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Review

Avian cathelicidins: Paradigms for the development of anti-infectives

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ABSTRACT

The broad-spectrum defense system based on host defense peptides (HDPs) is evolutionary very old and many invertebrates rely on this system for protection from bacterial infections. However, in vertebrates the system remained important in spite of the superposition of a very sophisticated adaptive immune system. The cathelicidins comprise a major group of HDPs in mammals. About six years ago it was first described that cathelicidins are also present in birds. Here we review the properties and biological activities of the recently discovered avian cathelicidins and their potential to be used as a paradigm for the development of anti-infectives. Like the mammalian cathelicidins, avian cathelicidins exert direct antimicrobial activities but can also selectively boost host immune responses by regulation of cytokine production and recruitment of immune cells. In addition, it was found that chicken cathelicidins bind endotoxins and dampen the endotoxin-mediated inflammatory response. Molecular dissection has allowed identification of different structural elements involved in bacterial killing and immunomodulation. These studies have enabled the design of small HDP-based antibiotics with specific functions, *i.e.* having primarily immunomodulatory or antimicrobial activities. Since the immunomodulatory effects may, to a certain degree, be species-specific, we hypothesize that poultry-specific antibiotics can be developed based on avian cathelicidins.

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1. Host defense peptides

HDPs can be defined as small peptides (10–50 amino acids in general), primarily enriched in hydrophobic and cationic amino acid residues (+2 to +9), which can display broad-spectrum antimicrobial and/or immunomodulatory activities and are widespread throughout the animal, plant and fungal kingdoms (Hancock and Sahl, 2006; Zasloff, 2002). In mammals, HDPs may be constitutively or regulatory expressed and are found at sites that routinely encounter pathogens, such as mucosal surfaces and skin, the crypts of the small intestine and within the granules of immune cells (Yang et al., 2004). HDP-containing immune cells may be attracted to the site of infection to ensure local delivery of the peptides (Froy, 2005). HDPs are important to bridge the gap between the onset of infection and sufficient clonal expansion of B and T cells necessary to mount an adequate adaptive immune response. The fact that protection against enteric Salmonellosis can be prevented by the introduction of a single HDP gene (human defensin 5) in transgenic mice indicates that even a single HDP gene can make the difference in the outcome of infection (Salzman et al., 2003). Currently, natural HDPs are increasingly being used as templates for the research and development towards new antibiotics due to their unusual broad-spectrum of antimicrobial and immunomodulatory activities. The aim of this review is to present our current knowledge on the biological functions of avian cathelicidins and to discuss possible applications in veterinary medicine.

2. Cathelicidins

Cathelicidins comprise a major group of HDPs and have been found in mammals (Zanetti, 2005), fish (Chang et al., 2006; Uzzell et al., 2003), reptiles (Zhao et al., 2008) and birds (Lynn et al., 2004; van Dijk et al., 2005; Xiao et al., 2006a). The name cathelicidin is derived from the similarity of the cathelicidin large middle domain to cathelin, a cathepsin L inhibitor originally isolated from porcine leukocytes (Ritonja et al., 1989). The number of cathelicidin variants within a species varies considerably between species, with humans, rhesus monkey, mice, rat, and guinea pig possessing only one gene, whereas eight or more cathelicidin genes have been found in pig, sheep and cattle (Braff et al., 2005; Frohm Nilsson et al., 1999; Nagaoka et al., 1997; Scocchi et al., 2009; Termén et al., 2003; Zhao et al., 2008).

2.1. Structural features

Cathelicidins are produced as inactive prepropeptides, consisting of a short signal peptide, a large cathelin-like propiece and a mature peptide. Upon release into the environment, the C-terminal mature peptide is activated by proteolytic cleavage and exerts its antimicrobial and immunomodulatory functions (Zanetti, 2005). Based on structural characteristics of their C-terminal mature HDPs, at least three subfamilies can be distinguished (Bals and Wilson, 2003; Zanetti, 2005): (i) α-helical peptides, linear peptides of 23–37 amino acid residues that adopt an amphipathic helical structure if in contact with environments mimicking biological membranes; (ii) β-hairpin peptides, short cyclic peptides (12–18 amino acid residues) formed by one or two intramolecular disulfide bridges; (iii) peptides containing an unusual proportion of one or two amino acids, such as tryptophan- (indolicidin, 13 amino acids) or proline/arginine-rich peptides. The α-helical cathelicidin peptides are the most widely spread of the three groups and found in all investigated mammalian species (Zanetti, 2005) and include all known chicken cathelicidins.

2.2. Modes of action

2.2.1. Antimicrobial activity

Mammalian cathelicidins possess potent antimicrobial activity against various bacteria, fungi, and enveloped viruses at micromolar concentrations (Zasloff, 2002). The cationic side chains of cathelicidins interact with the negatively charged components in the bacterial or fungal membrane. Subsequently, the hydrophobic side chains perturb the lipid bilayer resulting in transient pore-formation. Several models of pore formation have been suggested, *i.e.* the barrel-stave, carpet, and aggregate channel model. Microbial exposure to low peptide concentrations results in membrane permeabilization and a loss of proton motive force, while at higher concentrations complete lysis may occur. Peptides may internalize by self-promoted uptake and subsequently bind to negatively charged intracellular targets such as DNA, RNA and proteins leading to inhibition of DNA replication, protein synthesis and protein function. These mechanisms have been extensively reviewed elsewhere (Bals and Wilson, 2003; Hancock and Chapple, 1999; Nicolas, 2009; Shai et al., 2006) and will not be discussed in this review.

2.2.2. Immunomodulatory activity

Human cathelicidin LL-37 attracts neutrophils, monocytes and T cells by utilizing formyl peptide receptor like -1 (FPRL-1) as a receptor (Yang et al., 2000) and attracts mast cells via a G_i protein-phospholipase C signaling pathway (Niyonsaba et al., 2002). Besides their intrinsic chemoattractant properties, cathelicidins indirectly favor chemotaxis by inducing chemokine secretion, such as interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), in mononuclear phagocytes and epithelial cells (Oppenheim and Yang, 2005). In addition, several cathelicidins induce or enhance the production of pro-inflammatory cytokines in different cell types, enhance phagocytosis and stimulate maturation of dendritic cells (Bandholtz et al., 2006; Davidson et al., 2004; Scott et al., 2002; Yu et al., 2007). Combined, these effects augment uptake, processing and presentation of antigens, stimulate clonal expansion of T-lymphocytes and B-lymphocytes, and contribute to the clearance of microbes through phagocytosis (Yang et al., 2004). In addition, mast cell exposure to LL-37 may result in degranulation and the release of histamine and prostaglandins (Niyonsaba et al., 2001). Despite their structural diversity, many cathelicidins bind lipopolysaccharide (LPS) and lipoteichoic acid with high affinity and neutralize their biological activities (Scott et al., 2000). This anti-inflammatory activity of cathelicidins is of particular interest for the treatment of sepsis. Without neutralization bacterial endotoxins may enter the bloodstream resulting in the production of high levels of systemic pro-inflammatory cytokines, such as TNFα, IL-6, IL-1β that lead to septic shock. The ovine-derived cathelicidin OaBac5mini suppressed production of the inflammatory cytokine interleukin-12 by murine J774A cells that had been stimulated with *Staphylococcus aureus* strain cowan (Yu et al., 2010). Thus, cathelicidins exhibit multiple functions, by exhibiting direct antimicrobial activity as well as modulating immune cell functions to protect the host against potentially harmful pathogens, while simultaneously protecting the organism against the detrimental effect of an excessive inflammatory response.

2.2.3. Other activities

Human cathelicidin LL-37 and porcine PR-39 both possess angiogenic properties and are involved in wound healing. LL-37 stimulates angiogenesis by direct activation of the FPRL-1 receptors expressed on endothelial cells, resulting in their proliferation (Koczulla et al., 2003). In the case of PR-39, its *in vitro* angiogenic properties and capacity to stimulate vascularization *in vivo* are possibly mediated by inhibition of ubiquitin-proteasome-dependent degradation of hypoxia-inducible factor-1α (Li et al., 2000). The wound healing properties of LL-37 were demonstrated by treatment of skin wounds with LL-37-specific antibodies. This treatment inhibited re-epithelialization of skin wounds in a concentration-dependent manner, suggesting that LL-37 may play a role in epithelial cell proliferation (Heilborn et al., 2003). Introduction of PR-39 into a wound induces the expression of the cell surface heparan sulfate proteoglycans syndecan-1 and -4 as part of the wound repair process (Gallo et al., 1994). Cathelicidins have also been observed to potentially increase the lifespan of immune cells. Porcine cathelicidin PR-39 has been shown to inhibit macrophage apoptosis (Ramanathan et al., 2004), while human LL-37 is able to suppress neutrophil apoptosis (Nagaoka et al., 2006) and to promote secondary necrosis of apoptotic neutrophils without being pro-inflammatory to phagocytosing macrophages (Li et al., 2009).

3. Avian cathelicidins: biology

Currently four avian cathelicidin-like peptides have been described from the chicken (*Gallus gallus*), *i.e.* cathelicidin-1/fowlicidin-1 (Lynn et al., 2004), Chicken myeloid antimicrobial peptide 27 (CMAP27)/fowlicidin-2 (van Dijk et al., 2005), fowlicidin-3 (Xiao et al., 2006a) and cathelicidin-B1 (Goitsuka et al., 2007), which have been designated CATH-1 to -3 and CATH-B1 for clarity (Table 1). Recently, three cathelicidins were characterized in ring-necked pheasant (*Phasianus colchicus*), *i.e.* Pc-CATH1, -2 and -3, that share a high level of identity with chicken cathelicidins-1, -2 and -3, respectively (Wang et al., 2011).

3.1. Genomic organization and evolution

Chicken cathelicidin genes are encoded by four exons, similar to those described for mammalian cathelicidins (Scocchi et al., 2009; Zanetti, 2005; Zhao et al., 2008), the first exon encoding the 5′UTR, signal peptide sequence and part of the cathelin domain, the second and third exon the major part of the cathelin domain and the fourth exon encoding the last few amino acid residues of the cathelin domain, the mature peptide sequence and 3′UTR. The four chicken cathelicidin genes are clustered on chromosome 2 within a 7.7 kb region at less than 3.5 Mb from the proximal end (Goitsuka et al., 2007; Xiao et al., 2006a) with

Table 1
Nomenclature and amino acid sequences of avian cathelicidins.

Designation	Sequence	Genbank	References
CATH-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	AAS99323	Lynn et al. (2004), Xiao et al. (2006a)
CATH-2a	LVQRGRFGRFLRKIRRFRPKVTITIQGSARF-NH_2	AJ393748	Xiao et al. (2006a)
CATH-2b	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	AY817057	van Dijk et al. (2005)
CATH-3	RVKRFWPLVPVAINTVAAGINLYKAIRRK	AAZ42401	Xiao et al. (2006a)
CATH-B1	PIRNWWIRIWEWLNGIRKRLRQRSPFYVRGHLNVTSTPQP	AB307733	Goitsuka et al. (2007)
PcCATH-1	RIKRFWPVVIRTVVAGYNLYRAIKKK	GU143407	Wang et al. (2011)
PcCATH-2	LVQRGRFGRFLSKIRRFRPKFTITIQGSGRFG	GU143408	Wang et al. (2011)
PcCATH-3	RIKRFWPLVPVAINTVAAGINLYKAIKRK	GU171372	Wang et al. (2011)

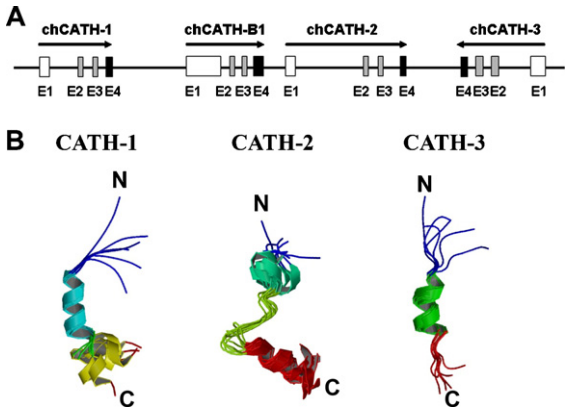

Fig. 1. Genomic organization and three-dimensional mature peptide structure of chicken cathelicidins. (A) Exon size and relative position in the genomic cluster are indicated with boxes: 5′ untranslated region and signal peptide sequence (E1, white); cathelin domain (E2 and E3, gray), mature peptide (E4, black). (B) Three-dimensional peptide structures as determined by NMR spectroscopy. RCSB Protein Data bank; http://www.rcsb.org/pdb/home/home.do; PDB structures 2AMN (Xiao et al., 2006b), 2GDL (Xiao et al., 2008) and 2HFR (Bommineni et al., 2007). N and C indicate N- and C-termini.

the CATH-1, -B1 and -2 genes oriented in the same direction and the CATH-3 gene positioned in a reverse orientation (Fig. 1). Comparative analysis divides mammalian, avian and fish cathelicidins in distinctly separated clusters (Goitsuka et al., 2007). Chicken cathelicidins show more sequence similarity to neutrophilic granule protein (NGP)-like cathelicidin, such as rabbit P15 and mouse, rat, pig and bovine NGP, than to other cathelicidins (Xiao et al., 2006a) and are suggested to share a common ancestor.

3.2. Tissue-specific gene expression

Pheasant CATH-1 mRNA is moderately to highly expressed in lung, heart, brain, testis and spleen with highest expression levels found in bone marrow and bursa of Fabricius and low levels in liver and thymus (Wang et al., 2011). Like Pc-CATH-1, the Pc-CATH-2 and -3 genes were cloned from a pheasant spleen cDNA library, but their expression levels in spleen and other tissues have not been investigated. Chicken CATH-1 and -2 mRNA are predominantly expressed in bone marrow (Lynn et al., 2004; van Dijk et al., 2005). Immunohistochemical staining showed abundant expression of CATH-2 protein in heterophilic granulocytes, but not in other blood cells (van Dijk et al., 2009b). Moderate to strong CATH-2 expression was observed in thymus and spleen (van Dijk et al., 2005). In the respiratory tract, low levels of CATH-1 and CATH-2 mRNA were detected in trachea with additional low expression of CATH-2 mRNA expression in lung tissue (Lynn et al., 2004; van Dijk et al., 2005). CATH-1 expression could not be detected in skin tissue and only weak CATH-2 expression was found in normal, intact, skin (Lynn et al., 2004; van Dijk et al., 2005). Similarly, cathelicidin expression in human and mouse skin is absent or low in normal skin, but induced upon injury and contributes to wound-repair (Dorschner et al., 2001; Heilborn et al., 2003). Interestingly, high CATH-2 mRNA expression was observed in uropygial (preen gland) tissue (van Dijk et al., 2005), a single large sebaceous gland located at the base of the tail in most birds. This gland secretes a waxy substance that is distributed onto skin and plumage during preening activity. In the digestive tract, the low CATH-2 expression detected in the proventriculus is probably linked to the proximity of the esophageal tonsil (van Dijk et al., 2005). Moderate expression levels of CATH-1 were found in gizzard, small and large intestine (Lynn et al., 2004), while its expression in liver, gall bladder and cloaca was low (Lynn et al., 2004). High CATH-2 mRNA levels were found in liver tissue (van Dijk et al., 2005) and apart from moderate CATH-2 expression in cecal tonsil tissue, low expression was found throughout the intestinal tract. High expression levels of CATH-1 and -2 were present in kidney, testis, and bursa of Fabricius (Lynn et al., 2004; van Dijk et al., 2005). These findings and the earlier observed high β-defensin expression levels in the urogenital tract (van Dijk et al., 2008) emphasize the importance of HDPs in protection of this region. The tissue distribution of CATH-3 has not yet been reported, but based on the similarities in expression patterns found for CATH-1 and -2, a predominant presence in myeloid and lymphoid cells and tissues is anticipated. Chicken CATH-B1 is exclusively expressed in the bursa of Fabricius, restricted to secretory epithelial cells that are in close proximity of M cells (Goitsuka et al., 2007).

In chickens, cathelicidin expression levels during embryonic development correlate with their phylogenetic relationship (Meade et al., 2009a). Relative to 3 days after laying, CATH-1, -2 and -3 expression levels increased 6- to 9-fold at day 6, declined at day 9, and were significantly increased at day 12 (CATH-1, 12- and 21-fold; CATH-2, 3-fold; CATH-3, 6- and 8-fold in head and abdomen, respectively), while CATH-B1 was not expressed until day 9 and like the other cathelicidins increased at day 12 (6- and 5-fold in head and abdomen, respectively). None of the cathelicidin genes showed preferential expression in the head or abdomen. The increasing levels of cathelicidin expression during embryonic development and the earlier reported elevated β-defensin expression levels during the first week after hatching (Bar-Shira and Friedman, 2006) suggest that in this life phase HDPs strongly contribute to host defense until adaptive immunity has matured.

3.3. Regulation of secretion

Biosynthesis of avian cathelicidins has only been studied for chicken cathelicidins-2 and -B1. Similar to many mammalian cathelicidins, CATH-2 is constitutively produced by bone marrow cells of the heterophil lineage. After biosynthesis is initiated at the early promyelocyte stage, the resulting precursor protein is sorted via the Golgi apparatus and stored into a subset of large rod-shaped granules (van Dijk et al., 2009b). Besides constitutive expression, cathelicidin expression may be induced in response to microbial infection or by pro-inflammatory stimulants (Frohm et al., 1997; Raqib et al., 2006; Wu et al., 2000). Oral challenge of 1-day-old broiler chicks with 10^4 CFU of *S. typhimurium* phage type 193 significantly increased cathelicidin expression levels at 3 days *p.i.*

(Akbari et al., 2008). A comparative *in vivo* study using 4-week-old Ross 308 broilers indicated differential expression of cathelicidin genes in response to *Salmonella* and *Campylobacter* infection. Peripheral blood leukocytes isolated at 6 h *p.i.* from *Campylobacter jejuni*-challenged animals exhibited significantly decreased CATH-2 and -3 mRNA expression levels, while expression levels of CATH-1, -2, -3 or -B1 were not altered by *S. typhimurium*-challenge (Meade et al., 2009b). Bacterial challenge of 4-day-old Ross 308 broilers with *Salmonella enteritidis* did not induce CATH-2 expression in intestinal tissues, but did result in recruitment of CATH-2 containing heterophils to the site of infection (van Dijk et al., 2009b). Immunosuppression of cathelicidin genes has also been reported for protozoan parasites, *e.g.* jejunal CATH-3 mRNA expression was significantly suppressed at 3 days *p.i.* in broilers infected with *Eimeria praecox* (Sumners et al., 2011).

It was shown that activation of cell surface receptors with LPS resulted in the extracellular release of CATH-2 via exocytosis and that released CATH-2 precursor was cleaved by simultaneously released metallo/serine protease(s) from a different subset of heterophil granules (van Dijk et al., 2009b). Serine proteases are known to be involved in activation of neutrophil-stored LL37 precursor (proteinase 3) and processing of bovine and horse neutrophil cathelicidins (neutrophil elastase) (Skerlavaj et al., 2001; Sørensen et al., 2001; Zanetti et al., 1990). Although avian heterophil elastase has yet to be discovered, putative elastase cleavage sites are present in CATH-1, -2, and -3. In the case of CATH-2 this may generate two products, a 31 amino acid peptide and a 27 amino acid truncated variant originally described as fowlicidin-2 and CMAP27, respectively. Based on mammalian cathelicidin processing, it is anticipated that, *in vivo*, the C-terminal glycine residue is most likely replaced by an amide group (Shinnar et al., 2003) thus generating a 30 amino acid and a 26 amino acid product, designated here as CATH-2a and CATH-2b, respectively.

CATH-B1 is the single avian cathelicidin of epithelial origin, being exclusively expressed by the bursal epithelium. The exact processing mechanism in which CATH-B1 precursor is converted into its mature form is unknown, however it is hypothesized that CATH-B1 precursor protein is secreted into the bursal lumen and after uptake via pinocytosis by neighboring M cells is cleaved to yield the bioactive mature peptide. Mature CATH-B1 peptide is found deposited on the fibrillar network surrounding the basolateral M cell surfaces covering the bursal lymphoid follicles and is thought to exert a protective antimicrobial role at the M cell gateway (Goitsuka et al., 2007).

4. Avian cathelicidins: structure–function relationships

Chicken CATH-1, -2, and -3 are all primarily α-helical peptides (Fig. 1). In NMR spectrometry studies CATH-1 was shown to be composed of two short and highly rigid helices (L^8-A^{15} and R^{21}-K^{25}) with a slight kink between G^{16} and Y^{20} (Xiao et al., 2006b). Similarly, the G^{17} residue in between both rigid helical regions of CATH-3 (V^9-A^{16} and N^{19}-R^{25}) allows peptide backbone flexibility (Bommineni et al., 2007). Instead of a glycine residue in its central region,

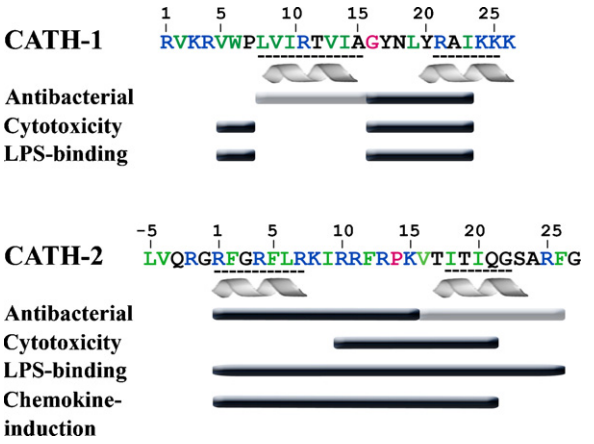

Fig. 2. Core elements involved in biological activities of chicken cathelicidins-1 and -2. Distribution of cationic (blue), hydrophobic (green) and hinge region glycin and proline residues (purple). Helical segments are underlined. Bars indicate the segments that are moderately (gray) or strongly (black) involved in each biological activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

CATH-2 contains a proline residue (P^{14}) that produces a more pronounced kink between its helical segments (R^1–R^7 and I^{18}-G^{22}) (Xiao et al., 2008). The structures of chicken CATH-B1 and pheasant cathelicidins have not been determined.

In the following sections the different biological activities of mature avian cathelicidins are described as well as the use of truncated and amino acid substituted peptides to examine the involvement of helical and central kink regions in their biological properties (Fig. 2).

4.1. Antimicrobial activity

In microdilution broth assays, broad antimicrobial activity against Gram-positive and Gram-negative bacteria, including multidrug-resistant strains of *S. typhimurium* DT104 and methicillin-resistant *S. aureus* (MRSA) were observed for pheasant CATH-1 (MIC < 3 μM) (Wang et al., 2011) and chicken CATH-1, -2 and -3 with MIC values ranging from 1 to 10 μM (Bommineni et al., 2007; van Dijk et al., 2009b; Wang et al., 2011; Xiao et al., 2006a). Chicken feces-derived *Salmonella* strains did not show enhanced resistance towards cathelicidin-mediated killing. Fungicidal activity against *Candida albicans* and *Saccharomyces cerevisae* was described for CATH-2b peptide (MIC values: 2.5–5 μM) (van Dijk et al., 2009b).

The truncated CATH-2b analogs corresponding to the highly cationic 15 amino acid long N-terminal segment, C2(C1–15), exhibited the most potent antibacterial activity (van Dijk et al., 2009a). This included killing of *Bacillus anthracis* and *Yersinia pestis*, microorganisms that potentially can be used as weapons in biological warfare (Molhoek et al., 2011). The activity could be further enhanced by substitution of Phe residues by Trp residues; a 50% reduction in survival of *Y. pestis* and *B. anthracis* was achieved with 2.5 and 1 μM C2(C1–15)F2W/F5W/F12W peptide compared to 20 and 4 μM C2(C1–15) peptide, respectively. Interestingly, *in vitro* exposure to peptide

C2(1–15)F2W/F5W/F12W prevented biofilm formation and impaired mature *Staphylococcus epidermidis* biofilms (Molhoek et al., 2011).

Killing kinetics studies showed a 32-fold decrease in *E. coli* ATCC 25922 survival at 2×MIC value of pc-CATH-1 peptide within 60 min (Wang et al., 2011), while chicken CATH-2b and its analog C2(1–15) reduced 10^6 CFU/ml *S. enteritidis* within 5 and 10 min, respectively, to below the detection limit (100 CFU/ml) (van Dijk et al., 2009a). Permeabilization studies using *E. coli* ML-35p suggest that membrane disruption was a major mechanism of bacterial killing by CATH-2a (Xiao et al., 2008).

Substitution of the central proline residue in CATH-2b by leucine, which linearizes the hinge region, abrogated antibacterial activity and killing kinetics, which indicates the importance of the hinge region for rapid penetration of the bacterial membrane (van Dijk et al., 2009a). In contrast, antibacterial activity of CATH-1 was mostly retained in the central helical region including the hinge region and was not affected by leucine-substitution of the central glycine residue. The C-terminal helical region and hinge region (G^{16}-L^{19}) alone proved to be critical for CATH-1 antibacterial activity against Gram-positive bacteria, but was less important for Gram-negative bacteria (Xiao et al., 2006b). Omission of the N-terminal tryptophan residue (W^6) reduced CATH-1 antibacterial efficacy (≥4-fold) (Bommineni et al., 2010) which is not surprising as tryptophan is known to facilitate membrane perturbation (Kang et al., 1999). The CATH-1 sequence contains a T^8-XXX-G^{12} motif that is reminiscent of sequence patterns that are important for the oligomerization of transmembrane helices in membranes (Walters and DeGrado, 2006). Indeed, peptide VK22, a 22 amino acid analog of CATH-1, was found to oligomerize into a predominantly tetrameric state in dodecylphosphocholine (DPC) micelles. In these micelles it adopted a barrel-shaped assembly of four helices arranged in an anti-parallel configuration with basic residues positioned at both termini. The tyrosine residues in all helical subunits interact mutually and create a central non-polar region that contains aromatic clusters and is located inside the lipid acyl chains (Saravanan and Bhattacharjya, 2011). Substitution of tyrosine by alanine resulted in loss of antibacterial activity and the capacity to self-associate in DPC micelles suggesting that tyrosine residues are necessary to stabilize the quaternary association of CATH-1 monomers in lipid micelles (Saravanan and Bhattacharjya, 2011).

The antibacterial activity of some HDPs may be greatly reduced at high ionic strength and also in serum by binding to serum proteins and proteolytic degradation (Maisetta et al., 2008). The antibacterial activity of pheasant CATH-1 and chicken CATH-1, -2 and -3 is not greatly affected by physiological salt concentrations (Bommineni et al., 2007; Wang et al., 2011; Xiao et al., 2006a), but C-terminal truncation of CATH-2b nearly completely abrogated its antibacterial activity in the presence of salt (Molhoek et al., 2010). After 72 h incubation in 90% human serum Pc-CATH-1 retained most of its antibacterial potency (Wang et al., 2011). Similarly, CATH-1 and -3 retained >80% of their activity against *E. coli* in the presence of 50% chicken or human serum (Bommineni et al., 2007) and killing of *S. aureus* by CATH-2a and C2(10–26) peptide was not affected by 50% human serum, whereas several C-terminal CATH-2a deletion analogs were inhibited (Xiao et al., 2008). The antibacterial activity of truncated CATH-2b analogs in 50% human serum was greatly improved by the use of cyclic or D-amino acid analogs, increasing stability up to 24 h (unpublished results).

4.2. Cytotoxicity

Pheasant CATH-1 peptide exhibited negligible cytotoxicity as determined by hemolytic activity (4% at 3 μM) against human erythrocytes (Wang et al., 2011). Fifty percent of human and chicken erythrocytes are lysed by ~6–10 μM CATH-1 and ~10–20 μM CATH-2, while at 10–20 μM, both peptides killed 50% of Madin-Darby canine kidney (MDCK) cells during 24 h exposure with similar effects for RAW264.7 macrophage cells (Xiao et al., 2006a). The two sites involved in CATH-1 cytotoxicity (Fig. 2) appear to work synergistically to lyse erythrocytes and MDCK cells. Lysis is primarily mediated by the hydrophobic C-terminal tail and to a lesser extent involves the N-terminal tryptophan residue (Xiao et al., 2006b). CATH-2 analogs lacking the C-terminal hydrophobic tail were much less or even non-toxic towards human erythrocytes and Caco-2 cells (van Dijk et al., 2009a; Xiao et al., 2008). Multiple substitutions of Phe by Trp residues in peptide C2(C1–15) strongly increased cytotoxicity to human PBMCs up to 75% cytotoxicity at 40 μM (Molhoek et al., 2010). Cyclic C2(1–15) peptide was non-toxic to human PBMCs, but its D-amino acid analog displayed ~35% toxicity at 40 μM. Likewise, cyclization of the C2(1–15)F2W/F5W/F12W peptide showed significantly reduced toxicity against human PBMCs (down to 16%), while toxicity was not altered for its D-amino acid analog (55% at 40 μM). Compared to CATH-1, CATH-3 displayed slightly more potent bactericidal activity, and was 4- to 6-fold less cytotoxic to human erythrocytes and MDCK cells (Bommineni et al., 2007). In the presence of 10% serum, 24 h exposure of human PBMCs to 20 μM CATH-2b analogs did not greatly affect cell survival (van Dijk et al., 2009a). The hemolytic activity of CATH-1 and -2a peptides against human and chicken erythrocytes was 2- to 5-fold reduced in the presence of 10% serum (Xiao et al., 2006a), and reduced CATH-3 hemolytic activity 9-fold (Bommineni et al., 2007).

4.3. LPS-binding

In *Limulus* amoebocyte lysate (LAL) assays 10–15 μM CATH-1 and -2a peptide completely inhibited LPS-induced (100 ng/ml) procoagulant activation (Xiao et al., 2006a). As has been observed for sheep cathelicidin SMAP29 (Tack et al., 2002), two high affinity LPS-binding core regions in CATH-1 (V^5-P^7 and G^{16}-I^{23}) are also involved in cytotoxicity (Xiao et al., 2006b). NMR experiments of CATH-1 analogs in LPS micelles show that the two short helical segments, L^8-A^{15} and N^{18}-K^{25}, align parallel to LPS with all aromatic residues, *i.e.* W^6, Y^{17} and Y^{20}, in close proximity to LPS (Bhunia et al., 2009). Not surprisingly, the tryptophan residue was found to be critically important for neutralization of LPS-induced production of NO and TNFα

(Bommineni et al., 2010). Like CATH-1, CATH-2 was shown to contain at least two cooperative LPS-binding sites, one high affinity binding site located in the N-terminal helical segment and a low affinity LPS binding site in the region containing the C-terminal helical segment with a cationic 4 amino acid hinge segment (R^{10}-F^{26}) (van Dijk et al., 2009a; Xiao et al., 2008). The importance of the hinge proline for immunomodulatory activities of the full-length CATH-2 peptide is emphasized by the markedly reduced LPS neutralization after substitution of the central proline by leucine (van Dijk et al., 2009a). LPS-induced expression (100 ng/ml) in RAW 264.7 cells of IL-1, MCP-1 and MIP-1α is 90% blocked by 20 μM CATH-1 and -2a peptide (Xiao et al., 2006a), while 10 μM CATH-1 or -3 block >95% of IL-1β and MIP-1α expression by RAW cells (Bommineni et al., 2007).

CATH-1 analog C1(6–26)-NH_2 fully retained its capacity to block LPS-induced TNFα production (95%) in RAW264.7 cells, while inhibition of NO production decreased by 30% (Bommineni et al., 2010). The LPS-induced production of TNFα, IL-6 and IL-10 in human PBMCs, but not of IL-8, was efficiently inhibited by CATH-2b (van Dijk et al., 2009a). The LPS-binding properties of peptide C2(1–15) were greatly improved by phenylalanine to tryptophan substitutions; 10 μM of C2(1–15)F2W/F5W/F12W peptide blocked 85% of LPS-induced IL-6 production by human PBMCs (Molhoek et al., 2010). Whereas cyclic analogs only retained LPS-neutralization activity, truncated D-amino acid CATH-2 analogs showed significantly improved neutralization of LPS-induced IL-6 production by human PBMCs (unpublished results). In the presence of 10% serum, most CATH-2 analogs nearly completely lost the capacity to neutralize LPS-induced NO production of RAW cells (Xiao et al., 2008).

Considering the LPS-binding properties of HDPs it is not surprising that pathogenic microorganisms have developed strategies to minimize their susceptibility to HDPs. For instance, the causative agent of fowl cholera, *Pasteurella multocida*, expresses LPS lacking a polymeric O side chain as a result of which it has its highly variable outer-core sugars well exposed. The complete LPS structure is required for full virulence of *P. multocida* in chickens (Harper et al., 2007). Challenge experiments with glycosyl transferase mutants of *P. multocida* have demonstrated that the heptose side chain is critical for *P. multocida* resistance against CATH-1 and other cationic peptides and its survival in chicken, while terminal phosphocholine residues in the LPS structure are necessary for maximal resistance against CATH-1 (Boyce et al., 2009). A different adaptation is used by *C. jejuni*, which reduces its susceptibility to cationic peptides, including CATH-1, by increasing the number of amide-bound acyl chains in its lipid A moiety (van Mourik et al., 2010).

4.4. Immunomodulation

Besides blocking LPS-induced cytokine expression, HDPs may directly stimulate the production of cytokines in immune cells. Stimulation for 24 h of human PBMCs with 15 μM CATH-2b or its analog C2(1–21) resulted in a significant induction of monocyte chemotactic protein 1 (MCP-1) production ($P < 0.01$) and did not induce production of TNFα, IL-6, IL-10 or IL-8 (van Dijk et al., 2009a). The capacity to induce MCP-1 was lost upon linearization of the hinge region by substitution of the central proline by leucine, demonstrating the importance of the hinge region for immunomodulatory as well as antibacterial activities (van Dijk et al., 2009a). Stimulation (24 h) of RAW264.7 cells with CATH-2a peptide had no effect on TNFα or NO production (Xiao et al., 2008), nor did 20 μM CATH-1 or -2a induce gene expression of IL-1β or MCP-1 in these cells (Xiao et al., 2006a). These findings indicate that peptide-mediated chemokine induction is cell type- and species-dependent.

5. Development of chicken cathelicidin-based anti-infectives

To date several HDP-related compounds are commercially developed and currently being tested in (pre)clinical trials (Hancock and Sahl, 2006). Bommineni et al. (2010) tested the *in vivo* efficacy of chicken CATH-1 derived peptides in protecting against lethal infection by MRSA. A lethal dose (10^7 CFU/mouse) of MRSA ATCC 33591 was administered with or without 10 mg/kg peptide CATH-1 analog C1(6–26)-NH_2 via intraperitoneal injection into neutropenic mice (Bommineni et al., 2010). This analog was chosen because of its potent *in vitro* serum stability, antibacterial and LPS-neutralizing activities. This single dose of peptide provided partial protection against MRSA, all infected control animals died within 36 h *p.i.*, whereas peptide-treated animals showed less severe symptoms, a delayed onset of disease and a 50% survival rate at 7 days *p.i.* Bacterial titers were significantly inhibited in the peritoneal cavity ($p < 0.01$) and positively correlated with a decrease in organ lesions. Similarly, subcutaneous administration of 10 mg/kg C1(6–26)-NH_2 peptide to mice resulted in a lower bacterial titer in the peritoneal cavity 7 days *p.i.*, suggesting that the peptide might work systemically.

5.1. Antimicrobial resistance development

Concerns have been raised that a widespread clinical use of HDPs would select for pathogens that are resistant to natural immune defenses. Indeed, many bacterial species already possess slightly effective resistance mechanisms. For instance, HDPs with simple linear or α-helical structures, *i.e.* α-helical cathelicidins, are susceptible to proteolysis and are cleaved by bacterial proteases, such as *Pseudomonas aeruginosa* elastase, and *S. aureus* aureolysin and V8 protease (Peschel and Sahl, 2006). A different bacterial resistance strategy is to prevent HDPs from reaching the bacterial cell membrane by secretion of exoproteins that bind to and inactivate cathelicidin peptides, *e.g.* streptococcal inhibitor of complement (SIC) from *S. pyogenes* (Frick et al., 2003), while others can actively extrude HDPs from their cell wall (Peschel and Sahl, 2006). The most widely adapted resistance strategy used by *S. aureus* sp. and many other bacterial species is modification of the bacterial cell membrane, for example by production of the cationic lipid lys-phosphatidylglycerol (lys-PG), leading to reduced affinity of cationic peptides to

the bacterial membrane (Peschel and Sahl, 2006). As Perron et al. (2006) have shown, resistance to HDPs can be selected in the laboratory, *i.e.* heritable resistance to the cationic HDP pexiganan evolved in *P. fluorescens* and *E. coli* after 600–700 generations. However, compared to the rapid microbial resistance development against conventional antibiotics, resistance development against HDPs is relatively inefficient. The "Achilles heel" of conventional antibiotics is their high specificity, *i.e.* they usually act via a single mechanism. A simple mutation in a particular target can occur within a few generations leading to increased resistance. In contrast, HDP-based antibiotics exert a more broad spectrum activity by attacking various extra- and intracellular targets. Furthermore, development of microbial resistance is not likely to occur against strictly immunomodulatory peptide antibiotics as it does not pose a selective pressure by which microbiota compete.

5.2. Stability of peptides

Peptide-based antibiotics possess in general unfavorable pharmacokinetics. Their relative short half-life is the result of their susceptibility to proteolytic degradation by bacterial and host proteases and the rapid renal excretion due to their small size. These shortcomings seriously limit their efficacy in non-topical applications, hence clinical trails of peptide therapeutics are generally focused on topical application. To increase peptide half-life, strategies can be designed in which the combination of formulations, routes of administration and chemical modifications are optimized. Typical modifications are N-terminal amidation (Bommineni et al., 2010), introduction of D-amino acids (Braunstein et al., 2004) or non-natural amino acids (Bikker et al., 2006; Chongsiriwatana et al., 2008) and cyclization (Monroc et al., 2006). Modifications may increase half-life from a few minutes to hours (Lin, 2009). It was shown that C-terminal amidation of the CATH-1 analog C1(6–26) increased the peptide stability in serum for at least 1 h (Bommineni et al., 2010) suggesting a prolonged half-life *in vivo*.

5.3. Production methods and costs

Compared to peptide production by organic synthesis, recombinant expression of peptides in yeast or by other cells is cheaper. However, potential difficulties involving cytotoxicity to cells and proteolytic degradation of non-structured peptides may occur. To reduce cytotoxicity, enhance product stability, and to facilitate product recovery and purification, HDPs can be expressed as fusion proteins in which the biologically active peptide is linked via a proteolytic cleavage site to a carrier protein that blocks activity of the peptide. A long-term strategy could be to incorporate HDP fusion proteins in plant expression systems, such as maize or potatoes, which can be directly supplemented to animal feed.

6. Perspectives

Numerous bacterial species, including important zoonotic species, such as *Salmonella* and *E. coli,* have demonstrated an ability to rapidly develop resistance to many antibiotics. Sadly, the abundant use of antibiotics in veterinary medicine has resulted in the creation of reservoirs of multidrug-resistant bacteria in farm animals. The increasing prevalence of resistant bacteria in farm animals and the possible transmission of this resistance to human pathogens is an important impetus to search for novel alternative antibiotics. Currently used antibiotics are derived from a very small group of molecular structures, and operate via an effective, but single, mechanism of action, such as inhibition of cell wall or protein synthesis. In addition, many of these compounds are relatively narrow-spectrum antibiotics, *i.e.* they are effective against a limited number of classes of microorganisms.

The multiple actions of HDPs have precluded the development of resistance despite the fact that these peptides are part of the natural host defense of animals for millions of years. Thus, cathelicidins use several mechanisms to kill bacteria, such as cell wall permeabilization, inhibition of DNA replication and protein synthesis. In addition, as has been discussed in this review, cathelicidins also exert immunomodulatory activities. These activities may be specific for the host, *i.e.* immunomodulation in chickens is most optimal if peptides are used that are derived from chicken cathelicidins.

Conflict of interest

The authors state that there is no conflict of interest.

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