Shedding light on antibacterial activities of cathelicidins

Viktoria Schneider

The research described in this thesis was performed at:
- the Department of Infectious Diseases & Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
- the Department of Biochemistry and Cell Biology, Center for Cell Imaging, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
- the Department of Cell Biology, Cell Microscopy Core, University Medical Center Utrecht, Utrecht, The Netherlands
- the Department of Medical Microbiology & Infection Control, VU University Medical Center, Amsterdam, The Netherlands

The research described in this thesis was financially supported by the ALTANT ASIA2 program (http://www.immunovalley.nl/altant/altant-asia-project/) of the Dutch Ministry of Economic Affairs.

Printing of this thesis was financially supported by the ALTANT ASIA2 program of the Dutch Ministry of Economic Affairs and the Infection & Immunity Program, Utrecht University (Graduate School of Life Sciences).

ISBN: 978-90-393-6526-7

Cover: Bergwerkskopf (Lechtal Alps, Austria) - Franz Preschern
Cover design, printing and lay-out: Guus Gijben - Proefschrift-AIO

Shedding light on antibacterial activities of cathelicidins

De activiteiten van cathelicidines aan het licht gebracht
(met een samenvatting in het Nederlands)

Die Aktivitäten von Cathelicidine an das Licht gebracht
(mit einer Zusammenfassung in deutscher Sprache)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 26 mei 2016 des middags te 12.45 uur

door

Viktoria Anna Frieda Schneider
geboren op 26 augustus 1986 te Rum/Tirol, Oostenrijk

Promotor: Prof. dr. H.P. Haagsman

Copromotor: Dr. E.J.A. Veldhuizen

CONTENTS

Chapter 1

GENERAL INTRODUCTION

CONVENTIONAL ANTIBIOTICS

The development of the first antimicrobial agents changed the course of infectious diseases in the 1940s. Antibiotics such as sulfonamide, penicillin, gramicidin and streptomycin initiated the beginning of a new period where bacterial pathogens could efficiently been targeted, and bacterial infections seemed to be under control[1,2]. However, this golden antibiotic era did not last very long since antibiotic resistant bacteria were discovered. Since the 1970s the number of antibiotic resistant infections is consistently increasing and antibiotic resistance has developed to a medical super-challenge with an enormous impact on public and veterinary health[2].

Resistant bacteria evolved due to mutations or acquisition of resistant genes from already resistant pathogens. This transfer of resistant material occurs via transformation, conjugation or transduction. Resistant bacteria can cause ineffectiveness of an antibiotic by using efflux pumps in order to pump out the antibiotic, changing its target sites or using a different metabolic pathway circumventing antibiotic binding, entrance or attack.

Over the years the selective pressure of antibiotics has caused the evolution of multi-drug resistant (MDR) bacteria. These so-called superbugs have developed several resistance mechanisms and are resistant against ≥ 3 antibiotic classes[3]. One example of an MDR pathogen is *Staphylococcus aureus*, which is the most common cause for community- and hospital-associated bacterial infections. *S. aureus* developed resistance against for example vancomycin, rifampin, fluoroquinoles and in fact all β-lactam antibiotics (penicillin, methicillin)[3,4]. In a recent report from the World Health Organization (WHO) several antibiotic failures are described, due to the resistance development of bacteria against their specific targeting antibiotics. For example Gonorrhea, a sexually transmitted pathogen, evolved to be resistant against third generation cephalosporin. Urinary tract infections caused by *Escherichia coli* have been treated with fluoroquinoles, however resistance developed against this extremely important antibiotic[5]. Also resistance against carbapenems has been detected. Carbapenems have been known to be the last resource in fighting resistant Gram-negative bacteria like extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. Nevertheless, the numbers of carbapenem-resistant *Enterobacteriaceae* (CRE) are increasing as bacteria started producing β-lactamases targeting and destroying carbapenems[4,6].

The conventional antibiotics exhibit different modes of action including interference with the bacterial cell wall synthesis, protein or nucleic acid synthesis or important metabolic pathways[3].

Antibiotics such as the β-lactams (penicillin, cephalosporin and monobactams) and glycopeptides (vancomycin and teicoplanin) inhibit the cell wall synthesis of

bacteria. β-lactam antibiotics specifically interfere with the enzymes necessary for the peptidoglycan layer synthesis, whereas glycopeptides affect the crosslinking of the peptidoglycan layer, which plays an important role for a stable cell wall synthesis[3,7,8].

Inhibition of protein synthesis is performed by macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins, and oxazolidinones. These antibacterial agents specifically target bacterial ribosomes, which are structurally different from eukaryotic ribosomes. The 30S ribosomal subunit is targeted by macrolides, aminoglycosides and tetracycline and the 50S subunit of ribosomes is bound by chloramphenicol[3,7,8].

Inhibition of DNA synthesis is exhibited by fluoroquinolones, which break the DNA synthesis leading to lethal double strand DNA replication[9,10].

The inhibition of the metabolic pathways has been described for sulfonamides and trimethoprim. These compounds inhibit DNA synthesis due to blocking of folic acid synthesis[3,11]. Over the years, bacterial membrane breakage has been described as an additional bactericidal mode of action, which is exhibited by polymyxin and the lipopeptide daptomycin. Membrane permeability induced by polymyxin leads to leakage of bacterial components, whereas the cyclic lipopeptide daptomycin triggers depolarization of the membranes due to the insertion of its lipid tail[12,13].

An overview of antibiotics and their bactericidal mode of actions is presented in Table 1.

Mode of action	Antibiotics
Inhibition of cell wall synthesis	β-lactams
	Glycopeptides
Inhibition of protein synthesis	Macrolides
	Aminoglycosides
	Tetracyclines
	Chloramphenicol
	Streptogramins
	Oxazolidinone
Inhibition of DNA synthesis	Fluoroquinolones
Inhibition of metabolic pathways	Sulfonamides
	Trimethoprim
Disruption of bacterial membrane	Polymyxin
	Daptomycin

Table 1. Overview of the described mode of action of conventional antibiotics.

Daptomycin, linezolid and avibactam are the only approved antibiotics since the beginning of the 21st century. Linezolid and daptomycin are used for the treatment of Gram-positive infections, whereas avibactam in combination with ceftazidime target Gram-negative bacteria[14]. However, it is only a question of time when bacteria develop resistance against these antimicrobial agents.

Currently used antibiotics mainly bind a single bacterial target and exhibit often only one mode of action, which contributed to target-specific resistance development of bacteria. New multi-targeting antimicrobials are needed to combat bacteria[15]. However, developing and discovering new broad-spectrum antimicrobials is challenging as these compounds need to fulfill several criteria including i.e. the absence of toxic effects against host cells, the ability to kill multiple pathogens and hinder resistance development by binding to multiple bacterial targets[16].

Unfortunately, industrial pharmaceutical companies have lost interest in putting effort and money in the development and research of new antimicrobials. Developing antibiotics is a laborious business as it takes up to 15-20 years until a new antibiotic reaches the market. Developing new antimicrobial agents is expensive and their efficacy against their target pathogens is usually only short-lived[17]. Once an antibiotic is approved, it will only be used occasionally (to prevent resistance development) as a last resource if conventional antimicrobials cannot handle the infection. Due to this, new antimicrobials will only be sold in low numbers, making it even less lucrative for companies to work in this research field.

Slowing down or even eradicating the development of antibiotic resistance should be another important goal for both the human and veterinary sector. Over-usage of antibiotics, often even without prescriptions, has played an important role in the emergence of resistant bacteria in human as well as in the livestock sector[18,19]. In the Netherlands the numbers of antibiotic usage for humans are the lowest in Europe, however in veterinary use of antibiotics, the Netherlands actually scored almost highest in Europe[20,21]. During the last years different regulations were introduced to diminish the use of antibiotics also in the animal sector. Better hygiene and the registered use of antibiotic are required to contribute to a moderate use of antibiotics. Furthermore, it is prohibited to use important human antibacterial drugs in animals[22].

Frequently, antibiotics have been used as growth promoters for various animals including chicken, cattle and pigs, which stimulated the development of resistant bacteria even more. Since 2006 the use of antibiotics as growth promoters is prohibited in the EU[23,24]. Animal pathogens can be transmitted to man, as this was already demonstrated with food-borne pathogens (*E. coli*, *Salmonella* and *Campylobacter*), which cause severe diarrhea, fever and abdominal cramps in humans[25-27]. Nowadays also the transfer of ESBL-producing bacteria from chicken to human has been confirmed. Genotyping

in chickens and infected humans indicated that the chicken ESBL reservoir may significantly contribute to the levels detected in human[28,29]. Furthermore, a correlation between against methicillin-resistant *Staphylococcus aureus* (MRSA) genotypes in pigs and human has been observed. Especially farmers, who are in close contact with animals, and their family members are at risk of becoming infected with these live-stock derived MRSA strains[30]. Based on these findings, eradicating antibiotic resistant pathogens in animals would definitely have a positive effect on human health as well.

HOST DEFENSE PEPTIDES

New antibacterial drugs are urgently needed to solve the problem of antimicrobial resistance. One attractive alternative to the conventional antibiotics, are host defense peptides (HDPs)[31]. These peptides have been around for millions of years, and resistance against them seems to be absent[32].

HDPs are natural products of vertebrates and play an essential role in the first line of defense against invading pathogens like bacteria, fungi, protozoa and viruses. Once a microorganism enters the host, HDPs are rapidly acting by exhibiting immunomodulatory and/or broad-spectrum antimicrobial activities[31,33,34]. These innate defense peptides are small consisting of less than 100 amino acids and are amphipathic containing hydrophilic and hydrophobic groups at opposite sides of the molecules. HDPs are cationic as they are rich in lysines and arginines and are therefore expected to perfectly interact with the negatively charged membranes of for example bacteria[35]. HDPs are antimicrobials and can directly eradicate bacteria via several mechanisms including cell wall permeabilization, or inhibition of DNA or protein synthesis[36,37]. Besides their direct killing activity, these peptides are also involved in the innate immune response towards infection regulating cell signaling, chemo- and cytokine production, phagocytosis, chemotaxis, cell differentiation, and wound healing[38-40].

Two main classes of HDPs have been identified: defensins and cathelicidins, which are both produced as prepropeptides and have to undergo post-translational modification to form the mature peptides. Although these two HDP classes differ structurally, functionally their activities seem to have a large overlap[33,38,41-43].

Defensins are cysteine-rich peptides with a common β-sheet core stabilized by three disulfide bridges. Defensins were first identified in bovine epithelial cells and are divided in α-, β- and θ-defensins, which vary in their presence in vertebrates[42-44]. α-defensins have been only identified in certain mammals (e.g. human neutrophil peptides, mouse cryptdins), the cyclic θ-defensins have been found in rhesus monkeys and baboons (e.g. rhesus θ-defensin-1), whereas the

β-defensins have been detected in all vertebrates including birds (e.g. human, porcine or avian β-defensins)[43,45].

Cathelicidins consist of a highly conserved N-terminal signal peptide, a cathelin domain and a C-terminal antimicrobial peptide domain. The C-terminal peptide part is variable in sequence, amino acid length and function[38]. These prepropeptides are stored in granules of immune and epithelial cells. Upon liberation, the bioactive peptide is cleaved off by simultaneously released serine proteases. This process has been shown to happen in neutrophils of humans and in chicken heterophils[38,46,47].

Depending on the structure of the active C-terminal peptide, cathelicidins are classified in three groups: α-helical peptides (23-37 residues lacking cysteines), β-hairpin peptides (12-18 residues including cysteines), and peptides rich in tryptophan or proline residues (39-80 residues without cysteines)[38]. Most cathelicidins belong to the α-helical group, examples of these are sheep SMAP-29, bovine BMAP-27, porcine PMAP-23, -36 and -37 and all four avian cathelicidins. These peptides target negatively charged membranes/molecules like lipopolysaccharide (LPS), lipoteichoic acid (LTA) and interact with the hydrophobic lipid domains in bacterial membranes[33,48]. The second class of cathelicidins has β-hairpins stabilized by disulfide bonds. Peptides with this conformation are for example porcine protegrins and bovine dodecapeptide. Instead of having cysteines the third group of the cathelicidin family contains high numbers of certain amino acids like tryptophan and/or proline. Sheep OaBacs, porcine PR-39, prophenin-1/-2 and bovine Bac5 and Bac7 are proline-rich peptides, whereas bovine indolicidin and water buffalo cathelicidin belong to the tryptophan-rich group[38].

From a production point of view cathelicidins are usually (chemically) synthesized easier than defensins or other disulfide-bridge containing peptides. This might be one reason why linear cathelicidins are more readily considered for application purposes than structurally more complex peptides.

Some examples of three dimensional structures of defensins and cathelicidins are illustrated in Fig. 1.

Antibacterial actions

HDPs have strong bactericidal activity against Gram-positive and Gram-negative pathogens. The bacterial membrane has been shown to play a key role in the antibacterial mode of action of several HDPs. The membrane composition of bacterial cells is different compared to mammalian cells. Mammalian cell membranes have mainly zwitterionic phospholipids on the outer leaflet of the membrane with phosphatidylcholine and sphingomyelin. Bacterial membranes, however, are more negatively charged containing anionic phospholipids (e.g. phosphatidylglycerol and cardiolipin), lipopolysaccharides (LPS; in Gram-negative bacteria) or teichoic acids (in Gram-positive bacteria)[36,49]. These negatively

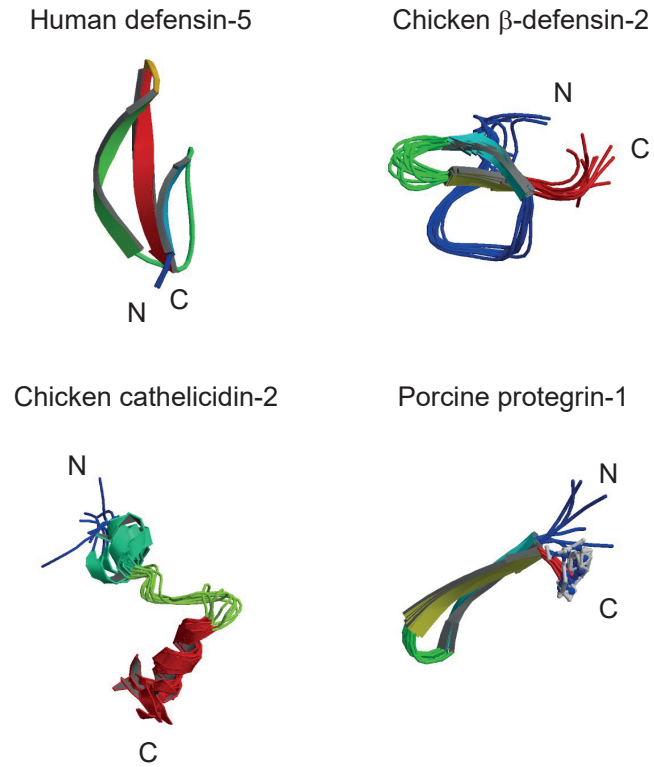

Figure 1. Structural organization of defensins and cathelicidins. Three-dimensional structures of human defensin-5 (α-defensin), chicken β-defensin-2 (β-defensin, AvBD2), chicken cathelicidin-2 (α-helical cathelicidin) and porcine protegrin-1 (β-hairpin cathelicidin) determined by NMR, were obtained from the RCSB Protein Data bank. N and C indicate the N- and C-terminus of the peptide, respectively.

charged components initiate an electrostatic interaction with cationic peptides which explains why AMPs have an initial preference for bacterial membranes. Besides charge and the cell surface, also the amino acid composition and the secondary structure of the peptides are essential for the interaction of the peptide with the membranes[36,49-52].

In the case of Gram-negative bacteria, antimicrobial peptides strongly interact with LPS and are suggested to pass this outer leaflet due to a self-promoted uptake mechanism. During the LPS binding the bulky peptide replaces membrane-stabilizing divalent cations Ca^{2+} and Mg^{2+}. As a consequence the membrane structure is disturbed and becomes permeabilized, which facilitates the uptake of the peptide and/or other molecules (e.g. proteins) across the outer

membrane[35,53]. This mechanism was first described by the group of Hancock and has been demonstrated for many antimicrobial peptides involving bovine indolicidin, cecropin mellitin hybrids and gramicidin S[54-56].

Once HDPs pass the outer membrane, they bind to the cytoplasmic lipid layer and cause permeabilization of the membrane, ultimately leading to leakage of internal cell content and bacterial cell killing. HDPs act in a non-receptor mediated way and four main models of membrane permeabilization have been proposed: aggregate, barrel-stave, toroidal and carpet model. An overview of the different mode of actions of peptides is illustrated in Fig. 2.

Peptides, which act according to the aggregate model, span the membrane as an aggregate consisting of peptide-lipid-micelle complexes without adopting any specific orientation[35,57]. The formed transient channels have no formal structure and differ significantly from each other in size and life-time, which ultimately leads to negative curvature strain and intracellular translocation of the peptide (based on an additional aggregate collapse)[35,57,58]. One peptide acting according to this membrane disrupting fashion is the horseshoe crab peptide polyphemusin[58] (Fig. 2A).

The second pore forming mechanism described is the toroidal pore model, which is relatively similar to the aggregate model[57]. After parallel membrane binding of the peptide to the membrane, the peptide distorts the alignment of the polar head groups of the lipids. This results in perturbation of the acyl chain interactions of the lipids, changes in membrane curvature and destabilization of membrane surface integrity. At certain peptide to lipid ratios, the peptides orient perpendicular to the membrane and induce the formation of transient toroidal channels. Peptides may subsequently translocate to the cytoplasm and attack intracellular targets[36,59]. LL-37, protegrin-1, mellitin and magainin 2 have been demonstrated to use this pore forming mechanism by bending the membrane inwards[60-63] (Fig. 2B).

The barrel-stave model was the first permeabilization mechanism proposed and has been considered to be the prototype in peptide-mediated transmembrane pore formation[64,65]. In this model peptides act as staves and vertically insert into the lipid bilayer forming barrels. Monomeric peptides, initially binding to the membranes, oligomerize and form bundles, leading to the formation of transmembrane pores. Hydrophobic groups from α-helical and/or β-sheet structures are essential during this process as these groups face the membrane lipids whereas the hydrophilic regions face the lumen of the channel[35,36,66]. One well-described peptide using the barrel-stave configuration is the cyclic peptide alamethicin[67] (Fig. 2C).

Another mechanism inducing membrane permeabilization is the carpet model. Peptides acting in such a fashion have been shown to cover the negatively charged membrane based on electrostatic attraction. After a certain peptide threshold concentration is reached, the membrane ruptures in a detergent-like

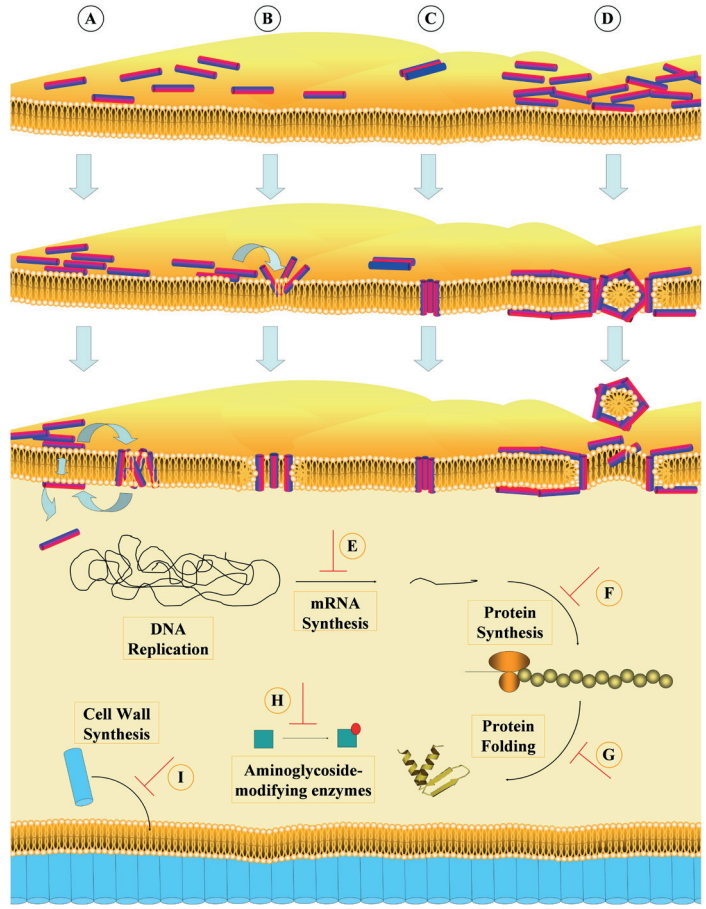

Figure 2. The four proposed mechanisms of membrane disruption are illustrated. Antimicrobial peptides (cylinders) with the hydrophilic regions (red) and hydrophobic regions (blue). Aggregate model (A), toroidal pore model (B), barrel-stave model (C) and carpet model (D). Peptides also can exhibit non-lytic killing mechanisms by translocating across the membrane and affecting cellular important processes including inhibition of nucleic acid synthesis (E), protein synthesis (F), protein folding (G), modification of aminoglycosides (H) and/or cell wall synthesis (I). Permission for using this figure was kindly granted by Dr. Håvard Jenssen and the American Society of Microbiology (Clinical Microbiology Reviews).

manner resulting in micelle formation of peptide with membrane lipids[35,36,48]. Compared to the toroidal and barrel-stave model, the carpet model is not based on the recognition of membrane bound peptide monomers, insertion into the membrane inducing pore formation or a certain peptide structure[48].

Due to this a broad range of peptides fulfill these criteria (e.g. aurein 1.2 and PMAP-23) and act based on a carpet-model-attack[68,69]. Peptides underlying a carpet pore forming mechanism have been shown to continuously bind the head group of phospholipids. Therefore it is suggested that the peptides induce transient channel formation in the membrane, which is comparable to a toroidal mechanism and indicates that the carpet model seems to be an extensive version of the toroidal model[36,48] (Fig. 2D).

Besides targeting the membrane, peptides can also cross the membrane and kill bacteria by binding specific (intracellular) targets[70]. This can lead to inhibition of nucleic acids synthesis (Fig. 2E), protein synthesis (Fig. 2F), protein folding (Fig. 2G), modification of aminoglycosides (Fig. 2H), and/or cell wall synthesis (Fig. 2I) depending on the specific targets bound by the peptide[35].

Peptidoglycan is an important component of the bacterial cell wall, which is only present in prokaryotic cells[51]. Several peptides have been described to inhibit the synthesis of peptidoglycan. Also interaction with the precursor of peptidoglycan, lipid-II, was shown to be a possible killing target for antimicrobial peptides like nisin[71,72].

Other peptides were shown to penetrate bacterial membranes in the absence of pore formation. For instance buforin II, a frog antimicrobial peptide, was demonstrated to bind DNA and RNA of *E. coli*[73]. Inhibition of protein folding was observed for the proline-rich peptides drosocin, apidaecin and pyrrhocidin, derived from insects. These peptides were initially thought to bind heat shock protein DnaK, resulting in production of misfolded proteins and subsequent cell death[74]. However, recent studies demonstrated that oncocin and apidaecin (derived peptides) bind 70S ribosome and less efficiently to DnaK[75].

In addition, dual modes of action for antimicrobial peptides have been investigated combining membrane permeabilization and intracellular targeting. This was first described for the bovine Bac7 peptide[76] and the results suggested that peptides not only exhibit one killing strategy but, instead, display several modes of action often dependent on the actual concentration of peptide.

Immunomodulatory actions

Since the identification of cathelicidins, most studies were aimed to study direct killing activities of these peptides. However, in recent years it was found that HDPs are multifunctional and are not only involved in the protection of the host against infections by targeting bacteria directly and/or inhibiting biofilm formation, but also have effects on host cells via several mechanisms, referred to as immunomodulation. In addition, these peptides promote wound healing and angiogenesis[40,77]. The best studied HDPs with respect to these latter activities are LL-37 (human), CRAMP (murine), BMAP-28 (bovine) and PR-39 (porcine)[78]. During an infection cathelicidins act as potent chemoattractants and directly recruit different cells like neutrophils, monocytes, and T-cells to the

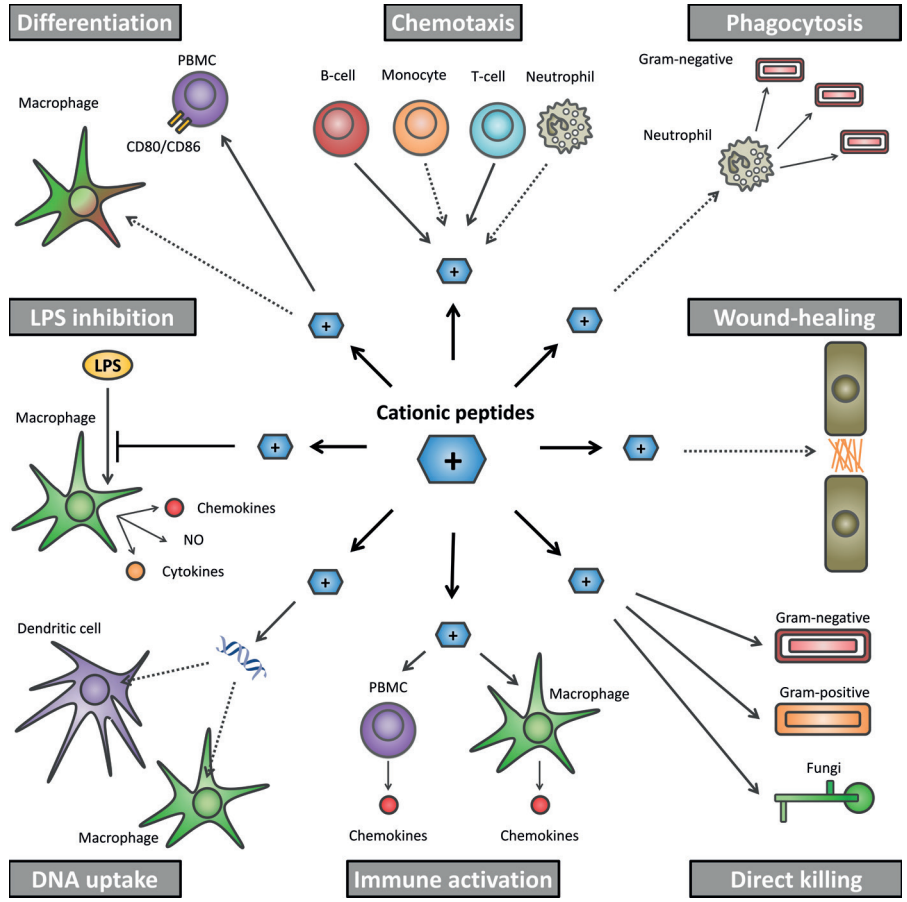

Figure 3. The functions of host defense peptides. Each peptide exhibits at least one task illustrated in the graph. Full arrows indicate functions found for avian HDPs, whereas dotted arrows represent the functions for HDPs from other species, however not yet described for avian species. Permission for using this figure was kindly granted by Tryntsje Cuperus MSc and Maarten Coorens MSc and Elsevier (Developmental and Comparative Immunology).

site of the infection[79,80]. Another route cathelicidins use to enhance chemotaxis is by triggering the secretion of chemokines (e.g. monocyte chemotactic protein-1 [MCP-1] and IL-8) from immune cells (mononuclear phagocytes) and epithelial cells. In addition, HDPs were demonstrated to enhance phagocytosis by dendritic cells, macrophages and neutrophils, which can contribute to a successful elimination of pathogens[40,78]. HDPs are involved in the activation and

differentiation of dendritic cells and macrophages and thereby provide a link to the adaptive immune response[81-83].

Besides direct effects on host cells, HDPs can also modulate immune activation through interaction with pathogen-associated molecular patterns (PAMPs). Invading pathogens possess different components, like for example LPS, which are recognized as molecular patterns by pattern recognition receptors (PRRs). Toll-like receptors (TLRs), which are PRRs, are expressed on innate immune cells and become activated after binding these inflammatory molecules[84]. Due to this activation, phagocytosis is increased and pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) are secreted. HDPs were also shown to stimulate the enhanced DNA uptake by macrophages, plasmacytoid dendritic cells (pDCs) and other mammalian cells[85-88]. LL-37, the best studied cathelicidin, modulates the pro- and anti-inflammatory responses during an inflammation. LL-37 specifically inhibits pro-inflammatory cytokine production (TNF-α and IL-1β) and enhances anti-inflammatory responses (IL-10)[89,90]. *In vivo* studies confirmed these *in vitro* findings, showing that knock out mice (Cathelicidin deficient male mice, Camp$^{-/-}$) have an enhanced inflammatory response compared to wild-type mice. In contrast, in the presence of cathelicidins a chronic allergic inflammation was suppressed, suggesting an anti-inflammatory role of the peptides[91]. The multifunctional effects of cathelicidins (highlighted in Fig. 3) may help to design peptide-based anti-infectives that do not elicit microbial resistance.

Chicken cathelicidin: CATH-2

In the 1980s cathelicidins were described first in bovine neutrophils and over the years these peptides were identified in various cell types and species including humans, horses, cattle, pigs, frogs, mice and chickens. In chicken more than 25 different types of β-defensins have been identified but only four cathelicidin-like peptides[92-94]. These four cathelicidins are termed chicken cathelicidin-1 (CATH-1, fowlicidin-1), chicken cathelicidin-2 (CATH-2, fowlidicin-2, chicken myeloid antimicrobial peptide 27 [CMAP-27]), chicken cathelicidin-3 (CATH-3, fowlidicin-3) and chicken cathelicidin-B1 (CATH-B1). All four peptides have a α-helical structure and are highly positively charged[94-97].

Localization of CATH-1, -2 and -3 were identified in high levels in bone marrow, lungs, cecal tonsils and bursa[95,98]. Protein expression of CATH-2 was determined to be exclusive for heterophils, the avian counterpart of mammalian neutrophils. CATH-B1, a rather elusive peptide, was shown to be expressed only in M-cells in the bursa of Fabricius, indicating a local protection function of this cathelicidin[97].

To date CATH-1, -2 and -3 are best studied. CATH-1 and -3 are highly similar in their primary and secondary structure, containing two α-helical parts linked through a glycine slight kink in the center and a flexible N-terminal fragment[99-101].

CATH-2 is a 26 amino acid long peptide. It has a prominent proline induced kink, which separates the α-helical segments[102]. Furthermore, CATH-2 is highly positively charged due to 9 cationic residues in its structure and also contains a flexible N-terminal end[103]. CATH-2 has shown to possess direct broad-spectrum killing and immunomodulatory features. Since its identification, the susceptibility of many pathogens was tested to this promising peptide antibiotic candidate. CATH-2 was demonstrated to kill bacterial pathogens, including ESBL-producing *Enterobacteriaceae*, MRSA, *Campylobacter* and different *Salmonella* strains, at micromolar level ranging from 1-10 μM[46,104,105]. Time course experiments have shown that this peptide kills pathogens rapidly, within 10-15 minutes[103]. Additionally, resistance experiments demonstrated that bacteria did not lose their sensitivity against CATH-2 and no major resistance mechanism occurred during ten-day induction experiments against *Klebsiella pneumoniae* and *S. aureus*[104]. The antibacterial mode of action of CATH-2 has not been investigated yet. However, its α-helical shape is suggested to be important in order to kill bacteria, like it has been demonstrated for cecropin-magainin hybrid peptides for instance[106]. The importance of the proline-rich hinge region (at position 14) for the antibacterial activity was highlighted in a study using different derivatives of CATH-2 and replacing proline with glycine or leucine. Glycine substituted peptides had slightly reduced activities compared to CATH-2, indicating that the flexibility provided by glycine likely still enabled kink-formation of the peptide. However the introduction of leucine for proline dramatically diminished antibacterial activity[103]. These studies show that the kink in the center of the CATH-2 peptide plays a pivotal role and is suggested to be involved in the peptide insertion in the bacterial membrane.

Truncated versions of CATH-2 have also been tested in another study, showing that the N-terminal C1-15 peptide exhibits similar antimicrobial activities compared to the full-length (1-26) CATH-2 peptide in phosphate buffer. However this shortened peptide was more susceptible to inhibition by physiological salt concentrations[103]. A replacement of phenylalanine residues with tryptophan (highly hydrophobic) even strengthened the antimicrobial activity of this shortened CATH-2 forms[107]. Additionally, it was shown that compared to N-terminal analogs, N-terminal truncations (losing important cationic amino acids) greatly weakened the bactericidal activity of the peptide[102,103].

CATH-2 was also reported to have several immunomodulatory functions[85,102,103]. Similar to LL-37, CATH-2 was shown to have LPS binding and neutralization activity[94,103,108-110]. In the mouse macrophage cell line RAW264.8 it was demonstrated that CATH-2 blocks the LPS induced release of cytokines (TNF-α, IL-1β, MCP-1) and NO[94,102]. Reducing the C-terminus or the N-terminus diminished the LPS inhibition activity of the peptide. However, if large parts of the N-terminus are retained, the LPS inhibitory activity is affected less[102,103].

Similar to truncated CATH-2 analogs, substitution of the proline position by leucine strongly influenced some of the peptide's activity, e.g. LPS neutralization, while MCP-1 production by PBMCs was not affected[103]. Recently, it was demonstrated that CATH-2 plays a key role in the DNA-induced macrophage activation. Due to the enhanced endocytosis of DNA-CATH-2 complexes, the DNA-induced mammalian and avian macrophage activation is triggered. During endocytosis CATH-2 is released from the complex and degraded resulting in TLR21 activation (or TLR9 in mammalian cells). In turn activated TLR21 induced cytokine expression and NO production[85].

Porcine cathelicidin: PR-39

In pigs, approximately 30 different HDPs have been identified, including 11 cathelicidins: three proline-rich peptides (proline-arginine-rich 39 amino acid peptide [PR-39], proline-phenylalanine-rich prophenin-1 [PF-1] and PF-2, five cysteine-rich protegrin like-peptides (PG-1 to PG-5), and three porcine myeloid antimicrobial peptides (PMAP-23, PMAP-36 and PMAP-37)[111].

Almost half of the amino acids of PR-39 are prolines and 24% consists of arginines[112]. Initially this proline-rich peptide was detected in the small intestines in pigs. Further research based on cDNA cloning has shown that PR-39 is expressed in the bone marrow and in porcine neutrophils[113,114]. PR-39 is stored as a 173-aa precursor and after enzymatic cleavage the mature biological active peptide (39 C-terminal amino acids) is released[112].

PR-39 has been shown to be highly multifunctional in its activity by covering almost all areas cathelicidins are known to be active in[115]. PR-39 is involved in angiogenesis, stimulates wound healing and inhibits inflammation[116,117]. Furthermore, PR-39 exhibits Ca^{2+}-dependent chemotactic effects on neutrophils and modulates the viability of macrophages in order to inhibit apoptosis[118,119]. Similar to other proline-rich peptides, PR-39 has been shown to be mainly active against Gram-negative bacteria (*E. coli* and *Salmonella typhimurium*). However, a few studies also demonstrated antibacterial activity of PR-39 against Gram-positive bacteria including Bacilli and Streptococci[120].

Interestingly, D-forms of the peptide showed altered or no antibacterial activity, although *S. aureus*, which was shown to be insensitive against L-PR-39, was killed[121]. With respect to the antibacterial mode of action of PR-39 it is reported that the peptide blocks protein and DNA synthesis, showing a non-lytic mode of action, by penetrating the cells without forming pores or membrane disruptions[122]. Truncations of PR-39 to 1-15 and 1-26 have shown that the N-terminal part is essential for the bactericidal activity of PR-39[123,124].

TECHNIQUES TO DETERMINE THE MECHANISM OF ACTION OF ANTIMICROBIAL HOST DEFENSE PEPTIDES

A vast set-up of different techniques has been developed to study the mechanism of action of HDPs. These techniques differ from each other, as each method focuses on different aspects of peptide activity[36]. The combination of available methodological tools is important to obtain a clear picture on the mode of action of HDPs. In this thesis various methods were used, which will be described including their advantages and drawbacks in the section below.

Assays to determine bacterial binding targets

Competition assays

The antibacterial activity of a peptide is usually tested by counting the colony forming units (CFU) after a peptide incubation period, or using broth dilution assays[125]. Competition assays (antagonization assays) are in principle similar to these tests. The difference is that the peptide is pre-incubated with the test compound (potential bacterial ligand) and, subsequently, the mixtures are exposed to bacterial cells. If binding between peptide and ligand occurs, it can antagonize the peptides killing activity resulting in higher observed minimal inhibitory concentration (MIC) values.

Nowadays various bacterial components are commercially available and can be tested in different ratios with the HDPs to obtain first insights on peptide binding to bacterial ligands. Another advantage using these assays is that they are easy to perform and rather cheap. A disadvantage of the method is that it is hard to determine which incubation time is required to obtain ligand-compound binding. An incubation time that is too long or too short can affect the outcome. Furthermore, it is an *in vitro* study and it is executed at controlled laboratory conditions. Another limitation of this method is that testing a single compound may not resemble peptide binding to whole bacteria, as on live cells the peptide may bind differently by having stronger binding preferences to other components.

Isothermal titration calorimetry (ITC)

Another method for detecting binding of peptides to bacterial components is based on isothermal titration calorimetry (ITC). With this method peptides are titrated into a cell chamber that contains the tested compound (usually 20 to 100 µM). The calorimeter determines the heat production when the peptide binds a second compound. From the value and pattern of heat production several variables can be determined such as association constant, enthalpy and entropy of the binding and the stoichiometry between the binding compounds[126]. This method offers several advantages. ITC studies are not restricted to any test compound size, can be used without labeling the compounds and can

be performed in a broad range of pH, salt concentration or other relevant conditions[127,128]. In addition, the binding is measured in solution and does not require solid phase preparation, as in the case with Biacore experiments, limiting time required for assay development. However, ITC also has several limitations. Although the calorimeters are quite sensitive, the production of heat has to be high enough to be measurable, which requires relatively high concentrations of the compounds[126].

Membrane potential assays

Antibacterial peptides that act via a membrane-attacking mechanism are proposed to also interfere with the membrane potential of bacteria. The membrane potential is created through a difference of charged ions intracellular (mainly K^+) and extracellular (mainly Na^+ and Cl^-), which are continuously crossing the cytoplasmic membrane through specific ion channels. The assortative ion movement and ion concentration gradients through the pumps establish a difference in voltage across the membrane. In order to measure the membrane potential, fluorescent dyes have been developed, which have to share the following criteria: 1. rapidly penetrating the membrane when a membrane potential is present, 2. accumulating and not binding to the membrane or other cellular components, 3. being measurable at low concentrations, and 4. being biologically inactive[129]. One dye that fulfills these criteria is DiSC3(5). This cationic cyanine fluorescent dye accumulates in the lipid bilayer and thereby auto-quenches its fluorescent signal. After a stable low fluorescent signal is reached, antibacterial compounds can be added[130]. If the peptide dissipates the membrane potential the dye is released from the membrane, and the fluorescent signal increases. Different antibiotics and antimicrobial HDPs such as valinomycin and melittin have been reported to exhibit such membrane depolarization effects[130,131]. This method senses the changes of the membrane potential on live bacteria; however, the assay is best feasible for Gram-positive bacteria. Due to the double membrane structure of Gram-negative bacteria, DiSC3(5) is hindered to translocate to the cytoplasmic membrane. Pre-treatment of the bacteria with EDTA, can permeabilize the outer membrane and allow the dye to enter, however this will also influence cell viability[130]. Another disadvantage is, that DiSC3(5) assay are quite laborious and have to be optimized in each lab and for each selected pathogen.

Microscopy

In order to study the effects of antimicrobial HDPs on membrane permeability or intracellular changes on (live) bacteria different imaging-techniques are available including confocal and electron microscopy. These techniques proved to be essential in revealing antibacterial mechanism(s) of action of peptides[132].

Confocal microscopy

Confocal microscopy is a great opportunity to investigate the first interaction of peptides with a living cell. Labeling of peptides and the use of fluorophore markers have become important tools to study the membrane permeabilization and the effect on intracellular components[132]. Based on confocal microscopy, bacteria-peptide interactions can be visualized with snap-shots or live-imaging. During live-imaging, the real-time attack of a peptide on bacterial cells can be monitored, which can be further determined due to membrane permeabilization and shrinkage of the cells[133]. With this technique live microorganisms can be studied in their physiological condition and no fixation of the samples is required limiting the occurrence of artifacts[134].

Confocal microscopes are correcting the out of focus images, thereby a laser illuminates the sample and the light is collected through a small pinhole. This microscopy method detects serial optical sections and has the ability to produce 3D images. Furthermore, the diminished background fluorescence and the enhanced signal-to-noise improve the contrast of confocal images compared to wide-field imaging[133,135]. Due to the various colors, confocal microscopy has the potential to do triple staining, which facilitates elucidation of the killing mechanism and/or membrane permeabilization induced DNA leakage[132].

Nevertheless, confocal microscopy has several disadvantages. For instance, it has a low power resolution and could cause fading and bleaching of fluorochromes. Another downside is the restricted condition of the objective lenses, therefore immersion oil and thickness of the glass slides should meet several criteria. A further inconvenience is the monitoring of the laser intensity, as too high intensity levels can affect the cell viability[134]. Another disadvantage is that the fluorescent-label can affect the biological activity of the peptide. Although the MIC values between unlabeled and labeled peptide are often the same, the mode of action of the peptide might be changed due to the fluorescent probes.

Transmission electron microscopy

Transmission electron microscopy (TEM) is an excellent method to study the morphological changes at high magnification, allowing the visualization of structures that are beyond the resolution of a wide-field or confocal microscope. Important features like membrane morphology or the distribution of ribosomes are readily visible.

After incubating bacteria with a peptide, the samples are chemically fixed and stained with heavy metals, which enhances the contrast in the electron microscope. After embedding the bacteria in a resin, ultrathin sections (50 - 70 nm) are prepared with an ultramicrotome which is evaluated in the electron microscope[132].

TEM requires an extensive training, in order to properly perform preparation, fixation, embedding, sectioning, visualization and analysis of the sample. As it is a laborious technique, it takes several days to obtain the first results. TEM is an expensive very specialized technique and requires a well maintained electron microscopy facility.

If an antibody against the peptide is available, the peptide localization can be determined using immuno-electron microscopy. In many cases the antibody does not recognize its antigen after resin embedding and other techniques are applied, like cryo-TEM[136]. However during cryo-TEM the ultrastructure of bacteria is less clear than conventional TEM. Therefore, immunogold-labeling of conventional TEM samples is an alternative method to detect the precise peptide localization on bacterial membranes or intracellular locations. In order to detect the peptide on the resin-section the antibody has to be strong and specific enough.

Zebrafish model to study HDP effects *in vivo*

In order to study the *in vivo* effects of HDPs, mostly mammalian models have been used, yet due to ethical and methodological limitations other models are desired. Recently, zebrafish embryos have come more into the spotlights, in order to model diseases or perform drug screening[137,138]. Zebrafish have an *ex utero* development and due to its optical transparency each cell stage can be monitored. Zebrafish have a rapid development, within 24 hours the fishes develop from a fertilized egg to a fish-looking animal (pigmentation of the eyes, straight tail, starting blood circulation and a beating heart), which makes it an interesting model to study host-pathogen interactions. High numbers of experiments can be performed as each female fish lays up to 200 eggs weekly and based on the research question different injection routes can be used[139,140]. Furthermore, ethical permission to use zebrafish embryos is not required until five days post-fertilization as afterwards these embryos require external feeding[141]. Nowadays also robotic injectors are available, which are yolk-injecting 2000 embryos within one hour[142].

Nevertheless, in order to perform microinjections manually an extensive training is required. Zebrafish embryos have been used as infection model, however not each pathogen infects the fish. Another disadvantage is the temperature differences. While mammalian pathogens grow at 37 °C, fishes are kept at 28-30 °C. Furthermore, there is a lack of cell markers and up to date no fish cells lines have been in culture[140]. Another limitation using this model is that fish have a different anatomy compared to mammalians: gills instead of lungs, a different reproductive system, hematopoiesis in the anterior kidneys instead of bone marrow and no visible lymph nodes[137].

Zebrafish embryos have been successfully used for drug screening and can be seen as a pre-selection tool to test huge numbers of compounds at micro- or milligram concentrations[143]. Another important advantage is that compounds can be tested simultaneously in *in vitro* studies in the early stages of drug development

and in this *in vivo* animal model, to obtain indications on the bioactivity and toxicity[138]. Clearly, zebrafish embryos may be a valuable model to study host-pathogen interactions. Nevertheless, the use of other vertebrate models (e.g. avian or mammalian models) is important to confirm the zebrafish findings and to determine which compounds/peptides show the desired activities.

OUTLINE OF THE THESIS

The increasing numbers of antibiotic resistance and the continuously declining numbers of new antibiotics getting into the market indicate that we are entering an era where we can no longer solely count on antibiotics to treat infections. The overuse of antibiotics has had a tremendous impact on the evolution of antibiotic resistant strains. Limiting the use alone will not solve this problem; therefore, there is an urgent need for new antimicrobials. HDPs have been shown to be potential candidates as alternative for conventional antibiotics to treat infections by these antibiotic resistant pathogens. HDPs are small cationic molecules and play an important role in innate immunity. These peptides have originally been shown to exhibit direct antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria. The past years different HDPs have been demonstrated to possess immunomodulatory activity and are able to boost the (innate) immune system.

The main focus of this thesis is to expand the knowledge on the antibacterial mechanism of CATH-2 *in vitro* and to gain first insights on the (possibly immunomodulatory) effect of the peptide *in vivo*. It is believed that the obtained results will deepen the knowledge about the action(s) of CATH-2, which are necessary for the development of this peptide as new anti-infective drug. Additionally PR-39 will be studied to elucidate its immunomodulatory and antibacterial effects *in vitro* in more detail.

This thesis shows the unique combination of various techniques to identify the mode(s) of actions of HDPs by using different pathogens, such as *Salmonella enteritidis*, *E. coli*, MRSA and *Bacillus globigii*. In **Chapter 2** important results are presented about the antibacterial mode of action of CATH-2 on the Gram-negative bacteria *E. coli* at sub-MIC and MIC values. In **Chapter 3** the mechanism of action of CATH-2 on MRSA is reported by studying morphological changes and peptide localization as a function of concentration. In **Chapter 4** the prophylactic effect of CATH-2 against bacterial infection in a zebrafish model is shown. The antibacterial and immunomodulatory function of PR-39 and its truncated forms are presented in **Chapter 5**. **Chapter 6** will provide a summarizing discussion with potential future perspectives of the work described in this thesis.

REFERENCES

1. White R. in *Antibiotic Discovery and Development* (eds Dougherty, T. & Pucci, M.) 3-31, 2011).
2. Centers for Disease Control and Prevention (2013) Antibiotic resistance threats in the united states, 2013. Accessed July 19, 2015; http://www.cdc.gov/drugresistance/threat-report-2013/.
3. Tenover, F. C. Mechanisms of antimicrobial resistance in bacteria. *Am. J. Med.* **119**, S3-10 (2006).
4. Arias, C. A. & Murray, B. E. Antibiotic-Resistant Bugs in the 21st Century - A Clinical Super-Challenge. *N. Engl. J. Med.* **360**, 439-443 (2009).
5. World Health Organization (2015) Antibiotic resistance, April 2015. Accessed, September 17, 2015; http://www.who.int/mediacentre/factsheets/fs194/en/.
6. Bush, K. Alarming beta-lactamase-mediated resistance in multidrug-resistant *Enterobacteriaceae*. *Curr. Opin. Microbiol.* **13**, 558-564 (2010).
7. Neu, H. The Crisis in Antibiotic Resistance. *Science* **257**, 1064-1073 (1992).
8. McManus, M. C. Mechanisms of bacterial resistance to antimicrobial agents. *Am. J. Health. Syst. Pharm.* **54**, 1420-33; quiz 1444 (1997).
9. Hooper, D. C. Mechanisms of action and resistance of older and newer fluoroquinolones. *Clin. Infect. Dis.* **31 Suppl 2**, S24-8 (2000).
10. Drlica, K. & Zhao, X. DNA gyrase, Topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* **61**, 377-392 (1997).
11. Petri, W. in *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (eds Brunton, L., Chabner, B. & Knollmann, B.) 1470-1476 (McGraw-Hill, New York, 2011).
12. Storm, D. R., Rosenthal, K. S. & Swanson, P. E. Polymyxin and related peptide antibiotics. *Annu. Rev. Biochem.* **46**, 723-763 (1977).
13. Carpenter, C. F. & Chambers, H. F. Daptomycin: another novel agent for treating infections due to drug-resistant gram-positive pathogens. *Clin. Infect. Dis.* **38**, 994-1000 (2004).
14. Shlaes DM. Research and Development of Antibiotics: The Next Battleground. *ACS infectious diseases* **1**, 232-233 (2015).
15. Evans D (2015) Designing smarter drugs to fight antibiotic resistance. *Elsevier*. Accessed, November 27, 2015; https://www.elsevier.com/connect/designing-smarter-antibacterials-to-fight-antibiotic-resistance.
16. Lewis, K. Challenges of Antibiotic Discovery. *Microbe Magazine* **10**, 363-369 (2015).
17. Conly, J. & Johnston, B. Where are all the new antibiotics? The new antibiotic paradox. *Can. J. Infect. Dis. Med. Microbiol.* **16**, 159-160 (2005).
18. Cully, M. Public health: The politics of antibiotics. *Nature* **509**, S16-17 (2014).
19. Reardon, S. Antibiotic resistance sweeping developing world. *Nature* **509**, 141-142 (2014).
20. Goossens, H., Ferech, M., Vander Stichele, R., Elseviers, M. & ESAC Project Group. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* **365**, 579-587 (2005).
21. Grave, K., Torren-Edo, J. & Mackay, D. Comparison of the sales of veterinary antibacterial agents between 10 European countries. *J. Antimicrob. Chemother.* **65**, 2037-2040 (2010).
22. Productschap Vee en Vlees. Jaarverslag 2012., 1-76 (2013).

23. Castanon, J. I. History of the use of antibiotic as growth promoters in European poultry feeds. *Poult. Sci.* **86**, 2466-2471 (2007).
24. Dibner, J. J. & Richards, J. D. Antibiotic growth promoters in agriculture: history and mode of action. *Poult. Sci.* **84**, 634-643 (2005).
25. Phillips, I. *et al*. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother.* **53**, 28-52 (2004).
26. Newell, D. G. *et al*. Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.* **139 Suppl 1**, S3-15 (2010).
27. World Health Organization (2015) WHO estimates of the global burden of foodborne diseases - foodborne diseases burden epidemiology reference group 2007-2015. Accessed, December 07, 2015; http://www.who.int/foodsafety/publications/foodborne_disease/fergreport/en/.
28. Overdevest, I. *et al*. Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. *Emerg. Infect. Dis.* **17**, 1216-1222 (2011).
29. Leverstein-van Hall, M. A. *et al*. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin. Microbiol. Infect.* **17**, 873-880 (2011).
30. van Cleef, B. A. *et al*. Livestock-associated MRSA in household members of pig farmers: transmission and dynamics of carriage, a prospective cohort study. *PLoS One* **10**, e0127190 (2015).
31. Hancock, R. E. & Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **24**, 1551-1557 (2006).
32. Yount, N. Y. & Yeaman, M. R. Immunocontinuum: perspectives in antimicrobial peptide mechanisms of action and resistance. *Protein Pept. Lett.* **12**, 49-67 (2005).
33. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395 (2002).
34. Steinstraesser, L., Kraneburg, U., Jacobsen, F. & Al-Benna, S. Host defense peptides and their antimicrobial-immunomodulatory duality. *Immunobiology* **216**, 322-333 (2011).
35. Jenssen, H., Hamill, P. & Hancock, R. E. Peptide Antimicrobial Agents. *Clin. Microbiol. Rev.* **19**, 491-511 (2006).
36. Brogden, K. A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **3**, 238-250 (2005).
37. Nguyen, L. T., Haney, E. F. & Vogel, H. J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* **29**, 464-472 (2011).
38. Zanetti, M. The role of cathelicidins in the innate host defenses of mammals. *Curr. Issues Mol. Biol.* **7**, 179-196 (2005).
39. Semple, F. & Dorin, J. R. beta-Defensins: multifunctional modulators of infection, inflammation and more? *J. Innate Immun.* **4**, 337-348 (2012).
40. Hilchie, A. L., Wuerth, K. & Hancock, R. E. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol.* **9**, 761-768 (2013).
41. Brockus, C. W., Jackwood, M. W. & Harmon, B. G. Characterization of beta-defensin prepropeptide mRNA from chicken and turkey bone marrow. *Anim. Genet.* **29**, 283-289 (1998).
42. Selsted, M. E. & Ouellette, A. J. Mammalian defensins in the antimicrobial immune response. *Nat. Immunol.* **6**, 551-557 (2005).

43. Yang, D., Biragyn, A., Hoover, D. M., Lubkowski, J. & Oppenheim, J. J. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu. Rev. Immunol.* **22**, 181-215 (2004).
44. Diamond, G. *et al*. Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3952-3956 (1991).
45. Cuperus, T., Coorens, M., van Dijk, A. & Haagsman, H. P. Avian host defense peptides. *Dev. Comp. Immunol.* **41**, 352-369 (2013).
46 van Dijk, A. *et al*. Chicken heterophils are recruited to the site of Salmonella infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS. *Mol. Immunol.* **46**, 1517-1526 (2009).
47. Gudmundsson, G. H. *et al*. The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur. J. Biochem.* **238**, 325-332 (1996).
48. Shai, Y. Mode of action of membrane active antimicrobial peptides. *Biopolymers* **66**, 236-248 (2002).
49. Matsuzaki, K. Control of cell selectivity of antimicrobial peptides. *Biochim. Biophys. Acta* **1788**, 1687-1692 (2009).
50. Yeaman, M. R. & Yount, N. Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* **55**, 27-55 (2003).
51. Guilhelmelli, F. *et al*. Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. *Front. Microbiol.* **4**, 1-12 (2013).
52. Gennaro, R. & Zanetti, M. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers* **55**, 31-49 (2000).
53. Hancock, R. E. Peptide antibiotics. *Lancet* **349**, 418-22 (1997).
54. Falla, T. J., Karunaratne, D. N. & Hancock, R. E. Mode of action of the antimicrobial peptide indolicidin. *J. Biol. Chem.* **271**, 19298-19303 (1996).
55. Piers, K. L. & Hancock, R. E. The interaction of a recombinant cecropin/melittin hybrid peptide with the outer membrane of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **12**, 951-958 (1994).
56. Zhang, L., Dhillon, P., Yan, H., Farmer, S. & Hancock, R. E. Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **44**, 3317-3321 (2000).
57. Laverty, G., Gorman, S. P. & Gilmore, B. F. The potential of antimicrobial peptides as biocides. *Int. J. Mol. Sci.* **12**, 6566-6596 (2011).
58. Powers, J. P., Tan, A., Ramamoorthy, A. & Hancock, R. E. Solution structure and interaction of the antimicrobial polyphemusins with lipid membranes. *Biochemistry* **44**, 15504-15513 (2005).
59. Carnicelli, V. *et al*. in *Microbial pathogens and strategies for combating them: science, technology and education* (ed Méndez-Vilas, A.) 1123-1134 (Formatex Research Center, Badajoz (Spain), 2014).
60. Henzler Wildman, K. A., Lee, D. K. & Ramamoorthy, A. Mechanism of Lipid Bilayer Disruption by the Human Antimicrobial Peptide, LL-37. *Biochemistry* **42**, 6545-6558 (2003).
61. Yamaguchi, S., Hong, T., Waring, A., Lehrer, R. I. & Hong, M. Solid-state NMR investigations of peptide-lipid interaction and orientation of a beta-sheet antimicrobial peptide, protegrin. *Biochemistry* **41**, 9852-9862 (2002).

62. Yang, L., Harroun, T. A., Weiss, T. M., Ding, L. & Huang, H. W. Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys. J.* **81**, 1475-1485 (2001).
63. Matsuzaki, K., Murase, O., Fujii, N. & Miyajima, K. An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry* **35**, 11361-11368 (1996).
64. Baumann, G. & Mueller, P. A molecular model of membrane excitability. *Journal of Supramolecular Structure* **2**, 538-557 (1974).
65. Ehrenstein, G. & Lecar, H. Electrically gated ionic channels in lipid bilayers. *Q. Rev. Biophys.* **10**, 1-34 (1977).
66. Shai, Y. & Oren, Z. From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides* **22**, 1629-1641 (2001).
67. Tieleman, D. P., Berendsen, H. J. & Sansom, M. S. An alamethicin channel in a lipid bilayer: molecular dynamics simulations. *Biophys. J.* **76**, 1757-1769 (1999).
68. Fernandez, D. I. *et al*. The antimicrobial peptide aurein 1.2 disrupts model membranes via the carpet mechanism. *Phys. Chem. Chem. Phys.* **14**, 15739-15751 (2012).
69. Orioni, B. *et al*. Membrane perturbation by the antimicrobial peptide PMAP-23: a fluorescence and molecular dynamics study. *Biochim. Biophys. Acta* **1788**, 1523-1533 (2009).
70. Nicolas, P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS J.* **276**, 6483-6496 (2009).
71. de Kruijff, B., van Dam, V. & Breukink, E. Lipid II: a central component in bacterial cell wall synthesis and a target for antibiotics. *Prostaglandins Leukot. Essent. Fatty Acids* **79**, 117-121 (2008).
72. Yount, N. Y. & Yeaman, M. R. Peptide antimicrobials: cell wall as a bacterial target. *Ann. N. Y. Acad. Sci.* **1277**, 127-138 (2013).
73. Park, C. B., Kim, H. S. & Kim, S. C. Mechanism of Action of the Antimicrobial Peptide Buforin II: Buforin II Kills Microorganisms by Penetrating the Cell Membrane and Inhibiting Cellular Functions. *Biochem. Biophys. Res. Commun.* **244**, 253-257 (1998).
74. Otvos, L.,Jr *et al*. Interaction between Heat Shock Proteins and Antimicrobial Peptides. *Biochemistry* **39**, 14150-14159 (2000).
75. Krizsan, A. *et al*. Insect-Derived Proline-Rich Antimicrobial Peptides Kill Bacteria by Inhibiting Bacterial Protein Translation at the 70S ribosome. *Angew. Chem. Int. Ed Engl.* **53**, 12236-12239 (2014).
76. Podda, E. *et al*. Dual mode of action of Bac7, a proline-rich antibacterial peptide. *Biochim. Biophys. Acta* **1760**, 1732-1740 (2006).
77. Overhage, J. *et al*. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* **76**, 4176-4182 (2008).
78. Choi, K. Y. & Mookherjee, N. Multiple immune-modulatory functions of cathelicidin host defense peptides. *Front. Immunol.* **3**, 149 (2012).
79. Yang, D., Chertov, O. & Oppenheim, J. J. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). *J. Leukoc. Biol.* **69**, 691-697 (2001).
80. Kurosaka, K., Chen, Q., Yarovinsky, F., Oppenheim, J. J. & Yang, D. Mouse cathelin-related antimicrobial peptide chemoattracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant. *J. Immunol.* **174**, 6257-6265 (2005).

81. Davidson, D. J. *et al*. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J. Immunol.* **172**, 1146-1156 (2004).
82. Bandholtz, L. *et al*. Antimicrobial peptide LL-37 internalized by immature human dendritic cells alters their phenotype. *Scand. J. Immunol.* **63**, 410-419 (2006).
83. van der Does, A. M. *et al*. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. *J. Immunol.* **185**, 1442-1449 (2010).
84. Takeda, K. & Akira, S. Toll-like receptors. *Curr. Protoc. Immunol.* **109**, 14.12.1-14.12.10 (2015).
85. Coorens, M., van Dijk, A., Bikker, F., Veldhuizen, E. J. & Haagsman, H. P. Importance of Endosomal Cathelicidin Degradation To Enhance DNA-Induced Chicken Macrophage Activation. *J. Immunol.* **195**, 3970-3977 (2015).
86. Lande, R. *et al*. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* **449**, 564-569 (2007).
87. Hurtado, P. & Peh, C. A. LL-37 promotes rapid sensing of CpG oligodeoxynucleotides by B lymphocytes and plasmacytoid dendritic cells. *J. Immunol.* **184**, 1425-1435 (2010).
88. Baumann, A., Demoulins, T., Python, S. & Summerfield, A. Porcine cathelicidins efficiently complex and deliver nucleic acids to plasmacytoid dendritic cells and can thereby mediate bacteria-induced IFN-alpha responses. *J. Immunol.* **193**, 364-371 (2014).
89. Mookherjee, N. *et al*. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J. Immunol.* **176**, 2455-2464 (2006).
90. Brown, K. L. *et al*. Host defense peptide LL-37 selectively reduces proinflammatory macrophage responses. *J. Immunol.* **186**, 5497-5505 (2011).
91. Morioka, Y., Yamasaki, K., Leung, D. & Gallo, R. L. Cathelicidin antimicrobial peptides inhibit hyaluronan-induced cytokine release and modulate chronic allergic dermatitis. *J. Immunol.* **181**, 3915-3922 (2008).
92. Hellgren, O. & Ekblom, R. Evolution of a cluster of innate immune genes (beta-defensins) along the ancestral lines of chicken and zebra finch. *Immunome Res.* **6**, 3-7580-6-3 (2010).
93. van Dijk, A., Veldhuizen, E. J. & Haagsman, H. P. Avian defensins. *Vet. Immunol. Immunopathol.* **124**, 1-18 (2008).
94. Xiao, Y. *et al*. Identification and Functional Characterization of Three Chicken Cathelicidins with Potent Antimicrobial Activity. *J. Biol. Chem.* **281**, 2858-2867 (2006).
95. Lynn, D. J. *et al*. Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. *Immunogenetics* **56**, 170-177 (2004).
96. van Dijk, A., Veldhuizen, E. J., van Asten, A. J. & Haagsman, H. P. CMAP27, a novel chicken cathelicidin-like antimicrobial protein. *Vet. Immunol. Immunopathol.* **106**, 321-327 (2005).
97. Goitsuka, R. *et al*. Chicken cathelicidin-B1, an antimicrobial guardian at the mucosal M cell gateway. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 15063-15068 (2007).
98. Achanta, M. *et al*. Tissue expression and developmental regulation of chicken cathelicidin antimicrobial peptides. *J. Anim. Sci. Biotechnol.* **3**, 15-1891-3-15 (2012).
99. van Dijk, A. *et al*. Avian cathelicidins: paradigms for the development of anti-infectives. *Vet. Microbiol.* **153**, 27-36 (2011).
100. Bommineni, Y. R. *et al*. Fowlicidin-3 is an alpha-helical cationic host defense peptide with potent antibacterial and lipopolysaccharide-neutralizing activities. *FEBS J.* **274**, 418-428 (2007).

101. Xiao, Y. *et al*. Structure-activity relationships of fowlicidin-1, a cathelicidin antimicrobial peptide in chicken. *FEBS J.* **273**, 2581-2593 (2006).
102. Xiao, Y. *et al*. The Central Kink Region of Fowlicidin-2, an α-Helical Host Defense Peptide, Is Critically Involved in Bacterial Killing and Endotoxin Neutralization. *J. Innate Immun.* **1**, 268-280 (2009).
103. van Dijk, A. *et al*. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. *Mol. Immunol.* **46**, 2465-2473 (2009).
104. Veldhuizen, E. J., Brouwer, E. C., Schneider, V. A. & Fluit, A. C. Chicken Cathelicidins Display Antimicrobial Activity against Multiresistant Bacteria without Inducing Strong Resistance. *PLoS One* **8**, e61964 (2013).
105. van Dijk, A. *et al*. *Campylobacter jejuni* is highly susceptible to killing by chicken host defense peptide cathelicidin-2 and suppresses intestinal cathelicidin-2 expression in young broilers. *Vet. Microbiol.* **160**, 347-354 (2012).
106. Oh, D. *et al*. Role of the Hinge Region and the Tryptophan Residue in the Synthetic Antimicrobial Peptides, Cecropin A(1-8)-Magainin 2(1-12) and Its Analogues, on Their Antibiotic Activities and Structures. *Biochemistry* **39**, 11855-11864 (2000).
107. Molhoek, E. M. *et al*. Chicken cathelicidin-2-derived peptides with enhanced immunomodulatory and antibacterial activities against biological warfare agents. *Int. J. Antimicrob. Agents* **36**, 271-274 (2010).
108. Larrick, J. W. *et al*. Human CAP18: a Novel Antimicrobial Lipopolysaccharide-Binding Protein. *Infect. Immun.* **63**, 1291-1297 (1995).
109. Nagaoka, I. *et al*. Cathelicidin Family of Antibacterial Peptides CAP18 and CAP11 Inhibit the Expression of TNF-alpha by Blocking the Binding of LPS to CD14(+) Cells. *J. Immunol.* **167**, 3329-3338 (2001).
110. Nan, Y. H., Bang, J. K., Jacob, B., Park, I. S. & Shin, S. Y. Prokaryotic selectivity and LPS-neutralizing activity of short antimicrobial peptides designed from the human antimicrobial peptide LL-37. *Peptides* **35**, 239-247 (2012).
111. Sang, Y. & Blecha, F. Porcine host defense peptides: Expanding repertoire and functions. *Dev. Comp. Immunol.* **33**, 334-343 (2009).
112. Gudmundsson, G. H. *et al*. Structure of the gene for porcine peptide antibiotic PR-39, a cathelin gene family member: Comparative mapping of the locus for the human peptide antibiotic FALL-39. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7085-7089 (1995).
113. Shi, J., Ross, C. R., Chengappa, M. M. & Blecha, F. Identification of a proline-arginine-rich antibacterial peptide from neutrophils that is analogous to PR-39, an antibacterial peptide from the small intestine. *J. Leukoc. Biol.* **56**, 807-811 (1994).
114. Storici, P. & Zanetti, M. A cDNA Derived from Pig Bone Marrow Cells Predicts a Sequence Identical to the Intestinal Antibacterial Peptide PR-39. *Biochem. Biophys. Res. Commun.* **196**, 1058-1065 (1993).
115. Ramanathan, B., Davis, E. G., Ross, C. R. & Blecha, F. Cathelicidins: microbicidal activity, mechanisms of action, and roles in innate immunity. *Microbes Infect.* **4**, 361-372 (2002).
116. Li, J. *et al*. PR39, a peptide regulator of angiogenesis. *Nat. Med.* **6**, 49-55 (2000).
117. Shi, J., Ross, C. R., Leto, T. L. & Blecha, F. PR-39, a proline-rich antibacterial peptide that inhibits phagocyte NADPH oxidase activity by binding to Src homology 3 domains of p47 phox. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6014-6018 (1996).

118. Huang, H. J., Ross, C. R. & Blecha, F. Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *J. Leukoc. Biol.* **61**, 624-629 (1997).
119. Ramanathan, B., Wu, H., Ross, C. R. & Blecha, F. PR-39, a porcine antimicrobial peptide, inhibits apoptosis: involvement of caspase-3. *Dev. Comp. Immunol.* **28**, 163-169 (2004).
120. Agerberth, B. *et al*. Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. *Eur. J. Biochem.* **202**, 849-854 (1991).
121. Vunnam, S., Juvvadi, P. & Merrifield, R. B. Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine. *J. Pept. Res.* **49**, 59-66 (1997).
122. Boman, H. G., Agerberth, B. & Boman, A. Mechanisms of Action on *Escherichia coli* of Cecropin P1 and PR-39, Two Antibacterial Peptides from Pig Intestine. *Infect. Immun.* **61**, 2978-2984 (1993).
123. Chan, Y. R., Zanetti, M., Gennaro, R. & Gallo, R. L. Anti-Microbial Activity and Cell Binding are Controlled by Sequence Determinants in the Anti-Microbial Peptide PR-39. *J. Invest. Dermatol.* **116**, 230-235 (2001).
124. Shi, J. *et al*. Antibacterial Activity of a Synthetic Peptide (PR-26) Derived from PR-39, a Proline-Arginine-Rich Neutrophil Antimicrobial Peptide. *Antimicrob. Agents Chemother.* **40**, 115-121 (1996).
125. van Dijk, A. *et al*. The β-Defensin Gallinacin-6 Is Expressed in the Chicken Digestive Tract and Has Antimicrobial Activity against Food-Borne Pathogens. *Antimicrob. Agents Chemother.* **51**, 912-922 (2007).
126. Velazquez-Campoy, A., Ohtaka, H., Nezami, A., Muzammil, S. & Freire, E. Isothermal titration calorimetry. *Curr. Protoc. Cell. Biol.* **Chapter 17**, Unit 17.8 (2004).
127. Ghai, R., Falconer, R. J. & Collins, B. M. Applications of isothermal titration calorimetry in pure and applied research--survey of the literature from 2010. *J. Mol. Recognit.* **25**, 32-52 (2012).
128. Doyle, M. Characterization of binding interactions by isothermal titration calorimetry. *Curr. Opin. Biotechnol.* **8**, 31-35 (1997).
129. Lolkema, J., Hellingwerf, K. & Konings, W. The effect of probe binding on the quantitative-determination of the proton-motive force in bacteria. *Biochim. Biophys. Acta* **681**, 85-94 (1982).
130. Breeuwer, P. & Abee, T. Assessment of viability of microorganisms employing fluorescence techniques. *Int. J. Food Microbiol.* **55**, 193-200 (2000).
131. Lv, Y. *et al*. Antimicrobial properties and membrane-active mechanism of a potential alpha-helical antimicrobial derived from cathelicidin PMAP-36. *PLoS One* **9**, e86364 (2014).
132. Marcellini, L., Giammatteo, M., Pierpaolo, A. & Mangoni, M. in *Antimicrobial Peptides, Methods and Protocols* (eds Giulliani, A. & Rinaldi, A.) 249-266 (Springer, New York, 2010).
133. Stephens, D. & Allan, V. Light microscopy techniques for live cell imaging. *Science* **300**, 82-6 (2003).
134. Quintas, V., Prada-López, I. & Tomás, I. in *Microscopy: advances in scientific research and education* (ed Méndez-Vilas, A.) 226-238 (Formatex Research Center, Badajoz (Spain), 2014).
135. Meyer, P. & Dworkin, J. Applications of fluorescence microscopy to single bacterial cells. *Res. Microbiol.* **158**, 187-194 (2007).
136. Slot, J. W. & Geuze, H. J. Cryosectioning and immunolabeling. *Nat. Protoc.* **2**, 2480-2491 (2007).

137. Tobin, D., May, R. & Wheeler, R. Zebrafish: a see-through host and a fluorescent toolbox to probe host-pathogen interaction. *PLoS Pathog.* **8**, e1002349 (2012).
138. Bowman, T. V. & Zon, L. I. Swimming into the future of drug discovery: in vivo chemical screens in zebrafish. *ACS Chem. Biol.* **5**, 159-161 (2010).
139. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of Embryonic Development of the Zebrafish. *Dev. Dyn.* **203**, 253-310 (1995).
140. van der Sar, A. M., Appelmelk, B. J., Vandenbroucke-Grauls, C. M. & Bitter, W. A star with stripes: zebrafish as an infection model. *Trends Microbiol.* **12**, 451-457 (2004).
141. Strahle, U. *et al*. Zebrafish embryos as an alternative to animal experiments - a commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reprod. Toxicol.* **33**, 128-132 (2012).
142. Spaink, H. P. *et al*. Robotic injection of zebrafish embryos for high-throughput screening in disease models. *Methods* **62**, 246-254 (2013).
143. Zon, L. I. & Peterson, R. T. In vivo drug discovery in the zebrafish. *Nat. Rev. Drug Discov.* **4**, 35-44 (2005).

Chapter 2

IMAGING THE ANTIMICROBIAL MECHANISM(S) OF CHICKEN CATHELICIDIN-2

Viktoria A.F. Schneider[a], Maarten Coorens[a], Soledad R. Ordonez[a], Johanna L.M. Tjeerdsma-van Bokhoven[a], George Posthuma[b], Albert van Dijk[a], Henk P. Haagsman[a], Edwin J.A. Veldhuizen[a]

[a] Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[b] Department of Cell Biology, Cell Microscopy Core, University Medical Center Utrecht, Utrecht, The Netherlands

Submitted

ABSTRACT

Host defense peptides (HDPs) have the potential to become alternatives to conventional antibiotics in human and veterinary medicine. The HDP chicken cathelicidin-2 (CATH-2) has immunomodulatory and direct killing activities at micromolar concentrations. In this study the antimicrobial mechanism of action of CATH-2 against *Escherichia coli* (*E. coli*) was investigated in great detail using a unique combination of imaging and biophysical techniques. Live-imaging with confocal fluorescence microscopy demonstrated that FITC-labeled CATH-2 mainly localized at the membrane of *E. coli.* Upon binding, the bacterial membrane was readily permeabilized as was shown by propidium iodide influx into the cell. Concentration- and time-dependent effects of the peptide on *E. coli* cells were examined by transmission electron microscopy (TEM). CATH-2 treatment was found to induce dose-dependent morphological changes in *E. coli*. At sub-minimal inhibitory concentrations (sub-MIC), intracellular granulation, enhanced vesicle release and wrinkled membranes were observed, while membrane breakage and cell lysis occurred at MIC values. These effects were visible within 1-5 minute of peptide exposure. Immunogold TEM showed CATH-2 binding to bacterial membranes. At sub-MIC values the peptide rapidly localized intracellularly without visible membrane permeabilization. It is concluded that CATH-2 has detrimental effects on *E. coli* at concentrations that do not immediately kill the bacteria.

INTRODUCTION

Since the 1980s the number of antibiotic resistant infections has consistently increased in both humans and animals[1,2]. To overcome this world-wide problem alternative treatments are required. Attractive alternatives to conventional antibiotics are host defense peptides (HDP)[3]. Chicken cathelicidin-2 (CATH-2), one of the four known chicken cathelicidin-like HDPs, is an arginine-lysine rich peptide with both immunomodulatory and strong broad-spectrum antibacterial activity, which is not inactivated by serum or high salt concentrations[4-6]. CATH-2 consists of two alpha-helical segments separated by a proline-induced kink, which is essential for the direct killing activity of the peptide[7]. Previous studies have already shown that CATH-2 exhibits broad-range bactericidal activity, however its antibacterial mode of action is still unrevealed.

The net positive charge and amphipathicity of antimicrobial HDPs enable a strong interaction with negatively charged outer (lipopolysaccharide; LPS) and inner (phospholipids) membranes of Gram-negative bacteria. This interaction can lead to destabilization and permeabilization of bacterial membranes, for which, depending on the exact interaction and peptide, several different models have been described. However, besides the described effects on the membrane, several studies have shown that peptides can actually cross membranes without membrane damage and reach intracellular targets such as ribosomes, DNA or other intracellular molecules. This can subsequently result in, among others, inhibition of DNA or RNA synthesis, protein synthesis, or protein folding. For extensive reviews on the multiple modes of action of HDPs see: Brogden and Nguyen, Haney and Vogel[8,9].

The aim of this study was to investigate the antibacterial killing mechanism of CATH-2. To achieve this, a unique combination of imaging techniques and binding assays was used during this study. Live-imaging fluorescence microscopy demonstrated the real-time attack of CATH-2 on *E. coli*. Morphological changes of the bacteria after peptide treatment were determined with transmission electron microscopy and immunogold electron microscopy was performed in order to determine peptide localization. Lastly, isothermal titration calorimetry and competition assays were used to study the peptide binding with LPS. Our results show that the bacterial membrane is an important (initial) target of CATH-2, but also that CATH-2 is present intracellularly at sub-MIC levels and has visible detrimental effects on bacterial cell structure.

MATERIAL AND METHODS

Peptide
CATH-2 was obtained from CPC Scientific Inc. (Sunnyvale, USA). Fluorescently labeled CATH-2 was made as described previously[10].

Bacterial strains and growth conditions
Six *Escherichia coli* (*E. coli*) strains were used in this study, i.e. *E. coli* ATCC 25922, ATCC 4157, K12, LMC500, MC4100 and 506 (O78K80), of which the latter was used as the reference strain in all experiments[11,12]. All bacterial strains were grown in Tryptone Soy Broth (TSB; Oxoid Limited, Hampshire, United Kingdom) and on Tryptone Soy Agar (TSA; Oxoid Ltd). For all experiments bacteria were inoculated and grown overnight in TSB at 37 °C. The next day bacteria were transferred to a fresh TSB tube and grown to mid-logarithmic phase.

Colony count assay
To measure the antibacterial activity of CATH-2 with or without FITC label, colony count assays were performed as described before[13]. In short, mid-logarithmic phase cultures were washed once in TSB and diluted to 2 x 10^6 CFU/ml. Subsequently, bacteria were exposed to peptide (0-40 µM) for 3 hours at 37 °C. Afterwards, the mixtures were diluted 50-5000-fold, spread plated on TSA plates and after 16 hours at 37 °C surviving colonies were counted.

Killing kinetics
To assess the time point of bacterial growth inhibition, killing kinetics with CATH-2 were performed. Peptide concentrations ranging from 0-20 µM CATH-2 were incubated with mid-logarithmic *E. coli* (2 x 10^6 CFU/ml). At 1, 5, 10, 20, 30, 60, 120 and 180 min, 100 µl aliquots were taken and immediately plated on TSA. Additionally, 20 µl aliquots were diluted 10- to 1000-fold and again 100 µl was plated. After 16 hours incubation at 37 °C the surviving bacteria were counted.

Confocal microscopy
To visualize peptide localization and permeability of the *E. coli* cytosolic membrane, time-lapse live-imaging was performed. Mid-logarithmic *E. coli* 506 was washed with 5 mM HEPES (pH 7.4, Sigma-Aldrich, Zwijndrecht, The Netherlands). Cells were pelleted and resuspended in 1 ml of HEPES buffer to obtain a high bacterial density. Silanized coverslips (Gerhard Menzel GmbH, Braunschweig, Germany) were prepared with 2% (3-Aminopropyl) triethoxysilane (SAA; Sigma-Aldrich) in acetone. Thirty microliters of 1% low-melting agarose (Agarose Type I low EEO, Sigma-Aldrich) was added in the center of the silanized coverslips. The same amount of bacteria was added and resuspended in the agarose. A second coverslip was placed on top. After 2 min the upper coverslip was removed and

the remaining agarose layer was fixed in an Attofluor cell chamber and 900 µl HEPES buffer was added. At this point the permeability marker propidium iodide (PI; 5.1 µM final concentration; Sigma-Aldrich) was added. After focusing the cells, the movie was started and 60 µl 20 µM FITC-labeled CATH-2 (0.9 µM final concentration) was added to the coverslip. Experiments were performed at the Leica SPE-II and Nikon A1R at the Center for Cell Imaging (CCI) at the faculty of Veterinary Medicine in Utrecht. FITC-CATH-2 and PI were detected with a Sapphire Blue Coherent laser (488 nm) and a Sapphire Yellow Coherent laser (561 nm). For data analysis ImageJ/FIJI software and NIS-Elements of Nikon was used.

Transmission electron microscopy
Concentration- and time-dependent bactericidal effects of CATH-2 were further investigated by performing transmission electron microscopy (TEM). Since higher bacterial densities were required (5 x 10^8 CFU/ml) for electron microscopy, additional colony count assays were performed to determine the antibacterial capacity of CATH-2 at this bacterial density. This yielded a MIC value of 40 µM. In order to determine the concentration-dependent effects of CATH-2, *E. coli* were incubated with various concentrations of the peptide (0, 2.5, 5, 10, 40 and 80 µM) for 30 min at 37 °C. Mixtures were fixed with 2% glutaraldehyde (Polysciences, Eppelheim, Germany), 5 mM $CaCl_2$, 10 mM $MgCl_2$ (both Merck, Darmstadt, Germany) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich) pH 7.4 overnight at 4 °C. In order to observe time-dependent effects CATH-2 (0, 5 or 40 µM) was incubated with bacteria (1, 5, 10, 30 min). Killing reactions were stopped by adding the fixative and keeping the cells overnight at 4 °C. After fixation, cells were washed for 3 x 10 min in sodium cacodylate buffer and embedded in 2% low-melting point agarose v/v (Sigma-Aldrich). Subsequently, cells were postfixed with 4% osmium tetroxide (Electron Microscopy Sciences; EMS, Hatfield, USA) and 1.5% $K_4Fe(CN)_6$-$3H_2O$ (Merck) in distilled water for 2 hours at 4 °C and after washing the cells for 5 x 10 min with distilled water, cells were incubated in 0.5% uranylacetate (EMS) for 1 hour at 4 °C. After rinsing for 3 x 10 min with distilled water, samples were embedded in Epon and ultrathin sections (50 nm) of each block were prepared on a Leica UCT ultramicrotome (Leica, Vienna, Austria). Lastly, sections were stained with uranyl acetate and lead citrate using Leica AC20 system (Leica). For all electron microscopy purposes a FEI Tecnai 12 electron microscope (FEI, Eindhoven, The Netherlands) at 80 kV was used.

Negative staining TEM
Negative staining TEM was used to detect bacterial surface changes after peptide treatment. Bacteria were incubated with various concentrations of CATH-2 (0-40 µM), as previously described for TEM. After fixation, bacteria were

rinsed with 0.1 M sodium cacodylate buffer for 3 x 10 min and 100 µl of each bacterial suspension was added on Parafilm. Copper grids (100 mesh) with a Formvar film were carbon coated shortly before use and placed for 5 min on the drop of bacterial suspension. Next, the grids were immersed in the staining solution uranylacetate (0.5%), the excess was removed with filter paper and dried overnight at RT.

Immunogold TEM

Epon blocks from the TEM experiments were used to determine the localization of the peptide. Sections (50 nm) were mounted on 100 mesh copper Formvar-carbon coated grids, incubated with PBS containing 0.5% fish skin gelatine (Sigma-Aldrich) and 0.1% Bovine Serum Albumin-c™ (AURION, Wageningen, The Netherlands) and immuno-labeled as described before[14]. Sections were incubated with CATH-2 antibody for 1 hour at RT, washed extensively with PBS (5 x 2 min) and exposed to protein-A gold (10 nm, Department of Cell Biology, University Medical Center Utrecht, The Netherlands) for 20 min at RT. Lastly, specimens were stained with 2% uranyloxalicacetate (pH 7; SPI, West Chester, USA) for 5 min at RT and finally embedded in methylcellulose-uranyl acetate (pH 4; 2% methylcellulose [Sigma-Aldrich] and 4% uranylacetate [SPI] in distilled water). In total 50 fields containing 1-3 cells were used for a quantitative analysis of the samples. The labeling densities were determined by counting the intracellular gold particles and gold particles on or nearby the bacterial membrane (all gold particles at a distance of 10 nm or less were considered to belong to the membrane), according to Griffiths[15]. The results from control cells were used to correct for background staining.

Isothermal titration calorimetry

Interaction between CATH-2 and different compounds (LPS and KDO_2-Lipid A) was tested using isothermal titration calorimetry (ITC). All ITC experiments were performed on a Low Volume NanoITC (TA instruments - Waters LLC, New Castle, USA). Smooth LPS (LPS-O111:B4) and rough LPS (LPS-K12) were obtained from Invivogen (Toulouse, France) and KDO_2-Lipid A was obtained from AdipoGen (Liestal, Switzerland). All compounds were diluted in phosphate-buffer (10 mM Na_2HPO_4-KH_2PO_4, pH 7.4). The syringe was filled with a 50 µl solution of 200 µM CATH-2 and the cell contained 190 µl of a solution of 25 µM LPS or Lipid A. Titrations were incremental with 2 µl injections at 300 seconds intervals. Experiments were performed at 37 °C. Data were analyzed with the Nano Analyze software (TA instruments - Waters LLC).

Competition assays

The inhibition by bacterial membrane compounds (LPS and Lipid A) on the antibacterial activity of CATH-2 was tested. CATH-2 (20 µM final concentration) was pre-incubated with different ratios of LPS-O111:B4 (1:0.25-1:3.25), LPS-K12 (1:0.25-1:2.5) or KDO_2-Lipid A (1:0.25-1:2.5) for 1 hour at 37 °C. Subsequently, the solutions were diluted two-fold in six-steps with distilled water. *E. coli* (2×10^6 CFU/ml) were added and the mixtures were then incubated for 3 hours at 37 °C as for a conventional MIC assay, described in the colony count assay section. After 16 hours at 37 °C, the lowest peptide/compound ratio causing inhibition of the antibacterial activity of CATH-2 was determined.

Statistical analysis

Statistical analysis was performed using a Student's t-Test. Significant differences were indicated as * ($P<0.05$), *** ($P<0.001$) or **** ($P<0.0001$).

RESULTS

CATH-2 rapidly kills bacteria at micromolar concentrations

CATH-2 and FITC-labeled CATH-2 were tested for their antibacterial activity using colony count assays. No differences in the minimal inhibitory concentration (MIC) values of the two peptides against *E. coli* were observed. Both peptides showed a MIC value of 5-10 µM. In addition, the killing kinetics of CATH-2 against *E. coli* was tested in order to detect the speed of killing at different peptide concentrations. At MIC level (10 µM) CATH-2 killed the bacteria within 10 minutes. At low peptide concentrations an initial decrease in the number of surviving bacterial cells was observed, however after 60 minutes surviving bacteria recuperated from the peptide attack and started growing again (Fig. 1).

CATH-2 binds and permeabilizes the bacterial membrane

Time-lapse imaging was performed using FITC-labeled CATH-2 and the permeability marker propidium iodide (PI). Instantly after adding the peptide, CATH-2 localized to the membrane of *E. coli* (Fig. 2A). Within approximately one minute, the first PI staining was observed indicating that CATH-2 had caused membrane leakage allowing PI to enter the cell and bind bacterial DNA (Movie S1; Fig. 2B). This order of events was seen with small variations, in all bacterial cells studied (Movie S2). Heat intensity plots confirmed the rapid membrane binding of CATH-2, especially at the bacterial septum of dividing cells, higher intensities levels were observed (Fig. 2C).

CATH-2 induces dose-dependent morphological changes of *E. coli*

Morphological changes of *E. coli* cells after treatment with different peptide concentrations were determined using transmission electron microscopy (TEM). Overall, non-peptide treated bacteria had intact membranes and a homogenous intracellular distribution of DNA and ribosomes (Fig. 3; light and darker area's respectively). At low peptide concentrations (2.5 and 5 µM, 1/16 and 1/8 MIC at the bacterial density used, respectively) CATH-2 exposure resulted in wrinkling of bacterial membranes and to some extent dissociation of membrane fragments. At 10 µM CATH-2 exposure induced membrane damage and strongly enhanced the amount of dissociated membrane fragments and number of ruptured cells. Interestingly, a marked release of small vesicles from the membrane was clearly visible in the presence of 2.5 µM CATH-2. At higher peptide concentrations no vesicle release was observed. At the same concentration intracellular effects were observed, as the DNA started to cluster in the center of the cell and ribosomes were directed towards the inner membrane of the bacteria (Fig. 3A-F).

Quantification of these effects showed that 5 µM CATH-2 caused strong membrane effects, i.e. almost 50% of the cells had wrinkled membranes and in 30% of the cells membrane ruptures were observed (Fig. 3G). Increasing the peptide concentration doubled the number of damaged membranes, enhanced the number of dissociated membrane parts in the section and ruptured cells (Fig. 3G-I). Interestingly, at the lowest peptide concentration used 40% of the cells showed redistribution of DNA and ribosomes and a 60% increase in vesicle release was observed (Fig. 3J and K). These results suggest that besides direct effects on the bacterial membrane, CATH-2 mediates intracellular effects, either directly through small amounts of translocated peptide or indirectly by the membrane-bound CATH-2. Similar results were observed with negative-staining TEM (Fig. S1A-E). At sub-MIC bleb-formation was observed, which is reduced at higher CATH-2 concentrations (Fig. S1F). At 10 and 40 µM complete membrane disruptions were observed (Fig. S1G). Additionally with ascending concentrations the flagella of *E. coli* cells disappeared (Fig. S1H).

To gain further insight in the order of events and the actual speed at which CATH-2 induced its effects, *E. coli* exposure to CATH-2 was examined at different time points (Fig. 4 and Fig. 5A-I). A one minute exposure of bacteria to 5 µM peptide resulted in wrinkled bacterial membranes. The number of wrinkled membranes per cell decreased with longer peptide incubation times, whereas simultaneously the number of damaged membranes and dissociated membrane fragments in the sections increased. Furthermore, DNA and ribosome redistribution and high vesicle release was observed after short (1-5 min) peptide incubation times. At the highest CATH-2 concentration tested, tremendous effects on the bacterial membranes were observed. After incubating the bacteria for one minute with 40 µM CATH-2 almost half of the cells had damaged membranes and in more than 70% of the analyzed sections, large dissociated membrane fragments were observed. These numbers consistently increased at later time points. To conclude, these results showed that

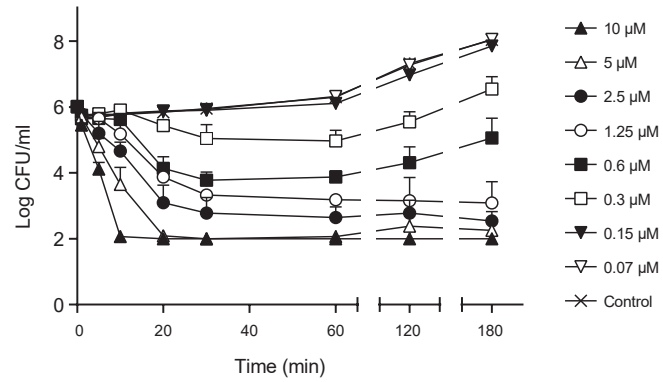

Figure 1. CATH-2 rapidly kills *E. coli*. *E. coli* 506 was co-incubated with different CATH-2 concentrations at 37 °C for different time points (1, 5, 10, 30, 60, 120 and 180 min). Each mixture was aliquoted at the various time intervals, serially diluted and spread plated on TSA plates. After 16 hours at 37 °C plates were counted for surviving bacteria. Data represent three independent measurements (means ± SEM).

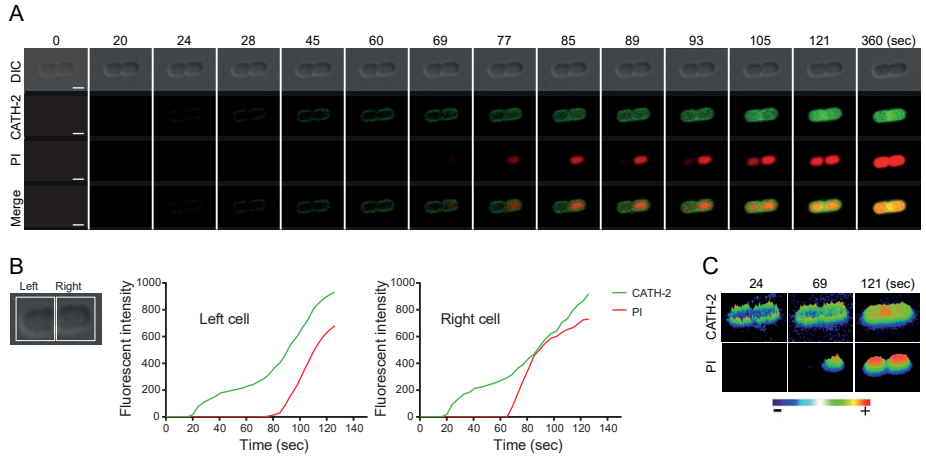

Figure 2. Snapshots of a single cell show the fast membrane binding and permeabilization of CATH-2. Various time lapses within a 6 min period are shown. Bars: 1 µm (A). Additionally, the fluorescent intensity of FITC-CATH-2 (0.9 µM) and PI (5.1 µM) was measured in both parts of a dividing bacterium, indicated as left and right cell (B). Heat intensity plots show the specific binding sites of FITC-CATH-2 and PI (C).

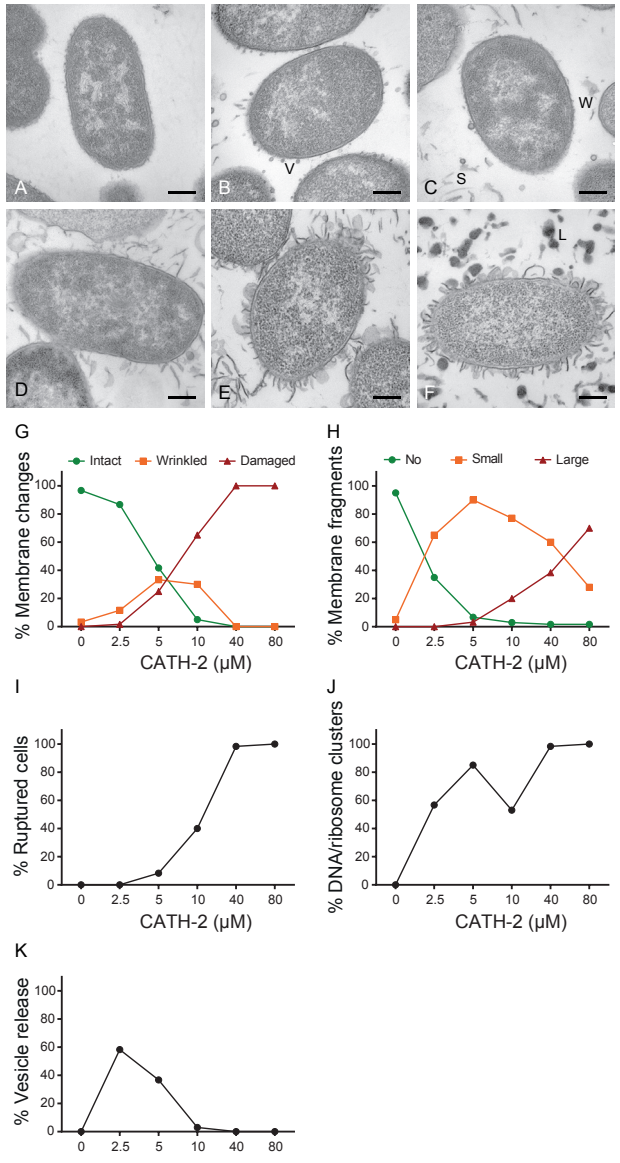

Figure 3. CATH-2 induced morphological changes of *E. coli* determined by TEM. Representative images are shown for 0 μM (A), 2.5 μM (B), 5 μM (C), 10 μM (D), 40 μM (E) and 80 μM (F). In total 60 cells of two independent experiments were analyzed per peptide concentration. V, vesicles; W, wrinkled membranes; S, small membrane fragments; L, large membrane fragments. Bars: 200 nm. Morphological changes were quantified in membrane intactness (G) and dissociated membrane fragments in the section (H). Additionally, morphological changes were classified in percentage of ruptured cells (I), DNA and ribosome clusters (J) and small vesicle release (K).

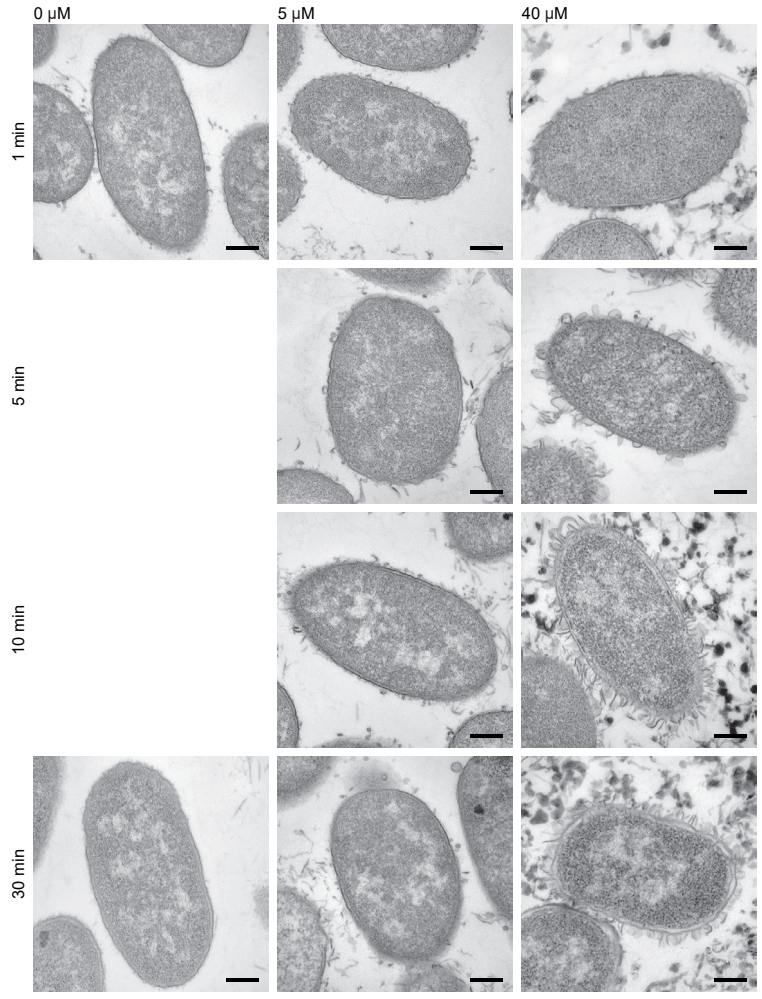

Figure 4. CATH-2 causes time-dependent morphological changes of *E. coli*. Bacteria were incubated with 5 and 40 μM CATH-2 and reactions were stopped after 1, 5, 10 and 30 min. Representative TEM images are shown for 0 μM, 5 μM and 40 μM. Bars: 200 nm.

prominent effects on the membrane and also inside the cell are visible after very short incubation times (1-5 min) even at low peptide concentrations.

CATH-2 localizes intracellularly at sub-MIC levels

To examine if CATH-2 translocates after adherence to the bacterial surface, TEM was combined with immunogold-labeling. Bacteria incubated for 30 minutes with 2.5 μM CATH-2, mostly showed localization of the peptide at the membrane. At 5 μM CATH-2 penetrated the bacterial membrane and CATH-2 was also found in

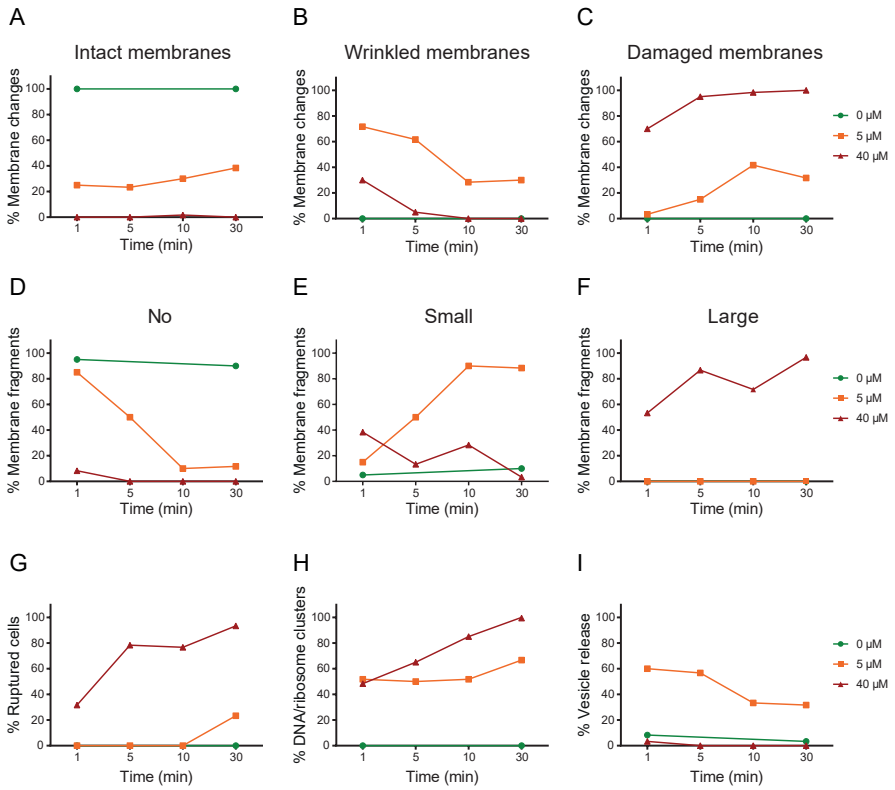

Figure 5. Time-dependent CATH-2 induced morphological changes of *E. coli*. Morphological changes were determined of two independent experiments by TEM. Sixty cells per condition were analyzed. Membranes were classified as intact (A), wrinkled (B) or damaged (C). Dissociated membrane fragments were identified as no (D), small (E) or large (F). Additionally, ruptured cells (G), DNA and ribosome clusters (H) and small vesicle release (I) were determined.

the cytoplasm. At higher CATH-2 concentrations increasing numbers of randomly distributed gold particles in the bacteria and on the bacterial membrane were observed (Fig. 6A, B and F). Compared to 5 µM, incubation of bacteria with 10 µM peptide resulted in an increased (7-fold; 1.44 gold particles/µm^2) number of intracellular electron-dense immunogold complexes and gold particles localized at the membrane (2-fold; 0.83 gold particles/µm^2). At 40 µM (MIC) and 80 µM, high numbers of gold particles (> 14 gold particles/µm^2) within the cells were seen (Fig. 6C-F).

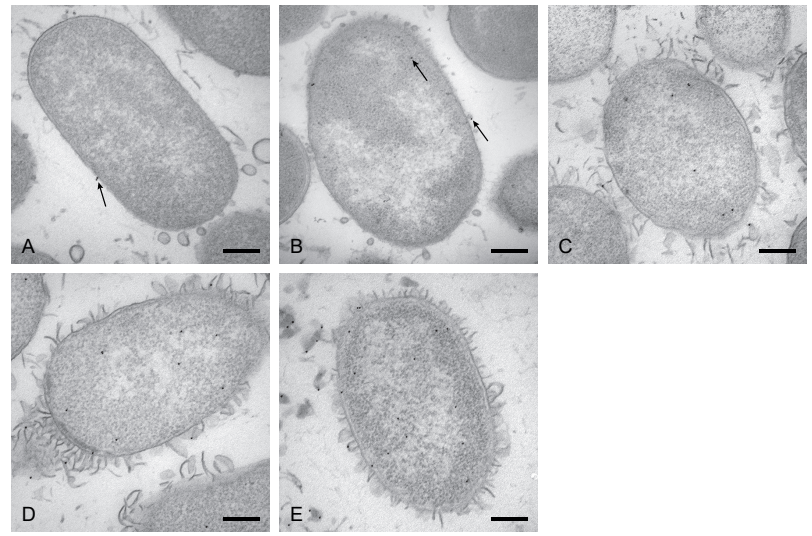

Figure 6. Localization of CATH-2 determined with immunogold labeling and quantification of gold particles. For each peptide concentration a representative TEM image is shown: 2.5 µM (A), 5 µM (B), 10 µM (C), 40 µM (D), 80 µM (E). Bars: 200 nm. The number of gold particles (black arrow) per µm^2 is divided in intracellular (grey bar) and at the intersection (membrane area; black bar) (F). The data are means ± SEM. ****P<0.0001 and *P<0.05 for intracellular vs intersection. All peptide-treated samples were corrected for background staining.

Furthermore, the kinetics of CATH-2 localization at two different peptide concentrations was studied. After a 5 minutes bacterial exposure to 5 µM peptide, CATH-2 was observed equally at the bacterial membrane or intracellularly, while at later time points (10 and 30 min) the number of intracellular electron-dense immunogold particles was strongly increased (Fig. 7 and 8A). At 40 µM, the concentration where the TEM experiments showed very rapid disruption of the bacterial membrane, immuno-electron microscopy showed that CATH-2

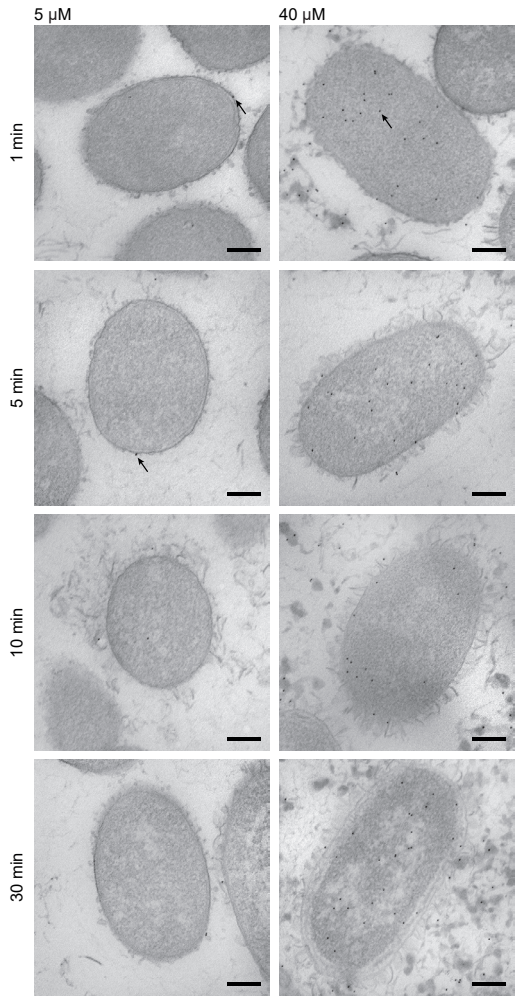

Figure 7. Localization of CATH-2 on *E. coli* as a function of time. For each time point a representative TEM image is shown: panel 5 µM and 40 µM both after 1, 5, 10 and 30 min peptide exposure. Black arrows indicate gold particles. Bars: 200 nm.

immediately enters the cell after a one minute exposure time (Fig. 7 and 8B). Longer incubation time did not further affect the CATH-2 localization indicating that at MIC values CATH-2 effects are almost instantaneous. To conclude, the TEM and immuno-TEM data combined showed that at sub-MIC level CATH-2 initially binds the bacterial membrane and then translocates intracellularly within a few minutes. This is accompanied by intracellular morphological changes of the bacterial cell as observed by redistribution of DNA and ribosomes.

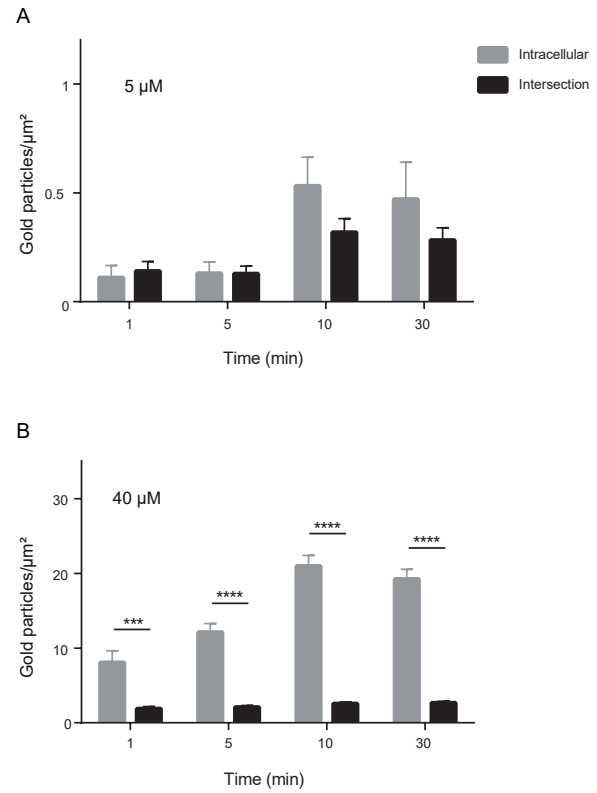

Figure 8. Time-dependent CATH-2 localization on *E. coli*. Electron-dense immunogold complexes were counted in two different concentrations, 5 µM (A) and 40 µM (B), and separated in intracellular (grey bar) and intersection (membrane, black bar). The data are means ± SEM. ****P<0.0001 and ***P<0.001 for intracellular vs intersection. All peptide-treated samples were corrected for background staining.

E. coli strain	MIC (µM)
506 (O78K80) (smooth)	10-20
ATCC 25922 (smooth)	10-20
ATCC 4157 (rough/smooth)	5
K12 (rough)	2.5-5
LMC500 (rough)	2.5-5
MC4100 (rough)	5

Table 1: MIC values of CATH-2 against *E. coli* strains with rough and smooth LPS

However, at MIC values these separate steps cannot be distinguished anymore and the observed morphological changes and CATH-2 localization seem almost immediate.

CATH-2 binds to LPS

Since the initial interaction between CATH-2 and *E. coli* is localized to the bacterial membrane, it is likely that LPS plays an important role in the early peptide localization. To determine whether differences in LPS types have an effect on the activity of CATH-2, antimicrobial activity tests of CATH-2 against several rough and smooth LPS containing *E. coli* strains were performed. MIC values of CATH-2 against *E. coli* ATCC25922 (smooth LPS) were 2-8-fold higher than rough LPS containing *E. coli* K12 strains, suggesting that CATH-2 binds to the O-antigen part of LPS (Table 1).

To determine whether differences in interaction between CATH-2 and LPS could explain the difference in killing efficiency of rough vs. smooth *E. coli*, isothermal titration calorimetry (ITC) experiments and competition assays were performed. ITC showed clear interaction between CATH-2 and smooth LPS, with an initial exothermic interaction followed by an endothermic one. Interestingly, titrations with CATH-2 into a rough LPS solution showed no indication of binding, while CATH-2 was able to interact with the even shorter Lipid A (Fig. 9A and B). Competition assays confirmed our findings, showing that the antibacterial activity of the peptide was inhibited by prior binding to smooth LPS (Fig. S2A) or Lipid A (Fig. S2B) but not to rough LPS (Fig. S2C).

DISCUSSION

In this study the antibacterial mechanism of action of CATH-2 was investigated using various imaging techniques involving confocal live-imaging and (immunogold) transmission electron microscopy. This combination of techniques, which has not been used before for mechanistic studies of antimicrobial peptides, provides a clear insight into the real-time localization and mechanism of action of CATH-2 at (sub-)MIC values.

CATH-2 was shown to have quick killing kinetics studies, as within 10 minutes bacterial killing was observed. Several studies have already shown that the bactericidal activity of peptides is usually within minutes of the first interaction with bacteria[16]. In addition, our study showed that lower peptide concentrations (1/16 MIC) result in reduced numbers of surviving bacteria, however, bacterial growth resumed after 60 minutes. These results indicated that the peptide is not bactericidal for the whole bacterial population, since surviving bacteria can overcome the peptide-induced damage and start growing again.

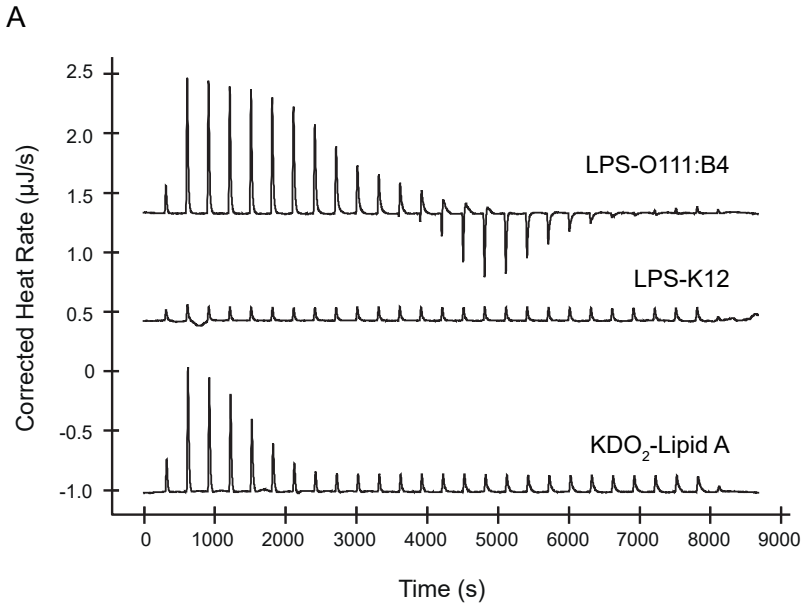

Figure 9. CATH-2 interaction with different LPS compounds. Thermograms of ITC experiments with titrations of CATH-2 into LPS-O111:B4, LPS-K12 and KDO_2-Lipid A (A). Released heat versus molar ratio of CATH-2 and LPS compounds (B).

Confocal fluorescence microscopy showed that FITC-labeled CATH-2 primary localizes at the membrane of *E. coli*, with a higher intensity of binding at the bacterial septum of dividing cells. This preferred localization of the peptide at the septum can be explained by the presence of a double membrane (and thus more binding area), but specific binding to proteins or lipids enriched in the bacterial septum cannot be excluded. Similar studies were performed with LL-37 and demonstrated that the peptide binds and permeabilizes the outer membrane first, resulting in release of periplasmic components, and then, after approximately 15-20 minutes reaches and permeabilizes the inner membrane of *E. coli*[17]. In our study, these separate steps, if present for CATH-2, could not be observed because CATH-2 seems to act much quicker with an inner membrane permeabilization of approximately one minute. Permeabilization of the bacterial membrane is a well-documented mechanism of action for many antimicrobial peptides. Several peptides, for example LL-37, magainin 2 and cecropin A, have shown to exhibit pore-forming activity by direct binding to the bacterial membrane, while other peptides permeabilize the membrane using a carpet model such as PMAP-23[18-22]. Time-lapse imaging does not allow to correlate peptide concentrations to sub-MIC or MIC values. Therefore, TEM was performed to study morphological changes of the cells after as a function of peptide concentration.

The TEM studies showed that, at MIC levels, bacteria were immediately disrupted and the peptide was localized both intracellularly and on the bacterial membranes. At this concentration (and speed of the killing process) no information about the order of events of CATH-2's antimicrobial mechanism of action could be determined. However, at sub-MIC levels these steps are more easily visualized. At 1/8 MIC, intracellular localization and morphological changes including, wrinkling of the membranes, relocalization of DNA and ribosomes, and increased vesicle release were observed. Gold particles were initially detected on the bacterial membrane and after 10 and 30 minutes immunogold complexes were also observed inside the cells without a clear disruption of the bacterial membrane. Similar findings were demonstrated with the proline-rich peptide Bac7 based on membrane permeabilization and immuno-electron microscopy. Bac7 (below MIC level) was suggested to bind to an intracellular target after entering the cell in a stereospecific manner in the absence of pore formation or membrane permeabilization[23]. In fact, the whole family of proline-rich peptides, the cell penetrating peptide P7 and buforin II are thought to act intracellularly instead of working via a lytic mechanism of action[24-30].

Our data indicated that CATH-2 quickly associates with the bacterial outer membrane, most likely with LPS. The observed LPS binding of CATH-2 was in line with previous studies demonstrating that CATH-2 neutralizes LPS induced cytokine production of PBMCs[5]. Recent studies on PMAP-23 have shown that at the MIC value of 10 µM the complete bacterial surface was covered by the peptide[31]. Considering the relatively similar peptide dimensions and MIC value

for CATH-2 compared to PMAP-23 it is anticipated that indeed a near complete (LPS) saturation of the outer membrane seems to be required for antimicrobial activity. However, the binding of CATH-2 to LPS does not necessarily contribute to the antimicrobial mechanism. It is more likely that LPS in the outer membrane is a barrier that the peptide has to overcome to reach its target, either the cytoplasmic membrane or an intracellular target or both. Similar binding effects were described for the proline-rich peptides drosocin, pyrrhocoricin and apidaecin[30]. LPS is their initial (docking) target, which plays an important role in the cell-mediated entry of these peptides to finally initialize the intracellular killing cascade[28,30,32].

Our results emphasize the importance of sub-MIC values in order to detect an antibacterial mode of action. In the case of CATH-2, at sub-MIC the peptide was not absorbed by LPS and showed membrane and intracellular changes, whereas at MIC values membrane leakage is observed after complete LPS saturation. Together, our current findings suggest that besides the membrane-permeabilizing effect of CATH-2, the peptide may trigger a killing cascade at sub-MIC values by escaping LPS. To date various antimicrobial peptides have been reported that exert a mechanism of action combining membrane and intracellular targeting[23,33-35]. Oligo-acyl-lysyl antimicrobial peptide C_{12}K-2β_{12} and Bac7 were demonstrated to bind to intracellular target(s) at sub-MIC values and exhibited a membrane-attacking mode of action at higher peptide concentrations[23,34]. These studies, together with our results, indicate that CATH-2 might exert similar dual activity on both intracellular targets and the bacterial membrane.

In conclusion, this report provides a detailed overview on the antibacterial mode of action(s) of a chicken cathelicidin using different imaging and molecular interaction studies. Visualization of the real-time attack of the peptide and the determination of peptide localization and morphological changes of the bacteria have shed light on the mechanisms of action at MIC and sub-MIC values. Based on these findings, future research on the antibacterial killing of (new) antimicrobials should not only focus on MIC values, as these concentrations will not tell the complete killing story of an antibacterial drug.

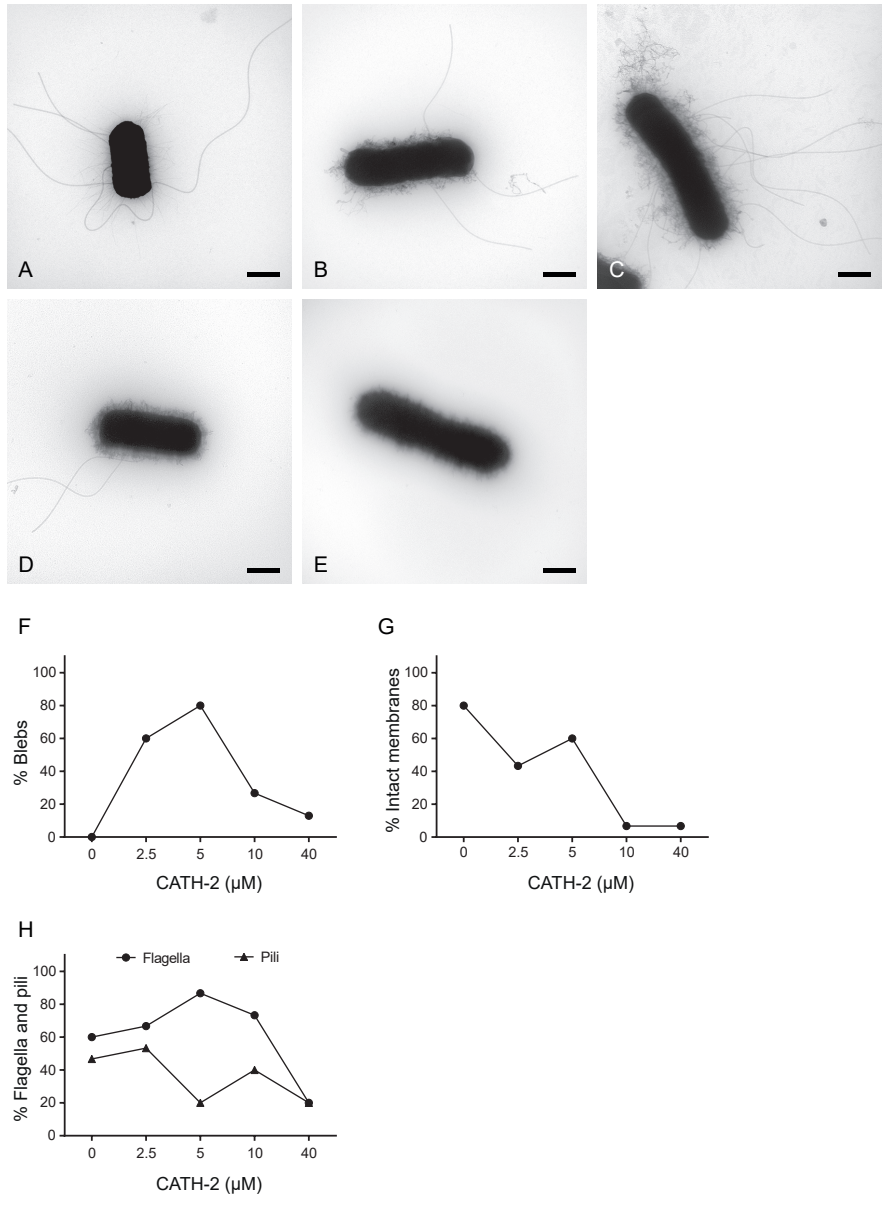

Figure S1. CATH-2 induces bleb-formation and at higher concentrations membrane damage as visualized by negative-staining TEM. 0 μM (A), 2.5 μM (B), 5 μM (C), 10 μM (D) and 40 μM (E) were incubated with *E. coli* cells for 30 min. Bars: 500 nm. Morphological changes were classified in bleb formation (F), membrane intactness (G) and amount of flagella and pili (H).

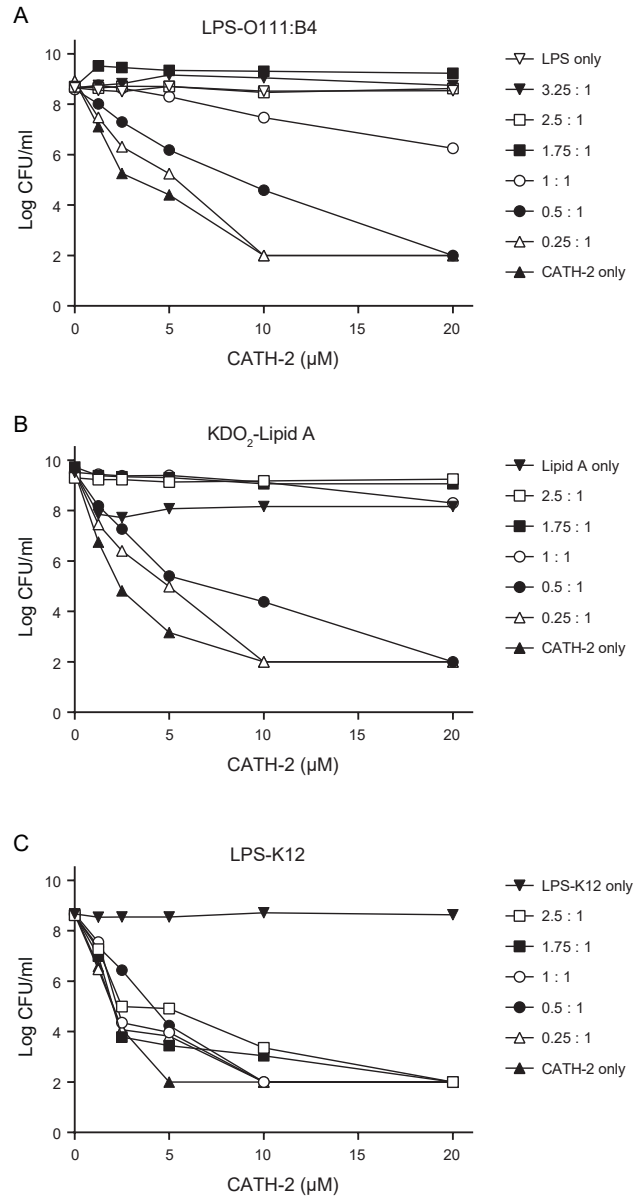

Figure S2. LPS and Lipid A inhibit CATH-2 induced killing of *E. coli.* Different ratios of LPS-O111:B4 (A), KDO_2-Lipid A (B) and LPS-K12 (C) were pre-incubated with CATH-2 for 1 hour at 37 °C. Subsequently mixtures were exposed to *E. coli* for 3 hours again at 37 °C and serially diluted and spread plated on TSA plates. Surviving colonies were counted after 16 hours. One representative figure per compound is shown (n=3).

Supplementary movies

Can be viewed at the following links:
Movie S1: https://youtu.be/rxVctQaNWkQ
Movie S2: https://youtu.be/HkTWOKNV_DY

Supplementary movie legends

Movie S1. CATH-2 binds and permeabilizes the bacterial membrane. Time-lapse imaging was performed with 0.9 µM FITC-CATH-2 and 5.1 µM PI. FITC-labeled CATH-2 (green fluorescence), PI (red fluorescence), DIC channel, and custom (merged channels). One representative cell is shown. Bars: 1 µm.

Movie S2. CATH-2 permeabilizes multiple E. coli cells. Live-imaging with FITC-labeled CATH-2 (0.9 µM) and PI (5.1 µM). Bars: 2 µm.

REFERENCES

1. Leeb, M. Antibiotics: A shot in the arm. *Nature* **431**, 892-893 (2004).
2. Centers for Disease Control and Prevention (2013) Antibiotic resistance threats in the united states, 2013. Accessed July 19, 2015; http://www.cdc.gov/drugresistance/threat-report-2013/.
3. Hancock, R. E. & Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **24**, 1551-1557 (2006).
4. Xiao, Y. *et al*. Identification and Functional Characterization of Three Chicken Cathelicidins with Potent Antimicrobial Activity. *J. Biol. Chem.* **281**, 2858-2867 (2006).
5. van Dijk, A. *et al*. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. *Mol. Immunol.* **46**, 2465-2473 (2009).
6. van Dijk, A., Veldhuizen, E. J., van Asten, A. J. & Haagsman, H. P. CMAP27, a novel chicken cathelicidin-like antimicrobial protein. *Vet. Immunol. Immunopathol.* **106**, 321-327 (2005).
7. Xiao, Y. *et al*. The Central Kink Region of Fowlicidin-2, an α-Helical Host Defense Peptide, Is Critically Involved in Bacterial Killing and Endotoxin Neutralization. *J. Innate Immun.* **1**, 268-280 (2009).
8. Brogden, K. A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **3**, 238-250 (2005).
9. Nguyen, L. T., Haney, E. F. & Vogel, H. J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* **29**, 464-472 (2011).
10. Ordonez, S. R., Amarullah, I. H., Wubbolts, R. W., Veldhuizen, E. J. & Haagsman, H. P. Fungicidal Mechanisms of Cathelicidins LL-37 and CATH-2 Revealed by Live-Cell Imaging. *Antimicrob. Agents Chemother.* **58**, 2240-2248 (2014).
11. van Eck, J. H. & Goren, E. An Ulster 2C strain-derived Newcastle disease vaccine: Vaccinal reaction in comparison with other lentogenic Newcastle disease vaccines. *Avian Pathol.* **20**, 497-507 (1991).
12. Aarsman, M. E. *et al*. Maturation of the *Escherichia coli* divisome occurs in two steps. *Mol. Microbiol.* **55**, 1631-1645 (2005).
13. van Dijk, A. *et al*. The β-Defensin Gallinacin-6 Is Expressed in the Chicken Digestive Tract and Has Antimicrobial Activity against Food-Borne Pathogens. *Antimicrob. Agents Chemother.* **51**, 912-922 (2007).
14. Slot, J. W. & Geuze, H. J. Cryosectioning and immunolabeling. *Nat. Protoc.* **2**, 2480-2491 (2007).
15. Griffiths, G. in *Fine Structure Immunocytochemistry* 413-424 (Springer, Berlin, 1993).
16. Hancock, R. E. & Lehrer, R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* **16**, 82-88 (1998).
17. Sochacki, K. A., Barns, K. J., Bucki, R. & Weisshaar, J. C. Real-time attack on single *Escherichia coli* cells by the human antimicrobial peptide LL-37. *Proc. Natl. Acad. Sci. U. S. A.* **108**, E77-81 (2011).
18. Henzler Wildman, K. A., Lee, D. K. & Ramamoorthy, A. Mechanism of Lipid Bilayer Disruption by the Human Antimicrobial Peptide, LL-37. *Biochemistry* **42**, 6545-6558 (2003).
19. Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N. & Miyajima, K. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. *Biochim. Biophys. Acta* **1327**, 119-130 (1997).

20. Rangarajan, N., Bakshi, S. & Weisshaar, J. C. Localized Permeabilization of *E. coli* Membranes by the Antimicrobial Peptide Cecropin A. *Biochemistry* **52**, 6584-6594 (2013).
21. Orioni, B. *et al*. Membrane perturbation by the antimicrobial peptide PMAP-23: a fluorescence and molecular dynamics study. *Biochim. Biophys. Acta* **1788**, 1523-1533 (2009).
22. Fernandez, D. I. *et al*. The antimicrobial peptide aurein 1.2 disrupts model membranes via the carpet mechanism. *Phys. Chem. Chem. Phys.* **14**, 15739-15751 (2012).
23. Podda, E. *et al*. Dual mode of action of Bac7, a proline-rich antibacterial peptide. *Biochim. Biophys. Acta* **1760**, 1732-1740 (2006).
24. Li, L. *et al*. A cell-penetrating peptide analogue, P7, exerts antimicrobial activity against *Escherichia coli* ATCC25922 via penetrating cell membrane and targeting intracellular DNA. *Food Chem.* **166**, 231-239 (2015).
25. Park, C. B., Kim, H. S. & Kim, S. C. Mechanism of Action of the Antimicrobial Peptide Buforin II: Buforin II Kills Microorganisms by Penetrating the Cell Membrane and Inhibiting Cellular Functions. *Biochem. Biophys. Res. Commun.* **244**, 253-257 (1998).
26. Scocchi, M., Tossi, A. & Gennaro, R. Proline-rich antimicrobial peptides: converging to a non-lytic mechanism of action. *Cell Mol. Life Sci.* **68**, 2317-2330 (2011).
27. Czihal, P. *et al*. Api88 Is a Novel Antibacterial Designer Peptide To Treat Systemic Infections with Multidrug-Resistant Gram-Negative Pathogens. *ACS Chem. Biol.* **7**, 1281-1291 (2012).
28. Kragol, G. *et al*. The Antibacterial Peptide Pyrrhocoricin Inhibits the ATPase Actions of DnaK and Prevents Chaperone-Assisted Protein Folding. *Biochemistry* **40**, 3016-3026 (2001).
29. Knappe, D. *et al*. Oncocin (VDKPPYLPRPRPPRRIYNR-NH_2): A Novel Antibacterial Peptide Optimized against Gram-Negative Human Pathogens. *J. Med. Chem.* **53**, 5240-5247 (2010).
30. Otvos, L.,Jr *et al*. Interaction between Heat Shock Proteins and Antimicrobial Peptides. *Biochemistry* **39**, 14150-14159 (2000).
31. Roversi, D. *et al*. How Many Antimicrobial Peptide Molecules Kill a Bacterium? The Case of PMAP-23. *ACS Chem. Biol.* **9**, 2003-2007 (2014).
32. Krizsan, A. *et al*. Insect-Derived Proline-Rich Antimicrobial Peptides Kill Bacteria by Inhibiting Bacterial Protein Translation at the 70S ribosome. *Angew. Chem. Int. Ed Engl.* **53**, 12236-12239 (2014).
33. Yi, T., Huang, Y. & Chen, Y. Production of an Antimicrobial Peptide AN5-1 in *Escherichia coli* and its Dual Mechanisms Against Bacteria. *Chem. Biol. Drug Des.* **85**, 598-607 (2015).
34. Makobongo, M. O., Gancz, H., Carpenter, B. M., McDaniel, D. P. & Merrell, D. S. The Oligo-Acyl Lysyl Antimicrobial Peptide C_{12}K-2β_{12} Exhibits a Dual Mechanism of Action and Demonstrates Sstrong *In Vivo* Efficacy against *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **56**, 378-390 (2012).
35. Lee, J. & Lee, D. G. Antimicrobial Peptides (AMPs) with Dual Mechanisms: Membrane Disruption and Apoptosis. *J. Microbiol. Biotechnol.* **25**, 759-764 (2015).

Chapter 3

VISUALIZATION OF THE ANTI-STAPHYLOCOCCI ACTIVITY OF CHICKEN CATHELICIDIN-2

Viktoria A.F. Schneider[a], Johanna L.M. Tjeerdsma-van Bokhoven[a], George Posthuma[b], Albert van Dijk[a], Edwin J.A. Veldhuizen[a], Henk P. Haagsman[a]

[a] Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

[b] Department of Cell Biology, Cell Microscopy Core, University Medical Center Utrecht, Utrecht, The Netherlands

In preparation

ABSTRACT

Chicken cathelicidin-2 (CATH-2) is a 26 amino acid antimicrobial host defense peptide (HDP) with potential to become a new antimicrobial agent. CATH-2 has immunomodulatory and direct killing features against a broad spectrum of Gram-positive and Gram-negative bacteria. However, its mode of action against Gram-positive bacteria is still unknown. In this study we explored the underlying antibacterial mechanism of CATH-2 against methicillin-resistant *Staphylococcus aureus* (MRSA) using live-imaging, (immuno-) transmission electron microscopy (TEM) and competition assays with bacterial ligands. These experiments showed that CATH-2 rapidly kills MRSA (within 10 minutes) and based on live-imaging analysis, it was demonstrated that CATH-2 binds bacterial membranes resulting in membrane permeabilization and cell shrinkage. Additional TEM studies showed that CATH-2 initiated pronounced morphological changes of the membrane (mesosome-formation) and ribosomal structures (clustering) in a dose-dependent manner. Immuno-labeling of these TEM sections demonstrated that CATH-2 binds to the bacterial membrane and passes the membrane at low peptide concentrations (2.5 and 5 μM). Furthermore, competition assays demonstrated that the bactericidal activity of CATH-2 is antagonized by prior binding of CATH-2 to lipoteichoic acid (LTA) and cardiolipin but not by peptidoglycan. It is concluded that CATH-2 instantly perturbs the bacterial membrane and that it subsequently enters MRSA and binds to intracellular components.

INTRODUCTION

Due to the overuse of antibiotics in the past years the numbers of antibiotic resistance pathogens are rising[1-3]. Host defense peptides (HDPs) are small effector molecules of the innate defense system and have the potential to be used as a paradigm to develop novel antimicrobials[4]. An interesting HDP is chicken cathelicidin-2 (CATH-2). CATH-2 consists of 26 amino acids, is rich in arginines and lysines and is one of the in total four identified chicken cathelicidins[5,6]. This highly cationic peptide is amphipathic (possessing spatially separated hydrophilic and hydrophobic groups) and has a proline-rich kink that was demonstrated to play an important role in the biological activity of the peptide[7]. CATH-2 exhibits different immunomodulatory properties and was shown to bind and neutralize LPS[6]. Recently it was described that the peptide also enhances the DNA-induced macrophage activation due to the increased endocytosis of DNA-CATH-2 complexes[8].

Furthermore, CATH-2 has salt-insensitive antibacterial activity and kills various types of Gram-negative and Gram-positive bacteria[5]. Recently, we tested the bactericidal effects of CATH-2 on Gram-negative *E. coli*, and could demonstrate that CATH-2 has distinctive effects on these bacteria at concentrations that do not immediately kill the bacteria (Chapter 2). However the mechanism of action of CATH-2 against Gram-positive bacteria has not been studied yet. Gram-positive bacteria have a thick peptidoglycan layer and a membrane, with lipoteichoic acids (LTA) and phospholipids[9,10]. Due to the positive charge of antimicrobial peptides these negatively charged membrane components induce electrostatic interactions and likely initiate the first interaction between membrane and peptide. Different antibacterial modes of action of HDPs have been described including membrane disruption or killing by inhibition of important cell functions (protein folding, nucleic acid and protein synthesis)[11-13].

This study focuses on the antibacterial activity of CATH-2 against the methicillin-resistant *Staphylococcus aureus* (MRSA). Based on various competition assays and different imaging techniques, involving live-imaging confocal microscopy and (immuno-) transmission electron microscopy (TEM) the antibacterial actions of CATH-2 were studied. Taken together, our findings suggest that CATH-2 binds and permeabilizes the membrane of MRSA and at sub-minimal inhibitory concentrations (sub-MIC) induces membrane ruffling and intracellular morphological changes including ribosomal structural changes.

MATERIALS AND METHODS

Peptide
CATH-2 was obtained from CPC Scientific Inc. (Sunnyvale, USA) and fluorescently labeled CATH-2 was prepared as described previously[14].

Colony counting assays
The antibacterial activity of CATH-2 was assessed by performing colony counting assays. MRSA WKZ-2 (human isolate) was grown to mid-logarithmic phase in Tryptone Soy Broth (TSB; Oxoid Limited, Hampshire, United Kingdom) and after a wash in TSB, diluted to 2 x 10^6 CFU/ml. Cells were exposed to different CATH-2 concentrations, ranging from 0 to 40 µM, for 3 hours at 37 °C. Subsequently, mixtures were serially diluted and spread plated on Tryptone Soy Agar (TSA; Oxoid Limited) petri dishes as described previously[15]. Surviving bacteria were counted after incubating the plates for 16 hours at 37 °C.

Time killing kinetics
The speed of killing of CATH-2 against MRSA was determined based on time kinetics assays. For this, 550 µl peptide (0.07-10 µM) was added to an equal volume of bacterial cells (2 x 10^6 CFU/ml) at 37 °C for several time intervals: 1, 5, 10, 20, 30, 60, 120 and 180 min. At each time point, 100 µl was immediately spread plated on TSA plates and 20 µl was serially diluted in minimum TSB medium (water containing 1/1000 TSB) and 100 µl of each dilution was plated on TSA. After 16 hours at 37 °C surviving bacteria were counted.

Live-imaging
Live-imaging was performed to visualize the action of CATH-2 on MRSA. MRSA cells grown to mid-logarithmic stage were washed and resupended in 1 ml 5 mM HEPES buffer (pH 7.4, Sigma-Aldrich, Zwijndrecht, The Netherlands). Low-melting agarose at 1% (30 µl; Agarose Type I low EEO, Sigma-Aldrich) were added in the middle of silanized coverslips (Gerhard Menzel GmbH, Braunschweig, Germany), which were previously prepared with 2% (3-Aminopropyl) triethoxysilane (SAA; Sigma-Aldrich) in acetone. Thirty-microliter of MRSA suspension was added and resuspended in the agarose drop. A sandwich was made by adding a second silanized coverslip on top of the first one. After 2 min the second coverslip was removed and a homogenous agarose-bacteria layer was formed. Coverslips were fixed in an Attofluor cell chamber, 450 µl HEPES buffer and the permeability marker propidium iodide (PI; 2.5 µM, final concentration) was added. When cells were focused, the movie was started and FITC-CATH-2 (0.7 µM final concentration) was added. Time-lapse imaging was performed at a Nikon A1R and at the Leica SPE-II at the Center for Cell Imaging (CCI) at the Faculty of Veterinary Medicine in Utrecht. Fluorescence of FITC-CATH-2 and PI were detected with a Sapphire Blue

Coherent laser (488 nm) and a Sapphire Yellow Coherent laser (561 nm). For data analysis the NIS-Elements software of Nikon was used.

Transmission electron microscopy
Transmission electron microscopy (TEM) was used to study the morphological changes induced by CATH-2 in detail. CATH-2 was incubated at various concentrations (0, 2.5, 5, 10, 40 and 80 µM) against MRSA WKZ-2. Due to the different experimental set-up (5 x 10^8 CFU/ml required) the MIC values were determined at this bacterial density (MIC = 40 µM). For TEM, peptide and bacteria were incubated for 30 min at 37 °C, and subsequently reactions were stopped by adding fixation buffer containing 2% glutaraldehyde (Polysciences, Eppelheim, Germany), 5 mM $CaCl_2$, 10 mM $MgCl_2$ (both Merck, Darmstadt, Germany) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich) pH 7.4. After overnight fixation at 4 °C, samples were washed for 3 x 10 min in 0.1 M sodium cacodylate buffer. Samples were embedded in 4% low melting point agarose (Sigma-Aldrich) and subsequently post-fixed with 4% osmium tetroxide (Electron Microscopy Sciences; EMS, Hatfield, USA) 1.5% $K_4Fe(CN)_6$-$3H_2O$ (Merck) in distilled water for 2 hours at 4 °C. Again, samples were rinsed for 3 x 10 min and stained with uranylacetate for 1 hour at 4 °C. Subsequently, samples were extensively washed (5 x 10 min) and embedded in resin. Fifty nanometer thick sections were prepared and mounted on 100 mesh copper Formvar-carbon coated grids and stained with uranyl acetate and lead citrate based on a Leica AC20 system (Leica, Vienna, Austria) Subsequently, cells were visualized on a FEI Tecnai 12 electron microscope (FEI, Eindhoven, The Netherlands) at 80 kV.

Immunogold TEM
In order to determine the peptide localization of CATH-2 on/in bacteria, the same Epon blocks, previously used for the morphology determination, were used. Sections (50 nm) mounted on Formvar coated copper grids were incubated with PBS containing 0.5% fish skin gelatine (Sigma-Aldrich) and 0.1% Bovine Serum Albumin-c™ (AURION, Wageningen, The Netherlands) and immuno-labeled as described earlier[16]. Subsequently, each section was incubated with the CATH-2 antibody for 1 hour at RT. After rinsing (5 x 2 min) with PBS, specimens were exposed to protein-A gold (10 nm, Department of Cell Biology, University Medical Center Utrecht, The Netherlands) for 20 min at RT. Finally, sections were stained with 2% uranyloxalicacetate (pH 7; SPI, West Chester, USA) for 5 min at RT and incubated with methylcellulose-uranyl acetate (pH 4; 2% methylcellulose [Sigma-Aldrich] and 4% uranylacetate [SPI] in distilled water).

Morphological changes and peptide localization were determined using a total of 60 cells, which were randomly selected, from two independent experiments. Gold particles localization was classified in three different positions: intracellular, membrane and peptidoglycan layer, according to Griffiths[17]. All peptide-exposed

samples were corrected for background staining (results from control non-peptide treated cells).

Competition assays

Inhibition of the bactericidal activity of CATH-2 by different membrane components of *S. aureus* was determined using competition assay. For this, the cardiolipin (Sigma-Aldrich), lipoteichoic acid from *S. aureus* and peptidoglycan from *S. aureus* (LTA-SA and PG-SA respectively, both obtained from Invivogen, Toulouse, France) were pre-incubated at different ratios (10:1-0.5:1) with CATH-2 for 1 hour at 37 °C. To determine, whether binding inhibited the bactericidal activity of the peptide, the mixtures were exposed to bacterial cultures, as described in the colony counting section. The following day survival bacteria were counted.

Statistical analysis

For statistical analysis SPSS 22 software (IBM, Armonk, USA) was used and differences between concentrations were assessed using non-parametric Kruskal-Wallis test, as data were non-normally distributed.

RESULTS

CATH-2 rapidly kills MRSA

The bactericidal activity of CATH-2 against MRSA was tested based on antibacterial colony counting assays. Our results showed that the minimal inhibitory concentration (MIC) of CATH-2 against MRSA (at 2 x 10^6 CFU/ml) was 5 μM (data not shown). Additional time point killings demonstrated that the peptide rapidly kills the bacteria as 10 μM of the peptide required 10 minutes to kill all bacteria. Due to the different experimental-set up compared to a conventional colony counting assay, the MIC value was 1.25 μM, showing complete bacterial killing at 60 minutes (Fig. 1). Interestingly, low peptide concentrations (0.6 μM) hampered the bacterial growth, however cells recovered and started to re-grow after 60 minutes.

CATH-2 targets the septum and permeabilizes the membrane

To visualize the effect of CATH-2 on bacteria, live-imaging with fluorescently labeled CATH-2 was performed. The peptide interacted with bacteria, with strong binding preference to the septum indicated by higher local fluorescence intensity. Subsequently, binding was followed by permeabilization of the cell leading to PI-binding to bacterial DNA. Simultaneously, bacterial shrinkage occurred, as was visible because cells were out of focus and lost their round structures (Movie S1 and S2; Fig. 2A). Compared to the start of the experiment

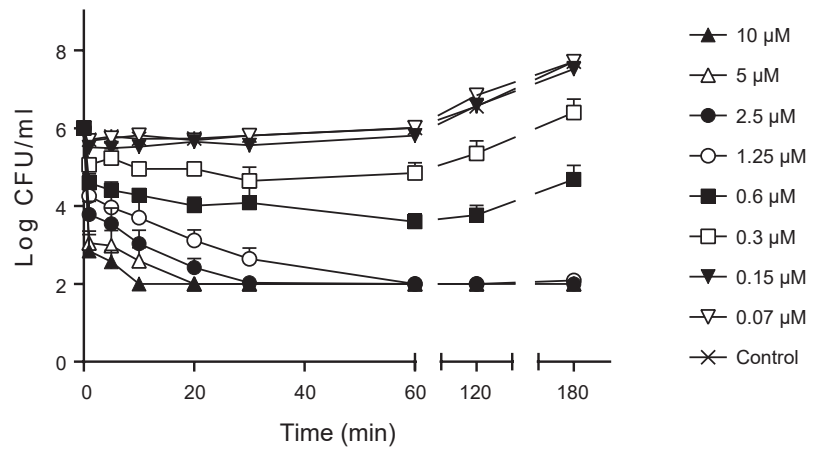

Figure 1. CATH-2 quickly kills MRSA. MRSA was incubated with CATH-2 at 37 °C (1, 5, 10, 30, 60, 120 and 180 min). Aliquots were taken, serially diluted and plated. After 16 hours at 37 °C viable bacteria were counted. Data represent three independent measurements (means ± SEM).

(0 sec), peptide treatment caused 0.15 μm shrinkage of the cells at 807 seconds (Fig. 2A). Single cell analysis was carried out to determine fluorescence intensity levels and showed first CATH-2 binding and after a few minutes intracellular PI (Fig. 2B). In addition, heat intensity plots confirmed the binding of the peptide especially at the bacterial septum (Fig. 2C).

CATH-2 causes membrane ruffling and ribosome clustering

The concentration-dependent effects of CATH-2 were determined by performing TEM studies. Representative pictures of the observed morphological changes at each CATH-2 concentration are shown in Fig. 3A-F, while quantification of the morphological changes are shown in Fig. 3G-J. At the lowest concentration used (2.5 μM), the peptidoglycan layers became fuzzy and the membrane started ruffling. Furthermore, structural changes of the ribosomes were observed compared to the untreated control cells (Fig. 3B and G-J). With 5 μM and 10 μM peptide exposure 20% and 40% of the cells showed major membrane ruffling, respectively and minor ribosome clustering was observed (Fig. 3C and D, Fig. 3H and I). Additionally at 10 μM, CATH-2 dissociated small bacterial fragments were found, which became larger and thicker at higher concentrations (Fig. 3J). At MIC and 2 x MIC (40 and 80 μM, respectively) pronounced effects on the cell wall were observed due to the major invaginations of the membrane (Fig. 3H). Inside bacteria the clustering of the ribosomes became stronger (Fig. 3I). Taken together these results suggest that CATH-2 attacks the membrane and induces intracellular changes.

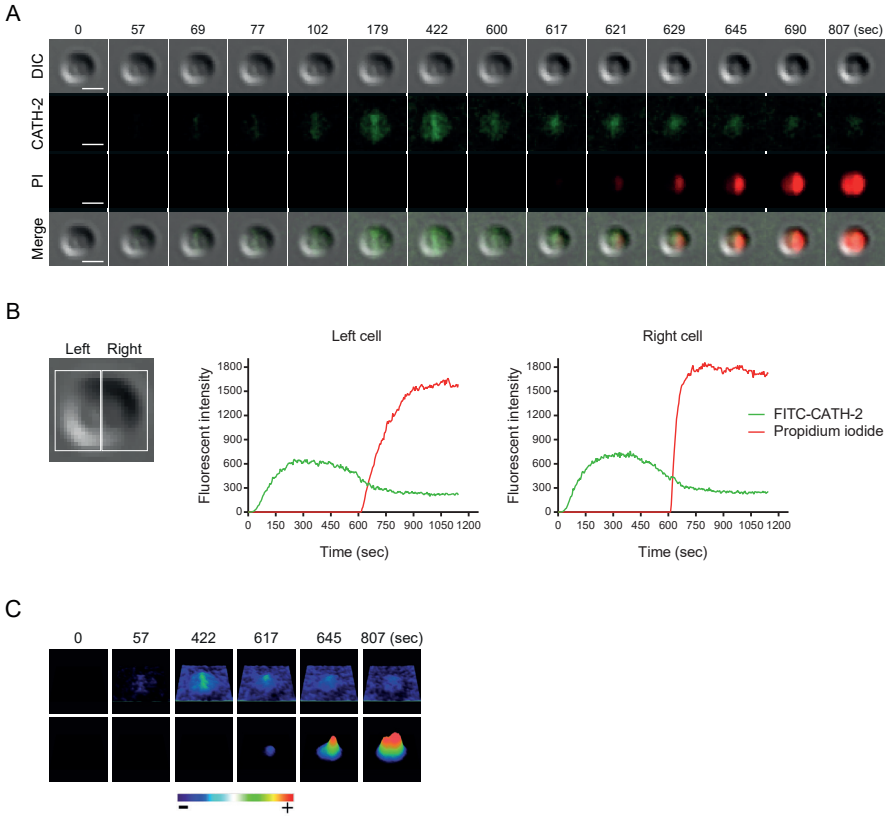

Figure 2. Time-lapse imaging demonstrates the CATH-2 binding and permeabilization of membranes. Peptide binding, entry of PI and cell shrinkage are shown at various time points. Bars: 1 µm (A). Fluorescence intensity of FITC-CATH-2 (0.7 µM) and PI (2.5 µM) was measured in a dividing cell (shown as left and right cell) (B). Heat intensity plots demonstrate the specific binding sites of FITC-CATH-2 and PI (C).

CATH-2 binds the membrane and enters the cells in high numbers at sub-MIC

To obtain more insights on the mechanism of CATH-2 against MRSA, immunogold labeling of the TEM sections with a CATH-2 specific antibody was performed (Fig. 4A-F). Interestingly, at a concentration as low as 2.5 µM, considerable numbers of gold particles were detected intracellularly and this accumulation strongly increased at higher peptide concentrations. At this concentration some binding of CATH-2 to the peptidoglycan layer and the membrane was observed (Fig. 4A and F). With increasing concentrations CATH-2 was shown to preferentially bind to the membrane. At each concentration tested the number of gold particles on membranes was always two-fold higher compared to the number of particles on the peptidoglycan layer (Fig. 4F). At 10 µM high numbers of electron-dense

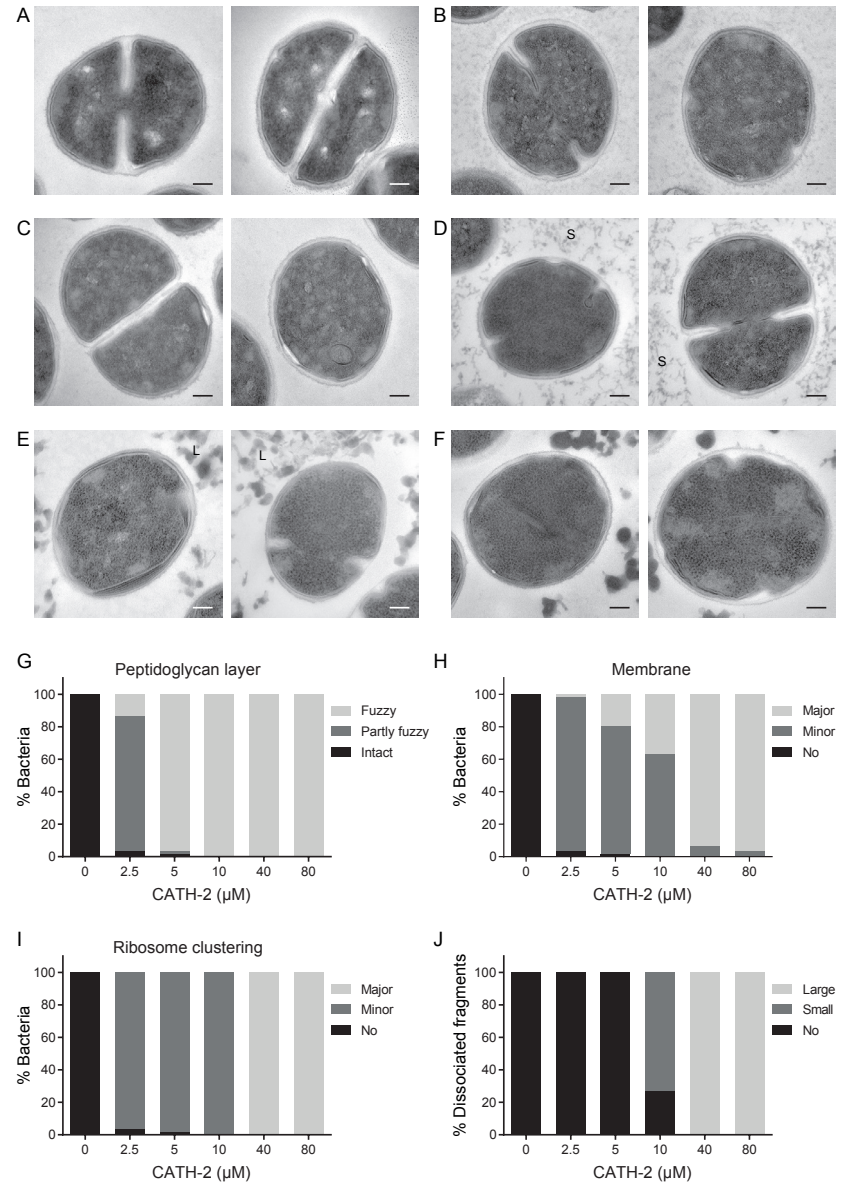

Figure 3. CATH-2 induced morphological changes of MRSA determined by TEM. Two representative images are shown for 0 µM (A), 2.5 µM (B), 5 µM (C), 10 µM (D), 40 µM (E) and 80 µM (F). S, small dissociated fragments; L, large dissociated fragments. Bars: 100 nm. Morphological changes were classified in peptidoglycan layer (G) and membrane ruffling (H). Additionally, morphological changes were categorized in ribosomal changes (I) and dissociated fragments (J).

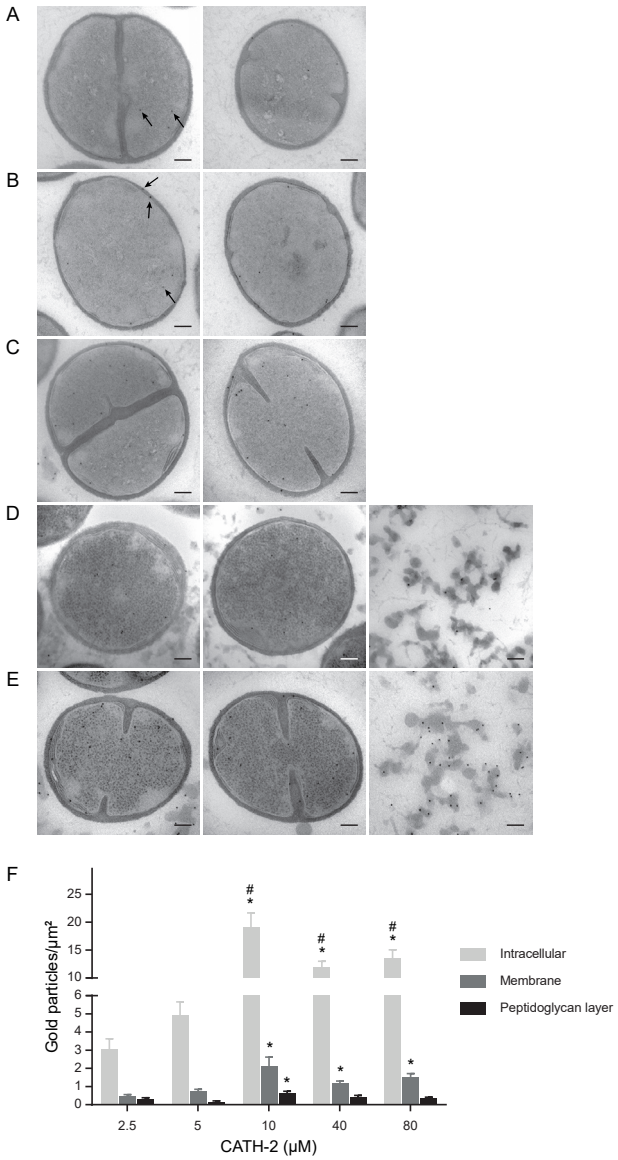

Figure 4. Localization of CATH-2 determined with immunogold labeling and quantification of gold particles. For each peptide concentration two representative TEM image is demonstrated: 2.5 µM (A), 5 µM (B), 10 µM (C), 40 µM (D), 80 µM (E). Bars: 100 nm. The number of gold particles (black arrow) per µm² is divided in intracellular, membrane and peptidoglycan layer (F). The data are means ± SEM. * indicates significant differences with 2.5 µM (P<0.001). # indicates significant differences with 5 µM (P<0.001). All peptide-treated samples were corrected for background staining.

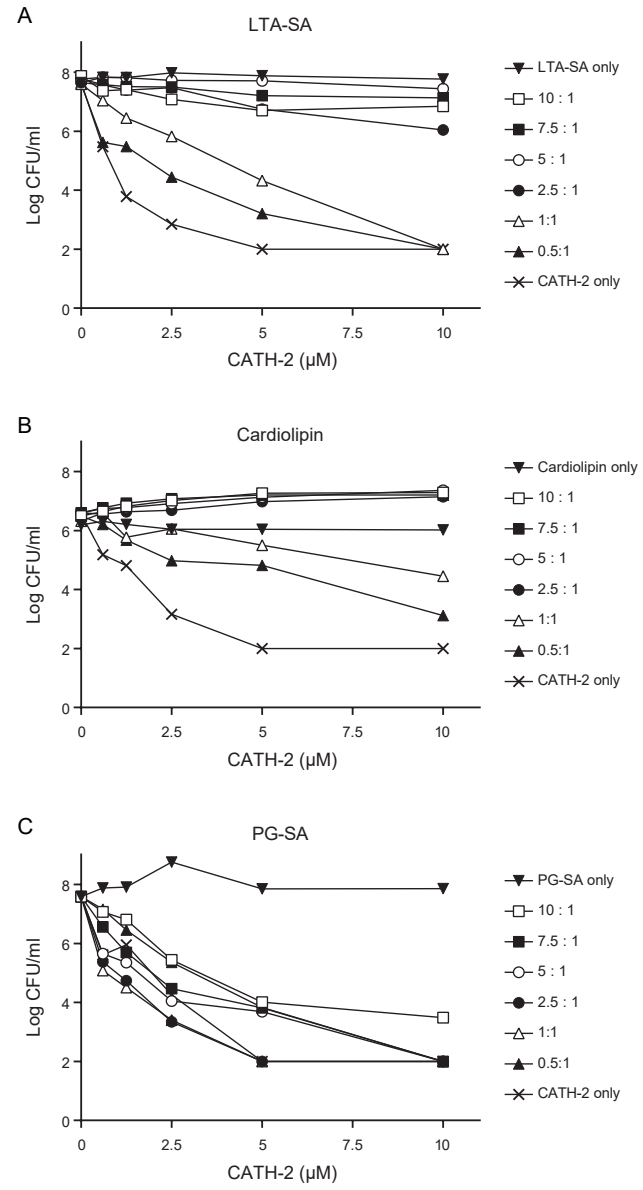

Figure 5. LTA and cardiolipin inhibit CATH-2 induced killing of MRSA. CATH-2 was pre-incubated with various ratios of LTA-SA (A), cardiolipin (B) and PG-SA (C) for 1 hour at 37 °C. Then mixtures were exposed to MRSA for 3 hours again at 37 °C and serially diluted and spread plated on TSA plates. Viable colonies were counted after 16 hours. One representative figure per compound is demonstrated (n=3).

immunogold complexes were observed intracellularly (19.1 gold particles/μm^2); however, these numbers decreased again with higher concentrations (11.8 gold particles/μm^2 and 13.6 gold particles/μm^2 for 40 and 80 µM, respectively) (Fig. 4C-F). Instead, at MIC levels and higher concentrations (≥ 40 µM) of CATH-2 high numbers of gold particles were present on the dissociated fragments, which were present throughout the section (Fig. 4D and 4E, third picture). These fragments are probably cytosolic remnants explaining why lower numbers of cytosolic gold particles were observed at 40 µM and 80 µM, compared to 10 µM (cell leakage).

Taken together, these electron microscopy studies demonstrated that at sub-MIC levels CATH-2 binds to the membrane and localizes intracellularly where it induces ribosomal changes and lamellar mesosome-like structures.

LTA and cardiolipin inhibit the antibacterial activity of CATH-2

To determine the potential interaction of CATH-2 to different cell wall components, competition assays were performed. Peptidoglycan, LTA and cardiolipin were pre-incubated in different ratios with CATH-2 peptide, and subsequently the antibacterial activity of the peptide was tested against MRSA. LTA and cardiolipin both showed strong peptide-binding, considering that already a ratio of 0.5:1 resulted in inhibition of the bactericidal activity of the peptide (Fig. 5A and B). In contrast, prior incubation of CATH-2 with peptidoglycan only showed a weak reduction in the killing activity of the peptide (Fig. 5C). These results correspond with our previous findings in the TEM study where relatively low binding of CATH-2 to peptidoglycan was observed.

DISCUSSION

Previously, we demonstrated the antibacterial mode of action of CATH-2 in *E. coli* cells (Chapter 2). However, in order to study new broad-spectrum antibacterial compounds both bacteria classes should be studied, as the mode of action of a peptide in Gram-negative bacteria might not resemble the actions of a peptide in Gram-positive bacteria and vice versa. The present study demonstrates for the first time the antibacterial mode of action of chicken CATH-2 against Gram-positive bacteria. On the basis of various imaging methods and competition assays, our data indicated that the peptide binds and folds the membrane. However, at low peptide concentrations, CATH-2 enters the cytosol and induces morphological changes of ribosomes.

CATH-2 is an important host defense peptide of the avian immune system and was shown to exhibit antibacterial activity against different bacterial pathogens (e.g. *Salmonella*, ESBL-producing *Enterobacteriaceae*, *Bacillus* and *Campylobacter*)[6,18-20]. Furthermore, earlier studies demonstrated that the peptide rapidly kills *Salmonella enteritidis*[6] and *E. coli* (Chapter 2). These

results were in line with the current findings as after a 10 minute exposure with 10 µM CATH-2 all MRSA cells were killed. Additionally, this study demonstrated that bacteria incubated with low peptide concentrations were initially suppressed in their survival, however recovered after 60 minutes and efficiently replicated again.

To elucidate the mechanism of action of CATH-2 live-cell imaging was performed on MRSA cells. Our data demonstrated the real-time binding of CATH-2 to the bacterial cell wall with higher binding intensities at bacterial septa, subsequently resulting in cell shrinkage and permeabilization of the cells due to PI entry. To our knowledge this is the first study using Staphylococci cells for live-imaging purposes. To date only one Gram-positive bacterium was used to study the real-time attack of an antimicrobial HDP. In this study Barns and colleagues demonstrated that LL-37, the only known human cathelicidin, shrinks and permeabilizes *Bacillus subtilis.* Bacterial shrinkage was suggested to occur due to a disorder of turgor pressure and membrane potential, induced by membrane permeabilization[21]. These results are in line with our findings and similar to other experiments in our group showing the permeabilization and shrinkage of *Bacillus globigii* and *subtilis* during CATH-2 exposure (unpublished data). The concentrations used in the current study were only for kinetic purposes and did not resemble sub-MIC or MIC levels. Therefore, no conclusions can be drawn on the speed of killing as doubling the peptide concentration resulted in quick membrane permeabilization, due to complete peptide coverage of coverslip and the focused cells.

Our TEM studies showed that already at 2.5 µM CATH-2 most morphological changes took place and became more pronounced with increasing concentrations. Two main effects of CATH-2 were the ruffling of membrane and the structural changes of the ribosomes. Similar morphological changes on the membrane have been demonstrated with Candida albicans. In these experiments CATH-2 treatment triggered the fungal plasma membrane detachment from the cell wall and the formation of pockets[14]. The membrane of Gram-positive bacteria has been shown to play a distinctive role in the antibacterial mechanism of action of antimicrobial peptides. Several studies have revealed the formation of mesosomes (membrane inclusions and folding) due to peptide exposure[22-26]. Studies on rat defensins and human neutrophil defensin-1 (HNP-1) showed that these peptides (600 µg/ml) target the membranes of *S. aureus* and induce lamellar mesosome formation. Interestingly, the exposure to higher peptide concentrations (3.2 mg/ml; close to MIC values) did not enhance the production of these mesosomal structures in *S. aureus*[22]. These results are in contrast with our findings, which show a CATH-2 concentration-dependent effect on the formation of mesosomes. At low peptide concentrations punctual invaginations were observed, whereas incubating bacteria with MIC values resulted in major lamellar mesosome formation as observed. The different results obtained with

defensins can have technical reasons or a different mode of action of these peptides.

The observed morphological changes correlate with the observed intracellular and membrane binding of the peptide at sub-MIC values. Comparable findings were obtained with small (~15 amino acids) alpha-helical peptides (MAw-1) by Azad and colleagues[27]. These peptides were shown to cause deformation of the membrane, the peptidoglycan layer and the outer acidic surfaces. Based on immuno-labeling of the TEM sections it was observed that the peptides are localized next to the induced membrane folds, suggesting that these membrane folds are aggregates of earlier produced toroid-like pores[27]. This situation might also happen for CATH-2, as in lower peptide concentrations similar peptide-bound folds were detected.

Further TEM studies demonstrated that at 2.5 µM the peptide induced a fuzzy peptidoglycan layer. However, competition assays and immunogold-TEM studies demonstrated that the peptidoglycan layer is only poorly interacting with the CATH-2 peptide, suggesting that peptidoglycan plays a minor role in the antibacterial mode of action of the peptide. Additional competition assays on LTA and cardiolipin showed that antibacterial activity of CATH-2 was strongly inhibited due to a pre-incubation of the peptide with both compounds, indicating a preference of the peptide to negatively charged membrane components. Different antimicrobial peptides have shown to possess similar LTA and cardiolipin binding properties[28-30].

This study provides important insights in the mechanism of action of CATH-2 on MRSA. Thereby, we demonstrated for the first time the combined use of time-lapse imaging and concentration-dependent (immune-) TEM studies of an antimicrobial HDP on MRSA. CATH-2 was found to act by a membrane-perturbing mechanism possibly due to the interaction with negatively charged membrane components such as LTA and cardiolipin, resulting in the formation of lamellar mesosomes and membrane permeabilization. However, the targeting of intracellular components at sub-MIC levels that results i.a. in ribosome clustering may contribute in killing of bacteria.

Supplementary movies

Can be viewed at the following links:
Movie S1: https://youtu.be/SaT9hguBpBI
Movie S2: https://youtu.be/tDaLGS2020g

Supplementary movie legends

Movie S1. CATH-2 instantly binds the bacterial septum and permeabilizes the membrane. MRSA live-imaging was performed using 0.7 µM FITC-CATH-2 and 2.5 µM PI. DIC channel, FITC-labeled CATH-2 (green fluorescence), PI (red fluorescence) and merged channels are demonstrated. One representative cell is shown. Bars: 500 nm.

Movie S2. CATH-2 permeabilizes multiple MRSA cells. Live-imaging with FITC-labeled CATH-2 (0.7 µM) and PI (2.5 µM). Bars: 1 µm.

REFERENCES

1. Cully, M. Public health: The politics of antibiotics. *Nature* **509**, S16-17 (2014).
2. Martin, M. J., Thottathil, S. E. & Newman, T. B. Antibiotics Overuse in Animal Agriculture: A Call to Action for Health Care Providers. *Am. J. Public Health* **105**, 2409-2410 (2015).
3. World Health Organization (2015) Antibiotic resistance, April 2015. Accessed, September 17, 2015; http://www.who.int/mediacentre/factsheets/fs194/en/.
4. Hancock, R. E. & Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **24**, 1551-1557 (2006).
5. Xiao, Y. *et al*. Identification and Functional Characterization of Three Chicken Cathelicidins with Potent Antimicrobial Activity. *J. Biol. Chem.* **281**, 2858-2867 (2006).
6. van Dijk, A. *et al*. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. *Mol. Immunol.* **46**, 2465-2473 (2009).
7. Xiao, Y. *et al*. The Central Kink Region of Fowlicidin-2, an α-Helical Host Defense Peptide, Is Critically Involved in Bacterial Killing and Endotoxin Neutralization. *J. Innate Immun.* **1**, 268-280 (2009).
8. Coorens, M., van Dijk, A., Bikker, F., Veldhuizen, E. J. & Haagsman, H. P. Importance of Endosomal Cathelicidin Degradation To Enhance DNA-Induced Chicken Macrophage Activation. *J. Immunol.* **195**, 3970-3977 (2015).
9. Shockman, G. D. & Barrett, J. F. Structure, function, and assembly of cell walls of gram-positive bacteria. *Annu. Rev. Microbiol.* **37**, 501-527 (1983).
10. Epand, R. M. & Epand, R. F. Lipid domains in bacterial membranes and the action of antimicrobial agents. *Biochim. Biophys. Acta* **1788**, 289-294 (2009).
11. Brogden, K. A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **3**, 238-250 (2005).
12. Jenssen, H., Hamill, P. & Hancock, R. E. Peptide Antimicrobial Agents. *Clin. Microbiol. Rev.* **19**, 491-511 (2006).
13. Nguyen, L. T., Haney, E. F. & Vogel, H. J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* **29**, 464-472 (2011).
14. Ordonez, S. R., Amarullah, I. H., Wubbolts, R. W., Veldhuizen, E. J. & Haagsman, H. P. Fungicidal Mechanisms of Cathelicidins LL-37 and CATH-2 Revealed by Live-Cell Imaging. *Antimicrob. Agents Chemother.* **58**, 2240-2248 (2014).
15. van Dijk, A. *et al*. The β-Defensin Gallinacin-6 Is Expressed in the Chicken Digestive Tract and Has Antimicrobial Activity against Food-Borne Pathogens. *Antimicrob. Agents Chemother.* **51**, 912-922 (2007).
16. Slot, J. W. & Geuze, H. J. Cryosectioning and immunominuteing. *Nat. Protoc.* **2**, 2480-2491 (2007).
17. Griffiths, G. in *Fine Structure Immunocytochemistry* 413-424 (Springer, Berlin, 1993).
18. Veldhuizen, E. J., Brouwer, E. C., Schneider, V. A. & Fluit, A. C. Chicken Cathelicidins Display Antimicrobial Activity against Multiresistant Bacteria without Inducing Strong Resistance. *PLoS One* **8**, e61964 (2013).
19. van Dijk, A. *et al*. *Campylobacter jejuni* is highly susceptible to killing by chicken host defense peptide cathelicidin-2 and suppresses intestinal cathelicidin-2 expression in young broilers. *Vet. Microbiol.* **160**, 347-354 (2012).

20. Molhoek, E. M. *et al*. Chicken cathelicidin-2-derived peptides with enhanced immunomodulatory and antibacterial activities against biological warfare agents. *Int. J. Antimicrob. Agents* **36**, 271-274 (2010).
21. Barns, K. J. & Weisshaar, J. C. Real-time attack of LL-37 on single *Bacillus subtilis* cells. *Biochim. Biophys. Acta* **1828**, 1511-1520 (2013).
22. Shimoda, M., Ohki, K., Shimamoto, Y. & Kohashi, O. Morphology of Defensin-Treated *Staphylococcus aureus*. *Infect. Immun.* **63**, 2886-2891 (1995).
23. Hartmann, M. *et al*. Damage of the Bacterial Cell Envelope by Antimicrobial Peptides Gramicidin S and PGLa as Revealed by Transmission and Scanning Electron Microscopy. *Antimicrob. Agents Chemother.* **54**, 3132-3142 (2010).
24. Friedrich, C. L., Moyles, D., Beveridge, T. J. & Hancock, R. E. Antibacterial Action of Structurally Diverse Cationic Peptides on Gram-positive Bacteria. *Antimicrob. Agents Chemother.* **44**, 2086-2092 (2000).
25. Rabanal, F. *et al*. A bioinspired peptide scaffold with high antibiotic activity and low in vivo toxicity. *Sci. Rep.* **5**, 10558 (2015).
26. Friedrich, C. L., Rozek, A., Patrzykat, A. & Hancock, R. E. Structure and Mechanism of Action of an Indolicidin Peptide Derivative with Improved Activity against Gram-positive Bacteria. *J. Biol. Chem.* **276**, 24015-24022 (2001).
27. Azad, M. A., Huttunen-Hennelly, H. E. & Ross Friedman, C. Bioactivity and the First Transmission Electron Microscopy Immunogold Studies of Short *De Novo*-Designed Antimicrobial Peptides. *Antimicrob. Agents Chemother.* **55**, 2137-2145 (2011).
28. Scott, M. G., Gold, M. R. & Hancock, R. E. Interaction of Cationic Peptides with Lipoteichoic Acid and Gram-Positive Bacteria. *Infect. Immun.* **67**, 6445-6453 (1999).
29. Scheinpflug, K., Krylova, O., Nikolenko, H., Thurm, C. & Dathe, M. Evidence for a Novel Mechanism of Antimicrobial Action of a Cyclic R-,W-Rich Hexapeptide. *PLoS One* **10**, e0125056 (2015).
30. Zhang, L., Rozek, A. & Hancock, R. E. Interaction of Cationic Antimicrobial Peptides with Model Membranes. *J. Biol. Chem.* **276**, 35714-35722 (2001).

Chapter 4

PROPHYLACTIC ADMINISTRATION OF CHICKEN CATHELICIDIN-2 BOOSTS ZEBRAFISH EMBRYONIC INNATE IMMUNITY

Viktoria A.F. Schneider[a], Albert van Dijk[a], Astrid M. van der Sar[b], Marina D. Kraaij[a], Edwin J.A. Veldhuizen[a], Henk P. Haagsman[a]

[a] Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[b] Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, The Netherlands

Developmental & Comparative Immunology (2016) **60**; 108–114

ABSTRACT

Chicken cathelicidin-2 (CATH-2) is a host defense peptide that exhibits immunomodulatory and antibacterial properties. Here we examined effects of CATH-2 in zebrafish embryos in the absence and presence of infection. Yolk-injection of 0.2-1.5 hours post-fertilized (hpf) zebrafish embryos with 2.6 ng/kg CATH-2 increased proliferation of phagocytic cells at 48 hpf by 30%. A lethal infection model was developed to test the prophylactic protective effect of CATH-2 peptide. Embryos (0.2-1.5 hpf) were injected with 2.6 ng/kg CATH-2, challenged with a lethal dose of fluorescently labeled *Salmonella enteritidis* pGMDs3 at 28 hpf and monitored for survival. Prophylactic treatment with CATH-2 was found to delay infection starting at 22 hours post-infection (hpi). At 18-20 hpi, significantly lower (two-fold) fluorescence intensity and decreased bacterial loads were detected in peptide-treated embryos. Thus prophylactic administration of low CATH-2 concentrations confer partial protection in zebrafish embryos by boosting the innate immune system.

INTRODUCTION

Host defense peptides (HDP) are key players in the primary defense of the innate immune system and are considered to have potential as new broad-spectrum antimicrobial agents[1]. Chicken cathelicidin-2 (CATH-2) is one of the four cathelicidins known in chicken[2-4]. CATH-2 is a short (26 amino acids), cationic peptide with amphipathic properties that has been shown to exhibit antibacterial activity at micromolar level[5,6]. Besides its microbicidal activity, this peptide is gaining considerable interest due to its immunomodulatory properties. *In vitro* it has been demonstrated that CATH-2 neutralizes LPS and induces human monocyte chemoattractant protein-1 (MCP-1) in PMBCs[5]. As *in vitro* cultures often do not resemble the complex *in vivo* situation, studies in animal models are crucial to investigate the function of HDPs.

Various HDPs have already been tested in *in vivo* models, showing that immunomodulation instead of direct microbial killing activity of a peptide is often essential in order to eliminate an invading pathogen[7]. A clear example of an immunomodulatory mode of action of HDPs *in vivo* was described for the innate defense regulator-1 (IDR-1) peptide. Despite its lack of antimicrobial activity *in vitro*, it can protect mice from bacterial infections. In these studies it was demonstrated that IDR-1 treated mice were more protected from bacterial infections, because the peptide elicited elaborate immune responses in macrophages and monocytes to clear the infection[8]. Similarly LL-37, the only human cathelicidin, is known to have low antimicrobial activity *in vitro* (MIC values 32-64 µg/ml). However, *in vivo* tests have shown that the bacterial load decreased in mice treated with LL-37, suggesting that triggering the immune system seems to be a key factor to eradicate invading pathogens[9].

Zebrafish are commonly used as an *in vivo* model, because they have a large number of offspring that develop externally, rapidly and are optically transparent[10]. Furthermore, zebrafish have an innate and adaptive immune system, whereby the latter is not fully functional in zebrafish embryos during the first weeks[11]. Their first line of defense involves various phagocytic cells, like macrophages and neutrophils, which emerge in the first two days post-fertilization[12-14]. Due to these immunological features, zebrafish embryos are a suitable host model to study the innate immunomodulatory properties of HDPs.

The objective of this study was to investigate the immunomodulatory activity of CATH-2 *in vivo*. Using the zebrafish embryo model we determined the toxicity of the peptide and the effect of CATH-2 on immune cells was explored. Lastly, the prophylactic protective effect of CATH-2 against bacterial infections in zebrafish embryos was determined. Our results showed a protective immunostimulatory effect of CATH-2 in *Salmonella enteritidis* infected zebrafish embryos, due to a peptide-induced increase of phagocytic cells.

MATERIALS AND METHODS

Peptides

Synthetic CATH-2 peptides (RFGRFLRKIRRFRPKVTITIQGSARF-NH2**)** were custom made at CPC Scientific Inc. (Sunnyvale, CA) and were of >95% purity. The peptides were endotoxin-free. Peptide concentrations were made by diluting CATH-2 with injection buffer, a phosphate buffered saline (PBS) containing 1.48 mM NaH_2PO_4, 8.06 mM Na_2HPO_4 and 20 mM NaCl (pH=7.27). For all experiments CATH-2 was used in its D-form. D-peptides have been shown to have a higher stability *in vivo*, as they are more resistant against proteolytic degradation than peptides in L-form[15,16].

Zebrafish embryos and injections

For all experiments embryos from the transparent mutant zebrafish line casper were used, which are lacking melanocytes and iridophores[17]. Zebrafish embryos were harvested from laboratory-breeding animals, which were maintained at 26 °C in aerated five liter tanks on a 10:14 hours light on/light off cycle. Embryos were collected and kept at 31.5 °C until 28 hours post-fertilization (hpf). Afterwards embryos were staged at 28 °C for their further development. Depending on the experimental set-up early stage embryos (1-16 cell stage, 0.2-1.5 hpf) or 28 hpf embryos were used for microinjection. Early stage embryos were transferred in agarose trays containing egg water (60 µg/ml instant ocean sea salts) immediately after harvesting the eggs. Zebrafish embryos at 28 hpf, were first mechanically dechorionated and then transferred to agarose trays containing egg water and 0.02% (w/v) buffered 3-aminobenzoic acid (Tricaine; Sigma-Aldrich), to anesthetize the embryos. After positioning early stage and 28 hpf embryos in the agarose trays, the embryos were used for injection purposes. Danio rerio (zebrafish) were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). The breeding of adult fish was approved by the local animal welfare committee (Animal Experimental licensing Committee, DEC) of the VU University medical center. All protocols adhered to the international guidelines specified by the EU Animal Protection Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 days after fertilization). Because embryos used in this study met these criteria, this specific study was therefore approved by the Animal Experimental Licensing Committee of the VU University medical center (Amsterdam, The Netherlands).

Toxicity studies of CATH-2

Two different stages (1-16 cell stage [0.2-1.5 hpf] weighed with chorion 2.5 mg and 28 hpf weighed without chorion 0.3 mg) of embryonic development were used for testing the toxic effects of CATH-2. Depending on the developmental

stage, embryos were injected in the yolk with various concentrations of the peptide: 6.4 fg (2.6 ng/kg in 0.2-1.5 hpf and 21 ng/kg in 28 hpf embryos), 64.1 fg (25.6 ng/kg in 0.2-1.5 hpf and 210 ng/kg in 28 hpf embryos), 6.41 pg (2.6 µg/kg in 0.2-1.5 hpf and 21 µg/kg in 28 hpf embryos) and 641 pg (256.4 µg/kg in 0.2-1.5 hpf and 2 mg/kg in 28 hpf embryos) or injection buffer. Embryo survival was controlled a few hours after injection and on a daily basis up to five days.

Whole-mount immunohistochemistry and confocal imaging of peptide treated embryos

To determine the number of phagocytic cells in the embryonic tail, cells were stained with L-plastin antibodies (a kind gift of Prof. P. Martin, Bristol University, United Kingdom). L-plastin is a leukocyte specific antibody, and tags both macrophages and neutrophils at 48 hpf[18,19]. To this end 1-16 cell stage (0.2-1.5 hpf) zebrafish embryos were microinjected with 2.6 ng/kg CATH-2 or injection buffer in the yolk. At 48 hpf the embryos were anesthetized and fixed with 4% paraformaldehyde (PFA; EMS) overnight at 4 °C. Embryos were rinsed several times for 10 min in PBTx (PBS with 1% Triton X-100) and permeabilized with a pre-chilled trypsin solution (0.24% in PBS buffer) on ice. After washing the embryos 10 x 10 min, the embryos were incubated for 3 hours with blocking buffer (10% normal goat serum diluted in 1% PBTx) at room temperature (RT). Zebrafish embryos were incubated with anti-L-plastin, diluted 1:500 in antibody buffer [PBTx with 1% (v/v) normal goat serum and 1% (w/v) bovine serum albumin] for 1 hour at RT. Subsequently, embryos were extensively washed with PBTx and blocked for 1 hour at RT. Finally, embryos were incubated overnight at 4 °C with fluorescently labeled Goat anti-Rabbit antibody (Alexa-Fluor-488, Invitrogen), diluted 1:200 in antibody buffer, and again washed in PBTx. Zebrafish embryos were embedded in 1% low-melting agarose (Sigma-Aldrich) and z-stack analysis was performed of the tail close to the posterior blood island on a Leica SPE-II confocal microscope at the Center for Cell Imaging, University Utrecht. Subsequently, all cells were analyzed with the Image J/Fiji software and green fluorescent phagocytic cells were counted by eye.

Bacterial strain, growth condition and transformation

Salmonella enteritidis phage type 13a was transformed with the pGMDs3[20]. Bacteria were stored in glycerol stocks and grown in LB-broth and LB-plates (Lennox, BD Difco™) containing 100 µg/ml ampicillin (Sigma-Aldrich). Injection stocks with 20% glycerol were made from mid-log phase bacteria in 10 ml ampicillin-LB-broth. Finally, bacteria were washed in Dulbecco's phosphate-buffered saline (DPBS; GIBCO) and diluted 1:100,000. For visualization purposes during injections, bacteria were resuspended 1:1 with 0.17% (v/v) phenol red (Sigma-Aldrich) in PBS (pH 7.4; 136.9 mM NaCl, 2.68 mM KCl, 6.46 mM Na_2HPO_4 and 2.94 mM KH_2PO_4).

Antibacterial activity of CATH-2

Colony counting assays were used to determine the direct antibacterial activity of CATH-2 against *Salmonella enteritidis* pGMDs3. Bacteria were grown to mid-logarithmic phase and after washing the cells once in ampicillin-LB-broth diluted to 2 x 10^6 CFU/ml. Mixtures of 25 µl bacteria and 25 µl peptide (0-40 µM) were incubated for three hours at 37 °C. After serially diluting (50-5000-fold) the mixtures, 100 µl per sample was spread plated on Tryptone Soya Agar (TSA; Oxoid Limited) plates containing 100 µg/ml ampicillin and after 16 hours at 37 °C colonies were counted.

Determination of optimal infection dose

In order to determine the optimal infection dose, 28 hpf embryos were infected via caudal vein injections with various concentrations of *S. enteritidis* pGMDs3, ranging from 1 to more than 500 CFUs[21]. Groups of 15 embryos were used per bacterial dose. Embryos were maintained after injection at 28 °C and monitored regularly for their rate of survival under the microscope the next day.

Prophylaxis studies

Immediately after harvesting 1-16 cell stage (0.2-1.5 hpf) zebrafish embryos were microinjected with 1 nl CATH-2 (2.6 ng/kg to 260 µg/kg). On average 28 embryos were used per peptide concentration, injection buffer or uninjected control. Embryos were kept at 31.5 °C overnight and at 28 hpf embryos were systemically infected with *S. enteritidis* pGMDs3 in the caudal vein. After keeping the embryos at 28 °C overnight, embryos were anesthetized and the severity of the *S. enteritidis* pGMDs3 infection was monitored using a Leica MZ16FA Fluorescence Stereo microscope. Fluorescence and bright-field pictures were taken with a Leica DFC42C camera and the fluorescent intensity of the pictures was analyzed with the eLaborant software (www.eLaborant.com). To determine the bacterial load per zebrafish embryo, each embryo was anesthetized and mashed in an Eppendorf tube containing egg water solution. The embryos were further diluted in egg water solution and plated on ampicillin LB plates. Plates were incubated for 16-20 hours at 37 °C and colonies were counted the next morning. In addition, rate of survival studies were performed. For this the survival (heart-beating) of *S. enteritidis* challenged embryos, which were pre-injected with buffer or CATH-2, was monitored regularly by eye.

Graphs and statistical analysis

The graphs in this manuscript were made with the GraphPad Prism 5.0 software. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Dunnett's post hoc test. Significant differences were indicated as * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). To analyze the survival curves for the prophylaxis studies, a cutoff value of 22 hours was used to dichotomize the interval from

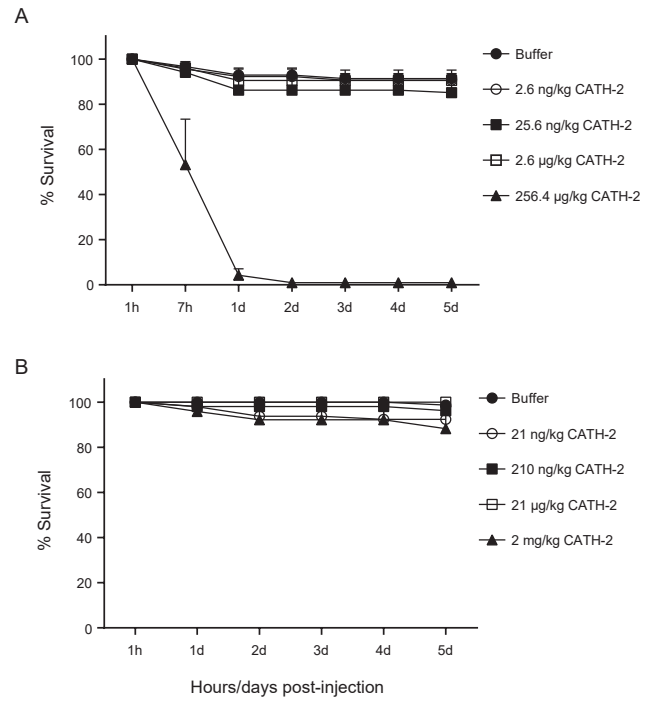

Figure 1. High concentrations of CATH-2 have toxic effects in early stage embryos. Pools of 20 embryos, at the 1-16 cell stage (0.2-1.5 hpf; A) and 28 hpf (B), were injected in the yolk with various concentrations of CATH-2. Embryos were monitored for survival (and development) seven hours after injection and afterwards daily for five days. All experiments were performed at least in triplicate (data represent the mean ± SEM).

injection of the first fish in the group till moment of death. A logistic regression analysis was applied with binary variable "death within 22 hours" as outcome and experiment as random effect to take the correlation between observations within experiments into account[22]. Treatment was added to the model as explanatory variable. The Akaike's Information Criterion was used to select the best model. R version 3.1.2 (R Core team, 2014) was used for this analysis.

RESULTS

CATH-2 toxicity studies in zebrafish embryos

In order to determine the toxicity of CATH-2, various concentrations of the D-amino acid analog of chicken cathelicidin-2 were yolk-injected in early stage (0.2-1.5 hpf) or 28 hpf embryos. Concentrations ranging from 2.6 ng/kg to 2.6

µg/kg did not affect the survival and development of early stage embryos. However, CATH-2 proved to be lethal to embryos at the highest concentration tested (260 µg/kg). At this dose 50% of early stage embryos died within seven hours after injection (Fig. 1A). Toxicity of CATH-2 was also tested in one-day old embryos (28 hpf). No toxic effects of the different peptide concentrations were observed. Five days post-injection more than 80% of the embryos survived even the highest concentration of 2 mg/kg CATH-2 used (Fig. 1B).

Phagocytic cell proliferation

To elucidate the immunomodulatory effect of CATH-2 injection in early stage (0.2-1.5 hpf) embryos, immunohistochemistry on phagocytic cells was performed on CATH-2 treated 48-hour old embryos (Fig. 2A). Macrophages and neutrophils were stained with the L-plastin antibody and their numbers quantified (Fig. 2B). Interestingly, relative to untreated or buffer injected embryos a 30% increase in phagocytic cells was observed in embryos that received 2.6 ng/kg CATH-2 (Fig. 2C). These results suggest that CATH-2 has an immunostimulatory effect by increasing the amount of phagocytic cells in embryos.

Embryo infection with *S. enteritidis* pGMDs3

In order to determine whether the observed increase in phagocytic cells could lead to increased protection against bacterial infections, a *S. enteritidis* pGMDs3 infection model was developed. To this end, embryos (28 hpf) were infected with various doses of *S. enteritidis* pGMDs3 via caudal vein injection. Concentrations exceeding 500 CFUs were lethal to embryos within 17 hours post-infection (hpi). Embryos infected with 51-500 CFUs had a very rapid infection progression and were therefore harder to monitor. With this infectious dose approximately 50% of the embryos had died already 17 hpi and at 25 hpi all embryos had succumbed to the infection. When challenged with 10-50 CFUs more than 80% of the embryos survived at 17 hpi, and 23% of these embryos were still alive at 25 hpi. In the lowest concentration tested (1-9 CFUs) 50% of the embryos survived even after 39 hpi, which may indicate self-clearance of the infection (Fig. 3). These results showed that *S. enteritidis* pGMDs3 is lethal for 28 hpf zebrafish embryos and 10-100 CFUs seems to be the appropriate bacterial dose to monitor the infection in zebrafish embryos one day after infection.

Prophylactic effect of CATH-2 on *S. enteritidis* infection

The proposed prophylactic effect of CATH-2 in zebrafish embryos was tested by injecting various concentrations of CATH-2 into the yolk of early stage (0.2-1.5 hpf) embryos and to subsequently systemically challenge these embryos with *S. enteritidis* pGMDs3 at 28 hpf. To determine the effect of CATH-2 in the early phase of infection, the bacterial infection in the embryos was analyzed by confocal microscopy. The fluorescent signal of *S. enteritidis* pGMDs3 in

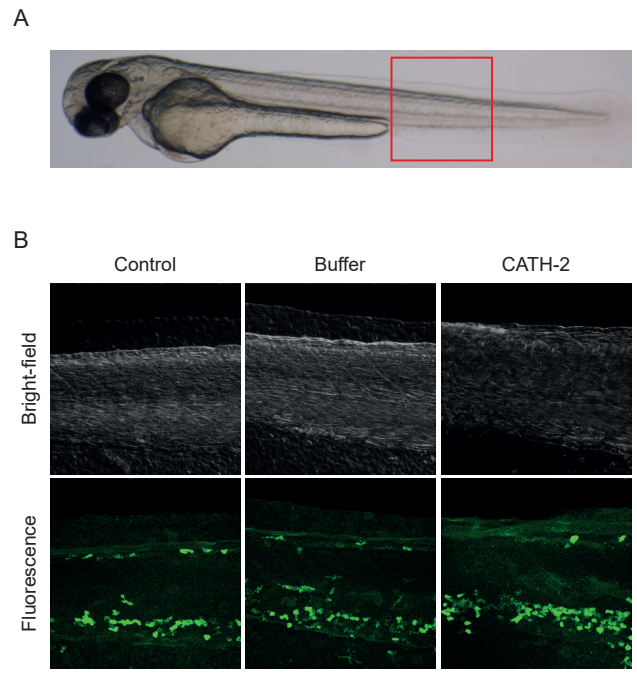

Figure 2. CATH-2 enhances the number of phagocytic cells in zebrafish embryos. Diagram of a 48 hpf embryo with the area, highlighted in a red box, which was used to measure phagocytic cells (A). Early stage (0.2-1.5 hpf) embryos were treated with CATH-2 and macrophages and neutrophils were visualized in green using a L-plastin antibody (B) and counted (C) (n=3, in average 9 embryos per group, bar = mean ± SEM is depicted). ***p<0.001, one-way ANOVA, multiple comparison.

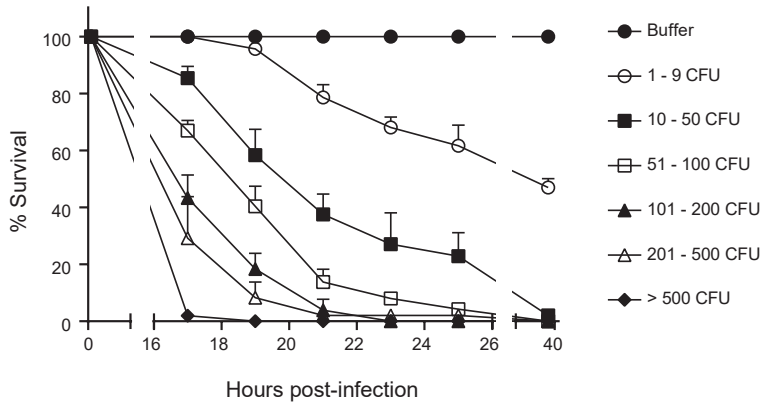

Figure 3. S*almonella enteritidis* pGMDs3 is lethal for zebrafish embryos. Groups of 10-15 transparent zebrafish embryos (28 hpf) were systemically infected with various doses of *S. enteritidis* pGMDs3. Embryos were monitored 17 hpi and at regular intervals thereafter. The survival curves represent the mean ± SEM of three independent experiments per inoculum class.

the embryos was monitored 18 to 20 hpi. Embryos, which were treated with CATH-2, had a lower fluorescent intensity compared to the control groups (Fig. 4A and Fig. S1). The measured fluorescent intensity level of CATH-2 injected embryos was significantly (two-fold) lower than observed for untreated and buffer injected embryos. The median values of the pixel count demonstrated even a two-log-reduction in CATH-2 pre-injected embryos compared to the control groups (Fig. 4B).

Additional plating of embryos at 20-22 hpi on agar media revealed that prophylactic administration of 2.6 ng/kg CATH-2 into the yolk resulted in a significantly two-fold reduction of bacterial survival in embryos. Median values confirmed these findings, as again a two-fold lower CFU count was observed in peptide pre-injected embryos (Fig. 4C).

Next the rate of survival of *S. enteritidis* infected embryos, which were pretreated with CATH-2, was monitored. Although prophylactic treatment with CATH-2 did not prevent mortality, a significant delay in the progression of infection could be observed 22 hpi for embryos treated with 2.6 ng/kg CATH-2 (Fig. 5). Quantification of the effect using logistic regression showed that the odds on death within 22 hours for untreated embryos was 1.9 times larger (95% CI: 1.1-3.2) than the odds for the buffer pre-injected group. For 2.6 ng/kg, 25.6 ng/kg and 2.6 µg/kg, odds were 0.5 (95% CI: 0.3-0.9), 0.7 (95% CI: 0.3-1.5) and 0.7 (95% CI: 0.4-1.3) relative to the buffer pretreated embryos respectively. Figure 5 is a result from six pooled independent experiments, only in one experiment the buffer injected embryos, showed a higher survival compared to the peptide injected embryos at 110 hpi.

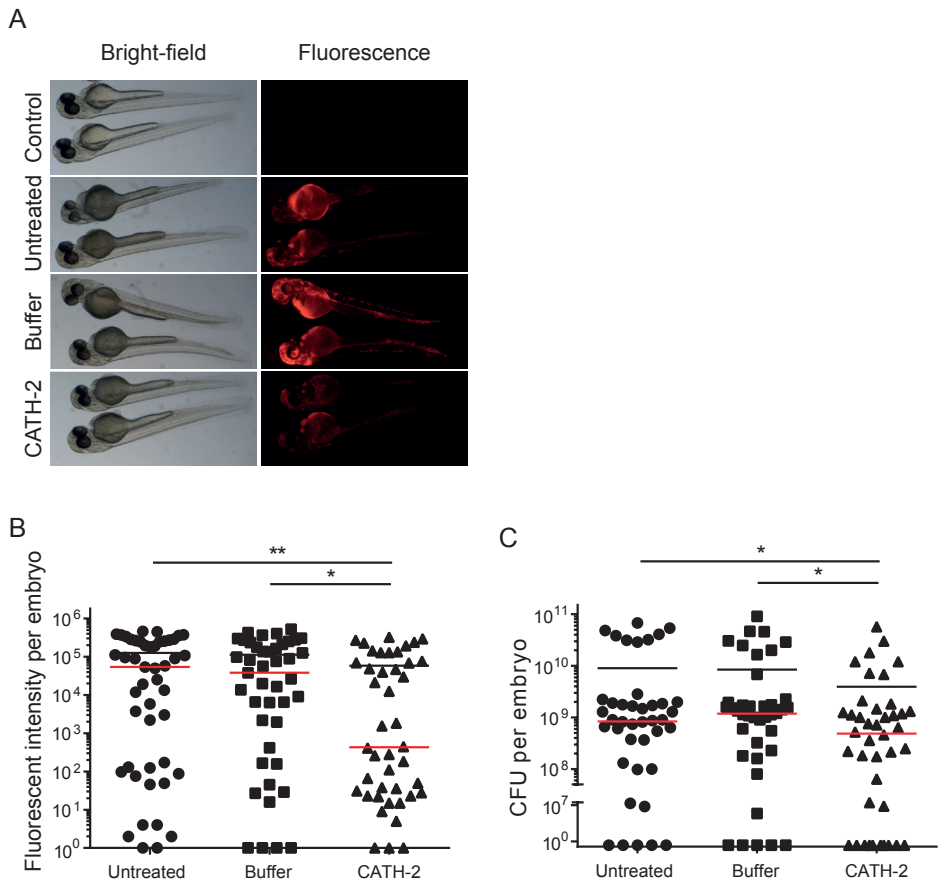

Figure 4. CATH-2 decreases the bacterial load in embryos. Groups of 10 embryos received either 2.6 ng/kg CATH-2 or buffer by yolk injection at 1-16 cell stage (0.2-1.5 hpf) followed by challenge with *S. enteritidis* pGMDs3 at 28 hpf. Fluorescence intensity was monitored at 18-20 hpi. Bright-field and fluorescent images of two representative embryos per condition are shown (A). Images were further analyzed based on their red fluorescent pixel counts (B) (n=4, 10 embryos per group, mean is depicted). CFUs of the *S. enteritidis* pGMDs3 infected embryos were determined 20-22 hpi (n=4, 10 embryos per group, mean and median are depicted black and red bar, respectively) (C). *p<0.05, **p<0.01. A log-transformation was applied on the CFU and pixel data to obtain normally distributed data. Statistical analysis was performed using one-way ANOVA, multiple comparison.

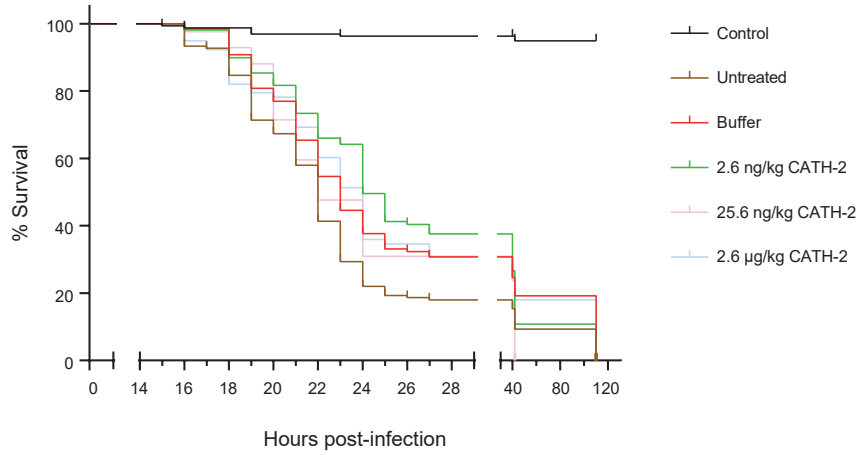

Figure 5. CATH-2 delays the infection in zebrafish embryos in their early phase of infection. Embryos were injected with various concentrations of CATH-2 or buffer at 1-16 cell stage (0.2-1.5 hpf) and were challenged with 10-100 CFUs of *S. enteritidis* pGMDs3 at 28 hpf. Embryos were monitored for survival at 16 hpi and at regular intervals thereafter. Kaplan Meyer survival analysis was based on the pooled data of six independent experiments (on average 28 embryos per group).

DISCUSSION

In this study we demonstrated for the first time the *in vivo* activity of CATH-2 peptide. Our data showed that prophylactic CATH-2 administration in the yolk of zebrafish embryos led to a partial protection against lethal bacterial infections. Zebrafish are an established *in vivo* model, which has been extensively used to study host-pathogen interactions and to model various diseases[23,24]. Furthermore, zebrafish embryos have been used to determine the toxicity and mode of action of various biochemical and biological compounds[25-28]. This model has, however, to the best of our knowledge, not yet been used to study the effect of HDPs on infection *in vivo*. To date only one study reports on the use of HDPs in zebrafish embryos to test cytotoxicity of such peptides. In this study magainin 2, pleurocidin and pleurocidin derived peptides were tested for their cytotoxic effects towards 4, 28 and 52 hpf zebrafish embryos, when added to the water[29]. Such compound screening on zebrafish embryos has indeed mainly been performed by adding the compound to the water[28,30-32]. This administration route has the disadvantage that compound uptake through the skin can be limited and is dependent on the biophysical characteristics of the compound. The yolk-injection of compounds, as performed in the current study, circumvents this problem of inadequate uptake efficacies.

Toxic effects of micro-injection of CATH-2 were analyzed at two different developmental stages of the zebrafish (1-16 cell stage and 28 hpf). Only a relatively high concentration of CATH-2 (260 µg/kg) was toxic for early stage embryos (Fig. 1A). At the early point of development embryos consist only of a few cells (1-16 cells). In contrast at 28 hpf embryos are more developed, at that time they have a beating heart, blood circulation, pigmentation of their eyes and a straight tail[21,33]. These later stage embryos were less susceptible to high peptide concentrations, exhibiting only low toxic effects with 2 mg/kg CATH-2 (Fig. 1B).

To investigate the immunostimulatory properties of CATH-2, the peptide was injected in early stage zebrafish embryos. Immunohistochemistry with L-plastin antibody was performed to detect macrophages and neutrophils in 48 hpf embryos upon pre-injection with CATH-2 at early cell stage. Injection of the peptide led to a significant 30% increase of these phagocytic cells in CATH-2 injected embryos compared to the control group (Fig. 2C).

To study the prophylactic activity of HDPs *in vivo*, a novel approach was developed by combining peptide micro-injections in early stage zebrafish embryos and challenging the same embryos at 28 hpf by caudal blood vein injection. These experiments showed a prophylactic potential of CATH-2 against bacterial infection. Both CFU and pixel counts demonstrated a significant two-fold decrease in CATH-2 pretreated embryos compared to the untreated control groups (Fig. 4). In addition, the survival rate of the embryos was monitored. At 22 hours post-infection a significant delay in the progression of infection could be observed in 2.6 ng/kg of CATH-2 zebrafish embryos (Fig. 5). Colony counting assays using *S. enteritidis* pGMDs3 have shown that CATH-2 has a minimal inhibitory concentration (MIC) value of 10 µM (Fig. S2). Due to the low peptide concentrations used for the zebrafish experiments a direct killing effect of CATH-2 *in vivo* is unlikely. Taken together these findings suggest that a peptide-induced increase in phagocytic cells may contribute to the prophylactic effect of CATH-2 in the infections experiments.

Interestingly, 2.6 ng/kg seemed to be the optimum concentration during this study as higher peptide concentrations led to a lower survival rate. At a dose of 2.6 ng/kg D-CATH-2 recruitment of phagocytic cells occurs. Chemotaxis has been reported to be highly concentration-dependent, i.e. immune cells will react to chemotactic ligands within a limited concentration range (De et al., 2000). It may well be that the higher concentrations tested are beyond this range. Several other explanations can be offered for the observation that higher peptide concentrations (25.6 ng/kg and 2.6 µg/kg) have less prophylactic effects. A high peptide concentration may result in specific detrimental effects on immune cells during infection. Another possibility is that at higher peptide concentrations the anti-inflammatory properties of CATH-2 prevail and cause disabling of the immature immune system.

During these studies also the L-form of CATH-2 was tested. Toxicity experiments with the L-form of CATH-2 showed similar results at all concentrations described as with the D-CATH-2. Only with the highest concentration of L-CATH-2 (260 µg/kg) toxic effects were observed in early stage embryos already seven hours post-injection (data not shown). In addition, the rate of survival of L-CATH-2 pre-injected embryos upon bacterial challenge was monitored, showing that the protective effect of L-CATH-2 against infection indicates the same tendency but was less strong compared to D-CATH-2 pre-injected embryos (data not shown). These findings support our hypothesis that the D-form of the peptide is more stable *in vivo* than the L-form.

Several studies in other animal models have described comparable immunomodulatory properties of HDPs *in vivo*[34-36]. One such peptide is human neutrophil peptide-1 (HNP-1), which has antibacterial activity and was proven to be effective in mice where it demonstrated high activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* infections. Low doses (0.4 ng) of HNP-1 were sufficient to rescue mice from bacterial infections and this activity was related to the presence of neutrophils since no effect was observed for neutrophil depleted mice[37]. Similarly, *in vivo* studies were performed on various innate defense regulator (IDR) peptides, which are derived from natural antimicrobial peptides but lack antimicrobial activity. These peptides show immunomodulatory properties by recruiting monocytes, macrophages or neutrophils during infection[8,38]. These studies including our current findings all point towards an important role for HDPs in innate immunity related to numbers and activity of neutrophils or macrophages and possibly other innate immune cells.

In conclusion, microinjection in zebrafish embryos was used for the first time as a method to study *in vivo* immunostimulatory and prophylactic effects of a HDP. At low doses CATH-2 was found to stimulate the immune system of zebrafish embryos by enhancing the proliferation of phagocytic cells. This increased amount of phagocytic cells is thought to be crucial in lowering bacterial survival and postponing the infection in *S. enteritidis* infected zebrafish embryos.

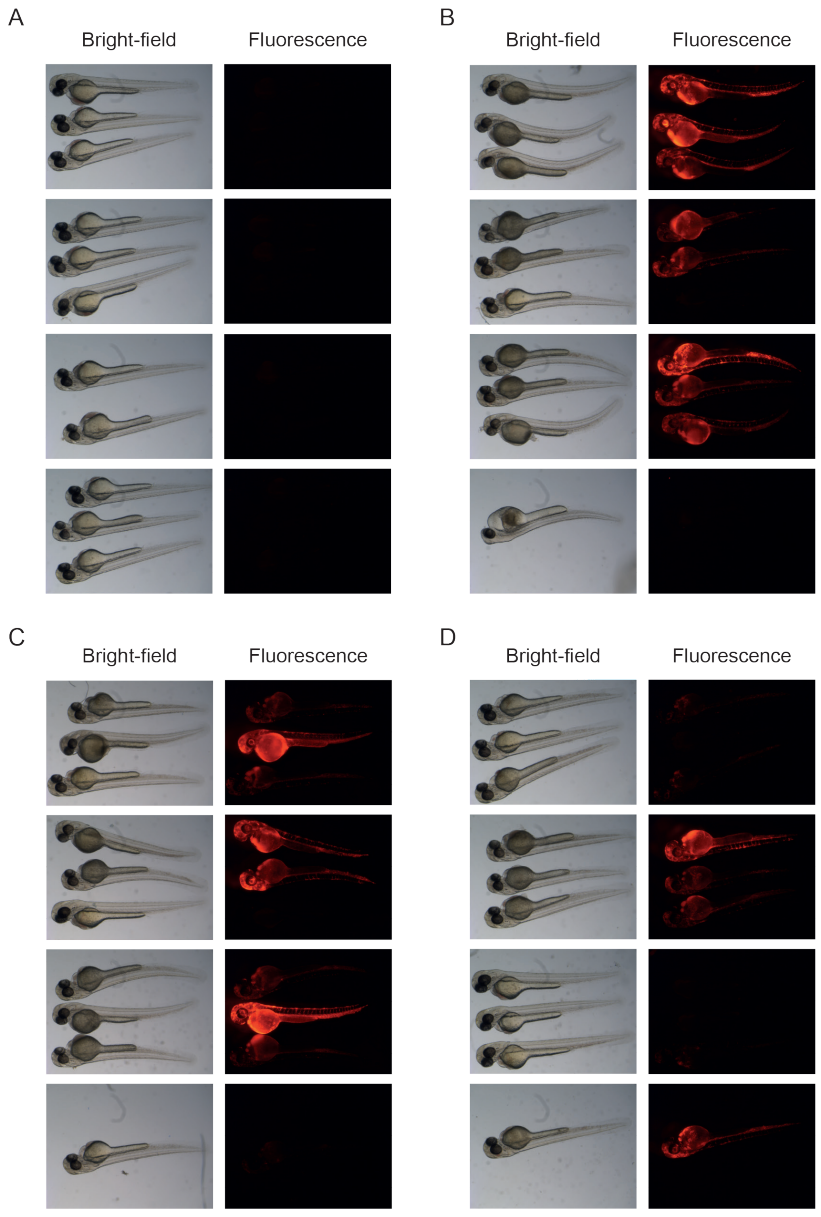

Figure S1. CATH-2 reduces the bacterial load in zebrafish embryos. Early cell stage (0.2-1.5 hpf) embryos were injected with 2.6 ng/kg CATH-2 or buffer and were challenged with *S. enteritidis* pGMDs3 at 28 hpf. At 18-20 hpi fluorescence intensity was monitored. Bright-field and fluorescent images of control (A), untreated (B), buffer injected (C) and CATH-2 injected (D) embryos of one representative experiment are shown (n=4, 10 embryos per group).

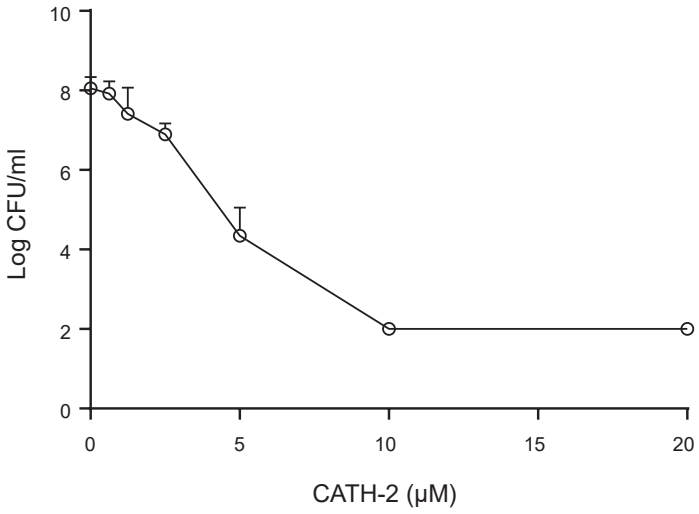

Figure S2. CATH-2 directly kills *Salmonella enteritidis* pGMDs3. Bacteria were incubated with different CATH-2 concentrations for 3 hours. Mixtures were serially diluted, spread plated and after 16 hours at 37 °C plates were counted for surviving colonies (n=4).

REFERENCES

1. Hancock, R. E. & Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **24**, 1551-1557 (2006).
2. Goitsuka, R. *et al*. Chicken cathelicidin-B1, an antimicrobial guardian at the mucosal M cell gateway. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 15063-15068 (2007).
3. Xiao, Y. *et al*. Identification and Functional Characterization of Three Chicken Cathelicidins with Potent Antimicrobial Activity. *J. Biol. Chem.* **281**, 2858-2867 (2006).
4. van Dijk, A., Veldhuizen, E. J., van Asten, A. J. & Haagsman, H. P. CMAP27, a novel chicken cathelicidin-like antimicrobial protein. *Vet. Immunol. Immunopathol.* **106**, 321-327 (2005).
5. van Dijk, A. *et al*. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. *Mol. Immunol.* **46**, 2465-2473 (2009).
6. Veldhuizen, E. J., Brouwer, E. C., Schneider, V. A. & Fluit, A. C. Chicken Cathelicidins Display Antimicrobial Activity against Multiresistant Bacteria without Inducing Strong Resistance. *PLoS One* **8**, e61964 (2013).
7. Hilchie, A. L., Wuerth, K. & Hancock, R. E. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol.* **9**, 761-768 (2013).
8. Scott, M. G. *et al*. An anti-infective peptide that selectively modulates the innate immune response. *Nat. Biotechnol.* **25**, 465-472 (2007).
9. Bowdish, D. M. *et al*. Impact of LL-37 on anti-infective immunity. *J. Leukoc. Biol.* **77**, 451-459 (2005).
10. van der Sar, A. M., Appelmelk, B. J., Vandenbroucke-Grauls, C. M. & Bitter, W. A star with stripes: zebrafish as an infection model. *Trends Microbiol.* **12**, 451-457 (2004).
11. Trede, N. S., Langenau, D. M., Traver, D., Look, A. T. & Zon, L. I. The Use of Zebrafish to Understand Immunity. *Immunity* **20**, 367-379 (2004).
12. Herbomel, P., Thisse, B. & Thisse, C. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* **126**, 3735-3745 (1999).
13. Crowhurst, M. O., Layton, J. E. & Lieschke, G. J. Developmental biology of zebrafish myeloid cells. *Int. J. Dev. Biol.* **46**, 483-492 (2002).
14. Willett, C. E., Cortes, A., Zuasti, A. & Zapata, A. G. Early Hematopoiesis and Developing Lymphoid Organs in the Zebrafish. *Dev. Dyn.* **214**, 323-336 (1999).
15. Molhoek, E. M., van Dijk, A., Veldhuizen, E. J., Haagsman, H. P. & Bikker, F. J. Improved proteolytic stability of chicken cathelicidin-2 derived peptides by D-amino acid substitutions and cyclization. *Peptides* **32**, 875-880 (2011).
16. Wade, D. *et al*. All-D amino acid-containing channel-forming antibiotic peptides. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4761-4765 (1990).
17. White, R. M. *et al*. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell. Stem Cell.* **2**, 183-189 (2008).
18. Le Guyader, D. *et al*. Origins and unconventional behavior of neutrophils in developing zebrafish. *Blood* **111**, 132-141 (2008).
19. Redd, M. J., Kelly, G., Dunn, G., Way, M. & Martin, P. Imaging Macrophage Chemotaxis In Vivo: Studies of Microtubule Function in Zebrafish Wound Inflammation. *Cell Motil. Cytoskeleton* **63**, 415-422 (2006).
20. van der Sar, A. M. *et al*. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. *Cell. Microbiol.* **5**, 601-611 (2003).

21. Benard, E. L. *et al*. Infection of Zebrafish Embryos with Intracellular Bacterial Pathogens. *J. Vis. Exp.* **(61). pii: 3781. doi**, 10.3791/3781 (2012).
22. Bates, D., Maechler, M., Bolker, B. & Walker, S. lme4: Linear mixed-effects models using Eigen and S4. *The Comprehensive R Archive Network* **1.1-7** (2014).
23. Meijer, A. H., van der Vaart, M. & Spaink, H. P. Real-time imaging and genetic dissection of host-microbe interactions in zebrafish. *Cell. Microbiol.* **16**, 39-49 (2014).
24. van Leeuwen, L. M. *et al*. Modeling tuberculous meningitis in zebrafish using *Mycobacterium marinum*. *Dis. Model. Mech.* **7**, 1111-1122 (2014).
25. Fleming A., A. W. K. Zebrafish in pharmaceutical industry research: finding the best fit fleming. *Drug Discovery Today: Disease Models* **10**, e43-e50 (2013).
26. Berghmans, S. *et al*. Zebrafish based assays for the assessment of cardiac, visual and gut function - potential safety screens for early drug discovery. *J. Pharmacol. Toxicol. Methods* **58**, 59-68 (2008).
27. Rubinstein, A. L. Zebrafish assays for drug toxicity screening. *Expert Opin. Drug Metab. Toxicol.* **2**, 231-240 (2006).
28. Ordas, A. *et al*. Testing Tuberculosis Drug Efficacy in a Zebrafish High-Throughput Translational Medicine Screen. *Antimicrob. Agents Chemother.* **59**, 753-762 (2015).
29. Morash, M. G. *et al*. The zebrafish embryo as a tool for screening and characterizing pleurocidin host-defense peptides as anti-cancer agents. *Dis. Model. Mech.* **4**, 622-633 (2011).
30. Zon, L. I. & Peterson, R. T. In vivo drug discovery in the zebrafish. *Nat. Rev. Drug Discov.* **4**, 35-44 (2005).
31. Goldsmith, P. Zebrafish as a pharmacological tool: the how, why and when. *Curr. Opin. Pharmacol.* **4**, 504-512 (2004).
32. Ridges, S. *et al*. Zebrafish screen identifies novel compound with selective toxicity against leukemia. *Blood* **119**, 5621-5631 (2012).
33. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of Embryonic Development of the Zebrafish. *Dev. Dyn.* **203**, 253-310 (1995).
34. Bommineni, Y. R., Pham, G. H., Sunkara, L. T., Achanta, M. & Zhang, G. Immune regulatory activities of fowlicidin-1, a cathelicidin host defense peptide. *Mol. Immunol.* **59**, 55-63 (2014).
35. Lisanby, M. W. *et al*. Cathelicidin Administration Protects Mice from *Bacillus anthracis* Spore Challenge. *J. Immunol.* **181**, 4989-5000 (2008).
36. Elahi, S. *et al*. The Host Defense Peptide Beta-Defensin 1 Confers protection against *Bordetella pertussis* in Newborn Piglets. *Infect. Immun.* **74**, 2338-2352 (2006).
37. Welling, M. M. *et al*. Antibacterial Activity of Human Neutrophil Defensins in Experimental Infections in Mice is Accompanied by Increased Leukocyte Accumulation. *J. Clin. Invest.* **102**, 1583-1590 (1998).
38. Nijnik, A. *et al*. Synthetic Cationic Peptide IDR-1002 Provides Protection against Bacterial Infections through Chemokine Induction and Enhanced Leukocyte Recruitment. *J. Immunol.* **184**, 2539-2550 (2010).

Chapter 5

ANTIMICROBIAL AND IMMUNOMODULATORY ACTIVITIES OF PR-39 DERIVED PEPTIDES

Edwin J.A. Veldhuizen[a], Viktoria A.F. Schneider[a], Herfita Agustiandari[a], Albert van Dijk[a], Johanna L.M. Tjeerdsma-van Bokhoven[a], Floris J. Bikker[b], Henk P. Haagsman[a]

[a] Department of Infectious Diseases and Immunology, Division of Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

[b] Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam, VU University Amsterdam, The Netherlands

PLoS ONE (2014) **9**(4): e95939

ABSTRACT

The porcine cathelicidin PR-39 is a host defense peptide that plays a pivotal role in the innate immune defense of the pig against infections. Besides direct antimicrobial activity, it is involved in immunomodulation, wound healing and several other biological processes. In this study, the antimicrobial and immunomodulatory activity of PR-39, and N- and C-terminal derivatives of PR-39 were tested. PR-39 exhibited an unexpected broad antimicrobial spectrum including several Gram-positive strains such as *Bacillus globigii* and *Enterococcus faecalis*. Of organisms tested, only *Staphylococcus aureus* was insensitive to PR-39. Truncation of PR-39 down to 15 (N-terminal) amino acids did not lead to major loss of activity, while peptides corresponding to the C-terminal part of PR-39 were hampered in their antimicrobial activity. However, shorter peptides were all much more sensitive to inhibition by salt. Active peptides induced ATP leakage and loss of membrane potential in *Bacillus globigii* and *Escherichia coli*, indicating a lytic mechanism of action for these peptides. Finally, only the mature peptide was able to induce IL-8 production in porcine macrophages, but some shorter peptides also had an effect on TNF-α production showing differential regulation of cytokine induction by PR-39 derived peptides. None of the active peptides showed high cytotoxicity highlighting the potential of these peptides for use as an alternative to antibiotics.

INTRODUCTION

Short cationic amphiphilic peptides have attracted considerable attention in the past years by both scientists as pharmacists due to their natural antimicrobial properties and the ability to modulate the immune responses of the host. These so-called host defense peptides (HDPs) are ubiquitously present in all classes of life and serve as the first line of defense against bacterial, fungal, and viral infections[1]. With few exceptions, HDPs are amphipathic (spatial separation of hydrophilic and hydrophobic residues), positively charged, and contain a high number of hydrophobic residues[2]. Based on their molecular properties and structural conformations, HDPs can be divided into several classes of which cathelicidins and defensins are the largest and best described[3].

The pig has a large reservoir of cathelicidins relative to other mammals[4]. Based on their primary amino acid compositions, porcine cathelicidins fall into three subgroups: linear proline-rich cathelicidins (PR-39, prophenin-1 and -2), disulfide-rich protegrins 1-5, and a arginine/histidine-rich myeloid subgroup[5]. PR-39 was originally isolated from porcine small intestine[6], but subsequent cDNA cloning showed that PR-39 is also expressed in bone marrow[7] and neutrophils[8]. PR-39 is secreted as a prepropeptide that undergoes post-translational modification by the cleavage of the N-terminal portion releasing the mature form of 39 C-terminal amino acids[9]. This mature PR-39 is active against a broad spectrum of bacteria, including multidrug resistant clinical isolates[10-12]. Similar to other proline-rich peptides, PR-39 does not only promote cell lysis by membrane perturbation, but translocates across the membrane and disrupts various cellular processes such as DNA and protein synthesis[13]. Besides its antimicrobial properties, PR-39 has been shown to induce migration of neutrophils in a calcium dependent manner[14], to modulate macrophage viability by inhibiting apoptosis[15], and to function as an anti-apoptotic factor in endothelial cells during hypoxia[16]. Many other biological processes such as regulation of angiogenesis[17], promotion of wound repair[18,19], and prevention of inflammation during tissue injury[18] have also been reported. The antimicrobial potential of PR-39 *in vivo* was elegantly demonstrated in a study where transgenic mice, expressing PR-39, were protected against Group A *Streptococcus* compared to the control group[20], although it is not clear whether this was achieved through direct or indirect effects of PR-39 on bacterial viability and virulence.

In this study we seek to find core elements of PR-39 involved in antimicrobial activity and immunomodulation. For this purpose, we synthesized N- and C-terminally truncated variants of PR-39 and determined their antimicrobial activities, cytotoxicity effects, and the ability to modulate the IL-8 and TNF-α response of porcine macrophages.

MATERIALS AND METHODS

Mammalian cell lines

Porcine intestinal epithelial IPEC-J2[21] and porcine alveolar macrophage 3D4/31 (American Type Culture Collection; ATCC-CRL-2844) were the two cell lines used throughout this study. Cells were cultured and maintained in advanced DMEM/F12 medium (Gibco; supplemented with 5% Fetal Calf Serum (FCS; vol/vol), 10 U/ml penicillin, 10 mg/ml streptomycin, 2 mM L-glutamine) and ATCC RPMI-1640 medium (supplemented with 10% FCS (vol/vol), 1% non-essential amino acids (Gibco), respectively. Both cell lines were grown at 37 °C under 5% CO_2 + 95% air condition. The medium was changed every other day until the cells reached 80% confluence prior to the next passage. The culture handling and sub-culture procedures for the 3D4/31 cell line were carried out according to the protocol provided by the distributor.

Bacterial strains

Four *Escherichia coli* strains (ATCC 25922, ATCC 4157, K12 and K88), 2 *Bacillus globigii* strains (BM013; and ATCC 6633), *Bacillus licheniformis* (ATCC 21424), *Bacillus cereus* (ATCC 9193), *Streptococcus pyogenes* (ATCC19616); *Enterococcus faecalis* (ATCC 29213), *Enterococcus faeceum* E155, *Staphylococcus aureus* (ATCC 29213) and MRSA (WKZ-2, human isolate) bacterial strains were all cultured in TSB at 37 °C (Tryptone Soy Broth; Oxoid).

Peptide synthesis

Peptides corresponding to C- and N-terminal domains of PR-39 were synthesized using Fmoc solid-phase synthesis as described previously[22]. All peptides were purified to a minimum purity of 95% by reverse phase high-performance liquid chromatography prior to biological testing. The sequences of the peptides used in this study are shown in Table 1.

Antimicrobial activity assays

Broth dilution assays

Initial screening for antibacterial activity of PR-39 was performed using broth dilution assays. Bacteria were grown to mid-logarithmic phase in MHB medium. The optical density was measured and bacteria were diluted to 2 x 10^6 CFU/ml. Subsequently 25 µl bacteria were incubated with 25 µl peptide in polypropylene 96 wells plates and incubated at 37 °C. After 3 hours 200 µl MHB medium was added and samples were further incubated overnight. Minimal inhibitory concentrations (MIC) were determined by turbidity measurement of the wells.

Colony counting assays

Overnight cultures of *E. coli* and *B. globigii* were grown in TSB medium at 37 °C to mid-logarithmic phase. The antimicrobial activity of the PR-39 derived peptides was tested using colony counting as described before[23]. In short, cells were collected via centrifugation at 900 x g for 10 min, and resuspended in 10 mM phosphate buffer, pH 7.0, containing 1/1000 TSB medium (Buffer A). Cells were further diluted to 2 x 10^6 CFU/ml in the same buffer. Next, 25 µl of cell suspension was mixed with an equal volume of different concentrations of peptides ranging from 0-80 µM, and incubated further for 3 hours at 37 °C. Ten-fold dilutions were prepared in minimal buffer containing 1/1000 TSB medium in distilled water, and 100 µl from each dilution was spread on TSA plates (Trypton Soy Agar; Oxoid). The plates were incubated at 37 °C and the number of colonies was counted 24 hours later to determine the number of surviving bacteria. Minimal bactericidal concentration (MBC) was defined as <100 CFU/ml corresponding to the detection limit of this assay. To determine the dependence of activity of PR-39 on the energy status of the cell, *E. coli* was incubated 30 min at 37 °C in the presence of the metabolic uncouplers 50 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP), or 5 mM 2,4-dinitrophenol (DNP). Subsequently, an antimicrobial activity assay was performed as described above.

ATP leakage measurements

B. globigii and *E. coli* were grown in TSB medium at 37 °C to mid-logarithmic phase. Bacteria were centrifuged, resuspended in buffer A and diluted to 2 x 10^7 CFU/ml. Sixty microliter bacteria were incubated with 60 µl 0.5 or 3 µM peptide for 5 min at 37 °C. The samples were centrifuged, supernatant was stored at 4 °C until further use and the bacterial pellet was resuspended in boiling 100 mM Tris, 4 mM EDTA and further incubated at 100 °C to lyse the cells. The lysed cells were centrifuged and the supernatant was kept on ice. Subsequently, both intra- and extracellular ATP levels were determined using the Roche ATP bioluminescence kit CSI II (Roche Diagnostics Nederland B.V., Almere, The Netherlands), according to the manufacturer's protocols.

Determination of membrane potential

B. globigii was grown in TSB medium at 37 °C to mid-logarithmic phase. Bacteria were pelleted and redissolved in buffer A to a density of 5 x 10^7 CFU/ml. To 2 ml of bacterial culture 20 µl 0.15 mM DiSC3(5) (3,3' - Dipropylthiadicarbocyanine iodide, Life Technologies, Europe BV, The Netherlands) and 50 µl 1M glucose were added until a stable fluorescence signal was reached. Peptide was added from a 1600 µM stock solution (final concentration depending on the peptide used) and fluorescence was continuously monitored (excitation 640 nm, emission 670 nm, slits 10 nM).

Cytotoxicity assay

The cytotoxic effect of PR-39 derived peptides on porcine intestinal epithelial IPEC-J2 and porcine macrophage 3D4/31 cells was determined using the cell proliferation reagent WST-1 (Roche), which measures cell viability based on glycolytic production of NAD(P)H, as described by the manufacturer. Approximately 5 x 10^4 cells were seeded into a 96-well microtiter plate and were incubated at 37 °C with 5% CO_2 until an 80% confluent monolayer was reached. Next the media were replaced with 50 µl of fresh media containing peptides to a final concentration ranging from 0-40 µM per well. After 24 hours incubation at 37 °C, the old peptide-containing medium was removed, and 100 µl of fresh medium containing 10% WST-1 reagent was added to each well, followed by 30 min incubation at 37 °C in the dark. Subsequently, the absorbance was measured at 450 nm using 650 nm as the reference wavelength.

Peptide induced IL-8 and TNF-α production

The ability of mature PR-39 and PR-39 derived peptides to modulate cytokine production in macrophage 3D4/31 cells was measured by means of an enzyme-linked immunosorbent assay (ELISA). Cells (5 x 10^4 cells) were seeded into 96-wells microtiter plates and grown to an 80% confluent monolayer prior to the assessment. After 24 hours the culture medium was replaced with fresh culture medium to remove non-adherent cells prior to peptide stimulation. On the next day the culture medium was discarded from each well and 100 µl of fresh medium containing 0 or 20 µM of peptides tested was added to the cells and incubation was continued at 37 °C. The culture supernatants were collected at 4 and 24 hours. The expression levels of porcine IL-8 and TNF-α were measured using the commercial available DuoSet ELISA kits (R&D Systems), following the protocols provided by the manufacturer. All samples were centrifuged briefly at 5000 rpm for 3 min at room temperature to remove cell debris prior to use. The microtiter plates were read at an absorbance of 450 using 550 nm as a reference wavelength to correct for optical imperfections of the microtiter plate.

Statistics

Statistical analysis of variance was performed using SPSS Version 20 for windows. All data were analyzed by one way ANOVA with a Bonferroni multi comparison post-hoc test. Significant differences between means were defined as $p < 0.05$.

RESULTS

Antimicrobial activity of PR-39 derived peptides

In order to determine the antibacterial spectrum of full-length PR-39, an initial screening was performed on multiple strains using broth dilution assays in 50%

				MBC (µM)			
				E. coli		*B. globigii*	
Peptide	Amino acid sequence	Length	Charge	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	39	+10	1.25	2.5	2.5	5
PR-39(1-26)	RRRPRPPYLPRPRPPPFFPPRLPPRI	26	+8	2.5	1.25	5	5
PR-39(1-22)	RRRPRPPYLPRPRPPPFFPPRL	22	+7	1.25	2.5	2.5	10
PR-39(1-18)	RRRPRPPYLPRPRPPPFF	18	+6	5	20	2.5	10
PR-39(1-15)	RRRPRPPYLPRPRPP	15	+6	5	>40	5	>40
PR-39(16-39)	PFFPPRLPPRIPPGFPPRFPPRFP	24	+4	10	>40	10	>40
PR-39(20-39)	PRLPPRIPPGFPPRFPPRFP	20	+4	5	>40	20	>40
PR-39(24-39)	PRIPPGFPPRFPPRFP	16	+3	40	>40	40	>40

Table 1: Activity of PR-39 derived peptides against *E. coli* and *B. globigii*.

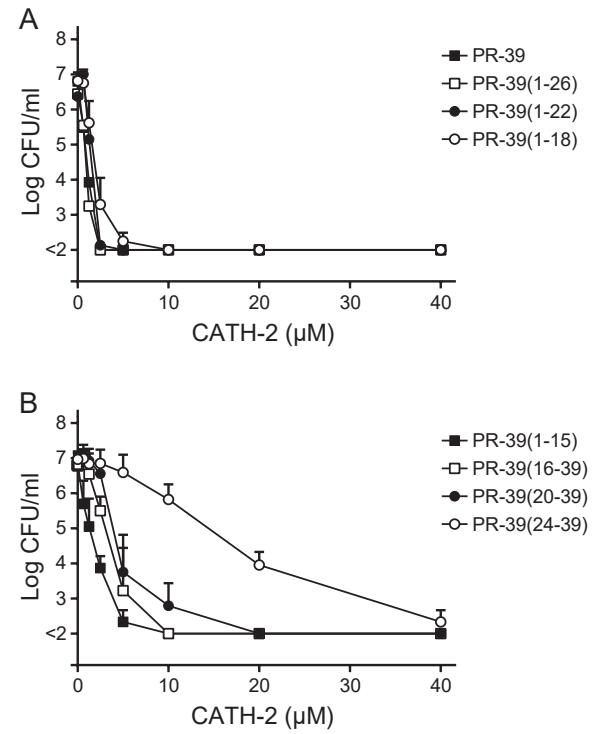

Figure 1. PR-39 derived peptides are active against *Bacillus globigii*. Peptides were incubated for 3 hours with 2 x 10^6 CFU/ml *B. globigii* in phosphate buffer (10 mM, pH 7; 1/100 TSB). Bacteria were serially diluted, plated on TSA plates and counted after 24 hours. Shown are mean ± SEM of n ≥ 3. For clarity, the data of eight peptides is divided over two figures.

MHB medium. Except for both *S. aureus* strains that were resistant to PR-39 at the tested concentrations, all other Gram-positive strains were as susceptible to PR-39 as *E. coli* (Table 2). To obtain a more detailed profile of the antimicrobial potency, mature PR-39 and PR-39 derived peptides were tested by means of colony counting assay against *E. coli* and *B. globigii*, (Fig. 1, Table 1). Only minor differences were observed between the two bacterial strains with respect to susceptibility to the PR-39 derived peptides. The MBC of mature PR-39 for *E. coli* was 1.25 µM while it was slightly higher for *B. globigii* at 2.5 µM. The truncated peptides were all antibacterial but with some differences in activity. N-terminal peptides PR-39(1-26), PR-39(1-22), PR-39(1-18) and PR-39(1-15) had activities close to mature PR-39, with some small increases in MBC values for the shorter peptides. C-terminal peptides all had reduced activity compared to the mature peptide, but only PR-39(24-39) had a severely reduced activity with complete killing of bacteria only at 40 µM. These results indicate that the N-terminus of PR-39 is contributing mostly but not exclusively, to the antibacterial activity of PR-39.

Effect of salt and energy status of the cell on antimicrobial activities of PR-39 derived peptides

The effect of salt on the antibacterial activities of the peptides was evaluated by the addition of 100 mM NaCl to the reaction buffer. The mature peptide was hardly affected by the higher ionic concentration, but the short PR-39 (1-15) peptide showed highly reduced antimicrobial activity against *E. coli* and *B. globigii* (Table 1). In addition, loss of functionality was also apparent for the C-terminal peptides e.g. PR-39(16-39), PR-39(20-39), PR-39(24-39). This result indicates a correlation between salt-induced inhibition of antimicrobial activity and total charge (but not charge density) of the peptide. The effect of the metabolic uncouplers CCCP and DNP on the antimicrobial activity of PR-39 was also determined. Incubation of bacteria with 5 mM DNP or 50 µM CCCP resulted in an inhibition of growth of *E. coli* (showing the effectiveness of the uncouplers), but did not lead to a changed susceptibility towards PR-39 (data not shown). This indicates that bacterial energy dependent uptake of peptide is not required for PR-39's activity.

ATP leakage

A five minute incubation of bacteria with 3 µM PR-39 and derived peptides resulted in a substantial loss of ATP from bacterial cells (Fig. 2). Full-length PR-39 exhibited the largest effect leading to approximately 80% extracellular ATP in both *E. coli* and *B. globigii*. ATP loss largely followed the antimicrobial activity of the peptides with low effects of C-terminal peptides and also smaller effects for shorter N-terminal peptides. Incubation of bacteria (both *E. coli* and *B. globigii*) with 0.5 µM PR-39 derived peptides did not have a significant effect on ATP release for any of the peptides (data not shown).

Bacterial strain	MIC (µM)
Bacillus subtilis (ATCC 6633)	2.5-5
Bacillus globigii (TNO)	1.25-5
Bacillus cereus (ATCC 9193)	5-20
Bacillus licheniformis (ATCC 21424)	5
MRSA (human clinical isolate)	>40
S. aureus (ATCC 29213)	>40
E. coli (ATCC 25922)	2.5-10
E. coli K88 403	1.25-2.5
E. coli K12	1.25-2.5
E. coli (ATCC 4157)	1.25-2.5
Streptococcus pyogenes (ATCC 19616)	2.5-5
Enterococcus faecalis (ATCC 29213)	10
Enterococcus faeceum E155	1.25-2.5

Table 2. Antibacterial activity of PR-39 against selected Gram-positive and Gram-negative bacteria.

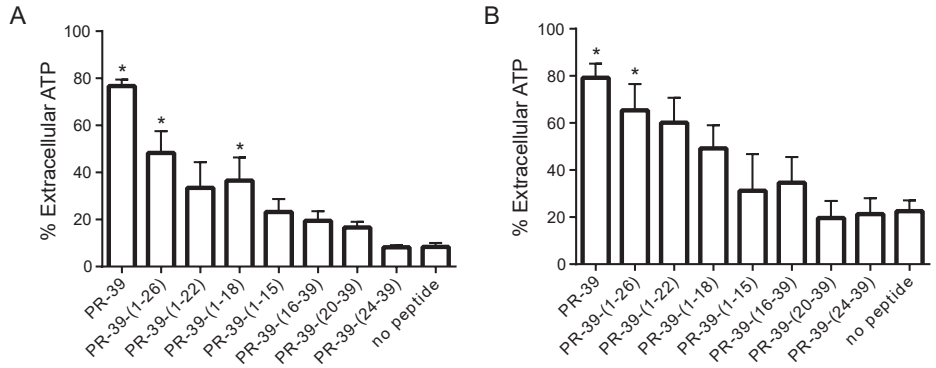

Figure 2. PR-39 derived peptides cause release of bacterial ATP. Bacteria were incubated for 5 min with 3 µM peptides. ATP in supernatant and cell pellet was determined using a luciferase bioluminescence kit. A: *E. coli*, B: *B. globigii*. Shown are mean ± SEM of at least three independent experiments in triplicate. *: $p<0.05$ compared to the no-peptide control.

Membrane potential

The effect of peptides on the membrane potential was measured by monitoring the membrane potential using the fluorescent dye DiSC3(5) (Fig. 3). Peptides were tested against *B. globigii* at concentrations close to the determined MBC values for each peptide in the buffer solution used. PR-39 (2.5 µM) caused a large and immediate increase in fluorescence (indicative of loss of membrane potential) that continued to slowly increase in the following minutes. Shorter N-terminal peptides showed a smaller effect, while C-terminal peptides and PR-39(1-15) did not have an effect. Viability assays after the measurement indicated that all samples contained > 10^6 CFU/ml except for PR-39 which had an average of 3 x 10^5 CFU/ml indicating that less bacteria were viable after incubation with the mature peptide. At lower concentrations (1 µM) no effect on membrane potential was seen for any of the peptides.

Cytotoxicity effects of PR-39 derived peptides

The toxic effect of PR-39 derived peptides towards alveolar macrophage 3D4/31 cells was assayed after a 24 hours exposure at 37 °C (Fig. 4). A relatively small reduction ($p>0.05$) in metabolic activity of these cells was seen upon addition of PR-39 derived peptides. At 40 µM, a concentration much higher than where PR-39 exerts its antibacterial effects, PR-39 lowered the metabolic activity to approximately 70% compared to non-treated control 3D4/31 cells. The same concentration of the other PR-39 derived peptides had a similar effect with only small changes in magnitude of the reduction, all not reaching statistical significance from the control. Similar results were obtained for porcine intestinal epithelial cells (IPEC-J2, Table S1), indicating that the low cytotoxic effect is a general characteristic of these peptides.

Induction of IL-8 and TNF-α production by PR-39 derived peptides

Besides antibacterial effects, HDPs are known to have immunomodulatory effects on host cells. The effect of PR-39 derived peptides on IL-8 and TNF-α production in 3D4/31 cells was investigated. After 4 hours incubation a significant ten-fold increase in IL-8 production was observed for the mature PR-39 peptide compared to the control (Fig. 5). PR-39 derived peptides did not induce a significant increase. After 24 hours of incubation, only PR-39 induced IL-8 production while all other peptides did not. Interestingly, the IL-8 level after 24 hours of the control samples increased to 4 ng/ml, indicating that the cells produced IL-8 under these conditions. PR-39 also induced TNF-α in 3D4/31 cells although to a ten-fold lower extent than IL-8. TNF-α induction was not limited to the full size peptide because some of the N-terminal peptides induced similar amounts of TNF-α. C-terminal peptides did not have any effect on TNF-α production indicating that the core element of the peptide required for this activity lies in the N-terminal part of the protein.

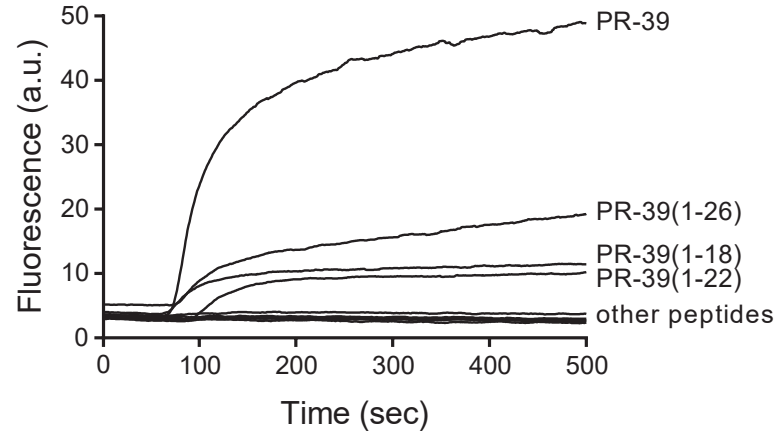

Figure 3. PR-39 is most active in disrupting the membrane potential of *Bacillus globigii*. The membrane potential sensitive dye DiSC3(5) was incubated with bacteria until a stable baseline was formed. Fluorescence increase upon addition of peptide was measured at Excitation/Emmission 640/670 nm. Shown are representative curves of: PR-39: 2.5 µM; PR-39(1-26): 2.5 µM; PR-39(1-22): 2.5 µM; PR-39(1-18): 2.5 µM; PR-39(1-15): 5 µM; PR-39(16-39): 10 µM; PR-39(20-39): 20 µM; PR-39 (24-39): 10 µM.

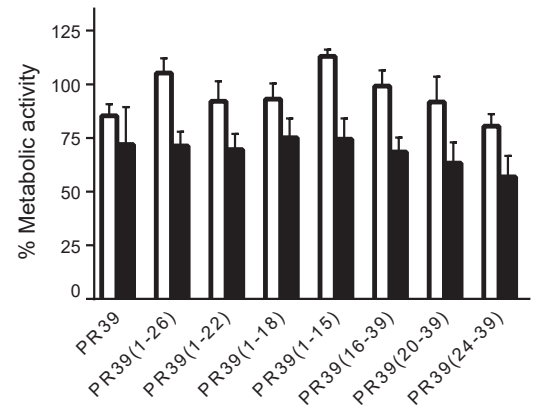

Figure 4. PR-39 derived peptides have low cytotoxicity. Porcine macrophages (3D4/31 cells) were incubated for 24 hours with 0-40 µM peptide. Metabolic activity was determined using WST-1 reagent. For clarity, the metabolic activity compared to the control (no peptide, 100%) is shown only for 5 (white bars) and 40 µM (black bars) peptide. Shown are mean ± SEM of at least 3 independent experiments. For full data set please see Table S1.

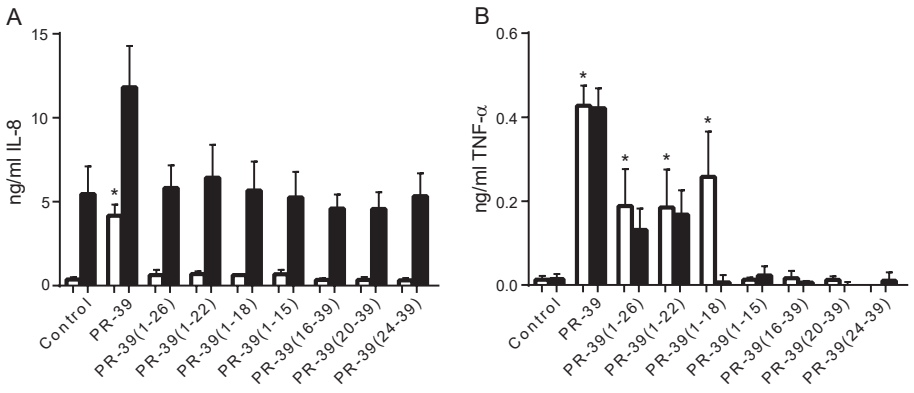

Figure 5. PR-39, but not shorter PR-39 derived peptides induce IL-8 production. Porcine macrophages (3D4/31 cells) were incubated with 20 µM peptides for 4 (white bars) or 24 hours (black bars). IL-8 (A) and TNF-α (B) production in the cell supernatant was determined using ELISA. Shown are mean ± SEM of at least three independent experiments. *: p<0.05 compared to the no peptide control.

DISCUSSION

Current literature on PR-39 has shifted from an initial focus on antibacterial activity to potential new roles in host defense mechanisms. In the search for new peptide antibiotics, PR-39 is, due to its high proline content, an excellent lead compound showing high stability in solution. PR-39 is resistant to serine proteases, elastase, and aminopeptidases, which results in a long half-life[5]. This could be an important feature if PR-39, or peptides derived thereof, are used for therapeutic purposes.

It has previously been demonstrated that PR-39 exhibits a broad antibacterial spectrum against Gram-negative bacteria excluding *Pseudomonas,* which has been described as insensitive towards PR-39[24]. Although Gram-positive strains are considered less susceptible, our study showed that seven Gram-positive strains, including four *Bacillus* strains, were all susceptible to PR-39. Only *S. aureus* showed resistance against PR-39 *in vitro.* This observation, showing that antibacterial activity of PR-39 is not completely restricted to Gram-negative bacteria is supported by only a small number of reports[25,26].

PR-39 is a member of a large family of proline-rich antimicrobial peptides (PR-AMPs). Mammalian PR-AMPs include the well-studied bovine Bac5 and Bac7 from bovine neutrophils and OABac11 and OABac6 from sheep. Many more members of the same family are found in for example insects and amphibians. For most of these members only antimicrobial activities have been determined, interactions with host cells as described for PR-39 have not been extensively

studied. More detailed information on the activity of the family of PR-AMPs can be found in an excellent review by Scocchi *et al*[27]. In general, most PR-AMPs are active against Gram-negative bacteria, indicating a comparable mechanism of action of all peptides within this group. Several short PR-AMPs for example, apidaecins and pyrrhocoricins from honeybee and fire bug, respectively[28,29] have comparable activity and specificity compared to mammalian PR-AMPs. This is largely in line with the observations in this report that short PR-39 fragments are also antimicrobial.

Several reports using PR-39(1-15) and PR-39(1-26) peptides have stated that the antibacterial activity of PR-39 is located in the N-terminus of the peptide. More notably, the positive charge of the first three amino acids of PR-39, as well as Leu9 and its following amino acids (PRPR) were found essential for full activity[25,26]. PR39(1-26) was described as slightly more active than full-length PR-39 while antibacterial activities of PR-39(1-15) and PR-39 were similar, although a direct comparison between the two peptides was only performed with one single strain; a PhoP-mutant of *Salmonella typhimurium*[30]. Our results confirm these previous observations, showing relatively small differences in MBC between the five N-terminal peptides, and extend these observations for activity against *B. globigii*. In addition, our results showed that C-terminal peptides indeed have lower activities than N-terminal peptides but that peptides lacking the 'essential' N-terminal amino acids, such as peptides PR-39(16-39) and PR-39(20-39) still have considerable antibacterial activity. Finally, the effect of ionic strength on the activity of peptides indicates that one has to be careful using MIC values as indicators for antimicrobial activity. The activity of full-length PR-39 was hardly affected by 100 mM NaCl while shorter peptides lost most of theirs. These effects are likely due to reduction of the initial electrostatic interaction between negatively charged bacterial membranes and positively charged peptides. Based on our data, the inhibiting effect seems to be related more to total charge of the peptide instead of charge density since PR-39(1-15) has a higher charge/length ratio than other peptides, yet still is inhibited strongly by salt.

The exact mechanism of PR-39's antibacterial activity is currently not exactly understood but it is generally thought that PR-39 and other PR-AMPs kill bacteria by a non-lytic mechanism[27]. For PR-39, this non-lytic mechanism was mainly based on original observations made by Boman *et al*, who noticed a lag-time between interaction of peptide with bacteria and actual killing[13]. More recently, it was shown that the activity of PR-AMPs, including PR-39 was reduced when mutations were formed in the *sbmA* gene of *E. coli*[31,32]. This gene is predicted to encode a component of an inner membrane transporter belonging to the ATP-binding-cassette superfamily of transporter proteins. It is hypothesized that this transporter is used to translocate PR-39 over the bacterial membrane. In support of this hypothesis, it was shown for Bac7, that the peptide was inactive against *E. coli* and *S. enteritidis* when the ATP-dependent transporter was inactivated

through the use of the metabolic uncoupler DNP[33]. Interestingly, *smbA* has been identified in several Gram-negative bacteria but not in *Pseudomonas aeruginosa*, a bacterial strain relatively resistant to PR-AMPs. In addition *smbA* has not been described in Gram-positive bacteria, which are generally also considered less susceptible to PR-39 and other PR-AMPs[32].

After energy dependent uptake, PR-AMPs bind to DnaK as their intracellular target[34]. DnaK belongs to the HSP70 family of chaperone proteins and binding of peptide interferes with normal protein folding in bacterial cells. For oncocin, an activity-optimized PR-AMP, it was shown that it binds with its N-terminal residues PPYLPR (AA 4-9) to the substrate binding site of DnaK[35]. Interestingly PR-39's N-terminal sequence contains an identical motif at the N-terminus (AA 6-11) indicating that it would bind similarly to DnaK. However, despite the binding evidence pointing towards a DnaK inhibiting function of PR-AMPs, DnaK deficient *E. coli* was found equally susceptible as wild type *E. coli* to Bac7(1-35)[27]. This indicates that other unidentified (intracellular) targets apart from DnaK could be involved in the bactericidal mode of action of PR-AMPs.

In our experimental set-up, we observed some contrasting results to the general hypothesis on the antimicrobial working mechanism of PR-39. Firstly, our PR-39 peptides are as active against Gram-positive strains (except *S. aureus*) as against Gram-negative bacteria. In addition we did not observe an effect of metabolic uncouplers DNP or CCCP on PR-39 activity, and finally we showed fast ATP leakage and loss of membrane potential upon incubation with PR-39. These data are more in line with a lytic mechanism or at least membrane perturbing mechanism of PR-39 than with a mechanism where ATP-dependent uptake of peptide is required. Interestingly, a lytic mechanism was also proposed by Vunnam *et al* based on clearance of an existing monolayer of *B. subtilis* on agar treated with PR-39[24]. In addition, the D-enantiomer was at least as active as the all-L isoform of PR-39 also indicating a non-chirality dependent mechanism of action for this peptide. For Bac7 it was shown that the peptide actually had a dual mode of action, energy and uptake dependent at sub-MIC concentrations, while a lytic mechanism was observed at higher concentrations[33]. At the moment it is unclear what causes the discrepancy in observed mode of action (lytic vs intracellular uptake) between the current study and earlier described experiments. However, the observed specific resistance of *S. aureus* instead of all Gram-positive strains towards PR-39, could possibly be explained by the presence of *S. aureus* specific proteases that cleave PR-AMPs, despite their proposed insensitivity towards proteases. The main indication for this hypothesis is the fact that *S. aureus* is very susceptible to the D-isomer of PR-39[24]. *S. aureus* is known to produce several proteases which help it to evade the immune system. For example, the secreted proteases aurelysin, V8 and Staphopain A & B are all involved in evasion of complement[36]. Another indication that protease activity could affect susceptibility towards PR-AMPs was recently described[37]. In this study, *E. coli*

oligopeptidase effectively cleaved several PR-AMPs and overexpression of this protease resulted in increased resistance towards PR-AMPs. It would be very interesting to determine further the susceptibility of PR-39 to *S. aureus* specific and other bacterial proteases.

The cytotoxicity measured for PR-39 and its derivatives was low towards porcine macrophages (3D4/31 cells) and intestinal cells (IPEC-J2), although at concentrations well above the MIC values some reduction in metabolic activity was observed. The low cytotoxicity for PR-39 is in agreement with the general feature of low cytotoxicity found in many PR-AMPs[32]. Low uptake of peptide by mammalian cells and lack of binding to mammalian HSP70 proteins are considered the main reason for this. However cytotoxicity of PR-39 seems to be cell specific, as was shown by Catrina *et al* who tested several different (mouse and human) cells[38]. Interestingly, using labeled PR-39 peptide they showed that cytotoxicity of PR-39 was related to the intracellular localization of the peptide indicating that only cells with an uptake mechanism suitable for PR-39 were affected. A similar cell dependency was observed for highly susceptible rat intestinal cells compared to MDCK cells. In this latter study PR-39(1-26) was also tested, which had a much lower cytotoxicity than the mature peptide, but no data on peptide uptake were shown.

Immunomodulatory activity of PR-39 derived peptides was determined by means of measuring expression of IL-8 and TNF-α in porcine macrophages upon peptide incubation. With respect to IL-8 only full-length PR-39 was capable of inducing expression of this cytokine. However, besides full-length PR-39 also some N-terminal derivatives were capable to induce TNF-α production, although to a lower extent than IL-8. This indicates that signaling cascades leading to TNF-α and IL-8 expression are not necessarily linked in this porcine macrophage cell line. The mature peptide and PR-39(1-26) were described to have chemotactic properties towards neutrophils (but not mononuclear cells)[14], but possibly the mature peptide also induces chemotaxis indirectly via the upregulation of IL-8 in macrophages. Some other studies showed immunomodulatory effects of PR-39. Delfino *et al* described protection by PR-39 treatment against septic shock in mice. It was thought that PR-39 has a dampening effect on TNF-α levels which resulted in septic shock[39]. The lack of effect in our macrophage stimulation model of the shorter peptides indicates that the full sequence is required for this stimulation. Recently, it was described that oncocin and apidaecin, two short (<20 amino acids) PR-AMPs also lack any immunomodulatory activity. No chemotactic activity towards DC, no modification of LPS induced immune responses or direct immune stimulating effects on macrophages were observed for these PR-AMPs, in contrast to the murine cathelicidin CRAMP used in the same study[40]. Taken together, our results show that N-terminal PR-39 derived peptides are sufficient for antimicrobial activity and stimulation of TNF-α production by macrophages, but that the full-length peptide is required for IL-8 production.

IPEC-J2 cells	μM peptide						
	0.6	1.2	2.5	5	10	20	40
PR-39	82 ± 4	81 ± 7	83 ±8	82 ± 12	74 ± 12	80 ± 16	68 ± 18
PR-39(1-26)	88 ± 8	94 ± 11	94 ± 11	93 ± 17	110 ± 18	88 ± 15	88 ±24
PR-39(1-22)	105 ± 12	113 ± 15	96 ± 16	97 ± 14	113± 22	101 ± 18	105 ± 15
PR-39(1-18)	115± 17	100 ± 7	115 ± 16	106 ± 15	111 ± 19	96 ± 10	90 ± 11
PR-39(1-15)	110 ± 12	107 ± 15	121 ± 15	103 ± 9	118 ± 15	119 ± 21	107 ± 9
PR-39(16-39)	112 ± 11	122 ± 11	123 ± 13	121 ± 15	107 ± 1	128 ± 6	109 ± 12
PR-39(20-39)	114 ± 16	116 ± 8	125 ± 18	119 ± 13	126 ± 11	122 ±13	107 ± 8
PR-39(24-39)	115 ± 16	107 ± 14	111 ± 11	108 ± 8	114 ± 16	117 ± 8	117 ± 8
3D4/31 cells	**μM peptide**						
	0.6	**1.2**	**2.5**	**5**	**10**	**20**	**40**
PR-39	83 ± 15	116 ± 2	96 ± 13	85 ± 7	94 ± 14	84 ± 12	72 ± 22
PR-39(1-26)	103 ± 11	108 ± 12	115 ± 6	105 ± 9	85 ± 10	78 ± 11	71 ± 9
PR-39(1-22)	126 ± 21	112 ± 10	99 ± 13	92 ± 12	92 ± 11	90 ± 15	70 ± 9
PR-39(1-18)	101 ± 8	101 ± 14	111 ± 5	93 ± 9	102 ± 14	92 ± 13	75 ± 12
PR-39(1-15)	116 ± 12	105 ± 10	112 ± 16	113 ± 4	90 ± 8	90 ± 9	75 ± 12
PR-39(16-39)	103 ± 10	106 ± 8	103 ±b8	99 ± 9	90 ± 9	93 ± 11	68 ± 9
PR-39(20-39)	99 ± 9	90 ±9	104 ± 7	92 ± 15	78 ± 6	78 ± 13	63 ± 13
PR-39(24-39)	82 ± 9	78 ±16	78 ± 10	80 ± 7	66 ± 6	72 ± 16	57 ± 13

Table S1. Cytotoxicity of PR-39 towards intestinal and macrophage cells.

REFERENCES

1. Hancock, R. E. & Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **24**, 1551-1557 (2006).
2. Powers, J. P. & Hancock, R. E. The relationship between peptide structure and antibacterial activity. *Peptides* **24**, 1681-1691 (2003).
3. Steinstraesser, L. *et al*. Host Defense Peptides as Effector Molecules of the Innate Immune Response: A Sledgehammer for Drug Resistance? *Int. J. Mol. Sci.* **10**, 3951-3970 (2009).
4. Wessely-Szponder, J., Majer-Dziedzic, B. & Smolira, A. Analysis of antimicrobial peptides from porcine neutrophils. *J. Microbiol. Methods* **83**, 8-12 (2010).
5. Sang, Y. & Blecha, F. Porcine host defense peptides: Expanding repertoire and functions. *Dev. Comp. Immunol.* **33**, 334-343 (2009).
6. Agerberth, B. *et al*. Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. *Eur. J. Biochem.* **202**, 849-854 (1991).
7. Storici, P. & Zanetti, M. A cDNA Derived from Pig Bone Marrow Cells Predicts a Sequence Identical to the Intestinal Antibacterial Peptide PR-39. *Biochem. Biophys. Res. Commun.* **196**, 1058-1065 (1993).
8. Shi, J., Ross, C. R., Chengappa, M. M. & Blecha, F. Identification of a proline-arginine-rich antibacterial peptide from neutrophils that is analogous to PR-39, an antibacterial peptide from the small intestine. *J. Leukoc. Biol.* **56**, 807-811 (1994).
9. Gudmundsson, G. H. *et al*. Structure of the gene for porcine peptide antibiotic PR-39, a cathelin gene family member: Comparative mapping of the locus for the human peptide antibiotic FALL-39. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7085-7089 (1995).
10. Fan, F., Wu, Y. & Liu, J. Expression and purification of two different antimicrobial peptides, PR-39 and Protegrin-1 in *Escherichia coli*. *Protein Expr. Purif.* **73**, 147-151 (2010).
11. Linde, C. M., Hoffner, S. E., Refai, E. & Andersson, M. *In vitro* activity of PR-39, a proline-arginine-rich peptide, against susceptible and multi-drug-resistant *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* **47**, 575-580 (2001).
12. Ramanathan, B., Davis, E. G., Ross, C. R. & Blecha, F. Cathelicidins: microbicidal activity, mechanisms of action, and roles in innate immunity. *Microbes Infect.* **4**, 361-372 (2002).
13. Boman, H. G., Agerberth, B. & Boman, A. Mechanisms of Action on *Escherichia coli* of Cecropin P1 and PR-39, Two Antibacterial Peptides from Pig Intestine. *Infect. Immun.* **61**, 2978-2984 (1993).
14. Huang, H. J., Ross, C. R. & Blecha, F. Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *J. Leukoc. Biol.* **61**, 624-629 (1997).
15. Ramanathan, B., Wu, H., Ross, C. R. & Blecha, F. PR-39, a porcine antimicrobial peptide, inhibits apoptosis: involvement of caspase-3. *Dev. Comp. Immunol.* **28**, 163-169 (2004).
16. Wu, J. *et al*. PR39 Inhibits Apoptosis in Hypoxic Endothelial Cells: Role of Inhibitor Apoptosis Protein-2. *Circulation* **109**, 1660-1667 (2004).
17. Li, J. *et al*. PR39, a peptide regulator of angiogenesis. *Nat. Med.* **6**, 49-55 (2000).
18. Shi, J., Ross, C. R., Leto, T. L. & Blecha, F. PR-39, a proline-rich antibacterial peptide that inhibits phagocyte NADPH oxidase activity by binding to Src homology 3 domains of p47 phox. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6014-6018 (1996).

19. Gallo, R. L. *et al*. Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11035-11039 (1994).
20. Lee, P. H. *et al*. Expression of an additional cathelicidin antimicrobial peptide protects against bacterial skin infection. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 3750-3755 (2005).
21. Schierack, P. *et al*. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. *Histochem. Cell Biol.* **125**, 293-305 (2006).
22. Bikker, F. J. *et al*. Evaluation of the Antibacterial Spectrum of Drosocin Analogues. *Chem. Biol. Drug Des.* **68**, 148-153 (2006).
23. Veldhuizen, E. J., Rijnders, M., Claassen, E. A., van Dijk, A. & Haagsman, H. P. Porcine β-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria. *Mol. Immunol.* **45**, 386-394 (2008).
24. Vunnam, S., Juvvadi, P. & Merrifield, R. B. Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine. *J. Pept. Res.* **49**, 59-66 (1997).
25. Chan, Y. R., Zanetti, M., Gennaro, R. & Gallo, R. L. Anti-Microbial Activity and Cell Binding are Controlled by Sequence Determinants in the Anti-Microbial Peptide PR-39. *J. Invest. Dermatol.* **116**, 230-235 (2001).
26. Shi, J. *et al*. Antibacterial Activity of a Synthetic Peptide (PR-26) Derived from PR-39, a Proline-Arginine-Rich Neutrophil Antimicrobial Peptide. *Antimicrob. Agents Chemother.* **40**, 115-121 (1996).
27. Scocchi, M., Tossi, A. & Gennaro, R. Proline-rich antimicrobial peptides: converging to a non-lytic mechanism of action. *Cell Mol. Life Sci.* **68**, 2317-2330 (2011).
28. Casteels, P., Ampe, C., Jacobs, F., Vaeck, M. & Tempst, P. Apidaecins: antibacterial peptides from honeybees. *EMBO J.* **8**, 2387-2391 (1989).
29. Cociancich, S. *et al*. Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug *Pyrrhocoris apterus*. *Biochem. J.* **300 (Pt 2)**, 567-575 (1994).
30. Chan, Y. R. & Gallo, R. L. PR-39, a Syndecan-inducing Antimicrobial Peptide, Binds and Affects $p130^{Cas}$. *J. Biol. Chem.* **273**, 28978-28985 (1998).
31. Pranting, M., Negrea, A., Rhen, M. & Andersson, D. I. Mechanism and Fitness Costs of PR-39 Resistance in *Salmonella enterica* Serovar Typhimurium LT2. *Antimicrob. Agents Chemother.* **52**, 2734-2741 (2008).
32. Mattiuzzo, M. *et al*. Role of the *Escherichia coli* SbmA in the antimicrobial activity of proline-rich peptides. *Mol. Microbiol.* **66**, 151-163 (2007).
33. Podda, E. *et al*. Dual mode of action of Bac7, a proline-rich antibacterial peptide. *Biochim. Biophys. Acta* **1760**, 1732-1740 (2006).
34. Otvos, L.,Jr *et al*. Interaction between Heat Shock Proteins and Antimicrobial Peptides. *Biochemistry* **39**, 14150-14159 (2000).
35. Knappe, D. *et al*. Rational Design of Oncocin Derivatives with Superior Protease Stabilities and Antibacterial Activities Based on the High-Resolution Structure of the Oncocin-DnaK Complex. *Chembiochem* **12**, 874-876 (2011).
36. Jusko, M. *et al*. Staphylococcal proteases aid in evasion of the human complement system. *J. Innate Immun.* **6**, 31-46 (2014).
37. Mattiuzzo, M. *et al*. Proteolytic Activity of *Escherichia coli* Oligopeptidase B Against Proline-Rich Antimicrobial Peptides. *J. Microbiol. Biotechnol.* **24**, 160-167 (2014).

38. Catrina, S. B., Refai, E. & Andersson, M. The cytotoxic effects of the anti-bacterial peptides on leukocytes. *J. Pept. Sci.* **15**, 842-848 (2009).
39. Delfino, D., Cusumano, V. V., Tomasello, F. & Cusumano, V. Beneficial effects of antibacterial peptide PR-39 in a neonatal murine model of endotoxic shock. *New Microbiol.* **27**, 369-374 (2004).
40. Fritsche, S. *et al*. Absence of *in vitro* innate immunomodulation by insect-derived short proline-rich antimicrobial peptides points to direct antibacterial action *in vivo*. *J. Pept. Sci.* **18**, 599-608 (2012).

Chapter 6

SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES

INTRODUCTION

Current antibiotic drugs often exhibit a single antibacterial killing mechanism and have a relatively narrow spectrum by (efficiently) killing only a small group of bacteria. The abundant use of antibiotics in the human and veterinary sector has contributed to the fact that many bacteria have developed resistance against conventional antibiotics[1-3]. The increasing numbers of (multi-drug) resistant pathogens enforced new research on alternative broad-spectrum antimicrobial compounds such as host defense peptides (HDPs).

The innate immune system produces a variety of these multi-functional HDPs and resistance against them seems to be absent. These peptides were shown to be active against a broad range of bacteria and directly kill bacteria based on various mechanisms (e.g. membrane permeabilization, inhibition of DNA or protein synthesis and/or interfering with protein folding)[4,5]. Moreover, HDPs possess immunomodulatory features by for instance activating immune cells, inducing chemotaxis or modulating the cytokine production[6].

For the development of anti-infectives based on HDPs, an in depth understanding of the antibacterial and immunomodulatory features is important. This thesis work was aimed to study the antibacterial mode of action and the (possible) immunomodulatory *in vivo* effects of chicken cathelicidin-2 (CATH-2). Furthermore, porcine PR-39 and its derivatives were studied to seek core elements involved in the antibacterial and immunomodulatory activity of this porcine peptide.

ANTIBACTERIAL MODE OF ACTION OF CATH-2

CATH-2 is one of the four described chicken cathelicidins and consists of 26 amino acids - rich in arginines and lysines[7,8]. This HDP was shown to be highly antibacterial against a wide range of different Gram-positive and Gram-negative bacteria[7,9-11]. Additional time kinetics studies have demonstrated that the peptide kills bacteria within a few minutes after exposure[7]. However, its antibacterial mode of action was not known when I started my thesis work.

HDPs have been shown to exhibit different modes of actions to kill bacteria including different ways of membrane permeabilization and/or intracellular targeting[4,5,12]. In this thesis, mainly based on various imaging and biophysical techniques, the antibacterial mode of action of CATH-2 at MIC and sub-MIC values was tested against Gram-negative (*E. coli*) and Gram-positive (MRSA) bacteria. For both strains tremendous intracellular and membrane effects were detected after CATH-2 exposure at sub-MIC levels (**Chapter 2** and **3**). CATH-2 was demonstrated, similar to LL-37[13,14], to bind and permeabilize the membranes of Gram-positive and Gram-negative bacteria, which was visualized using live-imaging. Transmission

electron microscopy (TEM) demonstrated that CATH-2 induced dose-dependent pronounced morphological changes on the bacterial (cytoplasmic) membrane. Membranes of *E. coli* cells were shown to start wrinkling at lower peptide concentrations and were completely disrupted at MIC levels. These results indicated that CATH-2 permeabilizes the membrane by forming pores or by using a carpet model. Both mechanisms have already been extensively studied for other cathelicidin-like peptides by other methods[15-19]. A different effect was observed for the Gram-positive bacterium MRSA. At sub-MIC values mesosomes (membrane folding) were observed, which became more pronounced at higher concentrations leading to a lamellar mesosomal structure. This mesosome formation due to peptide exposure is a well-described effect of antimicrobial peptides on Staphylococci[20-23].

In addition to the effects on bacterial membranes, intracellular morphological changes were observed already at low peptide concentrations both for *E. coli* and MRSA. For *E. coli,* ribosomes and DNA started to relocalize, whereas within MRSA cells clustering of ribosomes was observed. Interestingly, at low peptide concentrations intracellular CATH-2 was detected in *E. coli* and MRSA. The observed morphological changes correlated to the intracellular and (cytoplasmic) membrane binding of the peptide at sub-MIC values as determined with immuno-TEM. Intracellular targeting is a described mode of action for especially proline-rich antimicrobial peptides (PR-AMPs)[24-27]. A combined effect of membrane and intracellular targeting was earlier described for the bovine Bac7 peptide[28] and based on our results, we hypothesize that CATH-2 equally exhibits a dual mode of action.

Besides EM and confocal imaging, additional binding studies were performed which showed that CATH-2 is binding to LPS (Gram-negative bacteria) and LTA (Gram-positive bacteria). These findings are in line with other studies that the peptides bind to negatively charged components of the bacterial cell wall. For instance PMAP-23, a porcine cathelicidin with a similar structure as CATH-2, was described to bind and saturate the membrane in order to finally kill bacteria[29].

Although the morphology of Gram-positive and Gram-negative bacteria differs, several similarities in the mode of action of CATH-2 can be observed from studies in this thesis: 1. binding to bacterial membrane/septum, 2. permeabilization of the membrane, 3. dose-dependent morphological changes, and 4. intracellular peptide localization at low peptide concentrations.

Although the mode of action of CATH-2 has not been revealed yet, our findings provide important information on the understanding of the mechanistic features of CATH-2. The membrane seems to be an important (initial) binding target; however, based on the intracellular changes and peptide localization at low peptide concentrations a dual mode of action of the peptide cannot be excluded.

The use of different concentrations (sub-MIC and MIC values) and the combination of various techniques represent a new approach of testing

the mode of action of antimicrobial peptides. Important new insights were obtained, and we conclude that testing at sub-MIC is crucial in understanding the mechanism of action of a peptide.

IN VIVO EFFECTS OF CATH-2

Immunomodulatory activity is a well-described function for HDPs. Recent studies demonstrated that CATH-2 strongly enhances the uptake of bacterial DNA into endosomes of chicken macrophages. When the peptide is subsequently degraded, DNA is released and stimulates TLR-21 activation[30].

In vivo studies of several peptides have shown that immunomodulation rather than direct bacterial killing activity is often essential to eliminate bacteria[6,31,32]. The (possible) immunomodulatory effects of CATH-2 were tested for the first time in a zebrafish *in vivo* model. Zebrafish embryos are often used as disease models or for drug screens. However, only in one study the use of zebrafish embryos was described to test toxic effects of HDPs (magainin 2 and pleurocidin derived peptides)[33].

Testing anti-infective drugs is generally performed by adding the compounds to the fish water[34-36]; however, to circumvent inadequate uptake, peptides were yolk-injected in our studies. After peptide injection, the same embryos (at 28 hours post-fertilization) were challenged with *Salmonella enteritidis.* This combination of injection methods represents a novel approach to study *in vivo* effects of antibacterial compounds in zebrafish embryos.

In our studies, CATH-2 was shown to exhibit toxic effects only at relatively high peptide concentrations. Interestingly, yolk-microinjections of the peptide in early cell stage embryos resulted in an increase of phagocytic cells suggesting an immunostimulatory effect of CATH-2. Furthermore, prophylactic based studies were performed demonstrating that CATH-2 caused a delay of infection and significantly lowered fluorescence intensity and bacterial loads in peptide-treated embryos (**Chapter 4**). Several studies in other animal models have shown comparable immunomodulatory properties of HDPs *in vivo*[37-39]. For instance human neutrophil peptide-1 and various innate defense regulator peptides demonstrated immunomodulatory properties by recruiting monocytes, macrophages or neutrophils during infection[31,40].

Taken together, these findings highlight the importance of HDPs in innate immunity related to numbers and activity of phagocytic and possibly other innate immune cells. Although prophylactic administration of CATH-2 only confers partial protection in zebrafish embryos, these results provided important insights in the mode of action of CATH-2 *in vivo*, demonstrating that boosting the immune system is crucial to combat an infection.

PR-39

PR-39 is a proline-rich porcine cathelicidin and was shown to exhibit antibacterial properties against various types of bacteria. Moreover, PR-39 was reported to be involved in several immunomodulatory processes[41].

By using truncated variants of PR-39 the core domains of antibacterial and immunomodulatory activity were studied (**Chapter 5**). N-terminal truncations down to 1-15 amino acids showed similar antibacterial activity compared to full-length PR-39, whereas C-terminal variants had reduced antibacterial activity. Although the N-terminal part of PR-39 (and also that of other peptides) was shown to be important for the antibacterial activity, it was not exclusively this part that was involved in the antibacterial activity of PR-39. A peptide corresponding to amino acid 16-39 of PR-39, for example, was still active in killing *E. coli* and *Bacillus globigii* strains. The antibacterial activity of shorter PR-39 forms was dampened in the presence of salt, whereas the full-length peptide was still completely active. This salt dependency on peptide length was also described for CATH-2, while for many other HDPs also full-length peptides are inhibited by physiological salt concentration. The main examples of these peptides are the human cathelicidin LL-37 and the human β-defensin-1 that virtually lose all of their activity in high salt solutions[42,43]. Due to the salt-insensitivity of CATH-2 and PR-39, both host defense peptides are interesting candidates to become alternatives to antibiotics, as they will still function under physiological conditions.

PR-AMPs are suggested to act in a non-lytic killing mechanism by targeting intracellular bacterial components, e.g., 70S ribosome[27]. However, our results showed that full-length PR-39 induced fast ATP leakage and loss of membrane potential, which indicated a lytic or membrane perturbation mechanism. In contrast to other studies we observed antibacterial activity of PR-39 against several Gram-positive bacteria except *S. aureus*. Although PR-39 was shown to be stable against serum proteases, it is possible that the peptide is sensitive to specific proteases secreted by *S. aureus*.

In addition, the immunomodulatory properties of PR-39 and its truncated variants were studied. It was demonstrated that only the mature peptide induces IL-8 and TNF-α production in porcine macrophages. The shorter peptides did not induce IL-8 responses; however, N-terminal peptides triggered the TNF-α production indicating an altered regulation of these cytokines by PR-39 derived peptides. Other studies on full-length PR-39 and PR-39(1-26), demonstrated that both peptides were involved in chemoattraction of neutrophils[44]. These results suggested that PR-39 can be chemotactic itself or indirectly via production of IL-8, which acts as chemoattractant for neutrophils and possibly also for macrophages.

Taken together, we conclude that the N-terminal part of PR-39 has similar antimicrobial activity compared to the mature peptide. However, in order to induce strong immunomodulatory responses, including IL-8 and TNF-α production, full-length peptides are essential.

FUTURE PERSPECTIVES

The work in this thesis presents novel approaches to study the mode of action of host defense peptides. The methods used in these studies can be a new starting point in unravelling the biological features of (antimicrobial) HDPs. The complete antibacterial mode of action and the immunomodulatory functions for CATH-2 and PR-39 have not been revealed yet; however, the obtained results advance our understandings and are essential to develop anti-infectives based on HDPs.

Despite the exciting immunomodulatory activities of the peptides, the direct killing properties of the peptides should not be neglected. The use of different methods may aid the discovery of the antibacterial mode of action and the identification of the involved bacterial targets(s) of the peptides. The use of mutant bacteria, lacking specific membrane or intracellular components or performing immunoprecipitation of ligand-bound peptides could efficiently contribute to seeking possible antibacterial target(s) of the peptide. Another method, which could provide important insights in the antibacterial mechanism of the peptide, is the determination of the binding preferences of the peptides to isolated intracellular components (e.g. bacterial DNA or ribosomes).

Recently, also synergistic effects of antimicrobial peptides with conventional antibiotics have been described, leading to an enhanced antimicrobial killing activity. Testing CATH-2 and PR-39 in combination with conventional antibiotics could also strengthen the antibacterial potential of the peptides in order to successfully eliminate invading pathogens. Furthermore, extensive research (especially *in vivo*) is crucial in making these antibacterial and immunomodulatory compounds useful treatment candidates against (multidrug-resistant) bacteria in farm animals. Finally, in order to develop successful anti-infectives the stability of the peptide is an important issue. Peptides often are enzymatically degraded *in vivo* and lose their immunomodulatory and bactericidal activities. To avoid enzymatic breakdown, multiple strategies have been investigated, which could also be implemented for CATH-2 and PR-39. The use of D-peptides instead of the L-form can be of importance, which was also demonstrated in our zebrafish studies, as D-CATH-2 protected the embryos more effectively against infection than the L-variant of the peptide. Also modifying the peptides in a cyclic form could be beneficial to make them more resistant to proteases. Another beneficial application is the use of peptoids, which are less expensive to synthesize than D-peptides and are more stable than L-peptides. These

compounds mimic peptides and were shown to exhibit similar mechanisms as peptides.

These interesting challenges could potentially result in the development of HDP-based antimicrobials to improve health and well-being of poultry and swine via both antibacterial and immunomodulatory mechanisms.

REFERENCES

1. Cully, M. Public health: The politics of antibiotics. *Nature* **509**, S16-17 (2014).
2. Martin, M. J., Thottathil, S. E. & Newman, T. B. Antibiotics Overuse in Animal Agriculture: A Call to Action for Health Care Providers. *Am. J. Public Health* **105**, 2409-2410 (2015).
3. World Health Organization (2015) Antibiotic resistance, April 2015. Accessed, September 17, 2015; http://www.who.int/mediacentre/factsheets/fs194/en/.
4. Brogden, K. A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **3**, 238-250 (2005).
5. Jenssen, H., Hamill, P. & Hancock, R. E. Peptide Antimicrobial Agents. *Clin. Microbiol. Rev.* **19**, 491-511 (2006).
6. Hilchie, A. L., Wuerth, K. & Hancock, R. E. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol.* **9**, 761-768 (2013).
7. van Dijk, A. *et al*. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. *Mol. Immunol.* **46**, 2465-2473 (2009).
8. Xiao, Y. *et al*. Identification and Functional Characterization of Three Chicken Cathelicidins with Potent Antimicrobial Activity. *J. Biol. Chem.* **281**, 2858-2867 (2006).
9. Veldhuizen, E. J., Brouwer, E. C., Schneider, V. A. & Fluit, A. C. Chicken Cathelicidins Display Antimicrobial Activity against Multiresistant Bacteria without Inducing Strong Resistance. *PLoS One* **8**, e61964 (2013).
10. van Dijk, A. *et al*. *Campylobacter jejuni* is highly susceptible to killing by chicken host defense peptide cathelicidin-2 and suppresses intestinal cathelicidin-2 expression in young broilers. *Vet. Microbiol.* **160**, 347-354 (2012).
11. Molhoek, E. M. *et al*. Chicken cathelicidin-2-derived peptides with enhanced immunomodulatory and antibacterial activities against biological warfare agents. *Int. J. Antimicrob. Agents* **36**, 271-274 (2010).
12. Nguyen, L. T., Haney, E. F. & Vogel, H. J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* **29**, 464-472 (2011).
13. Sochacki, K. A., Barns, K. J., Bucki, R. & Weisshaar, J. C. Real-time attack on single *Escherichia coli* cells by the human antimicrobial peptide LL-37. *Proc. Natl. Acad. Sci. U. S. A.* **108**, E77-81 (2011).
14. Barns, K. J. & Weisshaar, J. C. Real-time attack of LL-37 on single *Bacillus subtilis* cells. *Biochim. Biophys. Acta* **1828**, 1511-1520 (2013).
15. Henzler Wildman, K. A., Lee, D. K. & Ramamoorthy, A. Mechanism of Lipid Bilayer Disruption by the Human Antimicrobial Peptide, LL-37. *Biochemistry* **42**, 6545-6558 (2003).
16. Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N. & Miyajima, K. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. *Biochim. Biophys. Acta* **1327**, 119-130 (1997).
17. Rangarajan, N., Bakshi, S. & Weisshaar, J. C. Localized Permeabilization of *E. coli* Membranes by the Antimicrobial Peptide Cecropin A. *Biochemistry* **52**, 6584-6594 (2013).
18. Orioni, B. *et al*. Membrane perturbation by the antimicrobial peptide PMAP-23: a fluorescence and molecular dynamics study. *Biochim. Biophys. Acta* **1788**, 1523-1533 (2009).
19. Fernandez, D. I. *et al*. The antimicrobial peptide aurein 1.2 disrupts model membranes via the carpet mechanism. *Phys. Chem. Chem. Phys.* **14**, 15739-15751 (2012).

20. Shimoda, M., Ohki, K., Shimamoto, Y. & Kohashi, O. Morphology of Defensin-Treated *Staphylococcus aureus*. *Infect. Immun.* **63**, 2886-2891 (1995).
21. Friedrich, C. L., Moyles, D., Beveridge, T. J. & Hancock, R. E. Antibacterial Action of Structurally Diverse Cationic Peptides on Gram-positive Bacteria. *Antimicrob. Agents Chemother.* **44**, 2086-2092 (2000).
22. Rabanal, F. *et al*. A bioinspired peptide scaffold with high antibiotic activity and low in vivo toxicity. *Sci. Rep.* **5**, 10558 (2015).
23. Friedrich, C. L., Rozek, A., Patrzykat, A. & Hancock, R. E. Structure and Mechanism of Action of an Indolicidin Peptide Derivative with Improved Activity against Gram-positive Bacteria. *J. Biol. Chem.* **276**, 24015-24022 (2001).
24. Scocchi, M., Tossi, A. & Gennaro, R. Proline-rich antimicrobial peptides: converging to a non-lytic mechanism of action. *Cell Mol. Life Sci.* **68**, 2317-2330 (2011).
25. Kragol, G. *et al*. The Antibacterial Peptide Pyrrhocoricin Inhibits the ATPase Actions of DnaK and Prevents Chaperone-Assisted Protein Folding. *Biochemistry* **40**, 3016-3026 (2001).
26. Otvos, L.,Jr *et al*. Interaction between Heat Shock Proteins and Antimicrobial Peptides. *Biochemistry* **39**, 14150-14159 (2000).
27. Krizsan, A. *et al*. Insect-Derived Proline-Rich Antimicrobial Peptides Kill Bacteria by Inhibiting Bacterial Protein Translation at the 70S ribosome. *Angew. Chem. Int. Ed Engl.* **53**, 12236-12239 (2014).
28. Podda, E. *et al*. Dual mode of action of Bac7, a proline-rich antibacterial peptide. *Biochim. Biophys. Acta* **1760**, 1732-1740 (2006).
29. Roversi, D. *et al*. How Many Antimicrobial Peptide Molecules Kill a Bacterium? The Case of PMAP-23. *ACS Chem. Biol.* **9**, 2003-2007 (2014).
30. Coorens, M., van Dijk, A., Bikker, F., Veldhuizen, E. J. & Haagsman, H. P. Importance of Endosomal Cathelicidin Degradation To Enhance DNA-Induced Chicken Macrophage Activation. *J. Immunol.* **195**, 3970-3977 (2015).
31. Scott, M. G. *et al*. An anti-infective peptide that selectively modulates the innate immune response. *Nat. Biotechnol.* **25**, 465-472 (2007).
32. Bowdish, D. M. *et al*. Impact of LL-37 on anti-infective immunity. *J. Leukoc. Biol.* **77**, 451-459 (2005).
33. Morash, M. G. *et al*. The zebrafish embryo as a tool for screening and characterizing pleurocidin host-defense peptides as anti-cancer agents. *Dis. Model. Mech.* **4**, 622-633 (2011).
34. Goldsmith, P. Zebrafish as a pharmacological tool: the how, why and when. *Curr. Opin. Pharmacol.* **4**, 504-512 (2004).
35. Zon, L. I. & Peterson, R. T. In vivo drug discovery in the zebrafish. *Nat. Rev. Drug Discov.* **4**, 35-44 (2005).
36. Ordas, A. *et al*. Testing Tuberculosis Drug Efficacy in a Zebrafish High-Throughput Translational Medicine Screen. *Antimicrob. Agents Chemother.* **59**, 753-762 (2015).
37. Bommineni, Y. R., Pham, G. H., Sunkara, L. T., Achanta, M. & Zhang, G. Immune regulatory activities of fowlicidin-1, a cathelicidin host defense peptide. *Mol. Immunol.* **59**, 55-63 (2014).
38. Elahi, S. *et al*. The Host Defense Peptide Beta-Defensin 1 Confers protection against *Bordetella pertussis* in Newborn Piglets. *Infect. Immun.* **74**, 2338-2352 (2006).

39. Lisanby, M. W. *et al*. Cathelicidin Administration Protects Mice from *Bacillus anthracis* Spore Challenge. *J. Immunol.* **181**, 4989-5000 (2008).
40. Nijnik, A. *et al*. Synthetic Cationic Peptide IDR-1002 Provides Protection against Bacterial Infections through Chemokine Induction and Eenhanced Leukocyte Recruitment. *J. Immunol.* **184**, 2539-2550 (2010).
41. Ramanathan, B., Davis, E. G., Ross, C. R. & Blecha, F. Cathelicidins: microbicidal activity, mechanisms of action, and roles in innate immunity. *Microbes Infect.* **4**, 361-372 (2002).
42. Turner, J., Cho, Y., Dinh, N. N., Waring, A. J. & Lehrer, R. I. Activities of LL-37, a Cathelin-Associated Antimicrobial Peptide of Human Neutrophils. *Antimicrob. Agents Chemother.* **42**, 2206-2214 (1998).
43. Goldman, M. J. *et al*. Human β-Defensin-1 Is a Salt-sensitive Antibiotic in Lung That Is Inactivated in Cystic Fibrosis. *Cell* **88**, 553-560 (1997).
44. Huang, H. J., Ross, C. R. & Blecha, F. Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *J. Leukoc. Biol.* **61**, 624-629 (1997).

Addendum

NEDERLANDSE SAMENVATTING

DE ACTIVITEITEN VAN CATHELICIDINES AAN HET LICHT GEBRACHT

Antibioticaresistentie is wereldwijd een toenemend probleem. Sinds 1970 groeit het aantal infecties door resistente bacteriën en dit heeft een grote invloed op de gezondheid van mens en dier. Onder andere het overmatig gebruik van antibiotica heeft ertoe geleid dat steeds meer bacteriën resistent zijn geraakt tegen de gebruikelijke middelen. Vergeleken met andere Europese landen is in Nederland het antibioticagebruik het laagst bij mensen, maar het was gedurende vele jaren het hoogst bij dieren. Alleen beperking van het gebruik van antibiotica zal het probleem waarschijnlijk niet oplossen dus zijn nieuwe antibacteriële stoffen hard nodig om de huidige antibioticaresistentie tegen te gaan. Een veelbelovend alternatief voor de gangbare antibiotica zijn afweerpeptiden van het aangeboren immuunsysteem (host defense peptides: HDPs). Deze peptiden zijn kleine eiwitten (max. 100 aminozuren lang), worden geproduceerd in alle gewervelde dieren en spelen een belangrijke rol in de natuurlijke afweer tegen micro-organismen. HDPs hebben een positieve lading en zijn daardoor effectief tegen bacteriën, die juist een negatief geladen membraan hebben. Het lijkt erop dat er verschillende manieren zijn waarop HDPs bacteriën kunnen doden. Zo kunnen HDPs de membraan van de bacterie lek maken, maar kunnen ze ook binnen in de cel effecten hebben zoals het remmen van DNA- of eiwitsynthese.

Verder hebben HDPs ook een belangrijke rol in het immuunsysteem. HDPs zijn bekend om hun immunomodulerende activiteiten waaronder activatie van immuuncellen, aantrekken van immuuncellen naar de plek van infectie (chemotaxis), binding en neutralisatie van endotoxinen (om septische shock te voorkomen), stimulatie van fagocytose en differentiatie van immuuncellen. Door hun variatie en ook combinatie van verschillende functionele eigenschappen om micro-organismen te doden en te neutraliseren is resistentieontwikkeling tegen deze stoffen niet aannemelijk.

Twee potentiële peptide-kandidaten waaraan in dit proefschrift is gewerkt zijn cathelicidine-2 (CATH-2) uit kippen en proline-rijk peptide PR-39 uit varkens. Het doel van het werk, dat in dit proefschrift beschreven is, was om het mechanisme waarmee CATH-2 bacteriën doodt vast te stellen en mogelijke immuunstimulerende effecten van CATH-2 in een zebravis model aan te tonen. Daarnaast werden de bacteriedodende en immunomodulerende activiteiten van PR-39 en kortere, daarvan afgeleide, peptiden bestudeerd.

CATH-2 is één van vier beschreven cathelicidines die voorkomen in de kip en bestaat uit 26 aminozuren waarvan vele postitief geladen zijn (arginine en lysine). CATH-2 werkt antibacterieel tegen Gram-negatieve en Gram-positieve bacteriën, bijvoorbeeld methicilline resistente *Staphylococcus aureus* (MRSA), *Bacillus*, *Salmonella* en *Escherichia coli*. CATH-2 doodt bacteriën binnen een

paar minuten, maar het antibacteriële mechanisme van het peptide is niet bekend.

In de **hoofdstukken 2** en **3** worden de werkingsmechanismen beschreven waardoor CATH-2 de Gram-negatieve bacterie *E. coli* en de Gram-positieve bacterie MRSA doodt. Deze twee bacterietypen verschillen in de opbouw van hun celwand. Gram-negatieve bacteriën hebben een complexe, uit twee lagen opgebouwde membraan, terwijl Gram-positieve bacteriën over een enkel membraan beschikken dat omhuld wordt door een dikke laag van een complex van suikers en eiwitten. Om het antibacteriële mechanisme van CATH-2 vast te stellen, is een aantal verschillende beeldvormende methoden en biochemische tests gebruikt. Dit leverde een unieke combinatie op van verschillende technieken: confocale fluorescentiemicroscopie (gebruik van fluorescerende peptiden en markers), elektronenmicroscopie (na inbedden van bacterie-peptide mengsels en het snijden van nanometer plakjes), het met goud markeren van deze plakjes door gebruik te maken van een antilichaam tegen het CATH-2 eiwit, antimicrobiële activiteitsmetingen en bindingsbepalingen met onderdelen van de bacteriële membraan.

De membraan was voor beide bacteriestammen een belangrijk doelwit. Door confocale microscopie hebben we aangetoond dat CATH-2 aan de membraan bindt (met sterke intensiteit bij het celdelingspunt, in het midden) en dit lek maakt. Met elektronenmicroscopie werden de veranderingen in vorm en structuur van de bacterie na een behandeling met CATH-2 bestudeerd. Tijdens deze studies zijn minimaal remmende concentraties (waarbij de bacteriegroei 100% geremd wordt) en lagere concentraties van CATH-2 (waarbij de bacteriegroei gedeeltelijk geremd wordt) gebruikt. Opvallend was dat met lagere peptideconcentraties binnen de cellen al ingrijpende veranderingen zichtbaar waren zoals een herverdeling van ribosomen en DNA. Ook membraaneffecten waren zichtbaar. In het geval van *E. coli* werd een rimpeling van de membranen bij lage concentraties en complete membraanbreuk bij hogere peptideconcentraties waargenomen. Bij MRSA veroorzaakte CATH-2 de vorming van mesosoom-achtige structuren (membraanvouwen), die bij hogere peptideconcentraties in vouwen van meerdere lagen overgingen. Aanvullend hebben we de snelheid van het doden van *E. coli* door CATH-2 bepaald, wat liet zien dat de meeste morfologische veranderingen al binnen enkele minuten optreden. Kleuring met CATH-2 antilichamen en goud om de plaats van het peptide in de bacterie te bepalen, gaf aan dat al bij lage peptideconcentraties CATH-2 binnen de cellen (intracellulair) te vinden was zonder de membranen te beschadigen. Deze resultaten duiden erop dat het peptide bacteriën niet alleen doodt door effecten op de membraan, maar door een combinatie van intracellulaire en membraan-gerichte mechanismen. Bindingsbepalingen met verschillende onderdelen van de bacteriële membranen lieten zien dat lipopolysaccharide (LPS) en lipoteichoïnezuur (LTA) belangrijke moleculen zijn waaraan CATH-2 kan binden.

Ondanks de morfologische verschillen tussen Gram-negatieve en Gram-positieve bacteriën werden meerdere overeenkomsten in effecten van het peptide geconstateerd. Bij beide bacteriën bond het peptide aan de membraan en werd het lek gemaakt; er traden in beide gevallen dosisafhankelijke morfologische veranderingen op en er was intracellulair peptide bij lage peptideconcentraties zichtbaar.

Naast hun antibacteriële activiteit, beschikken HDPs over immunomodulerende eigenschappen. Van CATH-2 is bekend dat het de opname van bacterieel DNA in endosomen van kippenmacrofagen kan versnellen. Verdere studies *in vitro (*buiten het lichaam) lieten zien dat CATH-2 endotoxine (LPS) neutraliseerde en het chemokine humaan monocyt chemoattractant proteïne-1 (MCP-1) induceerde in immuuncellen uit het bloed.

Om een eerste indicatie te krijgen hoe het peptide in een diermodel (*in vivo)* werkt, werd CATH-2 in embryo‘s van zebravissen getest. In **hoofdstuk 4** laten we zien dat een voorafgaande injectie van CATH-2 in de dooierzak van embryo‘s van een uur oud tot een immuunstimulerend effect leidt waardoor het aantal fagocyten met 30% verhoogd is in 48-uur oude embryo‘s. We hebben vervolgens een experimenteel infectiemodel ontwikkeld om het profylactische effect van CATH-2 tijdens een infectie vast te stellen. Hierbij werd CATH-2 ingespoten in de dooierzak van embryo‘s en de volgende dag (27 uur later) werd de rood-fluorescerende *Salmonella*-bacterie in het bloed *ge*ïnjecteerd. De resultaten tonen aan dat CATH-2 zebravis-embryo‘s gedeeltelijk tegen een *Salmonella*-infectie beschermt. In 48 uur oude embryo‘s die behandeld waren met peptide werd een lager aantal bacteriën gemeten, zowel door fluorescentiemetingen als door tellen van de bacteriën. Daarnaast hebben overlevingscurves aangetoond dat CATH-2 een vertraging van de infectie veroorzaakte.

In **hoofdstuk 5** zijn de antibacteriële en immunomodulerende activiteiten van PR-39 en daarvan afgeleide ingekorte peptiden van PR-39 beschreven. Het volledige peptide was sterk antibacterieel tegen verschillende Gram-negatieve en Gram-positieve bacteriën. N-terminaal ingekorte PR-39 varianten (tot 15 aminozuren) hadden vergelijkbare antibacteriële activiteit. Echter, in de aanwezigheid van zout waren kortere peptiden duidelijk minder (tot geheel niet) effectief, wat niet het geval was voor het complete PR-39 peptide. Met betrekking tot de immunomodulerende functie van de geteste peptiden, bleek dat alleen het volledige PR-39 IL-8 kon induceren terwijl ook TNF-α productie vooral door PR-39 en slechts enkele afgeleide peptiden werd verhoogd. Verder waren alle PR-39 varianten niet toxisch voor cellen van zoogdieren, wat een belangrijke vinding was om deze stoffen te gebruiken om nieuwe antibiotica te ontwikkelen.

Dit proefschrift presenteert een unieke benadering om de werkings mechanismen van afweerpeptiden te bestuderen. De hier ontwikkelde methoden kunnen toegepast worden om de biologische functies van antimicrobiële en afweerpeptiden te ontrafelen. Door de combinatie van verschillende technieken

werden belangrijke inzichten in het werkingsmechanisme van CATH-2 verkregen. Deze studies geven een duidelijk beeld van de volgorde van gebeurtenissen die optreden als CATH-2 bacteriën doodt en benadrukken het belang om ook lage peptideconcentraties in aanmerking te nemen. *In vivo* stimuleerde CATH-2 het aangeboren immuunsysteem van zebravis embryo‘s, hetgeen van cruciaal belang is bij het verlagen van bacteriële overleving en vertraging van de infectie in zebravis embryo‘s. Uit de PR-39 studies bleek dat het natuurlijke peptide de grootste antibacteriële effecten heeft. Vergeleken met de verkorte PR-39 versies was PR-39 sterk antibacterieel (zelfs in de aanwezigheid van zout) en had het immunomodulerende effecten.

De antibacteriële werkingsmechanismen en immunomodulerende functies van CATH-2 en PR-39 zijn niet helemaal opgehelderd, maar de verkregen resultaten bieden essentiële informatie voor de ontwikkeling van nieuwe middelen tegen infecties op basis van HDPs.

DEUTSCHE ZUSAMMENFASSUNG

DIE AKTIVITÄTEN VON CATHELICIDINE AN DAS LICHT GEBRACHT

Antibiotikaresistenz ist ein weltweit zunehmendes Problem. Seit 1970 wächst die Zahl der Infektionen, die von resistenten Bakterien verursacht werden und hat grossen Einfluss auf die Gesundheit von Mensch und Tier. Unter anderem der übermässige Antibiotikakonsum hat dazugeführt, dass immer mehr Bakterien gegen die herkömmlichen Medikamente resistent werden. Im Vergleich mit anderen europäischen Ländern ist der Antibiotikakonsum in den Niederlanden bei Menschen am niedrigsten, allerdings war er über viele Jahre bei Tieren am höchsten. Die Einschränkung des Antibiotikakonsums wird das Problem voraussichtlich nicht lösen, daher sind neue antibakterielle Stoffe notwendig, um gegen die steigende Antibiotikaresistenz zu kämpfen. Eine vielversprechenede Alternative zu den herkömmlichen Antibiotika sind Abwehrpeptide vom angeborenen Immunsystem (host defense peptides: HDPs). Diese Peptide sind kleine Eiweisse (max. 100 Aminosäuren lang), welche in Wirbeltieren produziert werden und eine wichtige Rolle in der natürlichen Abwehr gegen Mikroorganismen spielen. HDPs haben eine positive Ladung und sind daher wirksam gegen Bakterien, welche eine negativ geladene Membran besitzen. HDPs können Bakterien direkt töten, dabei können sie auf verschiedene Weise die Bakterienmembran undicht machen und/oder können sie intrazellulär-gezielte Effekte ausüben (Hemmug der DNA- und Eiweiss-Synthese).

Desweiteren spielen HDPs auch eine wichtige Rolle im Immunsystem. HDPs sind bekannt, um ihre immunmodulierende Aktivitäten, unter anderem Aktivierung von Immunzellen, Anziehung von Immunzellen zur Infektionsstelle (Chemotaxis), Bindung und Entschärfung von Endotoxinen (zur Verhinderung eines septischen Schocks), Stimulierung der Phagozytose und Differenzierung von Immunzellen. Durch ihre Vielfältigkeit und auch Kombination von verschiedenen, funktionellen Eigenschaften um Mikroorganismen zu töten und zu entschädigen, ist eine Resistenzentwicklung gegen diese Stoffe unwahrscheinlich.

Zwei potenzielle Peptid-Kandidaten mit welchen in dieser Dissertation gearbeitet wurde, sind Cathelicidin-2 (CATH-2) aus dem Huhn und das Prolin-reiche Peptid PR-39 aus dem Schwein. Das Ziel dieser Doktorarbeit war es, den antibakteriellen Tötungsmechanismus von CATH-2 zu ermitteln und etwaige immunstimulierende Effekte von CATH-2 in einem Zebrafischmodell anzuzeigen. Zudem wurden die antibakteriellen und immunmodulierenden Aktivitäten von PR-39 und seinen Nebenformen untersucht.

CATH-2 ist eines von vier beschriebenen Hühner Cathelicidine und besteht aus 26 Aminosäuren, wovon viele positiv geladen sind (Arginin und Lysin). CATH-2 arbeitet antibakteriell gegen gramnegative und grampositive Bakterien, z.B. Methicillin-resistenter *Staphylococcus aureus* (MRSA), *Bacillus*, *Salmonella* und *Escherichia coli* (*E. coli*). CATH-2 tötet Bakterien innerhalb von ein paar

Minuten, allerdings der antibakterielle Tötungsmechanismus vom Peptid ist nicht bekannt.

In den **Kapitel 2** und **3** werden die Wirkungsmechanismen wie CATH-2 das gramnegative Bakterium *E. coli* und das grampositive Bakterium MRSA tötet, beschrieben. Diese zwei Bakterienstämme unterscheiden sich in ihrem Zellwandaufbau. Gramnegative Bakterien haben eine komplexe, aus zwei Schichten aufgebaute Membran, während grampositive Bakterien eine einzelne Membran besitzen, welche durch eine dicke Schicht (bestehend aus Zucker und Eiweiss) umhüllt ist. Um den antibakteriellen Mechanismus von CATH-2 zu ermitteln, wurde eine Vielfalt von bildgebenden Methoden und biochemischen Tests verwendet. Dies ermöglichte eine einmalige Kombination von verschiedensten Techniken: konfokale Fluoreszenzmikroskopie (Verwendung von fluoreszierenden Peptiden und Marker), Elektronenmikroskopie (nach einbetten von Bakterien-Peptide-Mischungen und schneiden von Nanometer Schnitten), Goldmarkierung dieser Schnitte durch die Verwendung von Antikörpern gegen das CATH-2 Eiweiss, antimikrobielle Aktivitätsmessungen und Bindungstests mit Teilen der bakteriellen Membran.

Die Membran von beiden Bakterienstämmen war eine wichtige Zielscheibe für CATH-2. Mithilfe von Konfokalmikroskopie wurde gezeigt, dass CATH-2 die Membran bindet (mit starker Intensität beim Zellteilungspunkt in der Mitte) und diese undicht macht. Mit Elektronenmikroskopie wurden die morphologische Veränderungen (in Form und Struktur) von den Bakterien nach einer Behandlung mit CATH-2 bestimmt. Während dieser Studien wurden minimal hemmende Konzentrationen (wobei das Bakterienwachstum zu 100% gehemmt wird) und niedrige Konzentrationen (wobei das Bakterienwachstum nur teilweise gehemmt wird) verwendet. Auffällig war, dass mit geringer Peptidkonzentration innerhalb der Zellen einschneidende Veränderungen gefunden wurden, zum Beispiel eine Umverteilung von Ribosomen und DNA. Auch waren Membraneffekte sichtbar. Bei *E. coli*, wurde eine Zerknitterung von der Membran bei niedrigen Konzentrationen und ein kompletter Bruch der Membran bei höheren Peptidkonzentrationen beobachtet. Bei MRSA verursachte CATH-2 die Bildung von mesosomartigen Strukturen (Membranfaltungen), welche bei höheren Peptidkonzentationen zu mehrschichtigen Faltungen führten. Zusätzlich wurde untersucht, wie schnell CATH-2 *E. coli* tötet. Diese Tests zeigten, dass die meisten morphologischen Veränderungen innerhalb von ein paar Minuten auftreten. Dieselben Elektronenmikroskopieschnitte wurden zur Färbung mit Gold und Antikörpern verwendet, um die Lokalisation von dem Peptid zu bestimmen. Hierbei wurde festgestellt, dass bereits bei niedrigen Konzentrationen CATH-2 innerhalb der Zelle (intrazellulär) zu finden war ohne Membranschädigungen zu verursachen. Diese Ergebnisse deuteten daraufhin, dass CATH-2 Bakterien durch eine Kombination von intrazellulären und membran-gezielten Mechanismen tötet. Bindungstests mit verschiedenen Teilen von bakteriellen Membranen

zeigten, dass Lipopolysaccharide (LPS) und Lipoteichoic Säure (LTA) wichtige Bindungsziele für CATH-2 sind. Trotz der morphologischen Unterschiede zwischen gramnegativen und grampositiven Bakterien wurden mehrere Gemeinsamkeiten in den Peptid-auslösenden Effekten festgestellt. Bei beiden Bakterienstämmen band das Peptid die Membran und machte diese undicht. Desweiteren traten konzentrationsabhängige, morphologische Veränderungen auf und intrazelluläres Peptid war bei niedrigen Peptidkonzentrationen sichtbar.

Neben ihrer antibakteriellen Aktivität verfügen HDPs auch über immunmodulierende Eigenschaften. Von CATH-2 ist bekannt, dass es die Aufnahme von bakterieller DNA in Endosomen von Hühnermakrophagen beschleunigt. Weitere *in vitro* (ausserhalb eines Körpers/Organismus) durchgeführte Studien zeigten, dass CATH-2 das Endotoxin LPS entschärfte und das menschliche Chemokin Monozyten-chemoattractive Protein-1 (MCP-1) in Immunzellen aus dem Blut auslöste.

Um erste Hinweise zu bekommen, wie das Peptid in einem Tiermodell (*in vivo*) arbeitet, wurde CATH-2 in Zebrafischembryos getestet. In **Kapitel 4** wird gezeigt, dass eine vorherige Injektion von CATH-2 in den Dottersack der Embryos (eine Stunde alt), zu einem immunstimulierenden Effekt führt und dadurch die Anzahl von Phagozyten mit 30% in 48 Stunden alten Embryos erhöht. Ein experimentelles Infektionsmodell wurde entwickelt, um den vorbeugenden Effekt von CATH-2 während einer Infektion festzustellen. Dabei wurde CATH-2 in den Dottersack von den Embryos gespritzt und am nächsten Tag (27 Stunden später) wurde das rot fluorezierende Bakterium *Salmonella* in das Blut injektiert. Die Ergebnisse zeigen, dass CATH-2 Zebrafischembryos teilweise gegen eine Salmonelleninfektion schützt. In 48 Stunden alten Embryos (zuvor behandelt mit Peptid), wurde eine niedrigere Bakterienanzahl gemessen, sowohl durch Fluoreszenzmessungen, als auch durch das Zählen von *Salmonella*. Zudem zeigten Überlebungskurven, dass CATH-2 eine Verzögerung der Infektion auslöste.

In **Kapitel 5** werden die antibakteriellen und immunmodulierende Aktivitäten von PR-39 und den davon verkürzten Nebenformen beschrieben. Das vollständige Peptid war antibakteriell gegen verschiedene gramnegative und grampositive Bakterien. N-terminal gekürzte PR-39 Varianten hatten ähnliche, antibakterielle Aktivitäten. Jedoch in der Anwesenheit von Salz wurden die PR-39 Nebenformen weniger (bis gar nicht) wirksam, was für das vollständige PR-39 Peptid nicht der Fall war. Bezüglich der immunmodulierenden Funktionen der getesteten Peptide wurde festgestellt, dass nur das vollständige PR-39 Peptid IL-8 induzierte und PR-39 sowie einige wenige, verkürzte Nebenformen auch die TNF-α Produktion erhöhten. Desweiteren waren alle PR-39 Varianten für Säugetierzellen nicht giftig. Dies war eine wichtige Entdeckung, um diese Stoffe zu neuen potenziellen Antibiotika zu entwickeln.

Diese Dissertation präsentiert eine einzigartige Vorgangsweise, um den Wirkungsmechanismus von Abwehrpeptiden zu erforschen. Die hier

entwickelten Methoden können angewendet werden, um die biologischen Funktionen von (antimikrobiellen) Abwehrpeptiden zu identifizieren. Durch die Kombination von verschiedenen Techniken wurden wichtige Erkenntnisse im Wirkungsmechanismus von CATH-2 gewonnen. Diese Studien geben ein eindeutiges Bild in der Reihenfolge von Vorgängen wie CATH-2 Bakterien tötet und betonen die Wichtigkeit, um auch niedrige Peptidkonzentrationen zu testen. *In vivo* regte CATH-2 das angeborene Immunsystem von Zebrafischembryos an, welches von entscheidender Bedeutsamkeit beim Senken der bakteriellen Überlebung und der Infektionsverzögerung in den Zebrafischembryos ist. Die PR-39 Studien zeigten, dass das natürliche PR-39 Peptid die besten Effekte hat. Verglichen mit den (verkürzten) Nebenformen war PR-39 stark antibakteriell (auch in der Gegenwart von Salz) und hatte immunmodulierende Aktivitäten.

Der antibakterielle Wirkungsmechanismus und die immunmodulierende Funktionen von CATH-2 und PR-39 sind nicht vollständig abgeklärt, jedoch bieten die gewonnenen Erkenntnisse essenzielle Informationen für die Entwicklung von neuen Mitteln gegen Infektionen auf Basis von HDPs.

ACKNOWLEDGMENT
DANKWOORD
DANKSAGUNG

Seven years in the Netherlands, a beautiful and exciting journey! What initially only should have been a five-month bachelor internship in Amsterdam, led to a master studies and finally to a PhD. Due to nice coincidences my trip extended and achieved its top with the PhD degree. The PhD was a mountain hike with ups and downs and the mission is accomplished. It makes me proud and many people contributed to this!

Een bergwandeling die zonder mijn promotor **Henk** en copromotor **Edwin** nooit kon beginnen. Dank jullie wel dat ik aan dit project mee mocht werken en veel dank voor alle support die ik van jullie kreeg in de afgelopen 4 jaar. Dank jullie wel voor de leuke maandagochtendmeetings (om 9:07 uur), de opbouwende gesprekken om mij weer op het juiste spoor te zetten en dat jullie altijd in mij geloofden.

Henk bedankt dat je mij de kans gaf om op verschillende afdelingen te werken en dat ik de vrije ruimte kreeg om mijn werk te doen en zelf in te delen. Het was soms wel een hele uitdaging om het werk op verschillende afdelingen te combineren, maar het was een mooie en leerzame ervaring! Jij stond altijd achter me, herzlichen Dank dafür!

Edwin, ook al was ik nooit goed in het invullen van enquêtes, ik mocht alsnog bij jullie aan de gang onder jouw dagelijkse begeleiding. Het begon allemaal met mijn laatste masterstage en uiteindelijk mocht ik bijna 5 jaar met je samenwerken. Dank je wel dat je me het vertrouwen gaf een PhD aan te kunnen en dat je me altijd bijstond. Zelfs als ik weer handen te kort kwam op het lab, kwam je me helpen! Bedankt voor alle support die je gegeven hebt, vaak tot in de late uurtjes. Nu is het boek klaar en het maakt ons, volgens mij, allebei heel trots en we weten waarvoor we het deden.

Hanne ontzettend bedankt voor alle lab-hulp die ik van je kreeg. Ook al leek het soms alsof er nooit een einde aan kwam met al die stapels platen, maar uiteindelijk mogen we het resultaat in onze handen houden. Beste ASIA-mensen: **Albert**, **Maaike**, **Marina**, **Maarten** en **Tryntsje** – dank jullie wel voor de fijne samenwerking en de hulp als ik weer met computerprobleempjes zat of weer niet kon kiezen. Ik wens jullie heel veel succes en het beste voor de toekomst! **Weidong**, **Soledad** and **Martin** thanks for all the nice chats and inputs. All the best for you! Dear MHD-group, although I was mainly working in other departments, you guys were always there for me and my home-lab! Thank you!

Many other people from different departments joined my trip: the Infection Biology (UU, Veterinary faculty), Cell Microscopy Core (UMC Utrecht, Department of Cell Biology), Center for Cell Imaging (UU, Veterinary faculty) and the Zebrafish lab (VU Amsterdam, Department of Medical Microbiology and Infection Control). It was a great experience working in these different departments, meeting so many inspiring people and learning these unique techniques. Thanks that you were all so open and helpful when I was struggling with finding lab equipment or having questions. THANKS!!!

De **Infectiebiologen,** lieve buren bedankt voor alle leuke gesprekken, lab-trips en verjaardagstaarten-kringetjes! **Jos**, **Nancy**, **Marc**, **Linda**, **Claudia**, **Medi**, **Kasia**, **Anne-Xander**, **Anita**, **Carlos**, **Karin**, **Grazyna**, **Lieneke** en **Marcel**, dank jullie wel voor de gezamelijke WIP-meetings, discussies en ideeën, om elkaars projecten een nieuwe input te geven. To my **co-PhD students,** thanks for the gezellige borrels and dinners. All the best for your defences and future!

De CCI-groep met **Richard**, **Esther** en **Rob**. Voor jullie hulp bij de microscopen, het brainstormen en de data-analysen in de afgelopen jaren, hartelijk dank!

De CMC-groep: **Judith** bedankt dat ik de mogelijkheid kreeg in jouw lab deze mooie elektronenmicroscopie techniek te leren! **George**, jouw deur stond altijd open voor mij! Grote dank voor alle hulp als ik maar weer geen “licht” zag, alle leuke gesprekken met jou en je input en inzet! Jij hebt een groot deel hieraan bijgedragen en wat zijn de plaatjes mooi geworden hè! **Suzanne,** van jou en Viola mocht ik leren, dank je wel voor alle leerzame uren en dat ik altijd weer een antwoord op mijn vele vragen kreeg. **Cilia**, bedankt voor alle hulp met goud-labellen, de verwerking van de vele samples en de gezellige tijd! **René**, dank je wel voor alle tijd die je in het mooi maken van de foto’s investeerde, het waren echt heel veel plaatjes/blaadjes! **Tineke**, **Job** en **Nalan,** dank jullie wel voor alle hulp als ik weer eens niet wist hoe ik iets het beste moest aanpakken en als ik grids tekort kwam! Lieve CMC-group, bedankt voor de leuke samenwerking, ik heb heel wat uren bij jullie doorgebracht en ik voelde me altijd welkom!

De zebravisgroep: **Astrid**, zonder Henk en jouw idee was het nooit tot stand gekomen om ons peptide in zebravisembryo’s te testen. Het was heel bijzonder dat ik deze techniek mocht leren en het kwam volledig onverwacht op mijn pad. Dank je wel aan jou en **Wilbert** dat ik in jullie lab mocht werken en voor alle input van jullie! **Gunny**, bedankt voor alle tijd en hulp die je voor mij vrijmaakte om te leren met embryo’s te werken. **Theo**, dank je wel voor alle hulp bij het injecteren en al de leuke gesprekken! **Wim**, dank je wel voor het op tijd leveren van de vissen en dat je altijd je best deed dat ik al mijn proeven kon doen. **Lisanne**, **Maikel** en **Susanna**, bedankt voor de fijne tijd met jullie en alle support! Alle andere MMI-collega’s dank jullie wel voor jullie gastvrijheid.

Claudia die schönen Italiaanse bollen Mittagessen werde ich vermissen, danke für die schöne Zeit und deine Hilfe in meinem letzten Jahr! Ich wünsch dir viel Erfolg, dranbleiben, wird schon!!

Jeanique, mijn hart mocht ik wel altijd bij je luchten en kletste je soms de oren van je kop! Superbedankt voor alle plezierige etentjes en uitstapjes - mooi dat je op deze bijzondere dag mijn paranimf wilt zijn.

Lucie, wat fijn dat je mijn paranimf wilt zijn! Sinds mijn eerste dag in Nederland ken ik je en wat zal ik onze leuke ontmoetingen missen. Jij stond altijd voor me klaar en zorgde voor de nodige afleiding!

Clemens, mein kleiner großer Mann, schön, dass du an diesem großen Tag an meiner Seite sein wirst. Auch wenn es für dich oft nicht verständlich war warum

ich so oft und lange Weg war, das Resultat darfst du jetzt in den Händen halten. Ich freue mich auf unsere sportlichen Ausflüge, auch wenn ich dabei immer langsamer sein werde als du. :)

Liebe **Mama** es ist vollbracht! Du hast immer an mich geglaubt und mich aufs Neue motiviert. Du warst trotz der Entfernung immer für mich da und warst mir eine unglaubliche Stütze! Ohne die feinen Gespräche und dein Vertrauen in mich wäre ich wohl nie bis hierhin gekommen. DANKE für ALLES!

Dank jullie wel voor de mooie jaren!

Thank you for the wonderful past years!

Danke euch für die schönen letzten Jahre!

CURRICULUM VITAE

Viktoria Schneider was born on the 26th of August 1986 in Rum/Tyrol (Austria). After finishing high school (Bundesrealgynmasium Imst) in 2004, she started her Medicine studies at the Medical University of Innsbruck. In 2006 she switched to Biomedical Sciences at the Ausbildungszentrum West für Gesundheitsberufe (currently Fachhochschule - Gesundheit) in Innsbruck. During this study she enrolled in a Leonardo da Vinci programme, which funded a five-month internship at the Department of Clinical Chemistry (Metabolic Unit) of the Free University Medical Center (VUMC) in Amsterdam. Under the supervision of Dr. Desirée Smith and Prof. Henk Blom, she studied the influence of changed methionine-adenosyltransferase activity on global DNA methylation. In 2009 she started her Master Biomedical Sciences at the VU Amsterdam with the specializations cell biology and infectious diseases. Her first Master internship she performed at Sanquin Research, Department of Molecular Cell Biology supervised by Dr. Paula van Hennik and focused on the role of mitogen-activated protein kinases (MAPK) in the migration of hematopoietic cells. The second internship Viktoria performed at Utrecht University / Faculty of Veterinary Medicine in the Division of Molecular Host Defence supervised by Dr. Edwin Veldhuizen, determining the (combined) antibacterial activity of antimicrobial peptides and collectins against ESBL bacteria. In 2011 she finished her studies with writing her literature thesis supervised by Dr. Jim van Steenbergen (Leiden University Medical Center [LUMC]) entitled Hepatitis B vaccination: What are the minimum and maximum intervals to reach an effective long-lasting protection? On January 1st, 2012, she started her PhD track at the Department of Infectious Diseases and Immunology (Infection & Immunity Programme, Utrecht University) supervised by Prof. Henk Haagsman and Dr. Edwin Veldhuizen. The results of this PhD are presented in this thesis and published in different scientific journals.

LIST OF PUBLICATIONS

Edwin J. A. Veldhuizen, Ellen C. Brouwer, **Viktoria A. F. Schneider**, Ad C. Fluit. Chicken Cathelicidins Display Antimicrobial Activity against Multiresistant Bacteria without Inducing Strong Resistance. *PLoS One* 8, e61964 (2013)

Edwin J. A. Veldhuizen, **Viktoria A. F. Schneider**, Herfita Agustiandari, Albert van Dijk, Johanna L.M. Tjeerdsma-van Bokhoven, Floris J. Bikker, Henk P. Haagsman. Antimicrobial and immunomodulatory activities of PR-39 derived peptides. *PLoSOne* 9(4), e95939 (2014)

Viktoria A. F. Schneider, Albert van Dijk, Astrid M. van der Sar, Marina D. Kraaij, Edwin J. A. Veldhuizen, Henk P. Haagsman. Prophylactic administration of chicken cathelicidin-2 boosts zebrafish embryonic innate immunity. *Developmental & Comparative Immunology*, Vol. 60; p. 108–114 (2016)

Viktoria A. F. Schneider, Maarten Coorens, Soledad R. Ordonez, Johanna L. M. Tjeerdsma-van Bokhoven, George Posthuma, Albert van Dijk, Henk P. Haagsman, Edwin J. A. Veldhuizen. Imaging the antimicrobial mechanism(s) of chicken cathelicidin-2. *Submitted*

Viktoria A. F. Schneider, Johanna L. M. Tjeerdsma-van Bokhoven, George Posthuma, Albert van Dijk, Edwin J. A. Veldhuizen, Henk P. Haagsman. Visualization of the anti-staphylococci activity of chicken cathelicidin-2. *In preparation*

Martina E. C. Bluhm, **Viktoria A. F. Schneider**, Ingo Schäfer, Stefania Piantavigna, Tina Goldbach, Daniel Knappe, Peter Seibel, Lisandra L. Martin, Edwin J. A. Veldhuizen and Ralf Hoffmann. N-terminal Ile-Orn- and Trp-Orn-motif repeats enhance membrane interaction and increase the antimicrobial activity of apidaecins against *Pseudomonas aeruginosa*. *Accepted in Frontiers in Cell and Developmental Biology (section Membrane Physiology and Membrane Biophysics)*

Maarten Coorens, **Viktoria A. F. Schneider**, Marit A. de Groot, Marjolein Meijerink, Jerry M. Wells, Albert van Dijk, Edwin J. A. Veldhuizen, Henk P. Haagsman. Sensible sensing - How cathelicidins modulate interactions between Gram-negative bacteria and host immune cells. *In preparation*