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Review

Synthesis and structure–activity relationship of β-defensins, multi-functional peptides of the immune system

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Abstract: β-defensins are a large family of multiple disulfide-bonded peptides occurring in mammals and birds. They play an important role in the innate immune system, directly killing microbial organisms. Recent research has demonstrated that β-defensins are important for other biological functions beyond antimicrobial effects, including inhibition of viral infection, interaction with Toll-like receptors, chemotactic effects, and sperm function. The corresponding broad spectrum of activities makes this peptide class an important subject and tool in immunologic research. In this review, we summarize the current status of the routes to obtain synthetic β-defensins, their major structural properties and structure–activity relationship. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; beta-defensin; defensin; disulfide bond; immune system; structure–activity relation; synthesis

β-DEFENSINS: MULTI-FUNCTIONAL VERTEBRATE PEPTIDES OF THE IMMUNE SYSTEM

The immune system of multicellular organisms includes peptides that protect them from a broad range of microbes (bacteria, fungi, and protozoa) and viruses. Under the collective term 'defensins', a large number of cationic peptides that are 30–50 amino acid residues in size containing multiple disulfide bonds are known from vertebrates, insects, and plants. A particular subgroup emerging since the first description in 1993 [1], the β-defensins, occurs in mammals and birds. From a simplified structural view, β-defensins are characterized by six cysteine residues with a specific spacing forming three disulfide bonds in the arrangement Cys^1–Cys^5, Cys^2–Cys^4, Cys^3–Cys^6.

β-Defensins were isolated from neutrophils (bovine β-defensins [1]), blood ultrafiltrate (human β-defensin (HBD)-1 [2]), and psoriatic scales (HBD-2, HBD-3 [3,4]). Expression analysis revealed a predominant production of β-defensins in epithelial cells. Some β-defensins have been shown to express in adult heart, skeletal muscle, thymus, and placenta. While some β-defensins are constitutively expressed (HBD-1), the expression of others is induced by infectious challenges such as lipopolysaccharides (LPS) and bacteria (HBD-2, HBD-3, murine β-defensin (MBD)-2, MBD-3, MBD-6). MBD-specific mRNA has been detected in airways, esophagus, tongue, trachea, and other tissues. Recently, computational approaches led to the discovery of a large number of novel human and mouse genes located in five syntenic chromosomal regions [5]. The corresponding human genes have surprisingly been found to be almost exclusively expressed in the epididymis [6,7].

β-defensins, like other antimicrobial peptides, were initially considered as components of the innate immune system, directly disrupting microbial membranes. Several models for the interaction of the peptides with the bacterial cell wall have been considered, among them the barrel-stave and the carpet-like model [8]. Since β-defensins differ from each other in Gram specificity, strain specificity, and *in vitro* potency, it is clear that structural parameters such as primary structure, charge, hydrophobicity, and three-dimensional shape determine the interaction with the microbial cell wall. Some time after the discovery of the β-defensins it became evident that these peptides exhibit significant biological activities beyond the mere inhibition of microbial cells (Figure 1). 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), and on monocytes, macrophages (HBD-3), and mast cells (HBD-2)

Abbreviations: Acm, acetamidomethyl; BNBD, bovine neutrophil β-defensin; CBD, chinchilla β-defensin; EBD, enteric β-defensin; Defr-1, defensin-related gene 1; DLP, defensin-like peptide; DMSO, dimethyl sulfoxide; Gal, gallinacin; GBD, caprine β-defensin; GPV-1, gallopavin 1; HBD, human β-defensin; HPLC, high performance liquid chromatography; LAP, lingual antimicrobial peptide; LC-MS, liquid chromatography-mass spectrometry; LPS, lipopolysaccharide; MBD, murine β-defensin; 4-MeBN, 4-methyl benzyl; NMR, nuclear magnetic resonance; OSP-1, ostricacin 1; PBD, porcine β-defensin; RBD, rat β-defensin; SBD, ovine β-defensin; TAP, tracheal antimicrobial peptide; tBu, tert-butyl; TFA, trifluoroacetic acid; THP, turkey heterophil peptide; Trt, trityl.

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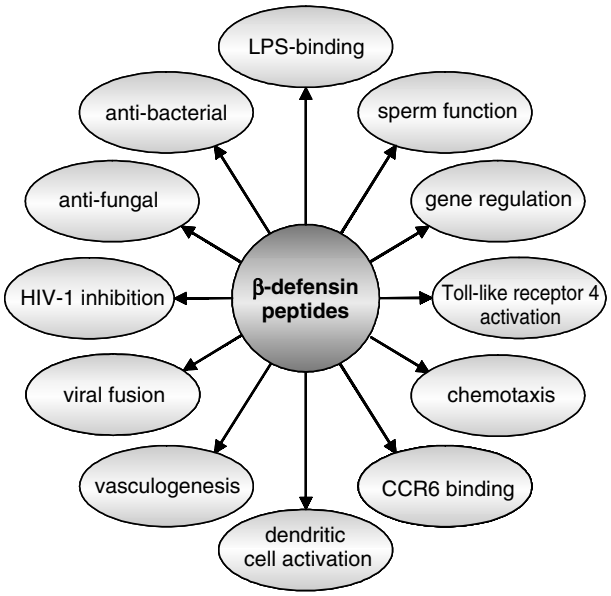


Figure 1 Peptides of the β-defensin family are involved in and mediate different biological processes based on microbicidal and non-microbicidal activities. Inflammatory mediators and microorganisms induce β-defensin expression. β-defensins have direct antimicrobial effects through the interaction with the microbial membrane, and have additional functions likely controlled by the interaction with specific receptors.

[9–14]. MBD-2 has been shown to activate dendritic cell maturation via Toll-like receptor 4 [15]. A further significant effect of HBD-2 and HBD-3 is the inhibition of HIV-1 replication *in vitro* [16–18]. Surprisingly, a large number of β-defensin peptides are specifically expressed in the epididymis [6,19–25]. The importance of a mouse β-defensin for sperm maturation has been reported recently [24,25]. Recent reviews comprehensively discuss the biological significance of β-defensins [26–33].

On the basis of the multi-functionality of β-defensins, the peptide family has been attracting a steadily growing interest, reflected by the increasing body of literature. Correspondingly, there is a great demand for procedures allowing the synthesis of diverse β-defensin analogs as research tools to unravel the structure–function relationship. In this review article, we summarize the current knowledge of chemical synthesis, structures, and structure-activity relationship of β-defensins.

PRIMARY STRUCTURES

Until now almost 50 β-defensins have been found in different mammals (primates, rodents and ungulates) and birds (Table 1), which identifies them as an evolutionary old class of antimicrobial peptides derived from ancient genes that existed before the diversification of the avian and mammalian line [34]. Some of the published sequences are based on peptides isolated from different organs such as skin (HBD-2 [3], HBD-3 [4]), blood plasma (HBD-1 [2]), neutrophils (bovine neutrophil β-defensin (BNBDs) [1]; gallinacins (Gal) [35]), tongue (lingual antimicrobial peptide (LAP) [36]), trachea (tracheal antimicrobial peptide (TAP) [37]), and stomach (spheniscins [38]). Other β-defensin sequences were deduced from genomic data after cloning of the corresponding cDNAs. Recently, the systematic analysis of the human and murine genome revealed a large number of genes encoding other putative β-defensin peptides [5,6]. While the longer-known human β-defensin genes encoding HBD-1, HBD-2 and HBD-3 are clustered on chromosome 8, most of the newly identified genes are located on chromosomes 6 and 20 (mouse: chromosomes 1, 2, 8, and 14). As an example for these β-defensins, Table 1 includes HBD-27 (gene: *DEFB127*) and HBD-28 (*DEFB128*) from human chromosome 20, which have been recently synthesized and characterized [39].

The most noticeable feature of the primary structure of β-defensins is the considerable diversity in the amino acid sequence and composition. While, in general, β-defensins are cationic peptides with a length of 35 to 50 amino acid residues, only eight of these residues can be considered as conserved. Among these, the six cysteine residues forming the characteristic intramolecular disulfide bonds are strictly conserved throughout. There are slight differences in the spacing between the cysteines. Furthermore, only two other residues are highly conserved. The first is a glycine residue located *N*-terminally at two positions from the second cysteine. Disregarding most of the novel human β-defensins, Table 1 contains three cases in which this glycine is replaced by an alanine (HBD-2, HBD-4, MBD-4). The second conserved residue is a glycine two positions *N*-terminally from the fourth cysteine. Only in turkey gallopavin 1 (GPV-1) and the newly discovered human β-defensins HBD-27 and HBD-28 this glycine is replaced by alanine or isoleucine, respectively. Interestingly, most of the new bioinformatically identified human β-defensins contain various amino acids at this position (for sequences, see [5,6]).

Although the amino acid composition varies considerably among β-defensins, some common characteristics are obvious. Cationic amino acid residues are generally clustered at the *C*-terminus. The presence of proline residues near the third and fourth cysteine residues might be of structural importance for the formation of β-hairpins [72]. Most of the recently identified β-defensin gene products, including HBD-3 and HBD-4, contain a glutamic acid residue located four amino acids *C*-terminally from the third cysteine, distinguishing them from other β-defensins [5]. The length of a mature form of β-defensins deduced from genomic data cannot be predicted. In some cases, e.g. HBD-4 or most of the

Table 1 Amino acid sequences of the cysteine-containing cores of β-defensins and β-defensin-like peptides

Type	Name	Species	Sequence[a]	Origin[b]	Net charge[g]	References
β-Defensins						
Primates[c,d]	HBD-1	Human	DHYNC--VSSGGQCLYSACPIFT**K**IQ**G**TC**Y**R**G**K**A**KCC**K**	P	+4	[2,40–42]
	HBD-2	Human	GIGDPVTC--L**K**SGAICHPVFCP**RR**Y**K**QI**G**TCGLPGT**K**CC**KK**P	P	+6	[3,43]
	HBD-3	Human	GIINTLQ**K**YYC--**R**V**R**GG**R**CAVLSCLP**K**EEQI**G**KCSTR**G**RKCC**RRKK**	P	+11	[4,13]
	HBD-4	Human	ELD**R**IC--GYGTA**R**C**R**-**KK**C**R**SQEY**R**I**G**RCPN-TYACCL**RK**	G	+7	[7]
	HBD-27	Human	EQL**KK**CWNNYVQGHC**RK**-ICRVNEVPEALC-ENG**R**YCCLNI**K**	G	+3	[39]
	HBD-28	Human	A**R**L**KK**C-FN**K**VTGYC**RK**-**K**C**K**VGE**R**YEIGC-LSG**K**LCCAN	G	+8	[39]
Rodents[d]	MBD-1	Murine	DQY**K**C--LQHGGFCL**R**SSCPSNT**K**LQ**G**TC**K**PD**K**PNCC**K**S	G	+4	[44,45]
	MBD-2	Murine	ELDHC--HTNGGYCV**R**AICPPSA**RR**P**G**SCFPE**K**NPCC**K**YM**K**	G	+3	[46]
	MBD-3	Murine	**KK**INNPVSC--L**RK**GG**R**C-WN**R**CIGNT**R**QI**G**SCGVPFL**K**CC**KRK**	G	+10	[47]
	MBD-4	Murine	QIINNPITC--MTNGAIC-WGPCPTAF**R**QI**G**NCGHF**K**V**R**CC**K**I**R**	G	+5	[48]
	MBD-6	Murine	QLINSPVTC--MSYGGSC-Q**R**SCNGGF**R**LGGHCGHP**K**I**R**CC**RRK**	G	+7	[49]
	MBD-7	Murine	NS**KR**AC--Y**R**EGGECLQ-**R**CIGLFH**K**I**G**TCNF-RF**K**CC**K**FQ	G	+6	[50]
	MBD-8	Murine	NEPVSC--I**R**NGGICQY**R**CIGL**R**H**K**I**G**TCGS-PF**K**CC**K**	G	+5	[50]
	RBD-1	Rat	DQY**R**C--LQNGGFCL**R**SSCPSHT**K**LQ**G**TC**K**PD**K**PNCC**R**S	G	+4	[51]
	RBD-2	Rat	TQSINNPITC--LT**K**GGVCW-GPCTGGF**R**QI**G**TCGLP**R**V**R**CC**KKK**	G	+7	[51]
	CBD-1	Chinchilla	GIINTIQ**R**YFC--**R**V**R**GG**R**CAALTCLP**R**ETQI**G**RCSV**K**G**RK**CC**R**T**RK**	G	+11	[52]
Ungulates	LAP	Bovine	GFTQGV**R**NSQSC--**RR**N**K**GICVPI**R**CPGSM**R**QI**G**TCLGAQV**K**CC**RRK**	P	+10	[36]
	TAP	Bovine	NPVSC--V**R**N**K**GICVPI**R**CPGSM**K**QI**G**TCVG**R**AV**K**CC**RKK**	P	+9	[37,53]
	EBD	Bovine	NPLSC--**R**LN**R**GICVPI**R**CPGNL**R**QI**G**TCFTPSV**K**CC**R**W**R**	G	+7	[54]
	BNBD-1	Bovine	DFASC--HTNGGICLPN**R**CPGHMIQIGICF**R**P**R**V**K**CC**R**SW	P	+4	[1]
	BNBD-2/3[e]	Bovine	*ZG*V**R**NHVTC--**R**IN**R**GFCVPI**R**CPG**R**T**R**QI**G**TCFGP**R**I**K**CC**R**SW	P	+9/+8	[1]
	BNBD-4	Bovine	Z**R**V**R**NPQSC--**R**WNMGVCIPFLC**R**VGM**R**QI**G**TCFGP**R**VPCC**RR**	P	+7	[1,55,56]
	BNBD-5	Bovine	ZVV**R**NPQSC--**R**WNMGVCIPISCPGNM**R**QI**G**TCFGP**R**VPCC**RR**W	P	+5	[1,55]
	BNBD-6	Bovine	ZGV**R**NHVTC--**R**IYGGFCVPI**R**CPG**R**T**R**QI**G**TCFG**R**PV**K**CC**RR**W	P	+8	[1]
	BNBD-7	Bovine	GV**R**NFVTC--**R**IN**R**GFCVPI**R**CPGH**RR**QI**G**TCLG**R**I**K**CC**R**	P	+8	[1]
	BNBD-8/9[e]	Bovine	*ZG*V**R**NFVTC--**R**IN**R**GFCVPI**R**CPGH**RR**QI**G**TCLAPQI**K**CC**R**	P	+8/+7	[1]
	BNBD-10	Bovine	GV**R**SYLSC--WGN**R**GICLLN**R**CPG**R**M**R**QI**G**TCLAP**R**V**K**CC**R**	P	+7	[1]
	BNBD-11	Bovine	GPLSC--**RR**NGGVCIPI**R**CPGPM**R**QI**G**TCFG**R**PV**K**CC**R**SW	P	+7	[1]
	BNBD-12/13[e]	Bovine	*SGIS*GPLSC--G**R**NGGVCIPI**R**CPVPM**R**QI**G**TCFG**R**PV**K**CC**R**SW	P	+6/+6	[1,56]
	GBD-1	Caprine	QGI**RS**RRSC--H**R**N**K**GVCALT**R**CP**R**NM**R**QI**G**TCFGPPV**K**CC**RKK**	G	+12	[57]
	GBD-2	Caprine	QGIINH**R**SC--Y**R**N**K**GVCAPA**R**CP**R**NM**R**QI**G**TCHGPPV**K**CC**RKK**	G	+10	[57]
	SBD-1	Ovine	QGV**R**N**R**LSC--H**R**N**K**GVCVPS**R**CP**R**HM**R**QI**G**TC**R**GPPV**K**CC**RKK**	G	+12	[58]
	SBD-2	Ovine	HGVTDSLSC--**R**W**KK**GICVLT**R**CPGTM**R**QI**G**TCGGPPV**K**CC**R**L**K**	G	+7	[58]
	PBD-1	Porcine	NSVSC--L**R**N**K**GVCMPG**K**CAP**K**M**K**QI**G**TCGMPQV**K**CCK**RK**	G	+9	[59]

(*continued overleaf*)

Table 1 (*Continued*)

Type	Name	Species	Sequence[a]	Origin[b]	Net charge[g]	References
Birds	Gal-1	Chicken	GRKSDC--FRKSGFCAFLKCPSLTLISGKCSR-FYLCCKRIW	P	+8	[34,35]
	Gal-1α	Chicken	GRKSDC--FRKNGFCAFLKCPYLTLISGKCSR-FHLCCKRIW	P	+8	[34,35]
	Gal-2	Chicken	LFC----KGGSCHFGGCPSHLIKVGSCFG-FRSCCKWPWNA	P	+4	[34,35]
	Gal-3	Chicken	GTATQC--RIRGGFCRVGSCRFPHIAIGKCAT-FISCCGRAY	G	+6	[34,60]
	CHP-1	Chicken	GRKSDC--FRKSGFCAFLKCPSLTLISGKCSR-FYLCCKRIR	P	+9	[34,61]
	THP-1	Turkey	GKREKC--LRRNGFCAFLKCPTLSVISGTCSR-FQVCC	P	+7	[34,61]
	THP-2	Turkey	LFC----KRGTCHFGRCPSHLIKVGSCFG-FRSCCKWPWDA	P	+5	[34,61]
	GPV-1	Turkey	GTPIQC--RIRGGFCRFGSCRFPHIAIAKCAT-FIPCCGSIW	G	+5	[34,60]
	Sphe-1	Penguin	SFGLC--RLRRGFCAHGRCRFPSIPIGRCSR-FVQCCRRVW	P	+9	[34,38]
	Sphe-2	Penguin	SFGLC--RLRRGFCARGRCRFPSIPIGRCSR-FVQCCRRVW	P	+10	[34,38]
	OSP-1	Ostrich	LFC----RKGTCHFGGCPAHLVKVGSCFG-FRACCKWPWDV	P	+4	[34,62]
β-Defensin-like peptides						
Epididymis-specific peptides[f]						
	HE2β1	Human	(51)GIRNTIC--RMQQGICRLFFCHSGEKKRDICSDPWNRCCVSNT(16)	G	+3	[19–22]
	HE2C	Human	(15)DLKVVDC--RRSEGFCQEY-CNYMETQVGYCPKKKDACCLH	G	−1	[22]
	Bin1b	Rat	GIRNTVC--FMQRGHCRLFMCRSGERKGDICSDPWNRCCVSSSIKNR	G	+6	[23]
Toxins and other defensin peptides[f]						
	Big defensin	Horseshoe crab	(40)DNHSCAGNRGWCRSKCFRHEYVDTYYSAVCGRYFCCRSR	P	+6	[63]
	DLP-1	Platypus	FVQHRPRDCESINGVCRHKDTVNCREIFLADCYNDGQKCCRK	P	+2	[64]
	DLP-2	Platypus	IMFFEMQACWSHSGVCRDKSERNCKPMAWTYCENRNQKCCEY	P	+1	[65]
	Crotamine	Rattle snake	YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG	P	+8	[66,67]
	Myotoxin A	Rattle snake	YKQCHKKGGHCFPKEKICIPPSSDLGKMDCRWKWKCCKKGSG	P	+8	[68]

[a] Z = pyroglutamyl; cysteine residues are boxed, highly conserved glycine residues are highlighted in grey; basic amino acid residues (Lys, Arg) are printed in bold. Gaps are inserted for alignment. The sequences of big defensin and β-defensin-like toxins are not aligned.
[b] P: peptide was isolated and sequenced; G: peptide sequence was deduced from genomic data.
[c] Primate β-defensins 1–3 are represented by human β-defensins since sequences of non-human primates differ only by few amino acids; for individual sequences of non-human primate defensins see Refs 29, 69–71.
[d] The recently discovered sequences of further human and MBDs are not listed; see Refs 5, 6 for their sequences.
[e] Some bovine β-defensins are cleavage variants: BNBD-2 is a N-terminally truncated form of BNBD-3, BNBD-8 is a variant of BNBD-9, and BNBD-12 is a variant of BNBD-13; the involved amino acids are printed in italics.
[f] Numbers in brackets denote the number of N- or *C*-terminal amino acid residues not shown.
[g] Net charge corresponds to the formal sum of negatively (Glu, Asp) and positively (Lys, Arg) charged amino acid residues.

novel human β-defensins [5,7], the *C*-terminus is considerably longer as shown in Table 1, which may point to a yet unknown functionality.

Another antimicrobial peptide with significant similarity to β-defensins is the big defensin isolated from hemocytes of the horseshoe crab, a 79-amino acid peptide, which contains six cysteine residues in the *C*-terminal region forming the β-defensin-typical disulfide pattern [63]. A further group of peptides with striking similarity to β-defensins consists of different toxins. The defensin-like peptides (DLP)-1 and -2 isolated from platypus venom contain the six characteristic cysteine residues but lack antimicrobial activity, possibly because of their less cationic character (Table 1) [64,65]. In addition, rattle-snake toxins like crotamine or myotoxin A closely resemble the β-defensins in size, net charge, and disulfide pattern [66–68].

On the basis of the length and sequence homology of the peptides and genes, the β-defensins can be divided into three groups. (i) The first group contains β-defensins with a relatively short prepro-peptide (63–64 amino acid residues) and, on the gene level, a short intron (<1.6 kbp) between the two β-defensin-encoding exons. This large group includes mammalian β-defensins such as HBD-2, MBD-3, TAP, LAP, enteric β-defensin, (EBD), BNBDs, ovine β-defensin (SBD)-1, SBD-2, porcine β-defensin (PBD)-1, rat β-defensin (RBD)-2, caprine β-defensin (GBD-1), and GBD-2. (ii) The second group of β-defensins contains longer prepro-peptides (68–69 amino acid residues) with a larger intron (>6.5 kbp) and includes HBD-1, MBD-1, MBD-2, and RBD-1. (iii) The third group is represented by avian β-defensins that in turn can be divided into two subgroups on the basis of sequence homology. Subgroup (a) includes Gal-1, Gal-1α, Gal-3, turkey heterophil peptide (THP)-1, GVP-1, and the spheniscins; subgroup (b) contains Gal-2, THP-2, and ostricacin 1 (OSP-1). Analysis of the evolutionary relationships supports this classification [34,57,73,74].

SECONDARY AND TERTIARY STRUCTURE

An essential parameter to assign a peptide to the β-defensin family is the typical intramolecular distribution of cysteine residues and the disulfide bonding pattern, which distinguish β-defensins from other defensin classes. The disulfide connectivity of β-defensins is Cys^1–Cys^5, Cys^2–Cys^4, Cys^3–Cys^6 and was first determined for bovine BNBD-12 by tryptic digestion combined with Edman degradation [75]. Since then very few investigations were carried out to determine the disulfide pattern of native β-defensins, and it is usually assumed that all β-defensin peptides contain disulfides determined for BNBD-12. Recently, the predicted disulfide connectivity of HBD-2 was confirmed by an analytical comparison of native and synthetic peptides [39]. In the group of β-defensin-like peptides, horseshoe crab big defensin and myotoxin A have been demonstrated to exhibit the β-defensin disulfide pattern [63,68].

The three disulfide bonds are thought to stabilize the three-dimensional structure of the β-defensins. The structure was first determined in 1995 for bovine BNBD-12 using nuclear magnetic resonance (NMR) spectroscopy, and was analyzed later for several other β-defensins. Figure 2 shows the backbone fold of all seven investigated β-defensins in comparison with the two β-defensin-like toxins DLP-1 and crotamine, and representatives of other three defensin classes (α-defensins, insect, and plant defensins).

The three-dimensional structure of β-defensins consists mainly of a triple-stranded antiparallel β-sheet. Although not observed for each β-defensin, there is a tendency to form an additional *N*-terminal α-helix. The length and regularity of this helix depend on the experimental conditions; it is more distinct in the crystalline state than in solution. It is not clear whether the formation of an α-helix is connected with the presence of particular amino acids, as was supposed for the *N*-terminal Asp^4 in HBD-2 [83]. The internal disulfide bridges reduce the flexibility of the peptide. While disulfide bond Cys^1–Cys^5 connects the α-helix to the third β-strand, β-strands 1 and 2 are held together by disulfide bond Cys^2–Cys^4. Disulfide bridge Cys^3–Cys^6 connects the loop between β-strands 1 and 2 with the third β-strand. β-Defensin-like toxins generally show a comparable overall fold, although the second β-strand is less distinct. Interestingly, the β-defensin fold shows some similarities to other defensin classes. The mammalian α-defensins exhibit an analogous triple-stranded β-sheet, but lack an *N*-terminal α-helix, probably because of their shorter peptide chain. This similarity in tertiary structure is remarkable since disulfide pattern and cysteine spacing differ from those of the β-defensins. The similarity between β-defensins and insect or plant defensins is lower. Although the secondary structural elements are comparable (α-helix and two or three β-strands), their spatial arrangement and the overall structure are different.

Interestingly, the tertiary structure of β-defensins is rather similar to that of chemokines like murine or human CCL20/MIP-3α [84,85]. Although they are larger than β-defensins (~70 amino acid residues), these peptides have a similar cationic character and are shown to exhibit antimicrobial activity [84]. On the other hand, β-defensins exhibit chemotactic properties via chemokine receptor CCR6 [14,32]. The chemokine tertiary structure of these chemokines consists of an antiparallel triple-stranded β-sheet with a long *C*-terminal α-helix.

The mode of action of cationic antimicrobial peptides is mostly described as the formation of pores by assemblies of molecules that insert into the microbial

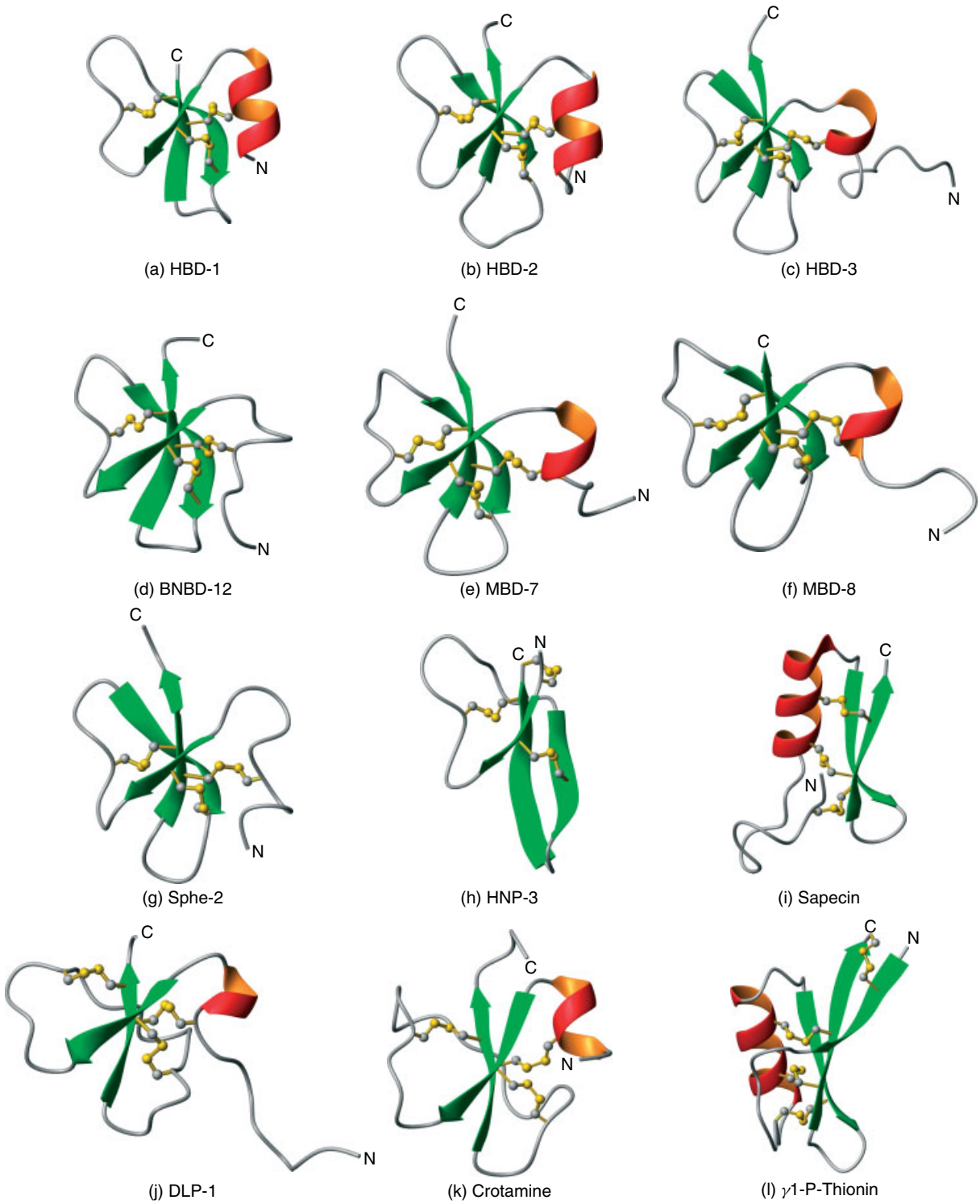
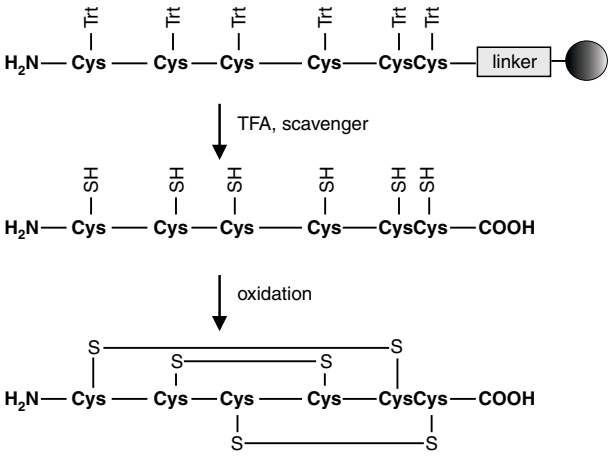


Figure 2 Three-dimensional structures of β-defensins and related peptides. The overall fold of seven β-defensins is compared with two β-defensin-like toxins (DLP-1 from platypus, crotamine), human α-defensin HNP-3, insect defensin sapecin, and plant defensin γ1–P-thionin. For each peptide, PDB entry number, peptide chain length, and method of structure determination are noted. Structures were drawn using MolMol [76]. (a) HBD-1, 36 amino acids (aa), PDB entry 1IJV, structure determined by X ray [77]; (b) HBD-2, 41 aa, 1FD3, X-ray [78]; (c) HBD-3, 45 aa, 1KJ6, NMR [79]; (d) BNBD-12, 38 aa, 1BNB, NMR [80]; (e) MBD-7, 37 aa, 1E4T, NMR [50]; (f) MBD-8, 35 aa, 1E4R, NMR [50]; (g) Sphe-2, 38 aa, 1UT3, NMR [81]; (h) HNP-3, 30 aa, 1DFN, X-ray; (i) sapecin, 40 aa, 1L4V, NMR; (j) DLP-1, 42 aa, 1B8W, NMR [64]; (k) crotamine (snake toxin), 42 aa, 1H5O, NMR [67]; (l) γ1-P-thionin, 47 aa, 1GPS, NMR. The disulfide bonds are plotted in yellow; secondary structural elements are in green (β-strands) and red (α-helix). N and C termini are indicated by capital letters. The Protein Databank (http://www.rcsb.org/pdb/) contains additional entries for HBD-1 (1E4S and 1KJ5, NMR structures [50,79]) and HBD-2 (1E4Q, NMR structure of synthetic 37aa peptide [50], and 1FQQ, NMR structure of recombinant 41 aa peptide [82]).

cell membrane [8,86]. Therefore, an important point concerning the antimicrobial activity is the ability of β-defensins to oligomerize. It appears that several β-defensins show a tendency for oligomerization, but the underlying mechanism and the spatial arrangement of monomers forming oligomers differ for the individual peptides. HBD-2 has been shown to dimerize by hydrophobic interactions within the first β-strand [78], while HBD-3 possibly dimerizes via the second β-strand involving hydrogen bonds between Glu, Lys and Gln residues, and a salt bridge between Arg and Glu [79]. The importance of these residues for the dimerization of HBD-3 has been shown by comparison with homologous, predominantly monomeric monkey β-defensins lacking these amino acids [69].



Scheme 1 Route for synthesis of β-defensins via oxidative folding. The cysteine protecting groups are indicated.

CHEMICAL SYNTHESIS

Different routes have been evaluated to develop specific strategies for the synthesis of β-defensin peptides. To facilitate disulfide bond formation in β-defensins, methods developed and used for other biologically active peptides, e.g. the conotoxins [87], have been applied [88,89]. Disulfide introduction to obtain β-defensins with the specific cystine pattern is discussed in detail below. Chain assembly to obtain the thiol precursors of β-defensins is achieved using both Boc and Fmoc chemistry. Not surprisingly, we and others found that, depending on the target amino acid sequence, the solid-phase assembly of some β-defensins is more straightforward than that of others [39,90,91]. In some cases a tendency for aggregation was observed, causing a significant formation of deletion and truncation products. However, optimized activation procedures and incorporation of Hmb-protected amino acids or pseudoproline building blocks are usually appropriate means to increase the quality of crude linear β-defensins if necessary.

Oxidative Folding of Hexathiol Precursors

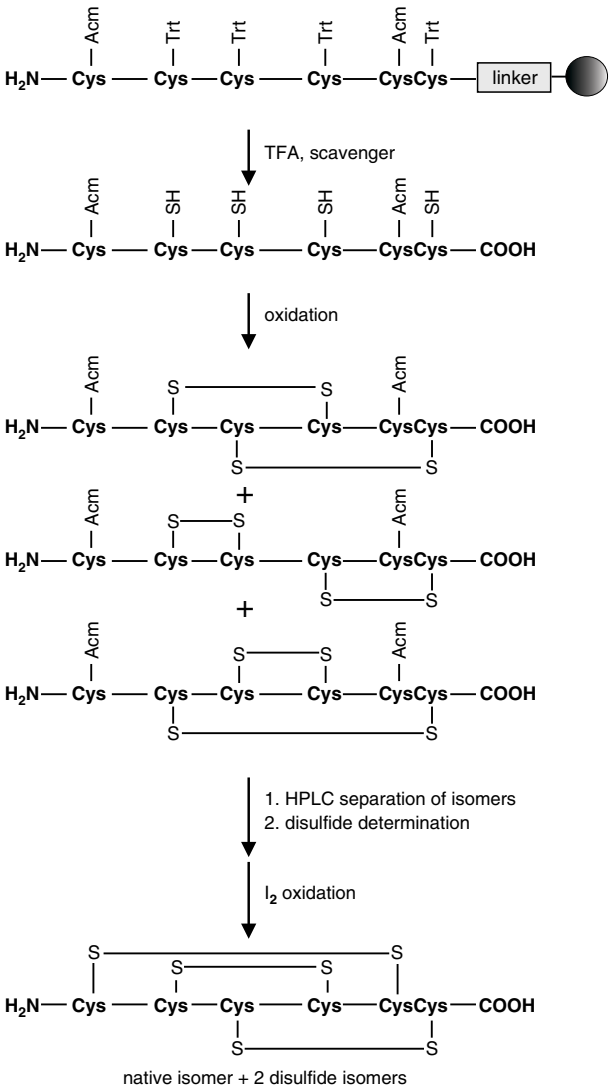
A straightforward approach to obtain correctly folded β-defensins with the disulfide connectivity Cys^1–Cys^5, Cys^2–Cys^4, Cys^3–Cys^6 is the oxidation of hexathiol precursor peptides (Scheme 1). This reaction occurs in diluted aqueous solution under oxidative conditions, e.g. in the presence of dimethyl sulfoxide (DMSO) or air. This procedure has been demonstrated to work well for HBD-1, HBD-2, HBD-27, MBD-7, MBD-8, BNBD-2, and BNBD-12 [10,39,72,90,92]. The thiol precursors of these peptides generate the desired disulfide isomer during oxidative folding as the dominating product. In some cases, an optimization of the folding conditions that include the support by a redox system such as cysteine/cystine, temperature, or other reaction conditions was necessary to suppress the formation of undesired disulfide isomers.

In contrast, the β-defensins HBD-3, HBD-23 (gene: *DEFB123*), and HBD-28 could not be obtained using oxidative folding. A complex mixture of disulfide isomers and, in some cases, other by-products such as peptide polymers tending to precipitate were formed [10,39]. Since the formation of a major product during an oxidative folding procedure does not necessarily mean that the desired disulfide bonds are present, the verification of the disulfide connectivity is an essential issue in the analysis of synthetic β-defensins. For the verification of the disulfide connectivity the fully oxidized peptides are usually treated with proteases, retaining all disulfide bonds, followed by liquid chromatography-mass spectrometry (LC-MS) analysis and Edman sequencing of cystine-containing fragments. The performance of such studies is often more laborious than the actual peptide synthesis procedure since β-defensins are, in some cases, e.g. HBD-2, remarkably stable against proteases used for the partial enzymatic degradation of the peptides (E. Klüver, unpublished results, [39]). In these difficult cases, the procedure of partial disulfide reduction and thiol alkylation had to be applied to determine the disulfide pattern of the synthetic peptide [39,93,94]. Some β-defensins such as HBD-3 and HBD-27 do not fold to the desired native structure [10,39]. Oxidative folding of these peptides yields a product mixture containing several fully oxidized isomeric species upon redox-controlled disulfide shuffling in aqueous solution.

Semiselective Formation of Disulfides

To reduce the product complexity in the synthesis of peptides with three or more disulfide bonds, some groups have successfully used selective protection of one pair of cysteine residues [87 and literature cited

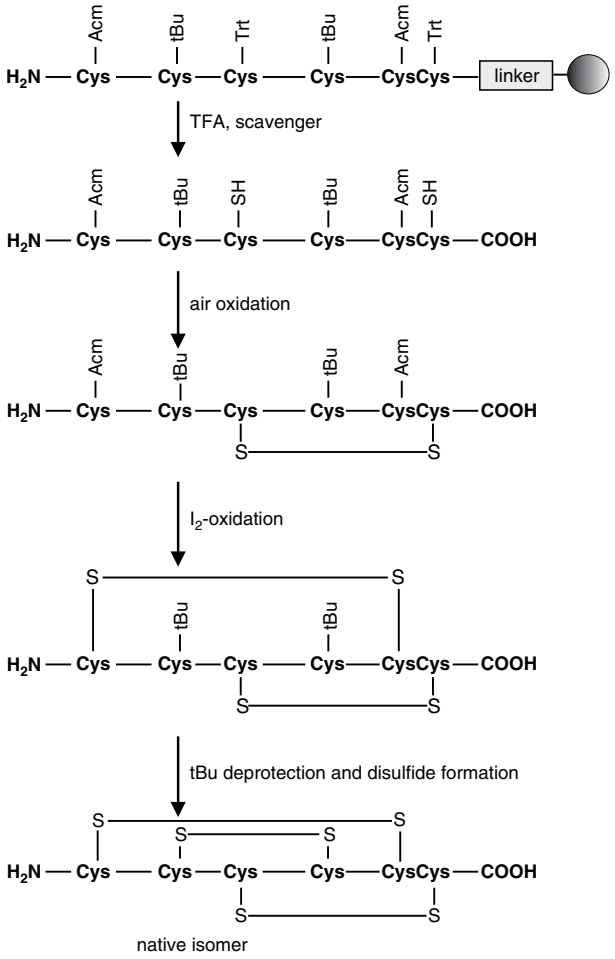
therein, [95,96]]. In such an approach, a peptide containing three disulfide bonds is assembled using four trityl (Trt)-protected cysteine residues while the remaining two are acetamidomethyl (Acm)-protected. In the first oxidation step, the thiols derived from the Trt-protected cysteines are converted to disulfides with a maximum of three disulfide isomers instead of 15, using six free cysteines. The isomers obtained can either be separated by high performance liquid chromatography (HPLC) methods, or the mixture can directly be used for the cleavage and oxidation of Acm-protected cysteines to introduce the third disulfide bond. Wu *et al.* employed this strategy for the synthesis of HBD-3 disulfide isomers (Scheme 2) [10]. They used Acm protection for Cys residues 1 and 5, and, alternatively, for Cys residues 1 and 6 to obtain non-native isomers. After cleavage from the resin, the linear peptides were oxidized in the presence of the redox system cysteine/cystine at basic pH, and three differently double disulfide-bonded HBD-3-peptides for each precursor were separated. Disulfide connectivities were determined after enzymatic cleavage of the products. In the second oxidation step, the third disulfide bond between cysteines 1–5 or 1–6 was formed with iodine at acidic pH generating 6 different isomers of HBD-3. Depending on the research goals, this strategy can be advantageous since it is possible to obtain disulfide isomer mixtures of limited complexity that can be separated by HPLC. However, in terms of achieving a high yield of a β-defensin, a semiselective approach does not appear to represent an appropriate strategy. Also, time and effort need to be considered, since it is impossible to predict the cysteine residues that have to be protected by Acm groups. In most cases, all three possible Acm-protected precursors are to be examined. While such synthetic route could be suitable for a single β-defensin peptide, it might not be applicable for another β-defensin.



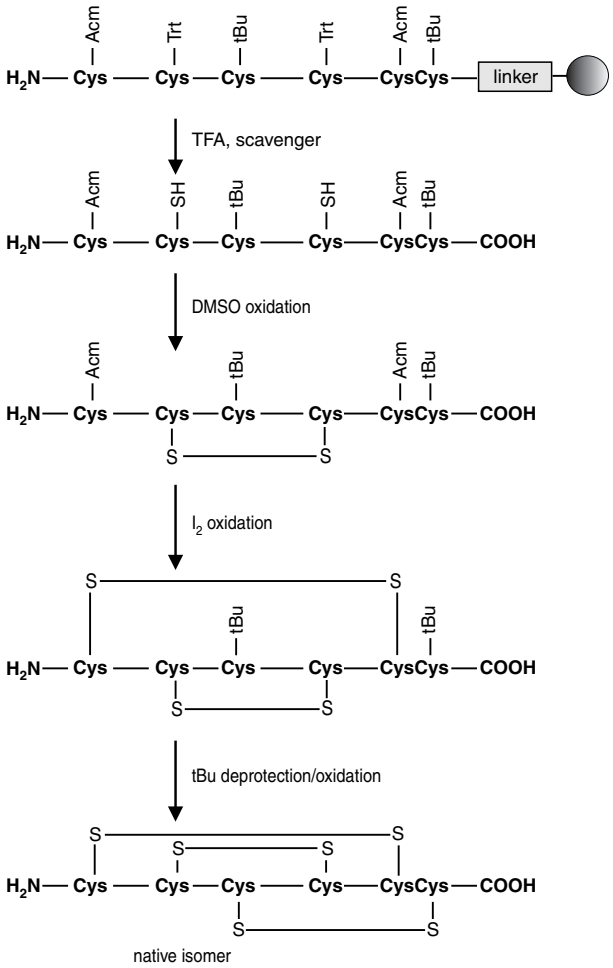
Scheme 2 Routes for synthesis of β-defensins HBD-3 and HBD-23 via a semi-selective approach using two Acm-protected cysteines. The cysteine protecting groups are indicated.

Orthogonal Protection of Cysteines and Stepwise Disulfide Formation

The only selective way to obtain β-defensins with the native disulfide bond pattern is the use of three orthogonal protective groups that allow the stepwise introduction of disulfides. Recently, the synthesis of novel β-defensins with the introduction of the disulfide bonds using three different cysteine protective groups has been examined [39]. The combination of the protective groups Trt/Acm/tert-butyl (tBu) proved to be successful (Schemes 3 and 4). Suitable reaction conditions for each single disulfide formation were examined. It was found that optimal reaction conditions strongly depended on the individual sequence of the peptide. The first disulfide bridge was introduced either by air oxidation (for HBD-2 and HBD-28) or DMSO (for HBD-3), yielding a single disulfide-bridged β-defensin intermediate with four pairwise protected cysteines in reasonable yields. In all cases, the second oxidation step involved iodine oxidation of the Acm-protected cysteines to the corresponding double disulfide-bridged peptide. After introduction of the second disulfide bridge, the oxidation of the two remaining cysteines protected by tBu groups required different reaction conditions for the three defensins examined. HBD-2 could be best obtained at a temperature of 37 °C and a peptide concentration of 0.1 mg/ml, whereas HBD-28 required an elevated reaction temperature of 60 °C and a lower peptide concentration of 0.05 mg/ml. The major side reaction observed was the generation of overoxidized material, probably resulting from the harsh reaction in concentrated trifluoroacetic acid (TFA) containing DMSO. Another protective group used in this study was 4-methyl benzyl (4-MeBn). The



Scheme 3 Route for selective synthesis of HBD-2 and HBD-28. The cysteine protecting groups are indicated.



Scheme 4 Route for selective synthesis of HBD-3 (40-mer). The cysteine protecting groups are indicated.

main advantage in using this protective group is its considerable stability, requiring elevated temperatures for cleavage. Cuthbertson *et al.* used 4-MeBn in the synthesis of three disulfide-containing peptides such as conotoxins and the bacterial heat-stable enterotoxin ST [97,98]. However, the 4-MeBn groups could not be cleaved in attempts to synthesize HBD-3 disulfide isomers. In the case of HBD-28, tBu and 4-MeBn groups were found to be not appropriately orthogonal since 4-MeBn was partially unstable under the conditions required for the cleavage of tBu groups.

These examples illustrate that there is a suitable strategy for cysteine protection to obtain β-defensins with the native 1–5/2–4/3–6 disulfide connectivity. In particular, peptide length, position of the disulfide bond to be formed, and amino acid sequence strongly influence these reactions. This is illustrated by attempts to synthesize HBD-3 derivatives of different *N*-terminal length. A variant with 40 amino acid residues could not be obtained via Trt-protected cysteines and oxidative folding of the reduced precursor peptide. It had to be synthesized by a selective approach, whereas, in contrast, a variant with 45 residues yielded the desired disulfide isomer upon oxidative refolding [39].

Recently, we investigated the synthesis of the epididymally expressed novel HBD-23 whose amino acid sequence was deduced from the corresponding cDNA sequence. For this peptide, only a semiselective protection strategy using two Acm-protected cysteines was successful (data not shown). The use of three orthogonal protecting groups such as Trt/Acm/tBu did not yield the desired product since the last oxidation step removing tBu protective groups resulted predominantly in overoxidized HBD-23 peptides under all conditions tested. This overoxidation occurred at a cysteine or tryptophan residue and was deduced from a mass shift by +16 Da.

The data available in the literature show that there is no general pathway for a successful synthesis of bioactive β-defensins with the native disulfide pattern. Some defensins readily fold to the desired product, whereas others do not. The yields of the chemically synthesized β-defensin peptides are generally in the range of 5 mg after purification, based on a standard scale of 0.1 mmol used in most laboratories. In some

cases a yield in the range of 25 mg was obtained. Although this is sufficient to carry out initial biological tests, it represents a constraint to perform more comprehensive studies requiring large amounts of the peptides.

There are very few reports about the synthesis of β-defensin peptides other than those from human origin, e.g. primate β-defensin 3 [69], BNDB-12 [92], DLP-1 [64], defensin-related gene 1 (Defr-1) [99], MBD-6 [49], and MBD-12 [100]. These peptides were synthesized by oxidative folding from hexathiol precursors. Data about the disulfide analysis of the corresponding products are limited.

RECOMBINANT EXPRESSION

Chemical synthesis technologies generally allow a faster and easier access to β-defensins including structural analogs than recombinant methods. However, the yield that can be obtained is often limited because of the specific problems of disulfide formation and multiple purification steps. The use of recombinant expression strategies might help to circumvent these restraints. Synthesis of β-defensins using recombinant methods in different host organisms is a challenging task, and major obstacles are low expression rates, high susceptibility towards degradation by host cell proteases, and a significant toxicity for the host organism [101–104]. For some β-defensins these drawbacks were overcome using expression systems in which the peptides are expressed as fusion proteins with different high molecular weight proteins such as thioredoxin and light meromyosin, or as a fusion protein linked to His_6-tags, thereby simplifying the chromatographic separation of the product [101–103]. For example, HBD-1 and HBD-2 have been studied for recombinant expression in *Escherichia coli*, and both peptides could be obtained in milligram amounts using the fusion protein expression strategy [101,102]. HBD-2 was also produced in a tandem expression system using *E. coli*. Multiple copies of the corresponding genes were linked [104], and the rate of HBD-2 expression was enhanced compared to the single copies of the genes. In another communication, it was reported that the expression level of a HBD-2-thioredoxin fusion protein can be enhanced by altering rare codons in the cDNA to those frequently used in *E. coli* to an amount of 50% of the total cellular protein, corresponding to a 9-fold enhancement of β-defensin expression over the wild type DNA [103]. Another drawback of bacterial expression is that in some cases recombinantly produced β-defensins are often found in both the soluble and insoluble fractions of bacterial extracts. Therefore, Cipakova *et al.* used the tendency of light meromyosin to form insoluble polymers at low ionic strength, which are dissolvable at high ionic strength [102,105]. It is noteworthy that in many cases, if not all, the disulfide connectivity of the recombinantly produced peptides was not reported. Cipakova *et al.* observed a lower antimicrobial activity of recombinant HBD-1 as reported for naturally occurring HBD-1 and speculated that this may be due to the presence of bacterially misfolded HBD-1 peptides [102].

STRUCTURE–ACTIVITY RELATION

So far, only few reports deal with a relation between β-defensin structure and biological activity (Table 2). In these studies, synthetic β-defensin peptides with defined structural variations were compared for their biological properties. Structural variations are artificial [10,69,72,83,91,92,99,106] or are based on naturally occurring mutations in related peptides [69,83,107]. The structural parameters of interest are the disulfide connectivity, single amino acid residue exchanges, length, charge, and hydrophobicity. In spite of their recognized biological multi-functionality, SAR studies with β-defensins have almost exclusively been performed considering antimicrobial activities. There are few reports dealing with the structural dependence of cytotoxic and chemotactic effects.

Concerning the antimicrobial activity of β-defensins, the results obtained in these studies support the widely accepted model of the mechanism of action of cationic antimicrobial peptides [8,86]. It has been shown for bovine BNBD-2 and BNBD-12 as well as for human HBD-3 that the presence or distribution of disulfide bonds is not essential for antimicrobial activity [10,72,91,92,106]. These authors tested several β-defensin peptides, differing only in the disulfide connectivity, and did not find significant differences in the antimicrobial activity. The same observations have been made with a naturally occurring five-cysteine variant of HBD-1, which contains only two intramolecular disulfide bonds, thus differing from the original HBD-1, and has comparable antimicrobial activity with HBD-1 [107]. This finding is expected for an unspecific, non-receptor-mediated mechanism. It is supported by computed models of the tertiary structure of truncated linear or disulfide-containing BNBD-2 peptides with different β-hairpin structure-inducing substitutions; despite the different predicted 3D conformations these derivatives exhibit comparable antimicrobial activity [72].

Important molecular factors for the antimicrobial potency of a peptide are charge and hydrophobicity. In recent studies, the activity of bovine BNBD-2 and BNBD-12 against different Gram-positive and Gram-negative germs was assigned to the highly cationic *C*-terminal part of the peptide which is structurally characterized by a β-hairpin structure [72,92]. For HBD-3 it was demonstrated that the cooperation of net charge and overall hydrophobicity determines

Table 2 Studies on the structure–activity relation of β-defensins

Peptide	Number of derivatives	Variants investigated	Measured parameters	References
HBD-1	2	Disulfide connectivity	Antimicrobial activity	107
HBD-2, mfaBD-2	3	Amino acid variations	Antimicrobial activity, secondary structure, membrane permeability, bacterial killing kinetics	83
HBD-3	7	Disulfide connectivity	Antimicrobial activity, chemotaxis	10
HBD-3	11	Disulfide connectivity, peptide length, charge	Antimicrobial activity	106
HBD-3	13	Disulfide connectivity, peptide length, hydrophobicity	Antimicrobial activity, cytotoxicity, secondary structure	91
HBD-3, hcBD-3	3	Amino acid variations	Antimicrobial activity, secondary structure, bacterial killing kinetics, dimerization	69
BNBD-2	8	Amino acid variations in peptide fragments	Antimicrobial activity, secondary structure, peptide conformation, membrane binding	72
BNBD-12	12	Disulfide connectivity, peptide length	Antimicrobial activity, secondary structure	92
Defr1	4	Covalent dimerization	Antimicrobial activity	99

mfaBD-2, *Macaca fascicularis* β-defensin 2; hcBD-3, *Hylobates concolor* β-defensin 3 (black gibbon); Defr1, MBD-related peptide with high homology to MBD-8, contains only 5 cysteine residues (DPVTYIRNGGICQYRCIGLRHKIGTCGSPFKCCK) [108].

the activity on prokaryotic and eukaryotic cells in such a way that antimicrobial and cytotoxic effects can be separated; the net charge of the peptide mainly influences the antimicrobial activity, while a greater hydrophobicity augments the cytotoxic effects. Thus, less cationic HBD-3 peptides with moderate hydrophobicity are virtually inactive, while peptides with a high positive net charge and a distinct hydrophobic moment are active against both cell types [91]. In the same study, it was reported that linear HBD-3 analogs with alanine or tryptophan substitutions for all cysteine residues exhibit comparable antimicrobial activity in low ionic strength medium compared to the native peptide. Interestingly, a hexa-tryptophan variant appeared to be still active in full medium.

Single amino acid mutations or *N*-terminal truncations, which do not significantly change the overall charge and hydrophobicity, have effects only on the specificity of the peptide against particular bacteria or the kinetics of membrane permeabilization, but not on the activity in general. This has been shown for HBD-2 [83], HBD-3 [91,106] and BNBD-2 [72], and is illustrated by comparison of the activity of β-defensins which occur naturally in different forms, such as bovine β-defensins and HBD-1 [1,40,41]. On the other hand, the antimicrobial activity of a β-defensin is considerably reduced when the truncation leads to short derivatives with low net charge and hydrophobicity, as was shown for HBD-3 [91,106].

It is further known that the antimicrobial activity of β-defensins depends strongly on the type and concentrations of ions, in particular, cations. Many studies report that most β-defensins lose their activity at higher concentrations of sodium chloride [40,41,43,44,51,69,81,108,109]. This is important for the physiological environment of β-defensins (150 mM NaCl). The inactivity of HBD-1 in the cystic fibrosis lung disease was ascribed to the increased mucosal level of NaCl [42]. The reduction of antimicrobial activity depends mainly on the charge of the ion. A study concerning ionic influences on the activity of HBD-2 shows that the effects of potassium and sodium are comparable, while divalent cations like Ca^{2+} and Mg^{2+} are significantly more effective [109]. In contrast, the concentration of anions has no detectable influence, as was shown for chloride and sulfate in this study. In some studies, β-defensin derivatives were reported to show a considerably lower salt sensitivity. Thus, it is described that a linear form of HBD-3 is more salt-resistant than the disulfide-linked peptide [10,106]. In another study, HBD-3 was compared with derivatives containing amino acid variations that caused a higher net charge and a reduced potential for dimerization [69]. These analogs were shown to be less salt sensitive

than the native HBD-3. On the other hand, a covalently dimeric β-defensin-related peptide is reported to be significantly more salt resistant than a monomeric variant [99]. Thus, in some cases the antimicrobial activity of β-defensins under high-salt conditions can be retained by a structural modification.

In contrast to the little importance for the antimicrobial activity, it was shown that the disulfide connectivity plays an essential role for the chemotactic functions of β-defensins. Six synthetic disulfide isomers of HBD-3 were characterized concerning their chemotactic effects on monocytes and CCR6-containing HEK293 cells [10]. The peptides differed significantly in the optimal peptide concentration for chemotaxis in a range of 1–10,000 ng/ml for monocytes and 10–1,000 ng/ml for CCR6/HEK293 cells, while a linear homolog was completely inactive. Interestingly, the chemotactic activity of the derivatives varied for different cell types. While the most active peptide on CCR6-containing cells was HBD-3 with the native disulfides, monocytes were more effectively attracted by a non-native disulfide isomer. These results confirm that receptor-mediated effects of β-defensins require a defined three-dimensional structure for binding and activation of the receptor.

CONCLUSIONS

After the first description of β-defensins in 1993, [1] a large body of knowledge about this peptide family has been created, including the elucidation of the structure of genes and the deduced peptides in different species, the expression and induction of expression of β-defensins, and, most importantly, their biological activities and roles in the innate and adaptive immune system. As summarized in this review, efficient methods facilitating the synthesis and investigation of β-defensin peptides are established. However, methods to produce larger amounts of β-defensins for *in vivo* tests of their functional properties and to generate peptide arrays containing a large number of structurally modified β-defensin analogs to examine the structure–activity relation are not yet sufficiently available. Corresponding future achievements should enable the evaluation of specific protein interactions of β-defensins and their biological role and therapeutic potential.

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