNBD-12, antimicrobial properties were assigned to certain C-terminal fragments of the peptide (*36*, *37*). Only little is known about the influence of structural properties of β-defensins on the interaction with eukaryotic membranes. On the basis of investigations with other cationic antimicrobial peptides such as magainin or indolicidin, it is generally assumed that a larger number of hydrophobic amino acid residues lead to increased hemolytic activity (*26*, *33*). Selectivity of effects of antimicrobial peptides including β-defensins on bacterial and eukaryotic cells may thus be determined by the balance of positively charged and hydrophobic surface regions. In contrast to the antimicrobial effect of hBD-3, the chemotactic receptor-mediated activity has been reported to be clearly susceptible to changes in the disulfide arrangement (*34*).

In the present work, we synthesized different hBD-3 peptides and investigated the influence of the location of disulfide bonds, sequence length, net charge, and overall hydrophobicity on the antimicrobial and cytotoxic properties of hBD-3. Suitable synthesis strategies to obtain disulfide isomers of hBD-3 were developed. The content of secondary structure elements and the antimicrobial, hemolytic, and cytotoxic properties of the synthetic peptides were determined.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Fluorenylmethoxycarbonyl (Fmoc)-protected L-amino acids were purchased from Orpegen (Heidelberg, Germany) or Merck Biosciences (Schwalbach, Germany) and were used with the following side-chain protective groups: Arg(Pbf)[1], Asn(Trt), Asp(OtBu), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu), Cys(Trt), Cys(Acm), Cys(tBu), and Cys(4-MeBn). Cysteine protection schemes for stepwise introduction of disulfide bonds are shown in Figure 1. Syntheses were carried out on a 433A peptide synthesizer (Applied Biosystems, Weiterstadt, Germany) and performed using preloaded TentaGel-R-PHB (Rapp Polymere, Tübingen, Germany) or

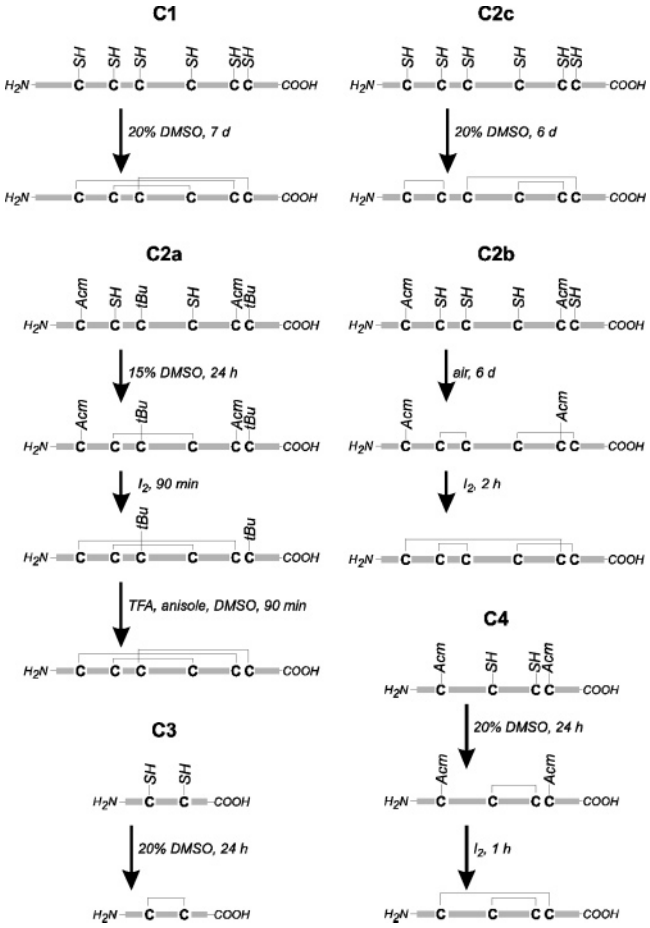

FIGURE 1: Routes for the chemical introduction of disulfide bonds in hBD-3 derivatives.

Wang resins (Merck Biosciences) for N-terminal hBD-3 fragments. Fmoc-Lys(Boc)-TentaGel-R-Trt resin (Rapp Polymere) was used for all other peptides. Acylation was carried out with HBTU-HOBt in NMP at a scale of 0.1 mmol. Fmoc deprotection was carried out with 20% piperidine in NMP. The resulting peptidyl resins were treated with a freshly prepared mixture of TFA/EDT/H_2O (94:3:3, v/v/v, 40 mL/g resin) for 3−4.5 h at room temperature. The crude peptides were precipitated by filtration into ice-cold TBME, separated by centrifugation, washed three times with TBME, and dried under vacuum. For purification, the crude products were dissolved in diluted acetic acid and loaded onto a preparative Vydac C18 column (The Separations Group, Hesperia, CA, 47 mm × 300 mm, 15 μm, 300 Å, flow rate 40 mL/min; eluent A, 0.07% TFA; eluent B, 0.07% TFA in MeCN−H_2O 80:20; UV detection at 215 nm; gradient, 35−60% eluent B in 50 min). The reduced precursors of peptides C2a and C4 were used for oxidation without purification. The thio-alkylated hBD-3 variant L1 was obtained from fully reduced 40-residue hBD-3 (C2c precursor). Reduced C2c was dissolved in 0.5 M Tris acetate/2 mM EDTA (pH 8) at a peptide concentration of 0.56 mg/mL. After addition of 15 equiv iodoacetamide, the mixture was stirred at room temperature for 2 h. The reaction was stopped by addition of the corresponding volume of 0.5 M citric acid, and the product was separated by preparative HPLC (conditions as above). Purified peptides were characterized by analytical HPLC (Vydac C18, 4.6 mm × 250 mm, 5 μm, 300 Å, flow rate 0.8 mL/min, eluent A 0.07% TFA, eluent B 0.07% TFA

[1] Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; Cam, carboxamidomethyl; CD, circular dichroism; DMSO, dimethyl sulfoxide; EDT, ethanedithiol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; FCS, fetal calf serum; Fmoc, fluorenylmethoxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; MHB, Mueller−Hinton broth; MIC, minimum inhibitory concentration; NMP, *N*-methylpyrrolidinone; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl; POPC, palmitoyloleoylphosphatidylcholine; PTH, phenylthiohydantoin; TBME, *tert*-butyl methyl ether; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TrisHCl, tris(hydroxymethyl)aminomethane hydrochloride; Trt, trityl; TSB, tryptic soy broth.

in MeCN/H_2O 80:20, UV detection at 215 and 230 nm, gradient 10−70% of eluent B in 30 min) and ESI-MS (Sciex API 100, Perkin-Elmer).

One-Step Oxidation (Peptides C1, C2c, and C3). Introduction of disulfide bonds, starting from a hexathiol precursor, was applied to the precursors of peptides C1, C2c, and C3. Reduced purified peptides were dissolved in 5% acetic acid (c = 0.6 mg/mL) with a pH adjusted to 6 by $(NH_4)_2CO_3$. Then, 20% DMSO (v/v) was added, and the mixture was stirred at room temperature. Reaction time was 6 days for C2c, 7 days for C1, and 24 h for C3. The reaction was stopped by dilution with 5% MeCN containing 0.05% TFA. Products were purified by preparative HPLC (conditions as above). Fractions containing the desired product, as confirmed by HPLC and ESI-MS analysis, were pooled, lyophilized, and further analyzed.

Two-Step Oxidation (Peptides C2b and C4). C2b and C4 were synthesized using two subsequent oxidative reactions. In the case of C4, the first disulfide bond between Cys^2 and Cys^3 was introduced using DMSO (conditions as described above for C3). After the reaction was stopped, the product was purified by preparative HPLC as described above and used as starting material for the introduction of the remaining disulfide bond. In the case of C2b, two disulfide bonds (Cys^2−Cys^3 and Cys^4−Cys^6) were introduced simultaneously. The purified, fully reduced peptide was dissolved in water (c = 0.1 mg/mL) and stirred at room temperature for 4−6 days. The solution was lyophilized and the dry peptide used for the next step to introduce the third disulfide bond. Mono-disulfide C4 or bis-disulfide C2b were dissolved in a mixture of acetic acid and 0.1 M HCl (4:1) at a concentration of 0.5 mg/mL. Iodine (20 equiv, 0.05 M in acetic acid) was added, and the mixture was stirred at room temperature. The reaction was stopped by addition of 0.2 M ascorbic acid. The reaction time was 1 h for C4 and 2 h for C2b. The products were purified on a semipreparative Vydac C18 column (22 mm × 250 mm, 5 μm, 300 Å, flow rate 7 mL/min; eluent A, 0.07% TFA; eluent B, 0.07% TFA in MeCN−H_2O 80:20; UV detection at 215 nm; gradient, 0.5% eluent B/min). Fractions containing the desired product, as confirmed by HPLC and ESI-MS analysis, were pooled and lyophilized.

Three-Step Oxidation (Peptide C2a). C2a was synthesized applying three subsequent steps of oxidation. Details of this method are reported elsewhere (*39*). In brief, to introduce the first disulfide bond (Cys^2−Cys^4), reduced crude C2a was dissolved in MeCN/H_2O (2:3) (c = 0.2 mg/mL) with the pH adjusted to 8 by diluted ammonia. Then, 15% DMSO (v/v) was added, and the mixture was stirred for 24 h. The reaction was stopped by addition of 2% TFA (v/v). The resulting product was purified by preparative HPLC (conditions as above). The second disulfide bond (Cys^1−Cys^5) was introduced by iodine oxidation of Acm groups (conditions as above for C2b and C4). Finally, the remaining disulfide bond (Cys^3−Cys^6) was formed by dissolving tBu-protected bis-disulfide C2a in TFA (c = 0.1 mg/mL). After addition of 400 equiv DMSO (4 mmol) and 40 equiv anisole (0.4 mmol), the mixture was stirred for 90 min, diluted with H_2O (1:7), and purified on a preparative Vydac C18 column (conditions as above). Fractions containing the desired product, as confirmed by HPLC and ESI-MS analysis, were pooled and lyophilized. Then, a second purification was performed at a column temperature of 70 °C on a semipreparative Vydac C18 column (conditions as above) to separate the desired product from dimeric byproducts. The collected fractions were analyzed by HPLC at 70 °C and ESI-MS, pooled, and lyophilized.

Assignment of Disulfide Connectivity. All synthetic hBD-3 peptides were thoroughly characterized concerning their disulfide pattern. The purified peptides (300 μg) were dissolved in 100 mM TrisHCl (500 μL, pH 8), and a solution of 12.5 μg trypsin and 12.5 μg chymotrypsin in 1 mM HCl (25 μL) was added. After 4 h at 36 °C, the reaction was stopped by addition of 50 μL TFA, followed by separation of the mixture on an analytical Vydac C18 HPLC column. The detected fragments were collected and analyzed by ESI-MS. The amino acid sequences of selected peptide fragments were then determined by Edman degradation (494 Procise peptide sequencer, PE Biosystems) (Table 1). In the case of C4, the disulfide pattern was confirmed by proteolytic cleavage of C4 and C2c and subsequent comparison of the corresponding cleavage products. A total of 100 μg of C4 and C2c each was dissolved in 120 μL of 100 mM TrisHCl (pH 8). A solution of 12.5 μg chymotrypsin in 1 mM HCl (20 μL) was added. After 4 h at 36 °C, the reaction was stopped by addition of 20 μL TFA and the mixture was analyzed by HPLC. Chromatographically detected fragments were collected. Fractions containing fragments with identical molecular weight were combined and analyzed for identity by capillary zone electrophoresis (Biofocus 3000, Bio-Rad, Hercules, CA, uncoated fused silica capillary, 30 cm × 50 μm; 0.1 M phosphate buffer containing polymer modifier, pH 2.5; constant voltage 20 kV; UV detection at 200 nm).

CD Spectroscopy. The hBD-3 derivatives C1, C2a, C2b, C2c, C3, C4, L2A, and L2W were analyzed by CD spectroscopy using the CD spectrometer AVIV Model 215 (AVIV Instruments, Inc., Lakewood, NJ). The samples were dissolved in water at a concentration of 25 μM directly before measurement. CD measurements were performed at room temperature within a wavelength range of 190−280 nm. Every sample was scanned three times. The smoothed and averaged data (AVIV CD spectroscopy software) were analyzed using the CDPro software package (*40*) (Figure 3).

Antimicrobial Activity. For antimicrobial testing, the microbroth dilution assay was used (*41*). *Staphylococcus aureus* ATCC25923, *Streptococcus pneumoniae* DSM11865, *Escherichia coli* DSM1103, *Klebsiella pneumoniae* DSM681, and *Pseudomonas aeruginosa* DSM1128, purchased from the Deutsche Sammlung für Zellkulturen und Mikroorganismen (DSZM, Braunschweig, Germany), were used as test organisms. For determination of the minimum inhibitory concentration (MIC), 2-fold serial dilutions of peptides starting with 200 and 300 μg/mL were prepared in full Mueller−Hinton broth (Difco, Heidelberg, Germany) and quarter-strength Mueller−Hinton broth. As positive control substance, the synthetic α-helical peptide MBI-28 (*42*) was used. Inocula of 2−5 × 10^5 cfu of bacteria (colony-forming units) growing in log phase were added to each well. After incubation for 18 ± 2 h at 37 °C, the bacterial growth was determined as OD at 570 nm. The MIC was defined as the lowest antimicrobial concentration at which there is no visible increase of the OD 570 after 18 ± 2 h.

Cytotoxicity Assay. THP-1 cells (human monocytic cell line, ATCC No. TIB-202) were cultivated in RPMI 1640

Table 1: Disulfide Bond Assignment of Fully Disulfide-Bonded hBD-3 Peptides: Fragments Obtained from Trypsin/Chymotrypsin Cleavage

Peptide		Sequence	M_r (exp)	M_r (calc)	Sequence analysis[a]
C1					
	1	CR SCLPK CCR	1199.8	1200.5	+
	2	VRGGR	543.5	543.6	n.d.
	3	GIINTL	629.5	629.8	n.d.
C2a					
	1	CR SCLPK CCR	1199.8	1200.5	+
	2	CR SCLPK KCCR	1328.5	1328.7	n.d.
C2b					
	1	EEQIGK	702.5	702.8	n.d.
	2	YYCR CSTR KCCR	1573.0	1573.8	+
	3	CAVLSCLPKEEQIGK	1615.8	1615.9	+
	4	CAVLSCLPK	930.8	931.2	+
C2c					
	1	EEQIGK	702.5	702.8	n.d.
	2	CLPK CSTR CCR	1300.8	1301.5	+
	3	SCLPK CSTR CCR	1387.8	1388.6	n.d.
	4	YYCR CAVLS	1092.8	1093.3	+
	5	YYCR CAVL	1005.8	1006.2	n.d.

[a] +, sequence confirmed by sequence analysis; n.d., not determined.

medium containing 2 mM L-glutamine and 10% FCS at 37 °C and 5% CO_2 atm for 5−7 days. Assays were performed by incubation of 2.5×10^4 cells with varying concentration of peptides for 19 h at 37 °C and 5% CO_2 in a 96-well plate. The cytotoxin cycloheximide (Calbiochem, Schwalbach, Germany) was used as positive control at a concentration of 50 μg/mL. The absorption of cells in pure medium was used as negative control. After the incubation period, 10 μL of WST-1 solution (Roche Diagnostics, Mannheim, Germany) was added per well and the incubation was continued for 1.5 h at 37 °C and 5% CO_2. The extinction (E) was measured at 450 nm with a reference wavelength of 630 nm, which corresponds to the absorption maximum of unreacted tetrazolium. The viability of the cells is reciprocally proportional to the cytotoxic activity of the tested material (*43*). The viability of cells was determined using the following formula: viability (%) = $100 \times (E_{Peptide} - E_{pos})/(E_{neg} - E_{pos})$. All experiments were performed in duplicate.

Hemolysis Assay. The release of hemoglobin from erythrocytes was used as a measure for the membranolytic activity of tested substances (*44*). As test medium, 1/10 TSB medium (3 g/L) with 287 mM glucose was used. Whole blood of a healthy test person (6−9 mL) was centrifuged for 10 min at 20 °C and 2770 rpm. The supernatant was discarded and the residue washed three times with test medium. The erythrocytes were then diluted 1:200 in test medium. An aliquot of 200 μL of this suspension was added to different amounts of the peptides in a V-bottomed 96-well plate, resulting in peptide concentrations in a range of 1−500 μg/mL. The plate was incubated for 1 h at 37 °C and then centrifuged for 5 min at 2800 rpm. A volume of 150 μL from each well was transferred to a flat-bottomed 96-well plate, and the extinction (E) was measured at 450 nm. Incubation with 1% Tween-20 was used as reference for total hemolysis; the antimicrobial peptide MBI-28 was used as positive control, whereas erythrocytes, incubated in pure test

Table 2: Amino Acid Sequences and Disulfide Connectivities of Synthetic hBD-3 Peptides

Peptide	Sequence[a]	Number of residues: Total	Hydrophobic[b] (aromatic)	Net charge	Overall hydrophobicity[c]
C1	GIINTLQ**K**YYC**R**V**R**GG**R**CAVLSCLP**K**EEQIG**K**CST**R**G**R**K**C**C**RRKK**	45	12 (2)	+11	-12.65
C2a	LQ**K**YYC**R**V**R**GG**R**CAVLSCLP**K**EEQIG**K**CST**R**G**R**K**C**C**RRKK**	40	10 (2)	+11	-12.70
C2b	LQ**K**YYC**R**V**R**GG**R**CAVLSCLP**K**EEQIG**K**CST**R**G**R**K**C**C**RRKK**	40	10 (2)	+11	-12.70
C2c	LQ**K**YYC**R**V**R**GG**R**CAVLSCLP**K**EEQIG**K**CST**R**G**R**K**C**C**RRKK**	40	10 (2)	+11	-12.70
C3	LQ**K**YYC**R**V**R**GG**R**CAVLS	17	7 (2)	+4	-0.98
C4	AVLSCLP**K**EEQIG**K**CST**R**G**R**K**C**C**RRKK**	27	6 (0)	+7	-11.53
L1	LQ**K**YYC**R**V**R**GG**R**CAVLSCLP**K**EEQIG**K**CST**R**G**R**K**C**C**RRKK** (Cam: Cys[1], Cys[2], Cys[3], Cys[5], Cys[6], Cys[7])	40	10 (2)	+11	n.c.
L2A	LQ**K**YYA**R**V**R**GG**R**AAVLSALP**K**EEQIG**K**AST**R**G**R**KAA**RRKK**	40	16 (2)	+11	-15.16
L2W	LQ**K**YYW**R**V**R**GG**R**WAVLSWLP**K**EEQIG**K**WST**R**G**R**KWW**RRKK**	40	16 (8)	+11	-3.04
L3A	LQ**K**YYA**R**V**R**GG**R**AAVLS	17	9 (2)	+4	-1.80
L3W	LQ**K**YYW**R**V**R**GG**R**WAVLS	17	9 (4)	+4	2.24
L4A	AVLSALP**K**EEQIG**K**AST**R**G**R**KAA**RRKK**	27	10 (0)	+7	-13.17
L4W	AVLSWLP**K**EEQIG**K**WST**R**G**R**KWW**RRKK**	27	10 (4)	+7	-5.09

[a] Cam, carboxamidomethyl; cationic residues are printed in bold. [b] The number of hydrophobic residues includes amino acids with aliphatic and aromatic side chains. [c] The overall hydrophobicity (ΔG, kcal mol^{-1}) was calculated based on the hydrophobicity scale of Wimley and White (*62*). Greater hydrophobicity is represented by a more positive ΔG; n.c., not calculated.

medium, served as negative control. Hemolysis is expressed relative to the total hemolysis caused by Tween-20 according to the following formula: hemolysis (%) = 100 × ($E_{\text{Peptide}} - E_{\text{neg}}$)/($E_{\text{pos}} - E_{\text{neg}}$). All experiments were performed in duplicate.

RESULTS

Synthesis of hBD-3 Peptides and Disulfide Bond Formation. The solid-phase synthesis of 13 hBD-3 derivatives was performed using Fmoc chemistry, as described in detail (Table 2). The use of cysteine derivatives with different protective groups or the replacement of cysteine residues by alanine and tryptophan did not cause a significant difference in purity and yield of the reduced products.

Disulfide bonds were introduced using different strategies, developed for each peptide. C1 and C2c were obtained in a one-step reaction from a completely unprotected, fully reduced hexathiol precursor peptide using DMSO at pH 6 as oxidizing agent. The longer N-terminus of the noncyclic C1 precursor significantly influenced the folding process. Although the oxidation conditions for C2c (40 residues) and C1 (45 residues) were identical, the composition of the obtained product differed (Figure 2). Oxidation of reduced C2c resulted in the formation of a mixture of disulfide isomers. At least four disulfide isomers were found by HPLC and ESI-MS analysis, and the appearance of peak shoulders

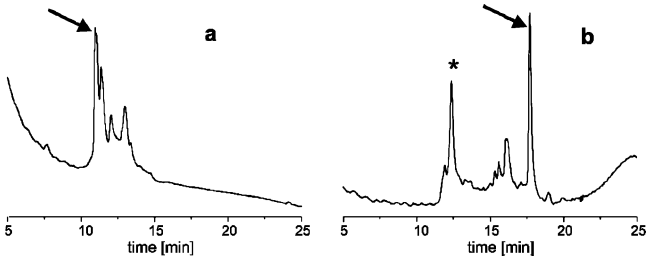

FIGURE 2: Analytical HPLC profile of (a) crude oxidized C2c (disulfide pattern Cys[1]−Cys[2], Cys[3]−Cys[6], Cys[4]−Cys[5]) and (b) C1 (disulfide pattern Cys[1]−Cys[5], Cys[2]−Cys[4], Cys[3]−Cys[6]) obtained with identical reaction conditions (see Experimental Procedures). Peaks corresponding to the fully disulfide-bonded products are marked by arrows. An asterisk indicates a nonpeptide contamination.

indicated the presence of even more isomers. The major component constituted less than 25% of the product as estimated by the corresponding peak area. The folding of the C1 precursor led to a mixture of disulfide isomers containing approximately 30% of the more separated product, thereby facilitating HPLC purification.

C2b was synthesized by a two-step synthesis strategy, starting with a tetrathiol precursor peptide containing two Acm-protected cysteine residues. Two disulfide bonds (Cys[2]−Cys[3] and Cys[4]−Cys[6]) were formed selectively by air oxidation. The remaining disulfide bond was subsequently introduced by iodine oxidation. Both oxidation steps resulted

in highly pure products. To obtain C4, a Trt/Acm-protected precursor was used since folding of a tetrathiol precursor failed. After introduction of the first disulfide bond by DMSO oxidation, the second disulfide was generated with iodine.

Attempts to synthesize a 40-residue hBD-3 with the predicted β-defensin disulfide pattern and an N-terminal length corresponding to earlier isolated β-defensins from precursors with four or six free thiol groups failed. Therefore, a strategy employing three different thiol protective groups allowing a regioselective introduction of disulfide bonds was developed, as discussed elsewhere in detail (*39*). The introduction of the three disulfide bonds was carried out with a noncyclic 40-residue hBD-3 peptide, whose cysteine residues were protected pairwise with trityl, acetamidomethyl, and *tert*-butyl groups (Figure 1). To form the disulfides Cys^2-Cys^4 and Cys^1-Cys^5, oxidation was carried out with DMSO and iodine. Yields were 25% and 40%, respectively. The third disulfide bond Cys^3-Cys^6 was introduced using a mixture of TFA/DMSO/anisole, which cleaves the tBu groups and subsequently forms the disulfide bond (*45*). We found that it is essential to meet exactly the reaction conditions provided above, since changes in concentrations, time, or temperature lead to byproducts such as unreacted or incompletely deprotected bis-disulfide starting material, overoxidized and isomeric compounds, or dimeric products. Even under optimized conditions, the last oxidation step led to the formation of covalently linked dimeric peptides coeluting with the desired peptide during C18 HPLC. However, dimers were separated in an additional purification step at elevated column temperature (70 °C), which decreased the yield of the last reaction step to 6%.

Assignment of Disulfide Bonds. All hBD-3 peptides containing two or three disulfide bonds were thoroughly characterized concerning their disulfide pattern, independently of whether a chemoselective or nonselective approach was used to introduce the disulfide bonds. The purified peptides were subjected to proteolysis with a mixture of trypsin and chymotrypsin, which preferentially cleave C-terminally to basic and to aromatic residues, respectively. The generated peptide fragments were then isolated by HPLC and analyzed by ESI-MS. The sequences of cystine-containing fragments were determined by automated Edman degradation. Analysis of the products of each sequencing cycle and identification of the corresponding PTH derivatives of cystine allowed the unambiguous localization of disulfide bridges. Table 1 shows the analytical data of fragments of hBD-3 peptides C1, C2a, C2b, and C2c with three disulfide bonds. In the case of C4, the disulfide pattern was confirmed by comparison of the disulfide connectivities of C4 and the identical C-terminal part of C2c. Fragments with a relative molecular mass of 2792 corresponding to the sequence of hBD-3 (22−45) were identified in both proteolytic cleavage mixtures. Their identity was demonstrated by capillary-electrophoretical comigration (data not shown).

CD Spectroscopy. The CD spectra of the hBD-3 derivates C1, C2a, C2b, C2c, C3, C4, L2A, and L2W agree very well with the CD spectrum of native hBD-3 (*46*). There are only minor differences among the spectra of the derivatives examined (Figure 3). Apparently, the positive band at 230 nm, present only in the CD spectra of C3 and L2W, does not contribute to a significantly different population of secondary structures as calculated with the CDPro package.

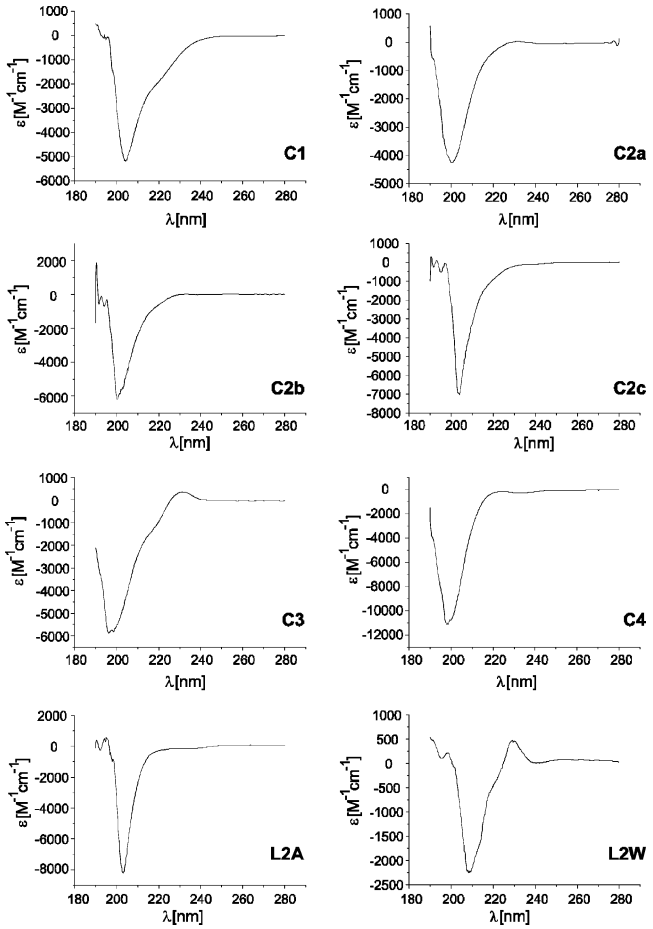

FIGURE 3: CD spectra of hBD-3 derivatives in water.

Thus, spectrally C1, C3, and L2W seem to be more ordered than the other peptides investigated. A computer curve analysis suggests that even the short peptides C3 and C4 contain together the same number of amino acids in a β-strand as is found in the long peptides (data not shown). This in turn demonstrates that the 3D structural features of the short peptides are not changed compared to the long peptides.

Antimicrobial Activity. The minimum inhibitory concentration (MIC) of the hBD-3 derivatives was determined against three Gram-negative (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) and two Gram-positive (*S. aureus*, *S. pneumoniae*) bacteria in media of different ionic strength (quarter- and full-strength Mueller−Hinton broth) (Table 3). MIC values of all hBD-3 peptides containing three disulfide bonds (C1, C2a, C2b, and C2c) were in the range of 10 μg/mL in medium of low ionic strength. MIC values seem to be slightly lower against Gram-positive bacteria compared to Gram-negative bacteria. Noncyclic hBD-3 variants, such as L1, L2A, L2W, and hBD-3 fragments did not show a preferential inhibition.

We have split the 40-residue hBD-3 variant C2c containing a non-native disulfide pattern into the fragments C3 with the disulfide Cys^1-Cys^2 and C4 with two disulfides Cys^3-Cys^6 and Cys^4-Cys^5. Peptides C3 and C4 exhibited a strongly reduced antibacterial activity against 4 out of the 5 germs tested (MIC $\geq$ 100 μg/mL). The reduction of activity was less drastic (14× for C3 and 3× for C4) with *S. pneumoniae*.

Table 3: Minimum Inhibitory Concentration (MIC) for hBD-3 Peptides against Gram-Positive and Gram-Negative Bacteria[a]

Peptide	Culture Medium[b]	*Escherichia coli* DSM 1103	*Klebsiella pneumoniae* DSM 681	*Pseudomonas aeruginosa* DSM 1128	*Staphylococcus aureus* ATCC 25923	*Streptococcus pneumoniae* DSM 11865
C1	1/4 MHB	9.4	12.5	12.5	3.13	4.7
	MHB	>300	300	*37.5 (80%)*	150	50
C2a	1/4 MHB	12.5	37.5	18.75	3.13	6.25
	MHB	>300	>300	>300	100	>300
C2b	1/4 MHB	9.4	18.75	12.5	4.7	4.7
	MHB	>300	>300	>300	>300	>300
C2c	1/4 MHB	9.4	25	18.75	4.7	4.7
	MHB	>300	>300	>300	>300	>300
C3	1/4 MHB	100	200	200	200	67.8
	MHB	>200	>300	>200	>200	>300
C4	1/4 MHB	150	200	200	100	13.4
	MHB	n.d.	n.d.	n.d.	n.d.	n.d.
L1	1/4 MHB	12.5	9.4	12.5	12.5	37.5
	MHB	>300	300	>300	>300	>300
L2A	1/4 MHB	18.75	9.4	12.5	12.5	25
	MHB	>300	>300	>300	>300	>300
L2W	1/4 MHB	18.75	9.4	12.5	12.5	9.4
	MHB	*25 (75%)*	>300	*25 (75%)*	18.75	25
L3A	1/4 MHB	150	>300	300	150	>300
	MHB	>300	300	>300	>300	>300
L3W	1/4 MHB	12.5	12.5	18.75	12.5	18.75
	MHB	>300	>300	*300 (75%)*	150	>300
L4A	1/4 MHB	75	300	>300	75	>300
	MHB	>300	>300	>300	>300	>300
L4W	1/4 MHB	9.4	9.4	9.4	9.4	9.4
	MHB	>300	>300	>300	>300	>300
MBI-28	1/4 MHB	6.25	6.25	6.25	6.25	12.5
	MHB	12.5	12.5	12.5	37.5	>300

[a] MIC values are given in μg/mL; italicized values correspond to incomplete inhibition (percentage of inhibition, as calculated from the measured optical densities, is given in parentheses); n.d., not determined. [b] MHB, undiluted Mueller−Hinton broth; 1/4 MHB, quarter-strength Mueller−Hinton broth.

In medium of low ionic strength, the three tested noncyclic 40-residue hBD-3 peptides were of similar potency compared to their cyclic counterparts. No dependence on the substitution of the cysteine residues was found; that is, replacement of cysteine by alanine, tryptophan, or carboxamidomethylated cysteine did not significantly change antimicrobial activity compared to that of the fully disulfide-bonded hBD-3 peptides. Interestingly, MIC values of variants of the N- and C-terminal fragment peptides were dependent on the type of amino acid by which cysteine residues were replaced. When tryptophan was used, MIC values of the same order of magnitude were found for the hBD-3 fragments L3W and L4W compared to the fully disulfide-bonded peptides C1, C2a, C2b, and C2c. In the case of the alanine-containing variants L3A and L4A, the peptides appeared to be active only at rather high concentrations.

Most hBD-3 derivatives lost their antibacterial activity almost completely when tested in medium of higher ionic strength, that is, with full Mueller−Hinton broth. The tryptophan derivative L2W showed slightly higher MIC values against *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. pneumoniae*, while it was inactive against *K. pneumoniae*. The activity was higher against the Gram-positive bacteria tested, since the inhibition of *E. coli* and *P. aeruginosa* was incomplete, and the MIC values listed correspond to only 75% inhibition. Another derivative with considerable antimicrobial activity at high ionic strength was C1, the peptide corresponding to the native hBD-3. While the activity of C1 was almost completely lost with Gram-negative bacteria (MIC values $\geq$ 300 μg/mL for *E. coli* and *K. pneumoniae*, and 37.5 μg/mL at 80% inhibition against *P. aeruginosa*), there was still a clear but reduced activity against Gram-positive bacteria. The activity was 50- and 10-fold lower against *S. aureus* and *S. pneumoniae*, respectively. Strongly reduced activity against *S. aureus* was also observed for C2a and L4W (MIC values of 100 and 150 μg/mL).

Cytotoxic Effects of hBD-3 Peptides. The potent antimicrobially active hBD-3 peptides C1, C2a, C2b, and C2c with three disulfide bonds and the analogues containing alanine and tryptophan for the cysteine residues were tested regarding their cytotoxic effects. Human THP-1 cells, a cell line of monocytic origin, were used as a model system. Cytotoxicity was examined by comparing the viability of THP-1 cells in the presence or absence of 10% fetal calf serum (FCS) using the WST-1 assay after treatment with hBD-3 peptides. This assay measures the metabolic activity of eukaryotic cells, which corresponds to the viability. Figure 4 shows the concentration dependence of the viability of THP-1 cells upon exposure to the above-mentioned hBD-3 derivatives. The 45-residue tris-disulfide peptide C1 reduced THP-1 cell viability to a significantly larger extent than the three 40-residue derivatives C2a, C2b, and C2c. A striking fact is that we observed a drop of THP-1 viability to almost zero after treatment of the cells with 50 μg/mL (Figure 4a). In an independent experiment, we compared the influence of the tris-disulfide 40-residue peptide C2c on the cell viability to the effects of the hBD-3 analogues L2A and L2W (Figure 4b). The results show that the alanine-substituted hBD-3 variant L2A shows activity comparable to the fully disulfide-bonded peptides of the same N-terminal length. In contrast, the tryptophan variant L2W exhibited a markedly higher potency in reducing the THP-1 cell viability starting

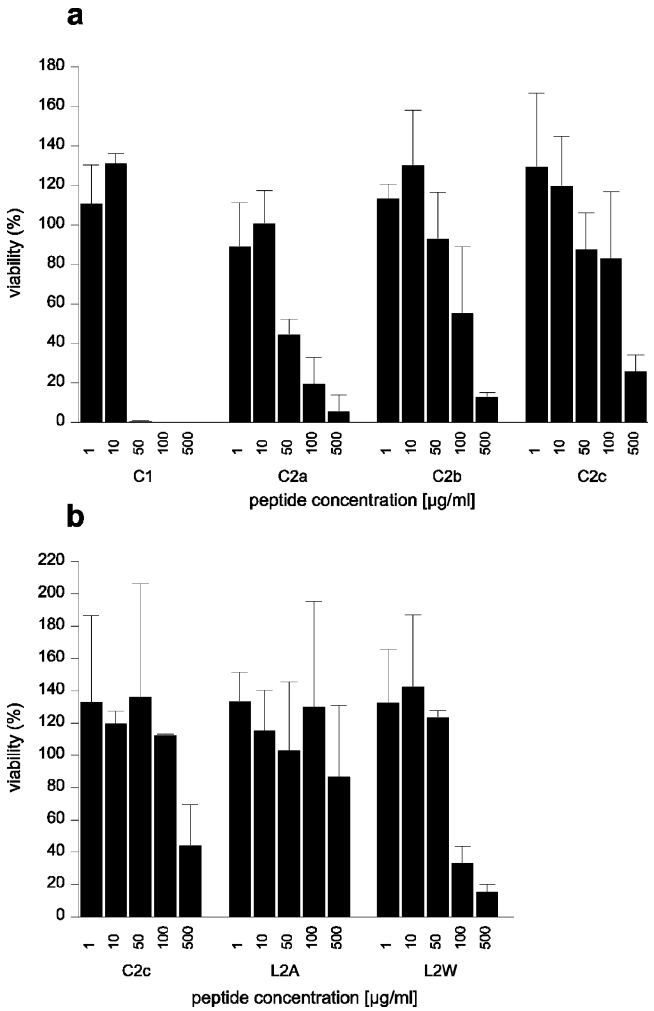

FIGURE 4: Dependence of the viability of THP-1 cells on the concentration of hBD-3 derivatives in the presence of 10% FCS. (a) Comparison of hBD-3 peptides C1, C2a, C2b, and C2c (three disulfide bonds). (b) Comparison of tris-disulfide 40-residue peptide C2c with the analogues L2A and L2W. Diagrams a and b are obtained from independent experiments.

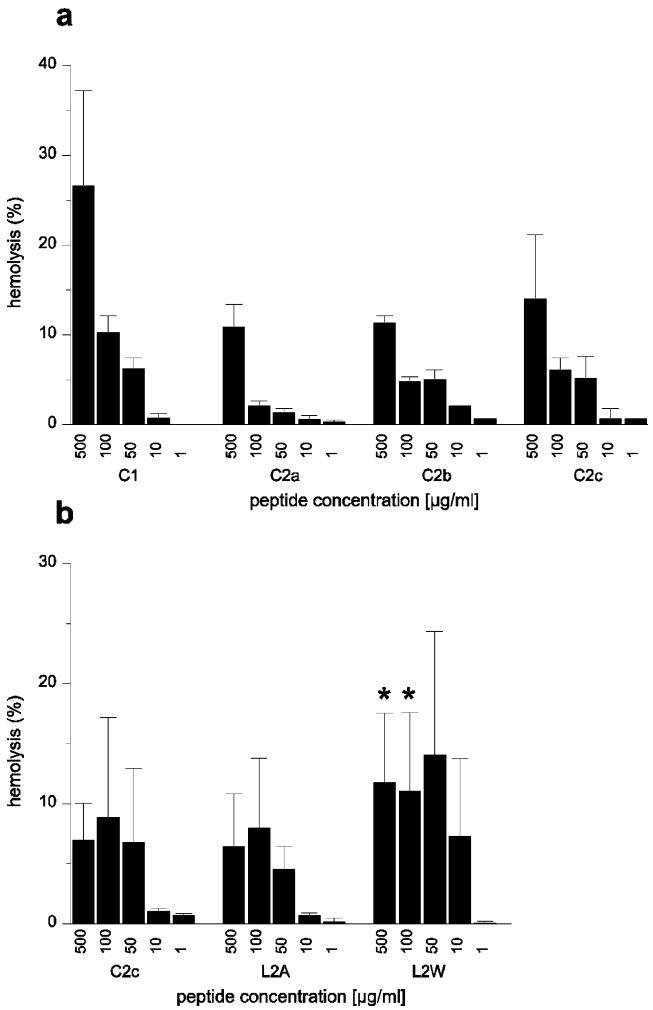

FIGURE 5: Concentration dependence of the hemolytic effects of synthetic hBD-3 peptides. (a) Comparison of hBD-3 peptides containing three disulfide bonds. (b) Comparison of the tris-disulfide 40-residue peptide C2c with the analogues L2A and L2W. Diagrams a and b are from independent experiments. Asterisks mark relative hemolysis values which are misleading due to an unexpected effect with L2W at higher concentrations: the disappearance of the peptide pellet indicated complete hemolysis, but concomitant discoloring of the supernatant did not allow the correct determination of the hemoglobin concentration. The hemolytic effect is therefore considered to be actually higher than is reflected by the measured values.

at concentrations between 50 and 100 μg/mL. For all peptides, cell viability was lower when the tests were performed without the addition of FCS. However, the extent of this effect depends on the particular peptide and is in the order of one concentration step (data not shown).

Hemoglobin Release by hBD-3 Peptides. The release of hemoglobin from erythrocytes was determined for the same hBD-3 peptides tested for their cytotoxic potential. Hemolysis was tested measuring the release of hemoglobin at different peptide concentrations. All peptides were compared with the hemolytic peptide MBI-28 and the detergent Tween-20, whose relative hemoglobin release was set at 100%. Figure 5 shows the dependence of the hemolytic activity of the six tested hBD-3 derivatives on the peptide concentration. Comparing the hemolytic effect among the four tris-disulfide hBD-3 derivatives C1, C2a, C2b, and C2c, it was found that the 45-residue peptide C1 is significantly more active than the three N-terminally smaller hBD-3 variants. In an independent experiment, the peptides L2A and L2W with substituted cysteine residues were compared with the fully disulfide-bonded hBD-3 variant C2c. While the hemolytic effect of the alanine-containing variant L2A was similar to that of the tris-disulfide C2c, the Trp analogue L2W containing six tryptophan residues showed a significantly higher hemolytic activity (Figure 5b). Notwithstanding, even the most hemolytic hBD-3 derivative exhibited a considerably lower hemolytic activity than the comparator peptide MBI-28. This peptide caused 50% hemolysis at 50 μg/mL, while hBD-3 peptides caused reduced hemolysis by 5- to 10-fold.

DISCUSSION

Synthesis of hBD-3 Peptides. The presented structure−activity study relies on the chemical synthesis of multiple disulfide-bonded hBD-3 peptides. As for most biologically peptides of this type, synthetic methods had to be established for each single peptide to introduce the desired disulfide connectivity. Our attempts to chemically synthesize the peptides C1 and C2a with the disulfide pattern predicted for β-defensins resulted in significantly different products. While a major product with the desired disulfide bonds was obtained

from the oxidative folding of the 45-residue C1 precursor, the conversion of the C2a precursor led to a mixture of fully disulfide-bonded isomers containing no significant amounts of the product with the β-defensin disulfides. Under identical reaction conditions of the oxidative folding, the N-terminal residues GIINT present in C1 thus facilitate the formation of the β-defensin-specific disulfide bond pattern. It is possible that these residues participate sterically in the formation of a key intermediate during the folding process.

Since C2b was not obtained by one-step oxidative folding, we synthesized the peptide using the orthogonal thiol-protective groups trityl and acetamidomethyl. The position of the Acm-protected cysteine residues determines the cysteine connectivity of the corresponding product. The hBD-3 isomer C2b was obtained from a precursor with Acm groups at Cys^1 and Cys^5 after two oxidation steps. First, the two disulfide bonds Cys^2−Cys^3 and Cys^4−Cys^6 were simultaneously formed by oxidative folding, as confirmed after proteolytic cleavage. Then, Cys^1−Cys^5 was formed by iodine oxidation. A corresponding approach with Acm groups at Cys^3 and Cys^6 resulted in the formation of C2c, whereas Acm protection of Cys^2 and Cys^4 did not lead to a homogeneous product after the first oxidation step. Such a semiselective strategy should therefore be considered for the chemical synthesis of β-defensins and related peptides in cases where straightforward one-step procedures fail.

The actual selective route to β-defensins with three disulfide bonds employs three orthogonal Cys protective groups. This strategy is the only route to obtain C2a. Among the several possibilities, we found that Cys^1 and Cys^5 protected with Acm, Cys^2 and Cys^4 protected with Trt, and Cys^3 and Cys^6 protected with tBu is a suitable positioning of protective groups. After three subsequent oxidation reactions, DMSO oxidation of free thiols, Acm cleavage and oxidation by iodine, and tBu cleavage and oxidation in the presence of DMSO/anisole, the peptide C2a was obtained. The use of other combinations examined led to unsatisfactory product quality and yields in the second and third oxidation step. As a consequence, the approach using three Cys-protective groups allows the synthesis of hBD-3 peptides with any disulfide pattern. However, a suitable positioning of protective groups is hard to predict.

Antimicrobial Activity. The majority of the hBD-3 derivatives tested here shows antimicrobial activity against Gram-negative and Gram-positive bacteria in low ionic strength medium. While the activity of the tris-disulfide peptides is higher against the tested Gram-positive bacteria, no Gram-favoring activity was observed for the noncyclic variants and fragments. An important observation was that neither the disulfide connectivity nor an N-terminal extension by five amino acid residues (peptide C1) significantly influences the antimicrobial activity of hBD-3 peptides. The disulfide isomers C2a, C2b, and C2c exhibited comparable MIC values. This is in agreement with earlier results, where similar data for 45-residue disulfide variants of hBD-3 were reported (*34*). On the basis of the disulfide variants examined in that study and on the isomers tested here, it appears that the positions of disulfide bonds do not generally influence the antimicrobial activity. The fact that the 40-residue variants L1, L2W, and L2A containing no disulfide bonds are as active as their cyclic counterparts shows that the presence of disulfide bonds is not necessarily required for antimicrobial activity. These observations are consistent with other studies (*35*, *37*). In contrast, reduced hBD-2 was earlier reported to be antimicrobially inactive (*9*).

The peptides C3 and C4 represent segments of C2c and contain the disulfides of the parent peptide. Earlier research assigned the antimicrobial properties of β-defensins to the C-terminal moiety containing most of the basic amino acid residues (Lys, Arg) (*36*, *37*). Our results show that the fragments C3 and C4 have a low activity compared to C2c. This indicates that hBD-3 peptides require a higher length for antimicrobial activity. Small changes at the N-terminus are tolerated as demonstrated by the comparison of activity of 40- and 45-residues hBD-3 variants (C2a, C2b, C2c, C1). The β-defensin core structure comprising the six cysteine residues should be intact to display antimicrobial activity.

The strongly reduced activity of the fragment peptide C3 cannot be explained merely by its smaller size. When comparing the primary structures, C3 shares some features with other short antimicrobial β-sheet peptides such as protegrins, tachyplesins, polyphemusins, gomesin, and the recently discovered θ-defensins (*1*, *3*, *4*, *47*−*51*). In Table 4, a comparison of corresponding sequences is shown. C3 resembles this class in size and amino acid composition. Moreover, it shares an accumulation of positively charged amino acid residues in the central region with related β-sheet peptides. The main differences are that C3 has only one disulfide, a lower net charge, and a C-terminal carboxyl. However, these difference should not cause a loss of activity, since it was shown for protegrins that changes of these parameters do not necessarily decrease antimicrobial activity (*52*). On the basis of CD spectral analysis, C3 shows a lower β-sheet content, probably due to lesser constraints in a single disulfide peptide.

The tryptophan-containing derivatives L2W, L3W, and L4W are potent antimicrobials. This property can be assigned to the presence of multiple tryptophan residues since the cysteine- or alanine-containing counterparts of L3W and L4W are antimicrobially inactive. The tryptophan derivatives can be compared with other tryptophan-containing antimicrobial peptides such as tritrpticin (*53*), indolicidin (*54*, *55*), and lactoferricin (*56*−*58*), for which tryptophan has been reported to be an essential constituent (*56*, *57*). The aromatic indolyl side chain of tryptophan is capable of π−π interactions and can participate in hydrogen bonding, particularly in an interfacial environment (*59*, *60*). Thus, in the derivatives studied here, the supporting effect of tryptophan in combination with further aromatic tyrosine residues and a higher net charge might cause a retained activity.

The antimicrobial activity of β-defensins depends on their ability to contact the bacterial cell mebrane by electrostatic interactions and to insert into the membrane by hydrophobic interactions. The potential of defensins for oligomerization may enhance the effects, as was shown recently for a murine β-defensin-related peptide forming covalent dimers (*61*). However, major determining factors for antimicrobial activity of hBD-3 are the positive net charge and the overall hydrophobicity. Figure 6 shows a correlation of these two parameters for the tested hBD-3 derivatives. The overall hydrophobicity of the synthetic peptides was calculated based on the hydrophobicity scale by Wimley and White (*62*). This scale presents values for the contribution of the 20 natural amino acids to the free energy ΔG of peptide transfer from

Table 4: Comparison of the hBD-3 Peptide C3 with β-sheet Antimicrobial Peptides

Peptide	Sequence[a]	Number of residues: Total	Hydrophobic (aromatic)[b]	Involved in β-sheet	Net charge	Overall hydrophobicity[c]
Protegrin I	RGGRLCYCRRRFCVCVGR*	18	5 (2)	10	+7	-1.44
Tachyplesin I	KWCFRVCYRGICYRRCR*	17	6 (4)	10	+7	1.01
Polyphemusin I	RRWCFRVCYRGFCYRKCR*	18	6 (5)	12	+8	-0.86
Gomesin	ZCRRLCYKQRCVTYCRGR*	18	4 (2)	14	+6	-3.02
RTD-1	GVCRCICTRGFCRCLCRR	18	4 (1)	10	+5	-0.84
C3	LQKYYCRVRGGRCAVLS	17	5 (2)	7	+4	-0.98

[a] An asterisk denotes an amidated C-terminus; Z, pyroglutamate; positively charged amino acids are printed in bold. [b] The number of hydrophobic residues includes amino acids with aliphatic and aromatic side chains. [c] The overall hydrophobicity (ΔG, kcal mol^{-1}) was calculated according to the hydrophobicity scale of Wimley and White (*62*); the value of glutamine was used for pyroglutamate.

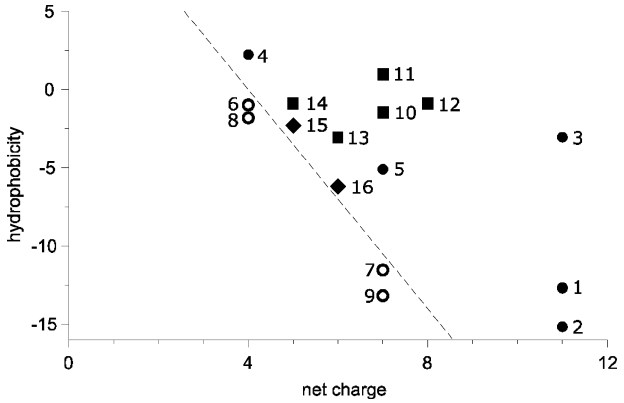

FIGURE 6: Correlation of the overall Wimley and White hydrophobicity score (ΔG, kcal mol^{-1} (*62*)) with the net charge of hBD-3 and other cationic antimicrobial peptides. Filled circles, antimicrobially active hBD-3 peptides (1, C1, C2a, C2b, C2c; 2, L2A; 3, L2W; 4, L3W; 5, L4W); open circles, antimicrobially inactive hBD-3 derivatives (6, C3; 7, C4; 8, L3A; 9, L4A); squares, β-sheet peptides from Table 4 (10, protegrin; 11, tachyplesin; 12, polyphemusin; 13, gomesin; 14, RTD-1); diamonds, 15, hBD-1 (33 residues, net charge +5, hydrophobicity −2.29); 16, hBD-2 (41 residues, net charge +6, hydrophobicity −6.16). The dotted line indicates a threshold below which hBD-3 derivatives are antimicrobially inactive in 1/4 MHB medium (for details see Discussion).

a bilayer interface into water. The values presented in Table 2 correspond to the sum of the particular hydrophobicity values of the contained amino acid residues, which is considered an approximation neglecting structural details. It is concluded from these values that the combination of net charge and hydrophobicity must fulfill a minimum requirement for a hBD-3 peptide to display antimicrobial activity. The dotted line in Figure 6 represents an estimated activity limit, below which hBD-3 derivatives are antimicrobially inactive, even in medium of low ionic strength. This correlation also explains the inactivity of the small monodisulfide C3. The hydrophobicity of C3 is not sufficient to compensate for its low net charge, but when cysteine is replaced by tryptophan, hydrophobicity is increased and activity is retained. The limit below which a cationic peptide is antimicrobially inactive depends on several parameters such as bacterial strain, medium, and mode of action of the corresponding peptide. Thus, the boderline of Figure 6 can only be applied for hBD-3, but the influence of the combination of positive charge and hydrophobicity is a general principle.

The loss of activity in test medium with higher ionic strength might be ascribed to the higher concentration of calcium and magnesium ions in undiluted Mueller−Hinton broth. An activity-decreasing effect of divalent cations has been reported for hBD-2 and hBD-3 against *E. coli* (*35*, *63*). Divalent cations compete with the peptides for the binding sites on the negatively charged bacterial cell wall. Thus, only peptides with a higher positive net charge can access the cell surface. Considering the charge−hydrophobicity ratio shown in Figure 6, the activity limit is shifted to higher net charges. It is plausible that, under these conditions, only the highly cationic and hydrophobic tryptophan-substituted peptide L2W retains activity. In the case of the smaller tryptophan-containing derivatives, the relatively low net charge cannot sufficiently be compensated by tryptophan.

The results of the CD spectroscopic analysis show that the disulfide connectivity has no significant effect on the secondary structural elements of hBD-3 derivatives. There were no changes even when substitutions with Ala or Trp for Cys residues take place. Thus, the secondary structure seems to be preformed in the peptides independent of the presence of a disulfide bridge. The number of amino acids involved in secondary structure elements is almost constant, and the sum of the number of these residues in the shorter peptides (C3, C4) is equal to that in the corresponding full-length peptides (C1, C2a, C2b, C2c, L2A, and L2W). This similar distribution of secondary structural elements in hBD-3 derivatives does not correspond with the considerable variety in their antimicrobial potency. This demonstrates that antimicrobial effects of β-defensins are due to the composition of the peptide in terms of hydrophobic and charged

amino acids, rather than to their particular spatial arrangement.

Effects on Eukaryotic Cells. It is well-established that antimicrobial peptides not only interact with microbial cells, but also display toxic potential to eukaryotic cells. The results of the hemolysis and cytotoxicity assays performed with hBD-3 peptides are generally consistent. In contrast to bacterial cell membranes containing anionic phosphatidylglycerol as a major component, eukaryotic cell membranes consist mainly of zwitterionic phosphatidylcholine and phosphatidylethanolamine susceptible to hydrophobic interactions (*26*). Therefore, it has been proposed that hemolytic effects of antimicrobial peptides are connected with the extent of hydrophobicity (*33*). Our results support this hypothesis. Differences in the hemolytic and cytotoxic activity of hBD-3 peptides are due to the sequence rather than the disulfide (Figures 4 and 5). The minor effect of secondary structural elements on the effects of hBD-3 peptides on eukaryotic cells is demonstrated by the results of the CD spectroscopic experiments. Differences in hemolytic or cytotoxic activity are not reflected in the distribution of secondary structural elements which does not differ among the hBD-3 derivatives.

Variations in the disulfide connectivities of the cyclic 40-residue derivatives C2a, C2b, and C2c do not change the overall hydrophobicity, and accordingly, a comparable hemolytic and cytotoxic activity was determined for these peptides. The greater activity of the 45-residue C1 is caused by the five additional N-terminal amino acids GIINT, two of which are bulky hydrophobic isoleucine residues. Considering the tertiary structure of hBD-3 (*13*) and the flexibility of the N-terminus, these residues are available for hydrophobic interaction. Replacement of cysteine residues by alanine causes a reduction in the overall hydrophobicity according to Wimley and White (*62*), which might explain the experimental finding that L2A is slightly less cytotoxic than the 40-residue tris-disulfide C2c (Figure 4). The higher hemolytic activity of L2W is reflected by the markedly increased hydrophobicity since tryptophan is the most hydrophobic residue in the Wimley and White scale. Correlations between the number and position of aromatic residues (especially tryptophan) and the hemolytic activity have been established for other antimicrobial peptides (*64*, *65*). For example, the above-mentioned tryptophan-rich indolicidin is highly hemolytic and cytotoxic due to the tryptophan residues contained in it (*51*, *52*). Since L2W contains six tryptophan and two tyrosine residues, the fraction of aromatic residues is 20%.

CONCLUSION

Since β-defensins exhibit an amphiphilic three-dimensional structure, the relation between positive charges and hydrophobic regions on the surface strongly influences antibacterial and cytotoxic properties. Our results for hBD-3 peptides show that the antimicrobial activity is elevated in vitro with increasing positive net charge and a higher overall hydrophobicity. This is demonstrated by the tryptophan-containing N- and C-terminal hBD-3 fragment peptides L3W and L4W, which compensate the low net charge through the hydrophobicity of the tryptophan residues contained in them and thus retain activity. The experiments performed with erythrocytes and THP-1 cells showed that an enhanced hydrophobic character allows a more efficient interaction with eukaryotic cells and causes cytotoxic and hemolytic effects.

The hBD-3 peptides investigated in this study can be classified into three groups based on the hydrophobicity−charge correlation (Figure 6): (i) peptides with a low net charge and moderate hydrophobicity are antimicrobially inactive; (ii) positively charged peptides with high hydrophobicity are potent antimicrobial agents exhibiting cytotoxic effects on eukaryotic cells, which is in agreement with other potent antimicrobial but cytotoxic peptides such as hBD-1, protegrin-1, and gomesin; and (iii) hBD-3 derivatives with high net charge and low hydrophobicity, which are potent antimicrobial peptides causing no significant cytotoxic effects. This general classification which is supported by the data obtained from hBD-3 peptides investigated in this study indicates that it is possible to separate interactions of β-sheet antimicrobial peptides with microbial cell walls from those with eukaryotic cell membranes. Correspondingly, potent antimicrobial peptides with no toxic effects on host cells might be designed.

SUPPORTING INFORMATION AVAILABLE

Mass-spectral data of products and synthetic intermediates, and a computer curve analysis of the CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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