Development of cathelicidin-derived peptides for the treatment of infectious diseases

The research described in this thesis was conducted at TNO Defense, Security and Safety, Rijswijk, the Netherlands and at the Department of Infectious Diseases and Immunology, Division Molecular Host Defense, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

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Development of cathelicidin-derived peptides for the treatment of infectious diseases

Ontwikkeling van op cathelicidine gebaseerde peptiden voor de behandeling van infectieziekten.

(met een samenvatting in het Nederlands)

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CHAPTER 1

General Introduction

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INFECTIOUS DISEASES

History of infectious diseases

Historically plague, measles and smallpox, have been the most devastating worldwide epidemic diseases. For instance, plague outbreaks were endemic in the cities of the Roman Empire and contributed to its final demise. Four centuries later, multiple waves of plague circulated, of which the one between 1347 and 1351 was the most disastrous with a total of 40 million deaths worldwide[1,2]. Nowadays, outbreaks like plague and measles cause local small outbreaks only or, in case of smallpox, are eradicated. However, other infectious diseases like cholera remain a global threat. During the 19th century cholera spread across the world, from its original reservoir in the Ganges delta in India, for the first time. Six subsequent waves killed millions of people across the continent. The current one started in South Asia in 1961, reached Africa in 1971 and the Americas in 1991[3,4]. Recently, in October 2010, cholera emerged in the Caribbean, as a consequence of an earthquake and a flooding caused by Hurricane Tomas, causing outbreaks in every section of Haiti, sickening more than 91.000 people and killing more than 2.000 and is still spreading[5,6].

In early epidemic studies it was recognized that infectious diseases were contagious, though knowledge of the true epidemiology of diseases was poor. As a result, efforts to control the spread of the diseases effectively were unsuccessful. In the 1860s Louis Pasteur and Robert Koch discovered that infectious diseases were caused by micro-organisms. In the subsequent 50 years, numerous micro-organisms were identified as the causative agents of specified infectious diseases. Among these was the causative agent of plague *Yersinia pestis*, identified in 1894 by Alexander Yersin and Shibasaburo Kitasato in humans and rats. Later, the link between rats and humans was found to be the rat flea which, once infected, passes on *Yersinia pestis* to humans. In 1898, Martinus Beijerinck demonstrated that other infectious diseases were caused by smaller objects, that could pass through bacteria stopping filters, later called viruses[2].

The new science of microbiology and the accompanying better understanding of infectious diseases provided a great impact on public health. This resulted in supply and consumption of purified water, sewerage, improved personal hygiene and so forth. Increased hygiene resulted in a decreased number of infected people. As a result the mortality ascribable to infectious diseases declined dramatically[2,7]. Consequently, a greater understanding of the biology of disease pathology led to better treatments and a further decline in infectious disease mortality. The first half of the 20th century was notable for their 'wonder drugs', the new antibiotics penicillin, streptomycin, chloramphenicol and a growing list of others that promised a reduction or, at times, an end to bacteria-based infections. Viral-based infections have offered fewer routes to remedies, except for vaccines. Effective vaccination was realized by massive public health campaigns. By the end of the 1970s, smallpox became the first and as yet only disease to be eradicated[7].

Antibiotic resistance

The euphoria over the potential conquest of infectious diseases, by using the discovered antibiotics, in the first half of the 20th century was short-lived. In case of bacterial treatment, as soon as the antibiotics were developed, bacteria responded by manifesting

forms of antibiotic resistance. Resistance to single antibiotics became prominent in organisms that encountered the first commercially produced antibiotics. The most notable example is resistance to penicillin among staphylococci, in particular by the enzyme penicilinase that degrades the antibiotic[8,9,10]. Over the years, continued selective pressure by different drugs has resulted in organisms bearing additional kinds of resistance mechanisms that led to multi-drug resistant pathogens. MDR pathogens are defined as pathogens resistant to three or more antibacterial drug classes[11]. Some of the most problematic MDR organisms that are encountered currently include *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant MRSA, *Klebsiella pneumonia* bearing extended-spectrum β-lactamases (ESBL), *i.e.* penicillin degrading enzymes, vancomycin-resistant enterococci and extensively drug resistant *Mycobacterium tuberculosis*[8,11].

Several factors contribute to the increase and number of antibiotic resistant strains. These include unnecessary prescription of antibiotics, incorrect diagnosis and improper use of antibiotics by patients. In addition, the non-prudent use of antibiotics in animal husbandry for prophylaxis, therapy and growth promotion has contributed to the alarming emergence of the spread of multi-drug resistant (MDR) pathogens[11]. Further, globalization plays a role in the spread of the antibiotic resistance[11].

Multi-drug resistance is a worldwide problem that is not restricted by international borders and can indiscriminately affect members of all socioeconomic classes. The costs associated with antimicrobial resistance are enormous. Therefore, there is a need for new antimicrobial compounds.

Biological Warfare

It is estimated that during the past century, over 500 million people died as a result of infectious diseases. Several tens of thousands of these deaths were due to the deliberate release of pathogens or toxins. The intentional use of disease-causing organisms or toxins to kill, injure or incapacitate humans and animals, to weaken resistance and reduce the will to fight is defined as biological warfare[1].

The use of biological warfare can be found far back in history. For example, ancient armies infected water supplies of entire cities that gave people horrible diarrhea. In addition, in the 14th century, when still little was known about the mechanism of infectious diseases, plague infected bodies were catapulted over city walls to infect the enemy. The foundation of microbiology by Louis Pasteur and Robert Koch offered new prospects for those interested in biological weapons because it allowed agents to be chosen and designed on a rational basis[12]. During World War II (WWII) it seemed like every country at war was interested in the development of biological warfare, but only few actually used them. For example, during WWII, the Japanese operated a secret biological warfare facility in Manchuria and carried out experiments on Chinese prisoners. They exposed more than 3000 victims to plague, anthrax and other agents and released these agents in a village in China. The U.S. started a biological weapon program in 1943 but this was stopped in 1969.

Traditional agents of offensive biological warfare programs have included the causative organisms of anthrax, plague, tularemia, brucellosis, glanders and smallpox. The Centers for Disease Control and Prevention (CDC) currently classifies biological warfare agents most likely to be used into three priority categories, A, B and C, depending on how

***Table 1**. Centers for Disease Control and Prevention classification of critical biological agents[1].*

CDC Category A agents	
• Can be easily disseminated or transmitted person to person • Result in high mortality rates and have the potential for major public health threat • Might cause public panic and social disruption • Require special action for public health preparedness	
Agents	Disease
Bacillus anthracis	Anthrax
Yersinia pestis	Plague
Variola major	Smallpox
Francisella tularensis	Tularemia
Ebola, Marburg, Lassa virus	Viral hemorrhagic fever
Clostridium botulinim toxin	Botulism
CDC Category B agents	
• Are moderately easy to disseminate • Result in moderate morbidity rates and low mortality rates • Require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance	
Agents	Disease
Vibrio cholerae	Cholera (Water safety threat)
Brucella species	Brucellosis
Salmonella species, *Escherichia coli* O157:H7, *Shigella*	Food safety threats
Burkholderia pseudomallei	Melioidosis
Burkholderia mallei	Glanders
Coxiella Brunetii	Q fever
Alphavirusses	Viral encephalitis

easily they can be produced and spread but also the severity of illness or death they cause, with category A (Table 1) having the highest priority[1]. The incubation period, symptoms, disease caused by and the medical tretament of the biological warfare agents, *Baccillus anthracis*, *Yersinia petis*, *Vibrio cholera*, used in studies of this thesis are presented in Table 2.

In 1972 the biological convention has entered into force, prohibiting the development, production and stockpiling of bacteriologic and toxin weapons[12]. This was signed by most of the world, 145 nations. However, even though the Soviet Union just signed the biological convention, they established Biopreparat, a gigantic state-sponsored biological warfare project in 1973. During this project the Soviet Union produced and stockpiled hundred of tons of anthrax bacilli and smallpox virus and engineered multi-drug resistant bacteria, including *Yersinia pestis.* Several large bioweapon production lines have been officially closed but it is thought that Biopreparat and other projects were continued and bioweapon research and development lasted at least through the 1990s[12,13]

Since biological agents are present in nature, easy to acquire and easy to use, there is a reason for concern that such agents will fall into hands of terrorist organizations. In the past, individual and non-governmental groups successfully used potentially dangerous micro-organisms. For example, in 1984, 751 persons were infected with

***Table 2**. Information on the biological warfare agents on which this thesis is focused*[1].

Disease	Dissemination/ infection route	Incubation period (days)	Effective dose	Transmission person to person	Symptoms	Medical treatment
Anthrax -Inhalational	Aerosol / inhalation of spores	1-6	10.000-50.000 spores	No	Flu-like symptoms (fever, fatigue, muscle aches, cough, headache), chest pain, respiratory failure and shock, meningitis	Ciprofloxacin, doxycycline
Anthrax- Cutaneous	Handling infected products / entering the skin through a cut	1-12		No	Intense itching, painless lesions, vesicular lesions, eschar surrounded by edema	Ciprofloxacin, doxycycline
Pneumic plague	Aerosol / inhalation	1-6	100-500 organisms	Yes	Flu like symptoms (high fever, cough, headache nausea, vomiting) respiratory failure	Streptomycin, gentamicin, ciprofloxacin, doxycycline
Cholera	Water / drinking contaminated water, eating contaminated food	1-3	10-500 organisms	Yes	Massive watery diarrhea	Fluid therapy and antibiotics (tetracycline, doxycycline, ciprofloxacin)

Salmonella typhimurium after an intentional contamination of restaurant salad bars in Oregon by a religious sect[12]. More recently, in October 2001, letters containing spores causing anthrax were sent to members of the U.S. Congress and media outlet, resulting in 22 cases of infection, five deaths and approximately 10.000 individuals being offered post exposure prophylaxis[14].

In case of the intentional release of a biological warfare agent, the attack itself may go unnoticed, because of the incubation time, until large groups of people begin exhibiting symptoms. Moreover, most biological warfare agents show non-specific clinical symptoms initially, making rapid diagnosis of the agent used difficult. In addition, commonly used antibiotic therapies to treat anthrax*, e.g.* doxycycline and penicillin, may be effective only when applied within 24 hour post-exposure. Therefore, rapid medical diagnosis and proper treatment of a patient exposed to the biological agent can be lifesaving. However, the currently used medical countermeasures: passive antibody therapy, vaccination and antibiotic therapy have major limitations. Immediate immunity can be provided by passive antibody therapy[15]. Antibodies specific for category A agents including anthrax, smallpox and plague have been used to develop antibody therapeutics. However, passive antibody therapy requires identification of the target agent, which is not always possible[16]. In case of vaccination, a sufficient protective immune response can often only be achieved by repeated vaccination during several months, while significant side effects may occur[16,17,18]. In addition, conventional antibiotics are less effective against drug-resistant strains of *Bacillus anthracis* and *Yersinia pestis*[16]. Because of the genomics revolution, there is an additional concern that a subsequent attack with a biological agent could be with an antibiotic-resistant strain. This genomic revolution makes it possible to enhance the resistance of pathogens to antibiotics or to transfer pathogenic properties among them. Such genetic engineering technologies could make pathogens harder to detect, diagnose and treat and thereby make it more useful for terrorists. Engineered strains of *Bacillus anthracis*, resistant to multiple antibiotics, including frontline agents, ciprofloxacin, doxycycline and β-lactam antibiotics have been constructed[19,20,21,22]. This threat underlines the motivation for the search for new, alternative broad-spectrum antimicrobial compounds.

CONVENTIONAL ANTIBIOTICS AND MECHANISM OF ANTIBIOTIC RESISTANCE

Conventional antibiotics

Antibiotics are compounds that kill or inhibit the growth of micro-organisms. Commonly, antibiotics are classified based on their mechanism of action, chemical structure or spectrum of activity. The primary mode of action is the inhibition of vital steps in the growth or function of the micro-organism. There are four general modes of antibiotic activity: 1) interference with the cell wall synthesis, 2) interference with nucleic acid synthesis, 3) interference with protein synthesis and 4) inhibition of a metabolic pathway[23].

Interference with the cell wall synthesis

Antibiotics that exhibit their antimicrobial mode of action by inhibiting bacterial cell wall synthesis include β-lactams, such as penicillins, cephalosporins and monobactams, and the glycopeptides vancomycin and teicoplanin. β-Lactam antibiotics inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer, which is the major structural component of the bacterial cell wall. Vancomycin inhibits cell wall synthesis via a different mechanism than β-lactam antibiotics. Its primary mode of action is inhibition of the cross-linking of peptidoglycan, required for a stable cell wall synthesis[23,24].

Interference with protein synthesis

Macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins and oxazolidinones exhibit their antibacterial action by inhibition of protein synthesis. Bacterial ribosomes differ in structure from eukaryotic ribosomes. Antibiotics take advantage of this difference by selectively inhibiting bacterial growth. Macrolides, aminoglycosides and tetracyclines bind to the 30S unit of ribosomes whereas chloramphenicol, streptogramins and oxazolidinones bind to the 50S unit[23,24,25].

Interference with nucleic acid synthesis

Fluoroquinolones exert their antibacterial effects by disrupting DNA synthesis and cause lethal double strand DNA replication[26,27].

Inhibition of a metabolic pathway

Sulfonamides and trimethoprim block the pathway for folic acid synthesis, which ultimately inhibits DNA synthesis[23].

Mechanisms leading to antibiotic resistance

The underlying mechanisms leading to antibiotic resistance can be divided in vertical and horizontal evolution. In vertical evolution, normally susceptible bacteria can acquire resistance to an antimicrobial agent via spontaneous, random mutations in the genes found on the bacterial chromosome. Such mutations may lead to resistance by alteration of a specified target of the antibacterial agent *e.g.* resulting from modifying binding sites, upregulation of inactivating enzymes such as β-lactamases, by downregulation or alteration an outer membrane protein channel that the drugs uses as entry or by up-regulation pumps that expel the drugs from the cell. In all these cases, strains of bacteria carrying resistance-conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive[8,23,24]. Bacteria can also develop resistance through the acquisition of new genetic material from other resistant organisms, termed horizontal evolution. This process may occur through exchange of genetic mobile elements. Although gene transfer among organisms within the same genus is common, this process has also been observed between very different genera, including transfer between such evolutionary distant organisms as Gram-positive and Gram-negative organisms[8,23,24].

HOST DEFENSE PEPTIDES

Alternative antibiotic agents

Host defense peptides (HDPs) are a universal feature of the defense system of virtually all forms of life ranging from bacteria to plants, invertebrates and vertebrates, including mammals. They form part of the ancient, nonspecific innate immune system, which is the principle defense system for the majority of living organisms[29,28]. HDPs display a wide range of biological activities against invading pathogens like bacteria, fungi, protozoa and enveloped viruses. Moreover, HDPs exhibit additional immunomodulatory activities essential for the orchestration of the innate immune and inflammatory response. During the last decades, HDPs have been widely studied as alternative to conventional antibiotics, especially for the activity against drug-resistant bacteria[9,28,30,31,32].

HDPs in vertebrates

HDPs have been isolated from a wide range of vertebrate species, including mammals, indicating that even in the presence of an adaptive immune response, these peptides play an important role in host defense[29]. In mammals, HDPs have been found at sites that routinely encounter pathogens, such as mucosal surfaces and skin, the crypts of the small intestine and within the granules of immune cells[33]. The expression of HDPs can be constitutively or their production and release may be induced by infectious and/or inflammatory stimuli, such as pro-inflammatory cytokines, bacteria or by conserved microbial structures, termed pathogen-associated molecular patterns (PAMPs), *e.g*. lipopolysaccharides (LPS)[34,33,35].

Cathelicidins

Two major classes of HDPs can be identified: defensins and cathelicidins. Whereas defensin structure is based on a common beta sheet core stabilized by three disulfide bonds, cathelicidins are diverse in their structure[28,33,36]. Cathelicidins are synthesized as inactive pre-pro-peptides. The N-terminal pre-region consists of a putative signal peptide (29-30 residues) followed by a highly conserved sequence of the peptide pro-region (94-114 residues) which is termed “cathelin” domain. The C-terminal domain represents the biological active peptide that varies considerably between individual molecules in sequence, length (12-100 residues) and function[33,37,38]. Cathelicidins have been isolated from multiple species and include cow indolicidin, pig PR-39, rabbit LL-37/CAP-18, sheep SMAP34, human hCAP18, mouse CRAMP, monkey RL-37 and chicken CATH-2 and display considerable interspecies conservation of their primary sequence[28,39,40, 41,42,43,44, 45,46].

Most cathelicidins are stored in an inactive pro-peptide state, mostly within the granules of circulating immune cells. However they are also expressed in various non-myeloid cells, particularly at sites such as skin and mucosal surfaces that face the external environment[33,47]. The processing of the pro-peptide to its mature, active peptide is shown to take place in activated circulating myeloid cells like neutrophils in human and heterophils in chicken[48,49,50,51].

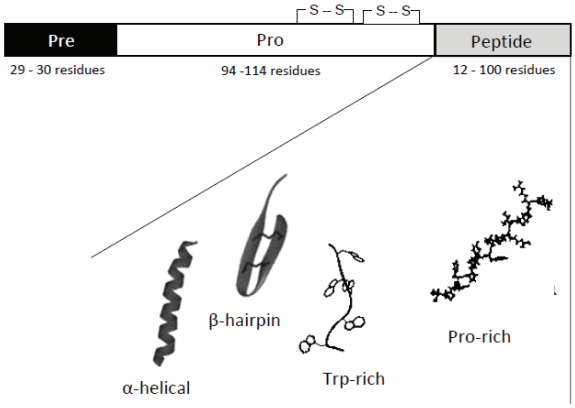

Figure 1. *Organization of mammalian cathelicidins and representative mature peptides. Peptide encoding regions for signal peptide (black bar), pro-piece or "cathelin" domain (white bar) and mature peptide (gray bar). The structural classes of mature cathelicidins; α-helical, β-hairpin, Trp-rich and Pro-rich peptide*

Beyond the common "cathelin" domain, mature cathelicidins are very heterogenic in sequence. However, they can be organized into three groups by structure. The first and most widespread groups of cathelicidins are linear peptides of 23-37 residues lacking cysteine residues that fold into amphipathic α-helices in environments mimicking biological membranes. The second group of the cathelicidin family consists of 12-18 residues with an even number of cysteines forming β–hairpin structures stabilized by disulfide bridges. The third group contains peptides that lack cysteine residues and are rich in certain amino acids like proline or tryptophan[37]. While structure of the three groups may vary, most peptides in their final folded conformations have a net positive charge of at least 2+ at neutral pH. In addition, peptides are amphipathic, having a hydrophobic face comprising non-polar amino-acid side chains and a hydrophilic face comprising of polar and positively charged residues[37].

In the search for alternatives for conventional antibiotics, cathelicidins are receiving particular attention as novel antimicrobials due to their simple structures and high antibacterial efficacy relative to defensins and other HDPs[38].

Cathelicidin mode of antibacterial action

The prominent function of cathelicidins is to inhibit growth of micro-organism including bacteria, protozoa, fungi and viruses. The killing activity is found to be fast at the concentrations in the micromolar range[9,28,30,31,32]. Among the investigations on the mechanism of action on cathelicidins, the majority of studies have been centered on bacteria.

The principle target of cathelicidins is the bacterial membrane. To reach this target, cathelicidins must traverse enveloping structures of varied complexity. Gram-positive bacteria possess a thick wall composed of heavily cross-linked polymers of teichoic or lipoteichoic acids (LTA) and peptidoglycan. Gram-negative bacteria do not produce teichoic acids but have a multilayered surface structure including a peptidoglycan matrix in the periplasmic space beneath an outer membrane. The outer membrane contains lipopolysaccharide (LPS). The structure of this molecule varies considerably

between strains of Gram-negative bacteria. LTA and LPS are negatively charged and facilitate the initial electrostatic attraction between the positively charged peptides and the bacterial cell[52,53]. Relatively little is known about the passage of cationic peptides across the LTA layer. Most research has been focused on Gram-negative bacteria. In the case of Gram-negative bacteria, passage of the cationic peptides is proposed to occur by a process, which was first described by Hancock[52,53], called 'self promoted uptake'. Due to their high affinity for LPS in the outer membrane, cationic peptides can competitively displace Ca^{2+} and Mg^{2+} ions, important for microbial membrane stability. Because the cationic peptides are bulky, this leads to disturbance of the outer membrane structure and permeabilization of the outer membrane and thereby promotes the uptake of the peptide itself. This results result in the arrival of the peptides at the inner membrane.

After passage of the outer membrane, peptides enter the interfacial region of the inner membrane, by a process that is driven by electrostatic and hydrophobic interactions, causing damage to the lipid bilayer. Phospholipids are key elements of most biological membranes. Because of their amphipathic nature, phospholipids organize into bilayers with their polar heads facing the aqueous face. The net charge of the membrane is based largely on the phospholipid composition. Bacterial membranes are highly electronegative because they are largely composed of acidic phospholipids such as phosphatidylglycerol and cardiolipin, which are responsible for the electronegative interaction. The events which take place following peptide interaction with the bacterial membrane remain under debate and several models have been proposed. These include the barrel-stave model, the carpet model, the detergent model, the toroidal pore model and aggregate model. Hence the mode of action of cathelicidins can be divided in membrane perturbation and impairment of intracellular processes.

Mechanism of membrane disruption

The functional integrity of the membrane is crucial to many essential functions of bacterial pathogens. Membrane dysfunction caused by cathelicidins, including breakdown of membrane potential, loss of metabolites and ions and altered membrane permeability, leads to death directly or indirectly. Three main mechanisms have been suggested for peptide permeation of the target membrane. The barrel stave model involves the formation of transmembrane channels in a voltage-dependent manner with non-polar domains of the peptides facing the membrane lipids thus forming a hydrophilic pore spanning the membrane[54,55]. The carpet model involves covering of the membrane by a layer of cathelicidins. When a threshold concentration of peptide is reached, the peptide monomers reorient towards the hydrophobic core of the membrane. This subsequently leads to collapse of membrane packing and micelle formation[56,57,58]. The aggregate channel model involves arrangement of peptides in unstructured clusters in the membrane allowing the dynamic formation of pores for short periods of time and the leakage of intracellular components. Cathelicidins can also enter the intracellular space through this mechanism.

Intracellular targeting

Evidence is found that other mechanisms exist than cathelicidin induced membrane dysfunction. In several studies the relationship between bacterial membrane permeabilization and cell death was evaluated, revealing that cell killing may proceed with

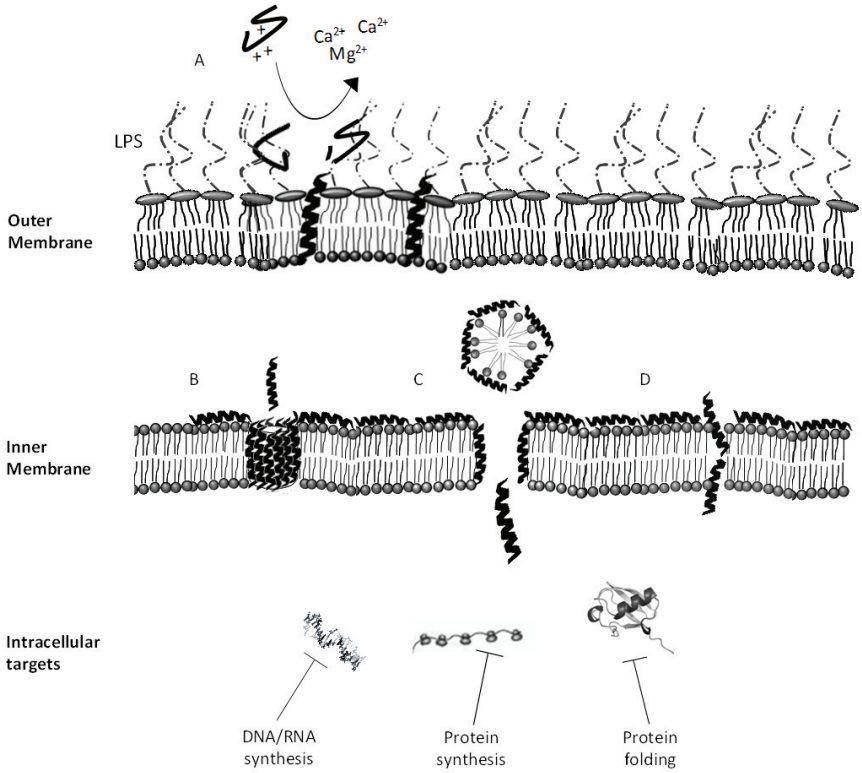

Figure 2. *Proposed mechanism of action for cathelicidins in Gram-negative bacteria. First, displacement of membrane-stabilizing ions in the outer membrane during the initial interaction with outer membrane components (A). Subsequently, the proposed models of membrane permeabilization are explained in the (B) barrel stave model (C) carpet model (D) aggregate model. The net effect is that some peptides will be translocated to the cytoplasm and affect intracellular targets.*

relatively little membrane disruption and suggests that some cathelicidins mainly interact with putative key intracellular targets[29,59,60,61]. Examples of intracellular activities include inhibition of DNA and RNA synthesis, inhibition of chaperone-assisted protein folding, inhibition of enzymatic activity and inhibition of cytoplasmic septum formation and cell wall synthesis[59].

Molecular basis of cathelicidin cell selectivity

One interesting property of cathelicidins is their cell selectivity for prokaryotic membranes over mammalian membranes. The main factors involved in this selectivity have been identified and attributed to both peptide and membrane characteristics. Concerning peptide structure, the net cationic charge and the presence of hydrophobic amino acids, have been reported to play an important role in cell selectivity. The net cationic charge of cathelicidins leads to a strong interaction with the anionic charged phospholipids present in the bacterial membranes. In contrast to bacterial membranes, outer leaflets of mammalian membranes are neutral in charge because they are enriched in zwitterionic phospholipids, such as phosphatidylcholine and sphingomyelin, although negatively

charged gangliosides are present in minor species. Acidic phopholipds are usually sequestered in the inner leaflets of the mammalian membranes. On the other hand, despite the presence of hydrophobic amino acids is essential for cathelicidin insertion into the bacterial membrane, a high level of hydrophobicity correlates with a strong affinity to the neutral mammalian membranes leading to a high level of toxicity. Regarding the mammalian membrane, the presence of cholesterol protects mammalian cells by stabilizing the membrane or by direct neutralization of cathelicidins. Therefore, mammalian membranes which are enriched with cholesterol are less susceptible to cathelicidins compared to bacterial membranes which do not incorporate cholesterol[62]. Furthermore, the transmembrane potential is usually higher and negative inside bacterial membranes, which is a driving force for the insertion and translocations of positively charged peptides into bacterial cell membranes[62].

Immunomodulatory characteristics of cathelicidins

In addition to their antimicrobial activity, many cathelicidins exhibit immunomodulatory activity. Generally, cathelicidins exhibit dual functionalities. At one hand they protect the host against potentially harmful pathogens by their antimicrobial activity and by stimulation of immune functions. At the other hand, they protect the organism from the detrimental effect of an excessive inflammatory response.

Immune stimulation

Cathelicidins generated at sites of infection form chemotactic gradients, which result in the recruitment of mononuclear phagocytes and other immune cells such as T cell lymphocytes, mast cells and neutrophils. Besides their intrinsic chemoattractant properties, which directly promote the attraction and arrival of cells to the site of injury, cathelicidins indirectly favor chemotaxis by inducing the secretion of chemokines, such as interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), by mononuclear phagocytes and epithelial cells[30,33,63,64,65]. In addition, **s**everal cathelicidins are able to induce or enhance the production of pro-inflammatory cytokines in different cell types[66,67,68,69,70,71] and are able to enhance phagocytosis by dendritic cells and enhance the maturation of dendritic cells[66,72]. All these effects will augment the uptake, processing and presentation of antigens, and stimulate the clonal expansion of T-lymphocytes and B-lymphocytes. B-lymphocytes will produce antibodies that are highly specific for pathogen antigens, contributing to the clearance of microbes through phagocytosis[33].

Inhibition of immune responses

In addition to these immune stimulating activities, cathelicidins have the ability to reduce the pro-inflammatory response. The pro-inflammatory response is a response of the innate immune system upon recognition of conserved molecular patterns, among them bacterial cell wall and outer membrane components, as signals of bacterial infection, *e.g*. LPS in Gram-positive bacteria and LTA in Gram-negative bacteria. Upon the release of these components as a result of cell division, death, or in particular antibiotic treatment against bacterial infection, these components are recognized by pattern recognition receptors (PRRs), *e.g*. Toll-like receptors (TLRs). These receptors are expressed on innate immune cells, mainly by mononuclear phagocytes, resulting in their activation that is

characterized by increased phagocytic activity, secretion of pro-inflammatory cytokines such as tumor necrosis factors-alpha (TNFα), interleukin 6 (IL-6) and induction of other pro-inflammatory responses such as inducible NO synthase (iNOS). Activation of inflammation is beneficial, and helps to clear the invading pathogen. However, prolonged uncontrolled inflammation is detrimental. When LPS enters the blood, the inflammatory response may lead to septic shock characterized by endothelial damage, loss of vascular tone and multiple organ failure often resulting in death. Many cathelicidins interact with bacterial membranes and cell wall components (*e.g*. LPS) and counteract the pro-inflammatory response[30,33,63,64,69,73,74,75]. In addition, cathelicidins selectively affects LPS-induced gene expression[76,77].

Mechanism of immunomodulatory action

Despite the wide range of immunomodulatory responses, there is still surprisingly little known of the precise molecular basis for these responses but a number of potential pathways have been proposed[78]. First, it has been suggested that cathelicidins can function as an alternative ligand for certain receptor proteins. This mechanism has been proposed for the interaction of human LL-37 and mouse CRAMP with the FPRL1 receptor, leading to direct signaling cascades and chemotaxis of different immune cells[17,68,79]. However, in view of the high propensity of cathelicidins to interact with lipid bilayers because of their amphipathic nature, it can not be ruled out that the activation of surface receptors may be mediated by a local perturbation of the membrane rather than through receptor-ligand binding. A different mechanism may rely on an indirect process, namely from scavenging of other signaling molecules of either host or external origin. For example, the charge of cathelicidins may contribute to binding of cathelicidins to the negatively charged LPS, thereby preventing LPS-induced phagocyte activation and pro-inflammatory cytokine secretion[80,81,82]. More detailed mechanisms at the molecular level are still a matter of debate and are very target-specific[78].

DESCRIPTION OF SELECTED CATHELICIDINS IN THIS THESIS

Human cathelicidin: LL-37/hCAP-18

Structure

The human cathelicidin, LL-37, is generated from the pro-peptide hCAP-18 encoded by the CAMP gene. Following excision of the signal peptide this pro-peptide is predominately stored in granules of neutrophils[43,83] but is also found in monocytes and specific lymphocyte populations, testis[33,37], human keratinocytes during inflammatory disorders[84], airway epithelium[85] and intestinal colon cells[86]. The hCAP-18 precursor protein is cleaved upon neutrophil degranulation by proteinase-3 to yield the 37 amino acid peptide LL-37[87]. LL-37 is a classical amphipathic α-helical peptide consisting of a disordered non-polar hydrophobic N-terminal following a bent α-helix in the middle. Glycine (G) residues are responsible for this bend. The C-terminal end is a short hydrophilic tail unit (Table 3, Figure 3).

Antimicrobial activity and toxicity to mammalian cells

LL-37 is active against a variety of pathogens, ranging from Gram-positive and Gram-negative bacteria to yeasts[88,89] and viruses[18,33,90]. The minimal inhibitory concentrations for a variety of Gram-positive and Gram-negative bacteria range from 1-10 μM. LL-37 is not as selective as some other α-helical HDPs, with toxicity to mammalian cells at 13-25 μM[91,92,93,94]. The most plausible model of antibacterial action for LL-37 appears to be the carpet model[95,96].

Chemotactic activity

LL-37 attracts neutrophils, monocytes and T cells using formyl peptide receptor-like 1 (FPRL1), and a distinct G_i-coupled receptor for mast cell attraction[79,97,98]. Besides the intrinsic chemoattractant properties, LL-37 indirectly favors chemotaxis by inducing or increasing the secretion of chemokines. LL-37 induced IL-8 secretion is observed in keratinocytes, airway epithelial cells[68], human airway smooth muscle cells[99] and myeloid cells[100]. LL-37-enhanced production of IL-8 is under control of MAPK p38 and extracellular signal-regulated kinase (ERK)[70,101]. In monocytes and macrophages LL-37 induces the chemokines IL-8, MCP-1, Gro-α, MIP-3α[76].

Immune stimulation

LL-37 enhances the cytokine responses of monocytes and macrophages to GM-CSF and IL-1β, but suppresses those to IFNγ. Furthermore, LL-37 affects macrophage development to a pro-inflammatory state. In addition, LL-37 bridges innate and adaptive immune responses by affecting monocyte to dendritic cell differentiation and enhances the GM-CSF/IL-4-driven differentiation of blood monocytes to immature DCs[66,71].

Inhibition of endotoxin induced pro-inflammatory response

In monocytic cells, LL-37 is capable of neutralizing the pro-inflammatory response to the TLR4 ligand LPS[30,64,69,73,74], the TLR2 ligand LTA[69,76,102] and the TLR9 ligand CpG[69]. The pathophysiological relevance of LL-37 in the treatment of septic shock was demonstrated in animal models, where this HDP was shown to protect mice and rats from LPS-mediated lethality[69,103,104]. The LPS neutralizing activities of LL-37 can partly be attributed to a strong binding to LPS accompanied by aggregate formation and subsequent prevention of LPS-binding to the carrier protein LPS-binding protein[74,105] as well as its ability to selectively suppress LPS-induced macrophage gene expression[70]. However, the precise mechanism by which LL-37 modulates gene expression remains unclear. Besides, LL-37 inhibits the pro-inflammatory cytokine production in monocyte-derived dendritic cells stimulated with the TLR4 ligand LPS, the TLR-2 ligand LTA and the TLR5 ligand bacterial flagellin[106].

Structure function relationship

An in depth understanding of the structure-activity relationship (SAR) is crucial for the development of artificial variants with optimized activity for therapeutic application. SARs for especially antibacterial and cytotoxic activities have revealed that the activities of amphipathic α-helical HDPs are not determined by the precise primary structure. Rather, activity was found to be determined by the subtle interplay of structural and physico-chemical parameters such as cationicity, hydrophobicity and amphipathicity[107].

Table 3. Chicken and human cathelicidins

Name	Synonym	Sequence*	Charge	Length
LL-37	hCAP18, FALL39[115]	*LLGDFF***RK**S**K**E**K***IG***K**E*F***KR***IVQ***R***I***K***DF*L***R***NLVPR*T*ES	6+	37
Cathelicidin-1	Fowlicidin-1[116]	**R***V***KR***VWPLVI***R***TVIAGYNLY***R***AI***KKK**	8+	26
Cathelicidin-2	Fowlicidin-2[116]	*LVQ***R**G**R***FG***R***FL***RK***I***RR***F***R***P***K***VTITIQGSAR***F***	10+	31
	CMAP27[46]	***R***FG***R***FL***RK***I***RR***F***R***P***K***VTITIQGSAR***FG***	9+	27
Cathelicidin-3	Fowlicidin-3[116,117]	**KR***FWPLVPVAINTVAAGINLY***K***AI***RRK***	6+	27

*cationic residues in **bold,** anionic residues are underlined, hydrophobic residues in *italic*.

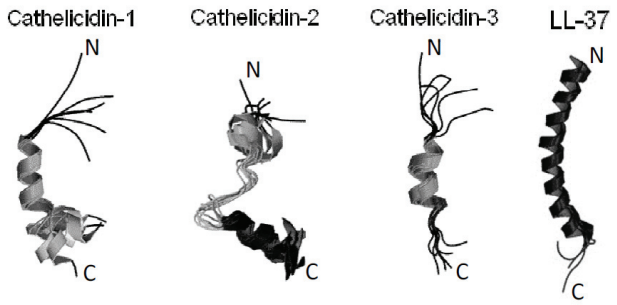

Figure 3. *Structural organization of Chicken Cathelicidins 1-3 and LL-37 (Adapted from the published 3-dimensional structure of the peptides as determined by NMR spectroscopy (RCSB Protein Data bank; http://www.rcsb.org/pdb/home/home.do). PDB structures 2AMN[120], 2GDL[75], 2HFR[117,] 2K6O[110]. N and C indicate N- and C-termini.*

By the use of truncated LL-37-variants, evidence has been gathered suggesting that the antibacterial activity of LL-37 is not located in the N-terminal of the peptide[64,108,109,110,111,112,113] but can be ascribed to the mid-region of LL-37[108,110,113] with as minimal antibacterial domain residues 17-33[110]. This mid-region consists of an amphipathic α-helix and 4 cationic residues. Although all the physico-chemical properties of LL-37 contribute to the antibacterial activity, cationic side chains on the hydrophilic surface of the peptide are most accountable[110].

Although discrepancies exist between studies that defined the part of LL-37 responsible for cytotoxic effects in eukaryotic cells, it is clear that hydrophobic residues play an important role in the toxicity of LL-37 to eukaryotic cells[64,109,110,111].

Because LL-37 has proven to be effective in binding of LPS, it has potential in the treatment of endotoxemia. In order to seek a viable therapeutic agent, there have been a number of attempts to dissect this activity from both immune-stimulatory and host cell toxicity properties of LL-37. Therefore, the minimal LPS neutralizing has to be known. The mid-portion of LL-37, amino acids 13-31, forms the minimal domain necessary for suppression of the LPS pro-inflammatory response[80,82,88,102,111,114].

Chicken cathelicidins

Structure

To date, 4 chicken cathelicidins have been identified: cathelicidin (CATH)-1, -2, -3 and B-1[46,116,117,118,119]. Chicken CATH-1 and -3 are also referred to as fowlicidin-1 and -3, respectively. CATH-2 is also referred to as fowlicidin-2 and chicken myeloid antimicrobial peptide 27 (CMAP27).

Comparable to mammalian cathelicidin, chicken cathelicidins are generated from their pro-peptide. The predicted signal peptide sequence of chicken cathelicidins is much shorter (~17 aa) compared to mammalian cathelicidins (~30 aa)[46]. Following excision of the signal peptide, the pro-peptide of CATH-2 is predominantly stored in heterophils and in their precursor cells in bone marrow[51]. Likewise, CATH-1 and -3 are found to be highly expressed in the bone marrow[118]. The pro-peptide is cleaved upon heterophil degranulation. Although the involved specific proteases in chicken cathelicidin processing still have to be identified, it was shown that a serine protease is responsible for CATH-2 release[51]. Compared to CATH-1, -2 and -3, B1 appears to be an outlier. Phylogenetic analysis revealed that the cathelin region of B1 belong to a different branch of the phylogenetic tree compared to the 3 other chicken cathelicidins. Further, B1 is expressed exclusively by a distinct subpopulation of bursal epithelial cells[119].

As mentioned before, amphipathicity is a characteristic of most α-helical cathelicidins. However, CATH-1 and CATH-3 are much less amphipathic, with no obvious segregation of hydrophobic residues from hydrophilic residues. Instead, the α-helical region is highly hydrophobic. CATH-1 and -3 have similar structures, consisting of 2 α-helical segments connected by a glycine induced slight kink in the center and a flexible unstructured region at the N-terminal end[117,120] (Table 3, Figure 3). CATH-2 consists of two well defined α-helices separated by a proline long extensive kink and a flexible region at the N-terminus. In contrast to most α-helical cathelicidins with a limited degree of kink near the center, the kink of CATH-2 in the central region is extensive due to the presence of the proline (P) (Table 3, Figure 3). The N-terminal α-helix adopts a typical amphipathic structure, while the C-terminal helix is highly hydrophobic. It is noted that the central kink region of CATH-2 carries strong positive charge, containing 5 cationic residues[63,116].

Biological activity

CATH-1, -2 and -3 display potent broad antibacterial activity against different Gram-positive and Gram-negative bacteria with similar efficiency against antibiotic-susceptible and antibiotic-resistant bacterial strains[46,116,117]. CATH-1, -2 and -3 have comparable minimal inhibitory concentrations (MICs) which range, dependent on the bacterial strain, between 1 - 10 μM[117,120]. Moreover, CATH-1, -2 and -3 bind LPS and block the LPS-induced cytokine release from mouse macrophages[116,117,120]. However, like other cathelicidins, chicken cathelicidins are toxic to mammalian cells in high concentrations. CATH-1 and -2 exhibit similar toxicity to mammalian cells. Fifty percent of chicken red blood cells (RBC) are lysed at ~20 μM peptide[63]. At 10 – 20 μM, fifty percent of Madin-Darby canine kidney cells were killed in the absence of fetal calf serum[116]. Similar results were obtained for the mouse macrophage cell line RAW264.7[116]. CATH-3 is the least toxic to mammalian cells, being 4-6 times less toxic compared to chicken CATH-1[117].

Structure-function relationship

The structure-activity relationship of chicken CATH-1[120] and CATH-2[63] was evaluated by using truncated variants. These studies revealed that the C-terminal helix after the kink of CATH-1 is more important in antibacterial activity then the N-terminal helix. The N and the C-terminal helix both have the same net cationic charge (4+). Therefore, this is probably caused by the more pronounced hydrophobic nature of the N-terminal helix. In addition, this region plays a role in LPS-binding and toxicity to mammalian cells. The N-terminal flexible region that participates in LPS binding and cytotoxicity is less important in bacterial killing[120]. In contrast to CATH-1, the N-terminal helix of CATH-2 is more important in antibacterial activity than the C-terminal helix. This can be probably ascribed to the high net positive charge present at the N-terminal part of CATH-2. For immune stimulation and strong LPS neutralizing activity, the N- and C-terminal helices are both required. Furthermore, the hinge region in CATH-2 was proven to be necessary for the biological activity of the peptide; substitution of proline-14 in the hinge region by leucine, strongly reduced the antibacterial, LPS-neutralizing and immune stimulating activity[63].

CHALLENGES IN THE DEVELOPMENT TO THERAPEUTICS

Host defense peptide resistance

Concerns have been raised that a widespread use of HDPs in the clinic would select for pathogens resistant to natural immune defenses. Indeed many bacterial species already posses slightly effective resistance mechanisms. HDPs with simple linear or α-helical structure, *i.e*. α-helical cathelicidins are susceptible to proteolysis and are targeted by several bacterial proteases[121,122]. For example, LL-37 is inactivated by cleavage by *Staphylococcus aureus* aureolysin and V8 protease[122,123], by *Pseudomonas aeruginosa* elastase[122,124] and by *Tannerella forsythia* produced karilysin[125]. A different mechanism of bacteria to resist HDPs can be the prevention of HDPs from reaching the bacterial cell membrane by bacterial secreted proteins like staphylokinase in *Staphylococcus aureus*[121]. In addition, there exist evidence that certain bacteria can actively extrude HDPs from the bacterial cells[121]. For example, an energy dependent efflux system termed *mtr* mediates resistance to HDPs of diverse structure in *Neisseria gonorrhoeae*. An other potential concern that has been noted is that *S. aureus* and a lot of other bacteria produce the cationic lipid lys-phosphatidyglycerol (lys-PG), which leads to a reduction in their net anionic charge of their cell envelope, reducing the affinity of HDPs to them[121,126]. In addition, it has been shown that resistance to HDPs can be selected in the laboratory; Perron and co-workers showed that heritable resistance to the cationic HDP pexiganan evolved in *Pseudomonas fluorescens* and *Escherichia coli* after 600-700 generations[127]. Even if these outcomes may raise several questions about safety in developing and introducing HDPs in the clinic, we cannot predict the time-scale required for the appearance of resistant strains in a natural situation. In addition, the mechanisms for resistance development are probably relatively inefficient compared to those that inhibit the effect of conventional antibiotics because they often use several microbial mechanisms simultaneously, targeting many microbial systems with low affinity rather

than having one specific target. Furthermore, only direct killing activity is affected by resistance mechanism of bacteria whereas immunomodulatory functions are untouched.

Stability

Peptides are relatively sensitive towards proteolytic degradation. The rapid clearance of peptides and unfavorable pharmacokinetics, due to proteolytic degradation, may severely restrict their success in systemic and oral administration and thereby their applicability. In this context, it is noteworthy that clinical trails of peptide therapeutics have been generally focused on topical application. To increase half-life, many strategies involving several formulations, ways of administration and different levels of chemical modifications are possible. The introduction of D-amino acids[128,129,130,131,132] and non-natural amino acids[134,135], amidation of the N-terminus[133], and cyclization[136,137] are the most common strategies to increase peptide stability.

Sensitivity to high ionic strength and plasma proteins

HDPs are highly active in non-physiological conditions (*e.g.* phosphate buffer), but a significant reduction in their antibacterial potency occurs in the presence of complex fluids such as plasma, serum, saliva and sputum[138,139,140]. The proposed inhibitory mechanisms exerted by biological fluids include cations and anionic proteins present in biological fluids. Monovalent cations such as Na^+ and K^+ (100 mM) and divalent cations like Mg^{2+} and Ca^{2+} (1-2 mM)[141] present in serum and other biological fluids may compete with cathelicidins for binding to the surface of bacterial cells and thereby inhibit their antibacterial activity[142,139]. Besides, anionic proteins, such as albumin and apolipoprotein A-1[143,144], which are abundantly present in biological fluids may scavenge HDPs and reduce their availability[139,140,145].

Selectivity

Systemic application would certainly expand the usefulness of HDP derived products. However, in general HDPs are usually toxic when injected into the bloodstream, although this issue has not been well documented. HDPs are believed to show cell selectivity, meaning selectively kill bacterial cells without significantly being toxic to host cells based on the difference in membrane composition described earlier. Several studies clearly showed that HDPs exhibit cell selectivity[62,146,147,148]. For example, a dye-labeled magainin, selectively bound to *Staphylococcus aureus* but not to surrounding epithelial cells[62]. However, HDPs can in addition to bacterial cell membranes, also interact with human cell membranes in the absence of bacteria. This interaction is at least partly responsible for toxicity to mammalian cells of some peptides. These setbacks led to the increased efforts to develop novel synthetic HDPs with less cytotoxic effects and improved antibacterial activity.

Production costs and production methods

Cathelicidins are easy produced by organic synthesis. However, at the moment, the cost price of synthetics is higher than that of conventional antibiotics. This hampers the full scale production of HDPs for therapeutic purposes. Costs may be reduced by focusing on linear peptides of minimal length, truncated variants and production in large quantities.

A cost effective alternative for organic synthesis to produce HDPs, may be by the production by recombinant expression methods. However, expression of HDPs faces large potential difficulties because their cytotoxicity to host cells and sensitivity to proteolytic degradation of non-structured peptides. Expression systems with an HDP fused to a partner protein may avoid these problems because of the reduced toxicity against host cells and enhanced product stability. Furthermore, the fusion protein can facilitate product purification. Several biological expression systems have been developed by fusing HDPs to a partner protein to mask the intrinsic antibacterial activity of the peptide in bacterial cells and toxicity to other host cells such as yeast or insect cells. However, purifying the peptide in a simple and cheap way is a common problem[149]. Nowadays, an *Escherichia coli* expression system is still the most commonly used because it is one of the most simple and cost-effective systems and suitable for large scale production of recombinant proteins[149,150]. During the last years many improvements have been made with respect to the expression and purification of HDPs like LL-37 in *Escherichia coli*[151,152,153,154]. This indicates that cost effective production of HDPs in the future will be possible.

THESIS OUTLINE

The increasing resistance of bacteria to conventional antibiotics has encouraged strong efforts to develop new antimicrobial agents. In addition, exposure to biological warfare agents may cause highly progressive, acute infections that may be lethal in some forms. Unfortunately, an attack by biological warfare agents may go unnoticed until large groups of people begin exhibiting symptoms and timely diagnosis is not always possible. Therefore, the early use of broad-spectrum antibiotics might be life saving.
Host defense peptides (HDPs) are widely considered to be excellent lead structures for the development of novel broad-spectrum antibiotics, because of their broad-spectrum of antimicrobial activity. In addition, they can modulate the innate immune response and boost infection-resolving immunity, while dampening potentially harmful pro-inflammatory (septic) responses. The aim of this thesis is the development of effective antibacterial and LPS neutralizing agents based on naturally occurring HDPs. It is expected that the new antibacterial peptides can be used *in vivo* to treat bacterial infections, including bacterial agents which could potentially be used for terroristic purposes. This thesis focuses on the *in vitro* biological activity of LL-37 and CATH-2 derived peptides. In **Chapter 2,** the minimal LPS-neutralizing domain of the best studied HDP in man, LL-37, is described and correlated with the physico-chemical properties to determine the features that are important in LPS-neutralizing. In **Chapter 3**, the structure-activity relationship of the newly discovered HDP chicken cathelicidin-2 (CATH-2) is evaluated. The N-terminal part of CATH-2 represents an attractive lead structure in the development of an antibacterial drug candidate. In **Chapter 4**, the improvement of the antibacterial and LPS-neutralizing properties of the N-terminal part of CATH-2, C1-15 by amino acid substitutions is presented. In **Chapter 5** the serum stability and stability to mammalian and bacterial proteases of the most promising C1-15 variant, $F_{2,5,12}W$, is described. In **Chapter 6** the inhibitory effect of the CATH-2 derived peptide $F_{2,5,12}W$ on *Staphylococcus epidermidis* biofilms is evaluated. **Chapter 7** provides a summarizing discussion.

CHAPTER 2

Structure - function relationship of the human antimicrobial peptide LL-37 and LL-37 fragments in the modulation of TLR responses

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ABSTRACT

Cathelicidins are effector molecules of the innate host defense system that establish an antimicrobial barrier at epithelial interfaces. The human cathelicidin LL-37 in addition to antimicrobial activity also exhibits immunomodulatory effects such as inhibition of pro-inflammatory responses to bacterial lipopolysaccharide (LPS) in human monocytic cells. In this report we show that LL-37 almost completely prevents the pro-inflammatory cytokine release by human peripheral blood mononuclear cells (PBMCs) following stimulation with Toll-like receptor (TLR)4 and TLR2/1 agonists while leaving TLR2/6, TLR5, TLR7 and TLR8 responses unchanged. Modulation of the TLR response by LL-37 occurred at least partly through the mitogen-activated protein (MAP) kinase pathway via inhibition of p38 phosphorylation. By using a LL-37 library with overlapping sequences, we identified the mid-region of LL-37, ranging from amino acid 13-31, as the active domain for modulation of TLR responses. The mechanism of immunomodulation of LL-37 and LL-37 fragments is LPS binding. Correlations between the capacity of LL-37 fragments to modulate TLR responses and their physico-chemical properties revealed that cationicity and hydrophobicity are essential for the modulation of LL-37 mediated TLR responses.

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INTRODUCTION

The efficacy of the mammalian host defense system against invading pathogens can be attributed to the capability of the immune system to quickly and specifically recognize and neutralize microbial invaders. The innate immune system plays a key role in the recognition of microbes. Toll-like receptors are primary cellular sensory molecules, which recognize conserved microbial structures, termed pathogen-associated molecular patterns (PAMPs). PAMP recognition leads to activation of signaling pathways resulting in the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8), which initiate both innate and adaptive immune responses[34,35]. In addition to the production of cytokines and chemokines, activation of the innate immune system leads to the synthesis and mobilization of host defense peptides (HDPs). HDPs are capable of directly killing a variety of pathogens, ranging from Gram-positive and Gram-negative bacteria to yeast and viruses[33].

In mammals cathelicidins are a well studied category of HDPs[33,37,121,155]. The sole human cathelicidin, hCAP18, is predominantly produced in granules of neutrophils[43] but is also found in monocytes and specific lymphocyte populations, testis[33], human keratinocytes during inflammatory disorders[84], airway epithelium[85] and saliva. The hCAP18 precursor protein is cleaved upon neutrophil degranulation by proteinase-3 to yield the 37 amino acid peptide LL-37[87]. As a result of proteolytic cleavage LL-37 derivatives KR-20, RK-31, KS-30 are generated. These three peptides exhibit stronger antimicrobial activity compared to full length LL-37[156]. In addition to its broad anti-microbial activity, LL-37 was found to counteract the development of septic shock[157,158]. Pro-inflammatory cytokine secretion is essential for the development of a proper inflammatory response. However, killing of bacteria by antibiotics, HDPs, phagocytes and/or the complement system can result in an exaggerated response. This can result in early sepsis, in which high levels of cytokines and inflammatory mediators become destructive, causing organ failure, cardiovascular shock and death[157,158]. LL-37 was reported to bind LPS with high affinity and is capable of neutralizing the pro-inflammatory response to the TLR4 ligand LPS[30,64,69,73,74], the TLR2 ligand LTA[69,76,102] and the TLR9 ligand CpG[69]. The pathophysiological relevance of LL-37 in the treatment of septic shock was demonstrated in animal models, where this HDP was shown to protect mice and rats from LPS-mediated lethality[69,103,104]. Mookherjee and co-workers indicated that LL-37 might act on the nuclear factor kappa beta (NF-κβ) pathway in inhibiting the endotoxin-stimulated pro-inflammatory cytokine expression[76]. However, the mechanisms by which LL-37 modulates the NF-κB pathway and its interaction with other signaling molecules remain unclear.

Although HDPs are diverse in their structure and size, they are mostly amphipathic, containing both cationic and hydrophobic faces. Structure activity-relationship (SAR) studies, especially for antibacterial and cytotoxic activities, have revealed that the activities of α-helical HDPs are not determined by their precise primary structure. Rather, the antibacterial and cytotoxic activities were found to be determined by the interplay of structural and physico-chemical parameters such as cationicity, hydrophobicity and amphipathicity[107].

The aim of the current study was to gain detailed insight in the mechanism by which LL-37 confers neutralization of TLR responses. For this purpose, the physico-

chemical characteristics of LL-37 fragments were correlated with the ability to neutralize TLR responses. In addition, we provide evidence that the main mechanism of action of LL-37 and LL-37 fragments in neutralizing TLR responses can be contributed to ligand binding.

MATERIAL AND METHODS

Cell lines and cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation from freshly prepared buffy coats supplied by Sanquin (Rotterdam, The Netherlands). PBMCs were washed twice, counted by trypan blue exclusion, resuspended in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (FCS) and seeded at 1. 10^6 cells per well into 96-well flat bottom plates (Costar, Corning, NY, USA) for stimulation experiments.

Human embryonic kidney (HEK) 293 cells stably transfected with human CD14/TLR2 and CD14/TLR4, a kind gift from Prof. Golenbock, were grown in dulbecco's modified eagle's medium (DMEM) supplemented with 10% FCS, 4.5 g/l glucose, 10 µg/ml ciprofloxacin and 5 µg/ml puromycin. Cultures were maintained at 37˚C in a 5% CO_2-humidified atmosphere and were subcultured twice a week. For stimulation experiments, cells were seeded in a 96-well flat bottom plate at a concentration of 4 x 10^4 cells per well and were used the next day. Stimulations of TLR4 were done in the presence of supernatants of HEK cells that were transfected with hMD-2. All stimulation experiments were done in the presence of 10% FCS.

Peptides

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and truncated variants (Table 1) were synthesized by Fmoc chemistry using a MiliGen 9050 peptide synthesizer (MiliGen, Bedford, MA, USA) as described previously[88]. Peptides were purified by preparative RP-HPLC to >90%. The identity of the peptides was confirmed by ion trap mass spectrometry with a LcQ Deca XP (Thermo Finigan, San Jose, CA, USA).

TLR agonists

Pam3CSK4 and Ultrapure LPS from *Porphyromonas gingivalis* were used as TLR2/1 agonists. FSL-1[159] and lipotechoic acid (LTA) from *Staphylococcus aureus*[160] were used to activate TLR2/TLR6. TLR4 activation was stimulated by ultrapure LPS from *E. coli* O111:B4. Purified flagellin from *S. thypimurium* was used as TLR5 agonist[161]. Imiquimod R837 was used to activate TLR7[162]. ssPolyU/LyoVec was used to activate TLR8[163]. All TLR agonist used were purchased from InvivoGen (San Diego, CA, USA).

Cell stimulation

PBMCs were stimulated with 15 ng/ml LPS from *P. gingivalis*, 200 ng/ml LTA, 0.5 ng/ml LPS *E. coli*, 60 ng/ml flagellin, 6 µg/ml R837 and 500 ng/ml ssPolyU. HEK293 cells stably transfected with CD14/TLR2 were stimulated with 125 ng/ml LPS from *P. gingivalis,* 10 ng/ml PAM3CSK4, 250 ng/ml LTA or 10 ng/ml FSL-1. HEK293 cells stably transfected with

CD14/TLR4 were stimulated with 50 ng/ml LPS *E. coli*. Cells were stimulated for 20 h in absence or presence of various concentrations of LL-37 fragments (0-5.5 µM).

Cytokine measurements

Following incubation of the cells under various treatment regimens, cell culture supernatants were collected and stored at -20°C until further analysis. Levels of IL-8 or IL-6 were determined in the supernatants by ELISA using the commercial PeliKine Compact™ human IL-8 or IL-6 ELISA kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations.

LPS binding of LL-37 fragments

Microtiter 96-well plates were coated with ultrapure LPS from *E. coli* O111:B4 (100 ng/well; Sigma, St. Louis, MO, USA) by incubating 50 µl of 2 µg/ml LPS in 0.1 M Na_2CO_3, pH 9.6, overnight at room temperature. The LPS solution was flicked out, and plates were washed. Excess binding sites were blocked with 200 µl/well phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) for 2 h and LL-37 fragments were incubated for 1 h in 50 µl/well at 37°C. After washing, 50 µl/well RPMI-1640 containing 10% FCS (which contains LPS-binding protein, LBP) was incubated for 1 h at 37°C. Subsequently, the plates were washed, and 50 µl/well 25 nM anti-LBP mAb6G3 in PBS containing 1% BSA (mouse-anti-human; Hycult Biotechnology, Uden, The Netherlands) was incubated for 1 h at 37°C. The mAb solution was rinsed out and replaced with HRP-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark; diluted 1000-fold in PBS containing 0.1% BSA) for 1 h at room temperature. Finally, the binding of LBP to immobilized LPS was quantified using 100 µl/well TMB as substrate. The assay was repeated at least three times.

Cytotoxic activity

The toxic effects of LL-37 fragments on HEK293 cells was evaluated by using WST-1 reagent. This method is based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells to a water soluble formazan dye (Roche Diagnostics, Mannheim, Germany). After 20 h of stimulation of HEK293 cells with medium or LL-37 fragments, 10 µl WST-1 reagents was added and cells were further incubated. After 30 min incubation time, absorbance was determined at 450 nm, with a reference wavelength at 650 nm.

Phosphorylation of MAPkinases

PBMCs were isolated as described earlier and seeded at 1. 10^6 cells per well into 24-well flat bottom plates. The next day, cells were stimulated with 1 µg/ml LPS from *P. gingivalis*, 2 µg/ml LTA and 10 ng/ml LPS from *E. coli* in the presence or absence of 5.5 µM LL-37 for 30 min. After stimulation, cells were fixed with 4% ultrapure paraformaldehyde in PBS, washed and resuspended in PBS containing 0.5% BSA (FACS buffer). For permeabilization, cells were incubated in 0.01% saponine for 5 minutes. Next, cells were washed and resuspended in FACS buffer. Signaling events in monocytes, defined by CD14 staining, were investigated using phospho-specific antibodies directed against ERK1/2 and p38. To

Table 1: *Sequences and physico-chemical characteristics of LL-37 and LL-37 fragments.*

Peptide	Sequence	Mw	Charge[a]	<μ>[b]	GRAVY[c]	% α-Helicity[d]	Cationicity[e]	Hydrophobicity[f]
	1 7 13 19 25 31 37							
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	3044	6	0.359	-0.724	68.6	100	100.0
LL-31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL	3824	6	0.427	-0.642	60.9	86.4	101.3
LL-25	LLGDFFRKSKEKIGKEFKRIVQRIK	3064	6	0.385	-0.752	65.8	63.1	69.1
LL-19	LLGDFFRKSKEKIGKEFKR	2327	4	0.371	-1.058	39.8	50.2	59.6
RK-25	RKSKEKIGKEFKRIVQRIKDFLRNL	3131	7	0.474	-1.168	53.7	78.9	85.1
RK-19	RKSKEKIGKEFKRIVQRIK	2372	7	0.435	-1.479	25.5	67.8	52.6
IG-25	IGKEFKRIVQRIKDFLRNLVPRTES	3044	4	0.437	-0.624	41.0	65.4	89.6
IG-19	IGKEFKRIVQRIKDFLRNL	2374	4	0.550	-0.458	32.7	60.7	92.5
IG-13	IGKEFKRIVQRIK	1615	1	0.539	-0.585	22.0	49.2	52.5
RI-19	RIVQRIKDFLRNLVPRTES	2341	3	0.434	-0.589	35.5	46.5	72.5

[a] *Net charge at pH7*
[b] *<μ>: calculated mean hydrophobic moment.*
[c] *Grand Average of Hydrophobicity, calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence*
[d] *Determined at 50% TFE*
[e] *Determined as relative retention on a SCX-HPLC column*
[f] *Determined as the relative retention time on a RP-HPLC column*

this end, cells were incubated for 2 h at room temperature with anti-CD14-PE (BD Biosciences, Mountain View, CA, USA), anti-phospho-p44/42 MAPK mouse mAb Alexa Fluor 488 conjugate (T202/Y204; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-p38 MAPK mouse mAb Alexa fluor 647 conjugate (T180/Y182, Cell Signaling Technology). After washing with FACS buffer, MAPK activation was determined by flow cytometry using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences) and analyzed using Cell Quest pro software (BD Biosciences).

HPLC

Retention in reversed phase (RP-) HPLC was used as a measure for the hydrophobic properties of each individual peptide, essentially as described by Hodges and co-workers[158]. The peptides were eluted from an analytical C-18 column (100 x 0.46 ID; BrownLee, Foster City, CA, USA.) using a gradient of 5% to 65% acetronitrile in 0.5 % TFA.

Retention in strong cation exchange (SCX-) HPLC was used as a measure of the cationic character of each individual peptide, essentially as described by Hodges and co-workers. The peptides were eluted from SCX column (Luna SCX, Phenomenex, Torrance, CA, USA.) using a linear gradient of 300 to 1150 mM KCl in 20 mM potassium phosphate buffer pH 6.5 containing 25% acetronitrile. Retention was expressed relative to LL-37 after correction for void time.

Circular Dichroism spectroscopy

CD spectra were recorded from 185 to 260 nm with a J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan), equipped with a Peltier temperature control system set at 20°C. Calibration was performed with an ammonium d-10-campersulfonate solution, of which to concentration was checked spectrophotometrically. Peptides were used at concentrations of 25 µM in 1 mM phosphate buffer (PPB) pH 7.0, trifluorethanol (TFE), or mixtures of both solvents. A 0.1 cm detection cell suited for far-UV CD measurements was used. Ten scans averaged for each sample. The response was 0.25 s and the step size was set 0.2 nm. Spectra were corrected for the background with an equally prepared sample without peptide and analyzed with the computer program CDNN.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 4.0. (GraphPad Software, San Diego, CA, USA). Differences were analyzed by Newman-Keuls multiple comparison test. Correlations were calculated by a two-tailed Spearman rank test. Differences were considered statistically significant if p-values were below 0.05.

RESULTS

Ligand dependent inhibition of induced cytokine secretion by LL-37 in PBMCs

Previous studies indicated that LL-37 has the ability to reduce pro-inflammatory cytokine production by macrophages and isolated monocytic cells induced by bacterial components like LPS and LTA[69,76]. We further explored the neutralizing effect of LL-37 on TLR

stimulation by bacterial PAMPs, and expanded these investigations to TLR7 and TLR8, that are known to be activated by viral compounds[163]. To this end PBMCs were stimulated with LPS *P. gingivalis* (TLR2/1), LTA (TLR2/6), LPS *E. coli* (TLR4), flagellin (TLR5), imiquimod (TLR7) and ssPolyU (TLR8), in the presence or absence of various concentrations of LL-37. The concentration of TLR agonists used represents the concentrations that lead to half of the maximum cytokine response. Although all TLR responses were slightly inhibited by LL-37, a significant effect was only seen for cytokine responses against LPS from *P. gingivalis* and LPS from *E. coli* (Figure 1). A dose of 5.5 μM LL-37 significantly inhibited 96% and 98% of the LPS from *P. gingivalis* and LPS from *E. coli* induced IL-6 production respectively, in contrast to 0-37% inhibition of IL-6 production induced by the other TLR ligands.

TLR dependent inhibition of induced cytokine secretion by LL-37 in HEK cells stably tranfected with TLRs

To explore whether LL-37 affects specific TLRs, HEK cells stably transfected with CD14/TLR2 or CD14/TLR4 were stimulated with agonists of TLR2/1 (LPS from *P. gingivalis*, PAM3CSK4), TLR2/6 (LTA, FSL-1) or TLR4 (LPS from *E. coli*) in the presence or absence of various concentrations of LL-37. As depicted in Figure 2, TLR2/1 and TLR4 activation was strongly inhibited while inhibition of TLR2/6 responses was not observed. In contrast to earlier reports[69] our data suggest that LL-37 has no general inhibitory effect on TLR stimulation but rather has a specific inhibitory effect on TLR2/1 and TLR4 stimulation.

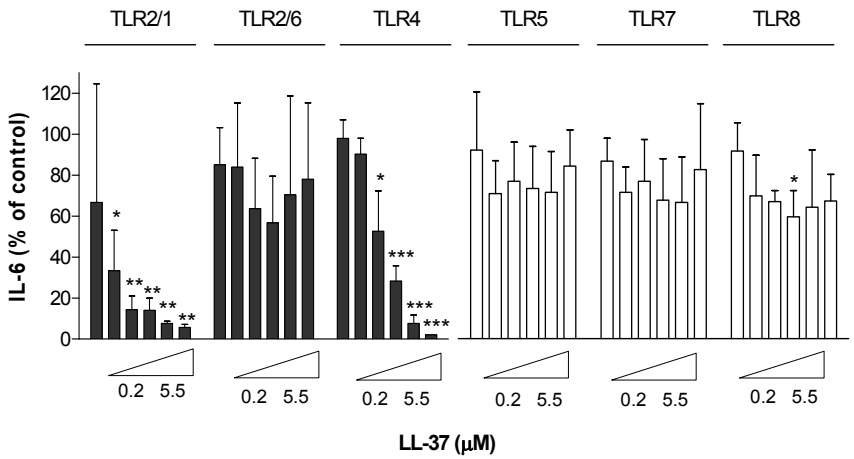

Figure 1. *LL-37 modifies the induced cytokine secretion by PBMCs in a ligand dependent manner. PBMCs were incubated with TLR agonists: LPS from P. gingivalis, LTA, LPS from E. coli, flagellin, imiquimod, and ssPolyU in the presence or absence of various concentrations of LL-37. The amount of IL-6 resulting from PBMCs incubated with TLR ligand alone was set to 100% (LPS P. gingivalis, 6.3 ± 0.9 ng/ml; LTA 9.6 ± 1.7 ng/ml; LPS E. coli, 19.2 ± 1.7 ng/ml; Flagellin, 26.7 ± 6.1 ng/ml; Imiquimod, 26.6 ± 3.4 ng/ml; ssPolyU, 22.7 ± 5.7 ng/ml). The results are average (±SEM) of 3 different donors.*

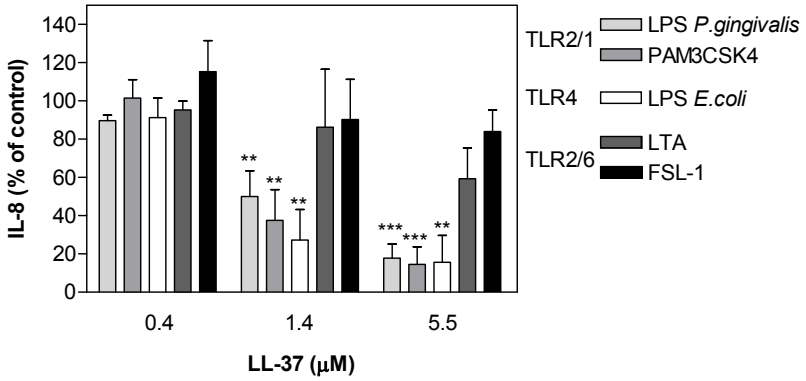

Figure 2. *LL-37 modifies the induced cytokine secretion by HEK cells stably transfected with TLRs in a TLR dependent manner. HEK cells stably transfected with CD14/TLR2 or CD14/TLR4 were stimulated with their corresponding TLR agonists: LPS from P. gingivalis, PAM3CSK4, LTA, LPS from E. coli in the presence or absence of various concentrations of LL-37. The amount of IL-8 resulting from cells incubated with TLR ligand alone was set to 100% (LPS P. gingivalis, 34.8 ± 9.1 ng/ml; PAM3CSK4, 28.2 ± 6.2 ng/ml; LTA 40.1 ± 7.8 ng/ml; FSL-1, 25.4 ± 5.9 ng/ml; LPS E. coli, 5.5 ± ng/ml). The results are average (±SEM) of at least 3 independent experiments.*

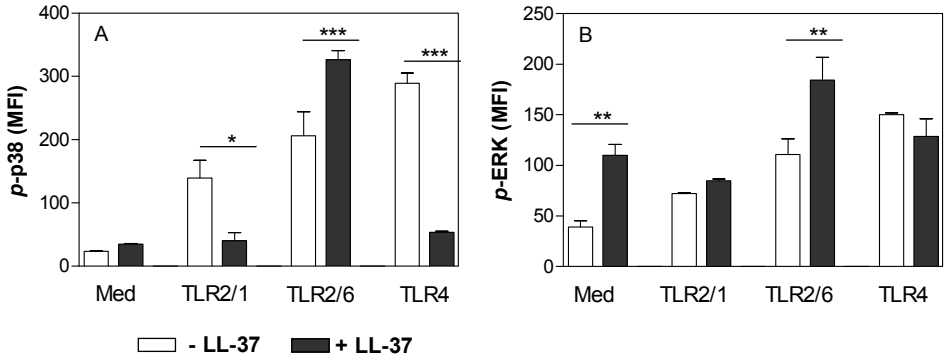

Figure 3. *LL-37 modifies the induced MAP kinase signaling in monocytes in a TLR dependent manner. PBMCs were incubated with TLR agonists: LPS from P. gingivalis, LTA, LPS from E. coli for 30 min in the presence or absence of LL-37. Phosphorylation of p38 (A) and ERK (B) was measured by FACs analysis. The results are average (±SEM) of 3 different donors.*

TLR dependent inhibition of induced MAP kinase inhibition by LL-37 in monocytes.

Cytokine measurements indicated that differences exist between different TLRs with respect to LL-37 induced inhibition of the pro-inflammatory response. To study this in more detail, we analyzed the effects of LL-37 on the intracellular activation of the mitogen-activated protein (MAP) kinases downstream of TLRs in stimulated monocytes. ERK (ERK1/2) and p38 are two effector kinases of the MAP kinase family and are known to play a role in the TLR induced expression of many pro-inflammatory cytokines and immune mediators[112,165,164]. Stimulation of monocytes with LL-37 did not lead to a general activation of MAP kinases itself but specifically leads to ERK phosphorylation. Phosphorylation of p38 and ERK was increased in monocytes 30 min after stimulation with TLR2/1, TLR2/6 and TLR4 ligands. LL-37 clearly suppressed TLR2/1 (LPS *P. gingivalis*) and TLR4 (LPS *E. coli*) induced phosphorylation of p38. In contrast, LL-37 induced an additive activation of p38 upon TLR2/6 (LTA) stimulation (Figure 3). ERK phosphorylation by TLR2/1 and TLR4 ligands was not influenced by LL-37, while LL-37 also induced an additive activation of ERK upon TLR2/6 stimulation.

Inhibition of TLR responses by specific LL-37 fragments

Attempting to identify a specific functional region of LL-37, which inhibits the pro-inflammatory response, a library of LL-37-truncated peptides was synthesized (Table 1). LL-37 fragments were screened for their ability to inhibit the pro-inflammatory response on various activated TLRs. HEK293 cells stably transfected with CD14/TLR2 or CD14/TLR4 were stimulated with agonists of TLR2/1 (LPS from *P. gingivalis*, PAM3CSK4) or TLR4 (LPS from *E. coli*) in the presence or absence of various concentrations of LL-37 or LL-37 truncated variants. After 20 hours stimulation IL-8 concentrations were determined in supernatants. The inhibitory effect of the different peptides was comparable for all TLR agonists. Active peptides showed a dose dependent inhibition of the pro-inflammatory TLR response (data not shown). The effect of 5.5 µM peptide on TLR responses is depicted in Figure 4. Truncation of LL-37 at the N-terminus with six residues or C-terminus with either six or twelve residues had little if any effect on the immunomodulatory effect of the resulting peptide (LL-31, RK-25, IG-25, IG-19). This suggests that residues 1-12 and 32-37 do not contribute significantly to the immunomodulatory effects of LL-37. Together, these data suggest that residues 13-31 form the minimal domain necessary for suppression of the pro-inflammatory response to bacterial components.

Previous studies indicate that LL-37 and its fragments showed poor cell selectivity, and therefore exhibit cytotoxic activity toward eukaryotic cells[109,112]. To exclude that the immunomodulatory effects of LL-37 and its fragments resulted from cytotoxicity, a WST-1 assay was performed on HEK cells stably transfected with CD14/TLR2 or CD14/TRL4. No cytotoxicity of the fragments was detected (data not shown).

LPS binding of LL-37 and LL-37 fragments

To verify whether the inhibitory effect of the LL-37 fragments could be contributed to LPS binding, LL-37 and LL-37 fragments were tested for binding to LPS according to a method described by Nagaoka and co-workers[80]. It was found that LL-37 and the LL-37 fragments LL-31, LL25, RK-25, IG-25 and IG-19 bound to LPS, revealing a plausible mechanism by which the peptides are capable to neutralize the LPS cytokine response (Figure 4).

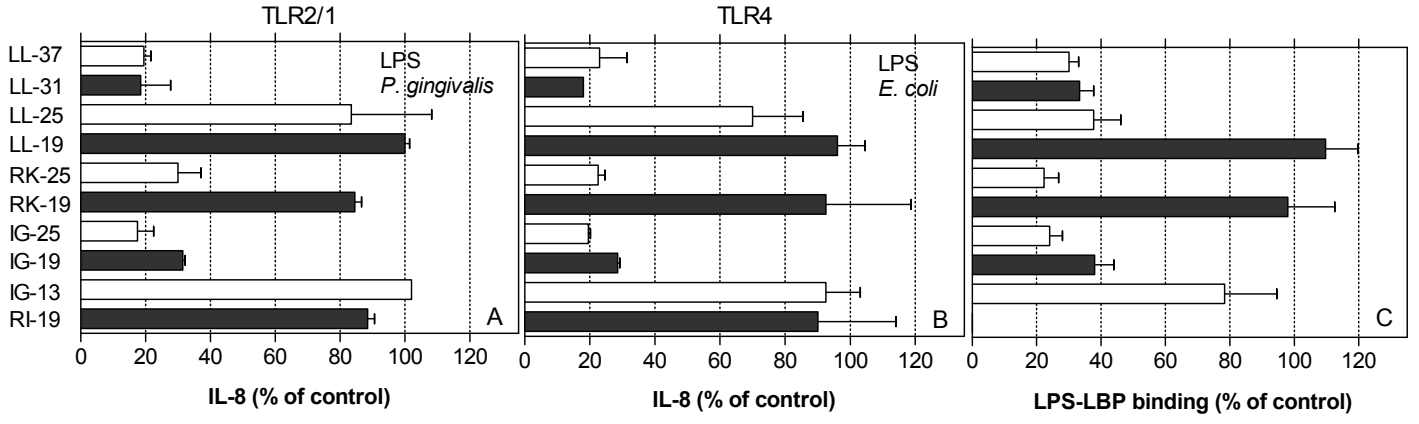

Figure 4. *Specific LL-37 truncated variants influence IL-8 production and LPS-LPB interaction. HEK cells stably transfected with CD14/TLR2 or CD14/TLR4 were stimulated with TLR agonists: LPS from P. gingivalis (A), LPS from E. coli (B) and stimulated with 5.5 µM LL-37 fragments. IL-8 concentration resulting from PBMCs stimulation with TLR ligand alone was set to 100% (LPS P. gingivalis, 9.8 ± 3.5 ng/ml; LPS E. coli, 1.5 ± 0.4 ng/ml). The results are the average (±SEM) of 2 independent experiments, each performed in duplicate. (C) Effects of LL-37 fragments on the interaction of LPS with LPB was examined by pre-incubation LPS coated microtiter plates with 5.5 µM LL-37 fragments in 50 µl RPMI. Thereafter, LPS-LBP binding was determined by incubating 50 µl RPMI containing 10% FCS. After incubating, bound LBP was detected by TMB reaction using anti-LBP MAb 6G3 and HRP-conjugated rabbit anti-mouse IgG. The results are average (±SEM) of 3 independent experiments.*

Physico-chemical properties of LL-37 and LL-37 fragments

Alteration of peptide structure by deletion of amino acids may change several physico-chemical properties simultaneously. For this reason, LL-37 fragments differ in their physico-chemical properties. To investigate if the physico-chemical properties of the peptides were related to the immunomodulatory function of the peptides, a correlation analysis was performed. The differences found in TLR inhibition by the different peptides could not directly be related to the physico-chemical characteristics that are supposed to play a pivotal role in the activity of HDPs. No correlation was found between the inhibition of the TLR response and the calculated hydrophobic moment as a measure of amphipathicity of the peptides (Table 1). Likewise, no correlation was found for Grand Average of Hydropathicity (GRAVY), which is a calculated measure of hydrophobicity of the peptides. Therefore, we decided to follow a more practical approach and correlated the TLR inhibition of the peptides with their physico-chemical properties.

The propensities of the peptides to adopt α-helical structures, essential for the amphipathic character, were determined using CD spectroscopy in aqueous solvent (PPB) with stepwise increased TFE content (Table 1). In PPB, all peptides showed CD spectra typical for random coils, with α-helical content of only 5 to 12%. In 50% TFE, the CD spectra of LL-37 indicated an α-helical content of 68.6% which was the highest value obtained in our experiments (Table 1). Similar percentages of α-helicity were observed for LL-31, LL-25, RK-25 (between 53.7 and 65.8% in 50% TFE). LL-19, RK-19, IG-25, IG-19, IG-13 and RI-19 showed decreased percentages of α-helicity (between 22% and 45.5% in 50% TFE). Although, no statistically significant relation was found, a trend was seen between the propensities of the peptides to adopt α-helical content and the capacity to inhibit the TLR dependent pro-inflammatory response (Spearman r = -0.6121, p= 0.0667, Figure 5A).
The calculation of the hydrophobic moment assumes 100% α-helicity, but as there is considerably variation between the peptides in the propensity to adopt an α-helix structure, the hydrophobic moment is not directly applicable as a measure for the association with hydrophilic and hydrophobic surfaces. Using HPLC, more practical measures for the affinities of the peptides for anionic surfaces and hydrophobic surfaces could be determined. To evaluate the peptides affinities for cationic surfaces we studied their retention on SCX-HPLC (Table 1). The cationic properties of the peptides were expressed relative to the retention of LL-37. LL-37, LL-31 and RK-25 exhibited the strongest retention on the SCX column and were therefore strongly cationic (between 79-100%). LL-25, LL-19, RK-19, IG-25, IG-19, IG-13 and RI-19 showed a decrease in the percentage of cationicity (between 46 and 67%). As shown in Figure 5B, the cationic properties of the peptides significantly correlated the capacity to inhibit the TLR dependent pro-inflammatory response (Spearman r = -0.7818, p= 0.0105). However, cationic properties could not completely predict immunomodulatory properties; IG-25 and IG-19 were as active in inhibiting the pro-inflammatory response as full length LL-37, whereas LL-25 which was slightly less cationic and RK-19 which was even more cationic were almost completely inactive. To evaluate the interaction of the peptides with their hydrophobic phase of membranes, we studied their retention on RP-HPLC (Table 1). The hydrophobic properties of the peptides were expressed relative to the retention time of LL-37. Similar hydrophobicity was seen for LL-37, LL-31, RK-25, IG-25 and IG-19 (between 101 and 85%). LL-25, RK-19, IG-13 and RI-19 showed a decreased percentage of hydrophobicity. The hydrophobic properties of the peptides were strongly correlated with

their capacity to inhibit the TLR dependent pro-inflammatory response (Spearman r= -0.8545, p= 0.0029, Figure 5C). Thus, hydrophobicity plays a dominant role with regard to the immunomodulatory capacity of LL-37 fragments.

DISCUSSION

Cationic host defense peptides are primitive and conserved components of the innate immune response. Expression of the sole human cationic host defense peptide LL-37 is induced upon exposure to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) or inflammatory mediators[84,85]. Although LL-37 has the ability to kill a broad range of microbes, including Gram-positive and Gram–negative bacteria as well as fungi and enveloped viruses, it also can modulate immune responses[31,33,37]. Mookerjee and co-workers reported that LL-37 has a general inhibitory effect on TLR stimulation in human peripheral blood mononuclear cells (PBMCs) stimulated with Toll-like receptor (TLR)2, TLR4 and TLR9 agonists, by specifically suppressing pro-inflammatory cytokine responses (interleukin 6, IL-6 and tumor necrosis factor-alpha, TNFα), which could lead to sepsis[69]. We further explored the inhibitory effects of LL-37 on TLR stimulation by PAMPs, and expanded these investigations to TLRs involved in defense against viruses. We found a specific effect of LL-37 on the TLR2/1 and TLR4 response. In human PBMCs as well as in human embryotic kidney (HEK) cell lines stably tranfected with TLRs, LL-37 suppressed pro-inflammatory cytokine production induced by agonists of TLR4 (LPS *E.coli*) and TLR2/1 (LPS *P.gingivalis*, Pam3CSK4), while little effect was seen on TLR2/6 (LTA *S. aureus*, FSL-1), TLR5 (flagellin), TLR7 (imiquimod) and TLR8 (ssPolyU) induced cytokine production. Surprisingly, in contrast to Mookerjee and co-workers no general inhibitory effect of LL-37 on TLR activation was observed, but rather found a specific effect of LL-37 on the TLR2/1 and TLR4 response. Despite our clear observations, repeated attempts and exact identical experimental conditions this observations seems inexplicable yet, but need to be addressed.

To study this effect of LL-37 in more detail, we analyzed the effects of LL-37 on TLR activated intracellular signaling pathways. Engagement of TLRs by microbial components triggers the translocation of nuclear factor kappa beta (NF-κβ), most commonly the NF-κB p50/p65 heterodimer, and the activation of mtitogen-activated protein (MAP) kinases, resulting in the expression of many pro-inflammatory cytokines and immune mediators[34,112,164,165]. Previous studies reported that LL-37 might directly act on the NF-κB pathway to inhibit the LPS stimulated pro-inflammatory cytokine expression. In those studies, LL-37 partially inhibited the p50/p65 translocation in LPS stimulated cells, however this could not fully account for the 95% reduction in cytokine release found. Therefore, mechanisms other than inhibition of NF-κB translocation are also required for LL-37 to regulate TLR-induced inflammation[69]. To gain insight into other intracellular signaling routes affected by LL-37 we focused on the MAP kinase pathway. Extracellular signal-regulated kinase (ERK) 1/2) and p38 are two effector kinases of the MAP kinase family and are known to be necessary for the TLR induced expression of many pro-inflammatory cytokines and immune mediators that are involved in innate immune responses against invading pathogens[112,164,165,166]. Likewise, we found activation of ERK and p38 MAP kinases in response to TLR activation. Addition of LL-37 specifically

suppressed TLR2/1 and TLR4 induced MAP kinase phosphorylation of p38 in human monocytes, while leaving ERK phosphorylation unaffected. Prior studies in monocytes have shown that the p38 MAP kinase pathway plays a role in LPS-induced cytokine release, and inhibition of this pathway attenuated the inflammatory response. In these studies, specific inhibition of the p38 kinase pathway by inhibitors such as SB202190[167], SB203580[166] and PCG[168], resulted in reduced IL-6 and TNFα mRNA expression and protein

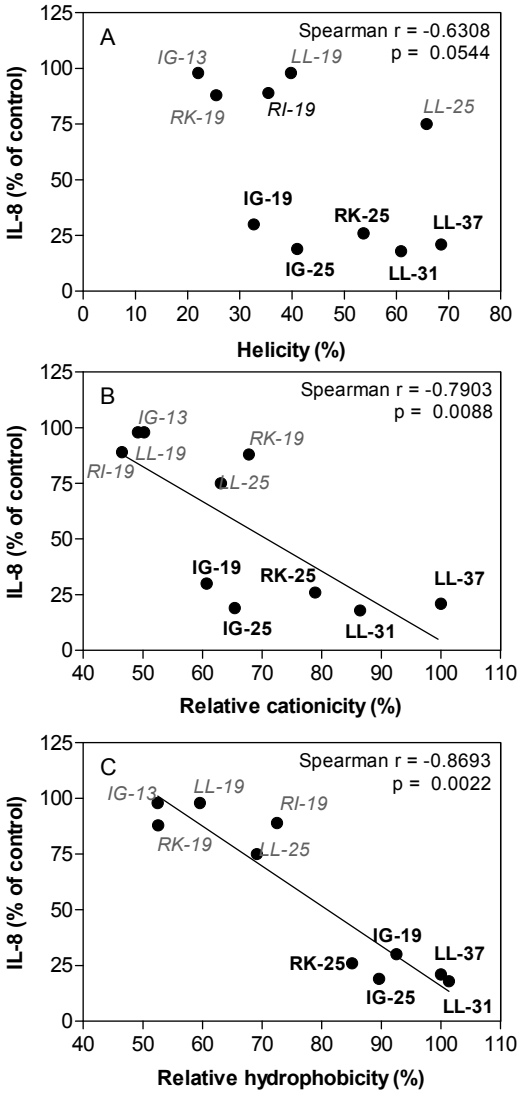

Figure 5. *Correlation of physio-chemical properties of LL-37 (-fragments) with the capacity to inhibit the pro-inflammatory response. The relationship between the potency to inhibit the TLR dependent (average of LPS P. gingivalis, LTA, LPS E. coli stimulation) cytokine response using 5.5 μM peptide and the percentage α-helicity (A), the relative cationicity to LL-37 (B) and the relative hydrophobicity to LL-37 (C) were shown. Each point represents a single peptide.*

levels. In addition, oral administration of BIRB 796 BS, a specific p38 MAP kinase inhibitor, attenuated the inflammatory response against LPS during experimental endotoximia in humans[169]. In addition to p38 MAP kinase activation, activation of ERK is necessary for optimal cytokine gene expression and production in LPS stimulated monocytes[165,166]. However, the contribution of each MAP kinase to the regulation of the cytokines release depends on the cell type. In this study we show that the regulation of IL-6 cytokine release is predominantly a p38 MAP kinase mediated effect in monocytes, a view that is supported by Tudhope and co-workers[168]. Inhibition of the ERK pathway is also associated with inhibition of LPS-induced IL-6 and TNFα release[168,170]. In total, these data suggest that although TLR activation requires multiple signaling events to elicit a cellular response, blocking a single MAP kinase is sufficient for inhibition. Stimulation of human monocytes with LL-37 alone resulted in the selective activation of ERK. ERK activation by LL-37 in PBMCs was also established by others[70]. In addition, the downstream transcription factor CREB, which is phosphorylated and translocated to the nucleus by ERK, was also found to be activated by LL-37[67]. These observations are in line with the capacity of LL-37 to stimulate the production of the chemokines IL-8, MCP-1 and MCP-3, which are responsible for attracting immune cells to the site of infection[69,70]. Although others reported that the MAP kinase p38 is also activated by LL-37[70], we could not confirm these observations. However, we did find an additive effect of LL-37 on p38 phosphorylation upon stimulation with a TLR2/6 ligand.

To gain detailed insight into the mode of action in the multiple functions of HDPs, structure activity (SAR) studies are used. SARs for especially antibacterial and cytotoxic activities have revealed that the activities of amphipathic α-helical HDPs, like LL-37, are not determined by the precise primary structure. Rather, activity was found to be determined by the subtle interplay of structural and physico-chemical parameters such as cationicity, hydrophobicity and amphipathicity[107]. There is evidence that the antibacterial activity of LL-37 is not located in the N-terminal of the peptide[64,108,109,110,111,112,113] but can be ascribed to the mid-region of LL-37[108,110,113]. Although all the physico-chemical properties of LL-37 contribute to the antibacterial activity, cationic side chains on the hydrophilic surface of the peptide are most accountable[110]. This implies that the electrostatic interaction of cationic residues of LL-37 with anionic phospholipids, such as phosphatidylglycerol and negatively charged liposaccharides, which are abundant in the bacterial cell membrane, are important for the antibacterial activity. Although discrepancies exist between studies that defined the part of LL-37 responsible for cytotoxic effects in eukaryotic cells, it is clear that hydrophobic residues are essential for the association of LL-37 with zwitterionic phospholipids, such as phosphatidycholine and sphingomeylin mainly present in the eukaryotic zwitterionic membrane[64,109,110,111]. We used LL-37 fragments to examine the structure-relationship for the immunomodulatory activity of LL-37. We revealed that the mid-portion of LL-37, amino acid 13-31, forms the minimal domain necessary for suppression of the pro-inflammatory response to bacterial components via TLR2/1 and TLR4. This observation is consistent with previous studies on LPS neutralization by LL-37[80,88,102,111,114]. Because antibacterial and cytotoxic activities of LL-37 are determined by the interplay of structure and physico-chemical properties we conducted parallel studies with regard to immunomodulatory action. We revealed that all physico-chemical properties of LL-37, such as α-helicity and cationicity moderately contribute to the immunomodulatory activity. Our data show that hydrophobicity plays a

significant role with regards to the immunomodulatory activity. This view is supported by a study of Nagaoka and co-workers, in which LPS neutralizing activities of LL-37 were enhanced by increasing the hydrophobicity[171]. It is often assumed that the inhibition of the pro-inflammatory response is mainly established by direct binding of LL-37 to the TLR ligand[73,74] which is mediated by electrostatic attraction between cationic residues of LL-37 and anionic residues of TLR ligands. Our data present evidence that LL-37 and LL-37 fragments bind to LPS, the TRL ligand, which could explain the observation of the downstream modulation of the of TLR responses.

In summary, LL-37 specifically suppresses TLR2/1 and TLR4 induced IL-6 production in human PBMCs, at least partly through suppression of the p38 MAP kinase pathway. The mid-region of LL-37 is responsible for this immunomodulatory effect resulting from direct binding to the TLR ligand. Hydrophobicity may be a very important physico-chemical characteristic in the immunomodulatory capacity of LL-37 fragments.

CHAPTER 3

Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities.

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ABSTRACT

Chicken host defense peptide cathelicidin-2 (CATH-2) is known to exert antimicrobial and immunomodulatory activities and consists of two α-helices connected by a hinge region. Here we report the biological properties of the separate α-helical segments and the importance of the proline residue in the hinge region. Substitution of proline-14 in the CATH-2 hinge region by leucine, but not by glycine strongly reduced antibacterial and hemolytic activity. Furthermore, substitution by leucine strongly reduced the neutralization of lipopolysaccharide (LPS)-induced cytokine production and peptide-induced monocyte chemotactic protein-1 (MCP-1) production by human peripheral blood mononuclear cells (PBMCs). This indicates that the hinge region is important for rapid penetration of the bacterial membrane as well as indirect and direct immunomodulatory activities. The highly cationic and amphipathic N-terminal segment (C1-15) exhibited very potent antibacterial activity and fast killing kinetics, while displaying low cytotoxicity towards chicken erythrocytes and PBMCs. The N-terminal and, to a lesser extent, the C-terminal helical regions potently neutralized LPS-induced release of TNFα, IL-6 and IL-10 by PBMCs, while IL-8 production was only moderately affected. These results indicate that core elements within mature CATH-2 can be identified that are linked to antibacterial and/or immunomodulatory activities. Further studies may lead to the development of peptide antibiotics with specific properties that can be used for prophylactic and/or therapeutic applications.

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INTRODUCTION

The increasing prevalence of antibiotic-resistant micro-organisms in farm animals and humans are of major concern and has elicited a world-wide ongoing search for novel antibiotics. Human pathogens, such as *Salmonella*, *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus* sp., have demonstrated the ability to rapidly develop resistance against antibiotics currently used for veterinary and human therapy[172,173]. Unfortunately, these antibiotics are still derived from a very small group of molecular structures, and operate often via an effective, but single mechanism of action, such as inhibition of cell wall synthesis and protein synthesis[174]. In addition, many of these compounds are effective against a relatively narrow-spectrum of micro-organisms[175,176] .

A promising new class of antibiotics is the so-called peptide antibiotics, peptides based on naturally occurring host defense peptides (HDPs) that exhibit potent antimicrobial and/or immunomodulatory properties. In contrast to 'classic' antibiotics, HDPs use multiple mechanisms to kill micro-organisms, including cell wall permeabilization, inhibition of DNA replication and protein synthesis and may exert potent broad-spectrum antimicrobial activity against bacteria, fungi, protozoa and enveloped viruses[28]. In addition, several mammalian cathelicidin HDPs have demonstrated to exert multiple immunomodulatory functions. These include the recruitment of neutrophils, monocytes and T cells[79], blocking of LPS-induced inflammatory responses[73], enhancement of antigen uptake and presentation by dendritic cells[66] and inhibition of apoptosis of macrophages and neutrophils[177,178].

Up to date, four avian cathelicidins (chicken CATH-1 to -3 and -B1) have been discovered[46,116,118,119] that, like mammalian HDPs, display potent antibacterial activity [51,116]. Moreover, CATH-1 to -3 have demonstrated to bind LPS and to block the LPS-induced cytokine release from a mouse macrophage cell line[116]. Unlike CATH-1 and -3, CATH-2 has a single proline residue at its center that destabilizes helical conformation and that may be of importance for its interaction with biological membranes.

The primary objective of this study was to identify core elements in chicken CATH-2 peptide linked to biological activities as a first step in the development of chicken-specific 'peptide antibiotics'. In addition, the role of the hinge region proline residue in biological activities of mature CATH-2 was examined. For these purposes, truncated peptides as well as hinge proline substituted analogs of CATH-2 were synthesized and tested for antibacterial, cytotoxic and immunomodulatory capacity.

MATERIALS AND METHODS

Cells

Chicken red blood cells (RBCs) were isolated from blood collected from healthy 17-week-old white leghorn hens. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation from freshly prepared buffy coats supplied by Sanquin (Rotterdam, The Netherlands). PBMCs were washed twice, counted by trypan blue exclusion, resuspended in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (FCS) and seeded at 1.0×10^6 cells per well into Costar 96-well flat bottom plates (Corning, NY, USA) for stimulation experiments.

Peptide synthesis

Peptides ranging from 15 to 32 amino acid residues, corresponding to structural domains of chicken cathelicidin-2 (CATH-2), were synthesized using Fmoc solid-phase synthesis on a Syro peptide synthesizer (MultiSyntech, Bochum, Germany). Synthesized peptides were purified by reversed phase (RP-) HPLC on a C18 column (Alltech, Altima, Deerfield, IL) eluted with a linear gradient of 5-80% acetonitrile in 0.01% (w/v) trifluoroacetic acid. Finally, RP-HPLC purified peptides were dissolved in distilled water and characterized by mass spectrometry, amino acid analysis and electrophoresis on Tris-tricine polyacrylamide gel electrophoresis (PAGE) gels. Peptide sequences are depicted in Table 1.

***Table 1**. Amino acid sequences and properties of CATH-2 derived peptides*

Peptide	Amino acid sequence	Length[a]	Charge	<H>[b]	μH[c]	Rf[d]
C1-27	**RFGRFLRKIRRFRPKVTITIQGSARFG**	27	+9	-1.34	0.320	11.35
C1-26*	**RFGRFLRKIRRFRPKVTITIQGSARF-NH$_2$**	26	+11	-1.30	0.326	11.31
C1-26	**RFGRFLRKIRRFRPKVTITIQGSARF**	26	+9	-1.30	0.326	11.46
C1-26(P14→G)	**RFGRFLRKIRRFRGKVTITIQGSARF**	26	+9	-1.39	0.319	11.48
C1-26(P14→L)	**RFGRFLRKIRRFRLKVTITIQGSARF**	26	+9	-0.92	0.309	11.28
C1-21	**RFGRFLRKIRRFRPKVTITIQ**	21	+8	-1.24	0.327	11.22
C1-15	**RFGRFLRKIRRFRPK**	15	+8	-2.27	0.421	10.67
C12-26*	**FRPKVTITIQGSARF-NH$_2$**	15	+5	-0.67	0.184	11.91

[a]*Length is measured in amino acids*
[b]*Global hydrophobicity (<H>) was calculated as a mean per residue value*
[c]*The amphipathicity was determined as the mean hydrophobic moments*
[d]*Relative retention time of peptides during RP-HPLC separation.*
**Peptides with C-terminal amidation*

Antimicrobial activity assays

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). *Salmonella enterica* serovar enteritidis 13367, originally isolated from chicken feces, was a gift from Dr. Jaap P. Wagenaar (Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University). *Bacillus globigii* BM013 was obtained from TNO collection at Rijswijk (The Netherlands). Bacteria were maintained in tryptic soy broth (TSB) medium at 37°C and grown to mid-logarithmic growth phase before testing. Colony count assays were used to evaluate the antimicrobial activity of peptides as previously described[179]. In brief, bacterial cultures were pelleted by centrifugation, resuspended in 10 mM sodium phosphate buffer (pH 7.0), containing 1/100 TSB medium and diluted to 2.5×10^6 CFU/ml. Twenty-five μl of peptide (0–40 μM) was then mixed with an equal volume of bacterial suspension and incubated for 3 h at 37°C. Ten-fold dilutions prepared in minimal TSB medium (1000-fold diluted TSB in distilled water) were spread plated onto tryptic soy agar (TSA, Oxoid Limited, Hampshire, UK) and after a 24 h incubation period at 37°C counted for surviving bacteria.

Killing kinetics

Thirty microliters of 20 µM CATH-2 derived peptides were mixed with an equal volume of 2.5×10^6 CFU/ml *S. enteritidis* mid-logarithmic phase culture bacteria in 10 mM sodium phosphate buffer (pH 7.0), containing 1/100 TSB and incubated at 37°C. As a negative control, bacterial suspensions were incubated with an equal volume of phosphate-buffered 1/100 minimal TSB medium. At various time points, a 50 µl aliquot was taken, diluted 5-, 50- and 500-fold in TSB medium of which 100 µl was plated on TSA medium. The number of colony forming units was counted after overnight incubation at 37◦C.

Hemolysis assay

EDTA anti-coagulated chicken blood was centrifuged for 10 min at 800 × g (20°C) to sediment the red blood cells (RBCs). Pelleted RBCs were washed three times in PBS, diluted 200-fold in PBS and counted using a hematocyte meter. In 96-well polypropylene plates, 75 µl of serial peptide dilutions (0-80 µM) were mixed with an equal volume of RBC suspension and incubated for 1 h at 37°C. PBS served as baseline and a 0.2% (v/v) Triton X-100 solution served as a control for complete lysis. Supernatants, collected after 10 min centrifugation at 1300 × g (20°C), were transferred into polystyrene 96-wells plates and absorbance was measured at 405 nm. Hemolysis (%) = $(A_{Peptide} - A_{Blank})/(A_{Triton} - A_{Blank}) \times 100$.

Cytotoxicity assay

The toxic effects of CATH-2 analogs on PBMCs were evaluated using the cell proliferation reagent WST-1 (Roche Diagnostics, Mannheim, Germany). In microtiter plates, PBMCs (1.0×10^6 cells/well) were resuspended in RPMI-1640 medium (supplemented with 10% heat inactivated FCS) containing 0 to 40 µM of CATH-2 analog in a final volume of 100 µl. After 24 h incubation at 37°C (5% CO_2), cells suspensions were centrifuged and 10 µl of WST-1 reagent was added to each well and further incubated (37°C; 5% CO_2). After 60 min absorbance was measured in a multiwell plate reader at 450 nm, using 650 nm as a reference wavelength.

Inhibition of LPS-induced cytokine release

To study the immunomodulatory effects of different peptides, PBMCs were stimulated with 1 ng/ml of ultra-pure *E. coli* O111:B4 lipopolysaccharides (LPS, Invivogen, San Diego, CA) in the absence or presence of peptide (0 to 40 µM) during 5 h (tumor necrosis factor alpha, TNFα) or 24 h (interleukins IL-6, IL-8 and IL-10). Cell supernatants were collected and stored at -20 °C until needed for analysis of cytokine levels.

Activation of PBMCs by CATH-2 analogs

PBMCs resuspended in RPMI-1640 medium supplemented with 10% heat inactivated FCS were incubated for 24 h in the presence of various concentrations (0 to 40 µM) of CATH-2 analogs. Following incubation, cell supernatants were collected and stored at -20°C until needed for analysis.

Cytokine measurement

Levels of TNFα, IL-6 and IL-8 were determined by ELISA using the commercial PeliKine Compact™ human ELISA kits (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations. Levels of monocyte chemotactic protein 1 (MCP-1) were determined by ELISA using the commercial ELISA kit from eBioscience (San Diego, CA).

Statistical analysis

Antimicrobial activity assays were performed at least 4-fold on different days. Cytotoxicity against erythrocytes and PBMCs was assessed in triplicate on different days. Cytokine levels were determined in triplicate. Data were analyzed in Excel and graphs were made using GraphPad Prism (Kaplan-Meier analysis, GraphPad Software, http://www.graphpad.com). Significance differences between mean values of groups were evaluated using a one-way analysis of variance (ANOVA) and Dunnet's posthoc test and were indicated as * ($P<0.05$) or ** ($P< 0.01$).

RESULTS

Antibacterial activity of chicken CATH-2 analogs

Peptides corresponding to different structural domains of mature CATH-2 (Figure 1) were evaluated in colony count assays against Gram-negative and Gram-positive bacteria. Peptide C1-15, corresponding to the N-terminal helix and hinge region, exhibited the most potent growth inhibition against all bacteria tested (MBC of 2.5–7.5 μM; Figure 2A).

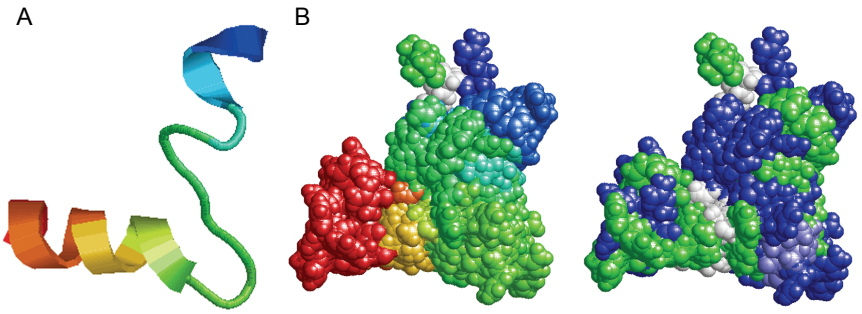

Figure 1. *Structural organization of the CATH-2 peptide. (A) Ribbon representation of the CATH-2 molecule, consisting of a double helical structure with a central "hinge" region (green). (B) Molecular surface representation of CATH-2 colored by group (left) and distribution of cationic (blue), hydrophobic (green) and neutral polar (white) amino acid residues (right). The proline residue located in the hinge region is colored purple. Adapted from the published 3-dimensional structure of CATH-2 as determined by NMR spectroscopy (RCSB Protein Data bank; http://www.rcsb.org/pdb/home/home.do. Molecular modeling was performed with RasWin molecular graphics (http://www.rasmol.org).*

Compared to mature CATH-2 peptide (C1-27), C-terminal amidation greatly augmented antibacterial activity against *E. coli* and to a lesser extent *S. aureus*, but not against *B. globigii*. Peptide C1-21, which lacks part of the C-terminal helical region and peptide C12-26*, corresponding to the hinge region and amidated C-terminal helix, displayed the lowest antibacterial activity. The antibacterial activity of C12-26* against *E. coli* was similar to that of mature CATH-2 peptide. Peptide C1-26, a mature CATH-2 analog lacking the C-terminal Gly residue, displayed antibacterial activities similar to that of mature CATH-2 (Figure 2B). Substitution of the hinge region proline (C1-26[P→G]) with a glycine residue abrogated antibacterial activity slightly against *E. coli*, *S. enteritidis* and *B. globigii*, while no differences were observed for *S. aureus* (Figure 2B). In contrast, leucine substitution (C1-26[P→L]) of the hinge region proline resulted in a marked overall reduction in antibacterial activity.

Killing kinetics of CATH-2 analogs

With the exception of peptide C12-26*, mature CATH-2 and other CATH-2 derived peptides displayed fast killing kinetics against *S. enteritidis*. CATH-2 and C1-21 exhibited identical killing kinetics, reducing *S. enteritidis* survival within 20 min to below the detection limit of 100 cells/ml in the presence of 10 μM peptide (Figure 3A). Even faster killing kinetics were observed for peptides C-1-26* and C-15, which were able to reduce *S. enteritidis* survival within 5 and 10 min, respectively. C12-26* exhibited the slowest killing kinetics and needed 60 min to reach the detection limit. C1-26 also killed bacterial cells more rapidly; within 10 min *S. enteritidis* survival was reduced to below the detection limit (Figure 3B). Although in colony count assays little difference was observed in killing efficiencies between C1-26 and its Proline to Gly substituted analog, the kinetics of *S. enteritidis* killing proved to be dramatically slower, *i.e*. 60 min instead of 10 min. Sixty minutes of exposure to 10 μM leucine-substituted C1-26 peptide was insufficient to reduce survival to below the detection limit, as the number of surviving *S. enteritidis* cells decreased by 3 log units.

Cytotoxicity of chicken CATH-2 analogs

To determine the toxic effects of CATH-2 analogs towards eukaryotic cells, chicken RBCs and human PBMCs were incubated with different concentrations of peptide. C1-27 was relatively toxic towards chicken RBCs (Figure. 4), lysing 50% of the RBCs at 40 μM. At the same peptide concentration, the C-terminally amidated analog (C1-26*) exhibited 60% hemolysis. Truncation of the C-terminal helical region (C1-21) reduced hemolytic activity to 40% at 40 μM, whereas the most truncated peptides, C1-15 and C12-26*, were not or only slightly hemolytic towards chicken erythrocytes. Long time exposure (24 h) of human PBMCs to CATH-2 or CATH-2 derived peptides did not greatly affect cell survival at concentrations up to 20 μM. At 40 μM only peptides C1-26* and C1-15 resulted in 9% and 16% cell death, respectively. By comparison, incubation with the human cathelicidin LL-37, which is known to be relatively toxic to host cells, resulted in more than 56% cell death at 40 μM. While mature CATH-2 peptide did not affect PBMC viability at lower peptide concentrations, at 40 μM it appeared to promote cell proliferation. Additionally, the effects of hinge region substitution on cell cytotoxicity were investigated. Incubation of chicken erythrocytes in the presence of 40 μM C1-26 resulted in approximately 26% lysis

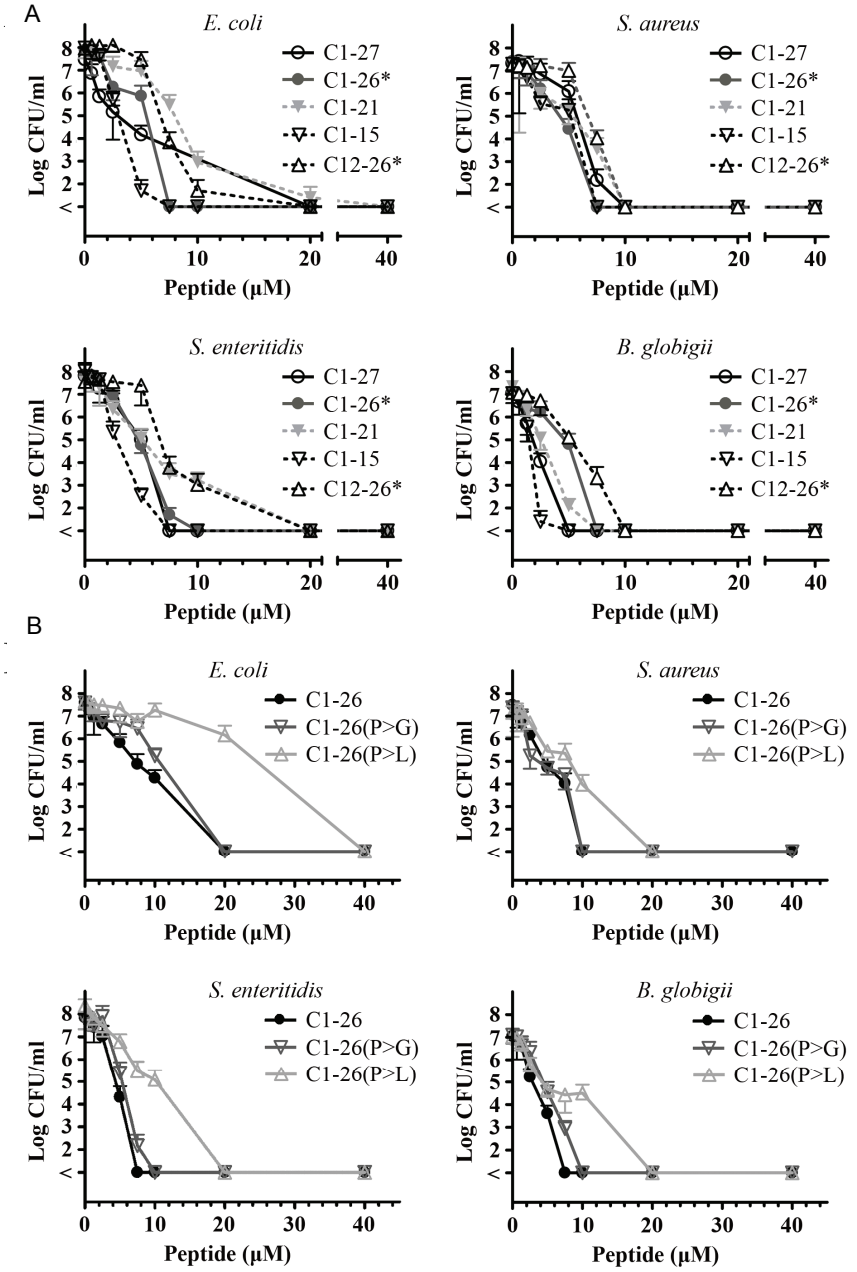

Figure 2. *Antibacterial activity of CATH-2 derived peptides. In colony count assays, bacteria were incubated for 3 h with various concentrations of CATH-2 derived peptides, serially diluted and spread plated on agar media. (A) Truncated CATH-2 peptides. (B) Glycine or leucine substitution of the CATH-2 hinge region proline. Bacterial survival was evaluated after 24 h of incubation at 37◦C. The data are means ± SD of four measurements performed on different days. The symbol "<" on the y-axis indicates means below the detection limit of 100 CFU/ml.*

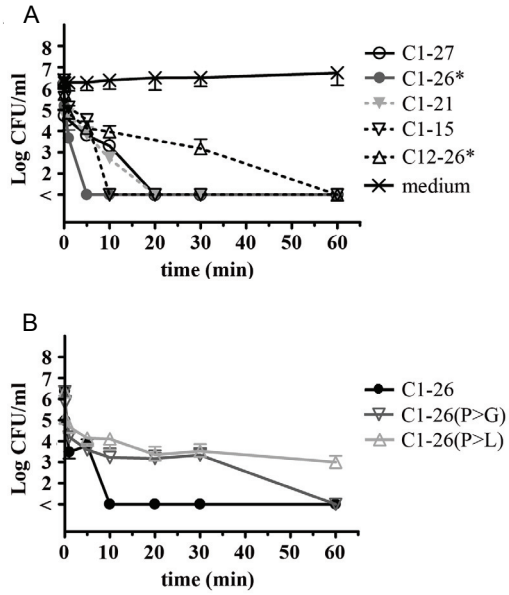

Figure. 3. *Killing kinetics of CATH-2 derived peptides against S. enteritidis. (A) Mature CATH-2 and truncated CATH-2 peptides. (B) Hinge proline substitutions. Log-phase S. enteritidis 13367 cells were incubated at 37◦C in the presence of 10 μM peptide. Aliquots were taken at different time intervals, serially diluted, spread plated on agar media and evaluated for growth after 24 h incubation at 37◦C. The data are means ± SD of three measurements performed on different days.*

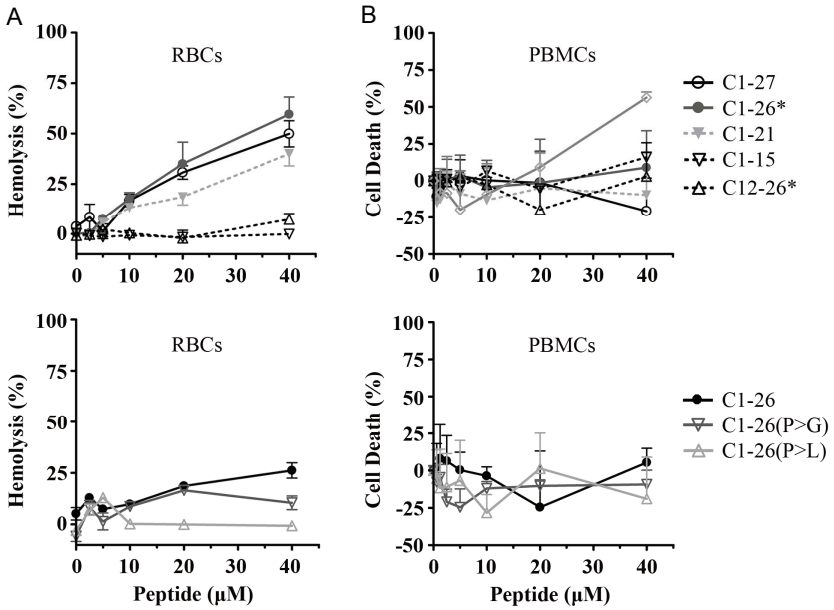

Figure. 4. *Cytotoxicity of CATH-2 derived peptides. (A) Hemolytic activity of truncated and substituted CATH-2 analogs towards chicken erythrocytes (RBCs) after 1 h of incubation at 37◦C. (B) Cytotoxic activity of truncated and substituted CATH-2 peptides towards human peripheral blood mononuclear cells determined by the WST-1 method. The data are means ± SD, determined in triplicate.*

(Figure 4). Substitution of proline by glycine reduced hemolysis to 10% at this concentration, whereas no hemolysis occurred during exposure to the leucine-substituted peptide. Contrary to chicken erythrocytes, CATH-2 derived peptides only displayed marginal effects against human PBMCs.

Inhibition of LPS-induced cytokine production by CATH-2 analogs

Peptide-mediated inhibition of LPS-induced cytokine production was investigated using human PBMCs. All peptides showed reduced activity compared to CATH-2 (Figure 5A). Especially the truncated peptides C1-21 and C12-26*, and to a lower extent C1-15, showed a highly reduced activity compared to the mature peptide for all cytokines measured. The C-terminally amidated C1-26 peptide (C1-26*) showed an interesting effect on the inhibition of cytokine release. This peptide efficiently inhibited TNFα and IL-6 production, but did not inhibit IL-8 production. Substitution of proline by glycine (C1-26[P→G]) did not affect LPS neutralization, while the leucine-substituted peptide (C1-26[P→L]) displayed reduced activity (Figure 5B).

CATH-2 analog mediated stimulation

MCP-1 production by human PBMCs was significantly stimulated by mature CATH-2 ($P<0.01$), its C-terminal amidated analog C1-26* ($P < 0.01$) and truncated peptide C1-21 ($P<0.01$). The stimulation was higher than that of LL-37, a known immunostimulatory cathelicidin (Figure 6). Peptides C1-15 and C12-26* had a highly reduced effect on MCP-1 stimulation. Likewise, MCP-1 production by human PBMCs was significantly ($P<0.01$) stimulated by peptide C1-26 and its glycine-substituted analog C1-26[P→G] while the leucine-substituted C1-26 peptide did not stimulate MCP-1 production.

DISCUSSION

It has been demonstrated by NMR spectroscopy analysis that mature chicken cathelicidin peptides CATH-1, -2 and -3 adopt a helix–hinge–helix structure in an environment mimicking biological membranes[116,120,180]. Similar winged helical configurations have been described for porcine PMAP-23 and ovine SMAP-29 and are thought to be important for membrane perturbation[181,182]. In contrast to CATH-1 and CATH-3, where a central glycine residue generates a slight kink between helices, CATH-2 contains a proline residue at position 14 that produces a more pronounced kink between both helical segments. To investigate the functional importance of the central proline residue for biological activities of mature CATH-2 peptide, CATH-2 peptide was compared with analogs in which the hinge proline was substituted by glycine or leucine. Substitution by leucine considerably reduced antibacterial activity, hemolytic activity, LPS neutralization and the ability to significantly induce MCP-1 production by PBMCs. Apart from greatly reduced killing kinetics against *S. enteritidis*, proline to glycine substitution did not affect other biological activities. Proline, and to a lesser extent glycine, are known to have a destabilizing effect on α-helix formation[183], whereas leucine stabilizes helix formation[184]. The low helix forming propensity of glycine is due to the high entropy costs associated with its backbone conformational flexibility and its lack of hydrophobic stabilization[185]. It is known that in

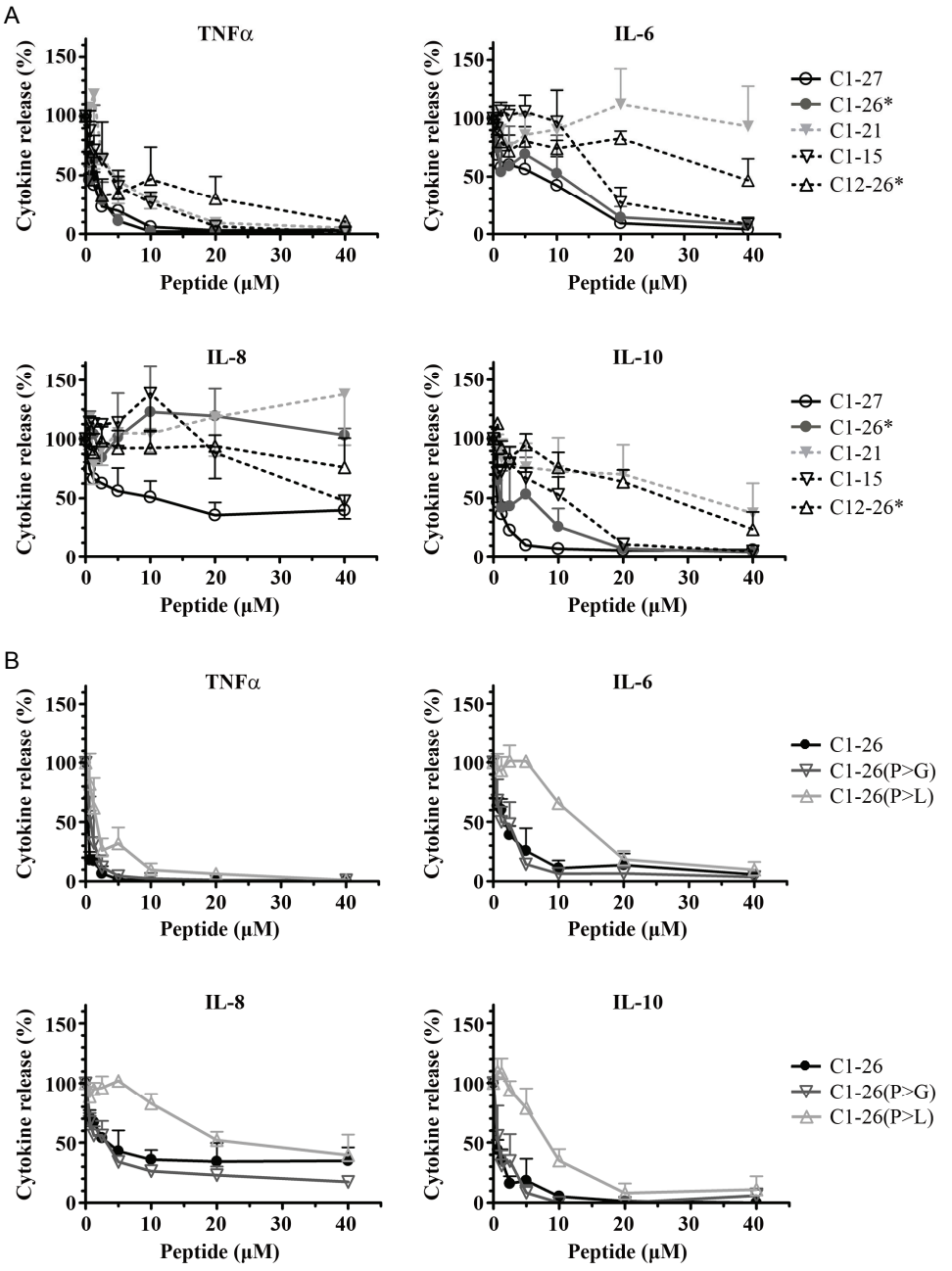

***Figure 5**. Inhibition of LPS-induced cytokine production. (A) Mature CATH-2 and truncated CATH-2 peptides. (B) Hinge proline substitutions. Human PBMCs were stimulated for 5 or 24 h with 1 ng/ml E. coli O111:B4 LPS in the presence of various concentrations of CATH-2 derived peptides. Concentrations of TNFα, IL-6, IL-8 and IL-10 in cell supernatants were determined by ELISA. In the absence of peptide, LPS-stimulation of human PBMCs resulted in 1135 ± 239 pg/ml TNFα after 5 h incubation, 38 ± 7 ng/ml IL-6, 321 ± 57 ng/ml IL-8 and 505 ± 143 pg/ml IL-10 after 24 h incubation. Per donor, cytokine levels were expressed relative to the cytokine levels released by PBMCs stimulated by LPS alone. The data are presented as mean values ± SEM obtained from 3 donors.*

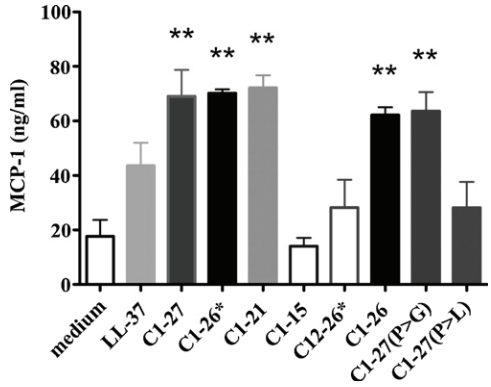

***Figure 6**. Peptide-induced MCP-1 production. Human PBMCs were directly stimulated with 15 µM of LL-37, CATH-2, truncated peptides or hinge proline substituted peptides for 24 h after which MCP-1 levels were determined by ELISA. MCP-1 production was found to be significantly (P<0.01) enhanced in the presence of CATH-2, C1-26* and C1-21 peptide. Substitution of the hinge region proline by leucine strongly reduced peptide-induced MCP-1 production. The data are presented as mean values ± SEM obtained from 3 donors.*

solution, a proline kink motif within an α-helical structure not only distorts the α-helical structure, but also produces significant local flexibility, which enables different conformations of the N-terminal and C-terminal helices around this region[186,187,188]. Compared to the highly cationic and amphipathic N-terminal segment, the C-terminal segment shows a higher helix formation propensity. We hypothesize that after initial electrostatic interaction of the N-terminal segment with the bacterial membrane, due to the proline induced kinked conformation the C-terminal will be inserted more rapidly and deeper into the lipid bilayer. This corroborates with reported effects in other host defense peptides; proline substitutions in piscidin-1, buforin II and PMAP-23 significantly affected membrane specificity, cell penetration and antimicrobial activity[181,187,188]. The differential effects for proline to leucine and proline to glycine substitutions described in our study implicate that the proline induced bending and swiveling of helical termini around this molecular hinge is necessary for fast penetration of bacterial membranes as well as crucial for immunomodulatory activities.

Next, to identify core elements involved in the biological activities of mature CATH-2 peptide, we tested truncated CATH-2 peptides in a variety of functional assays. The higher antibacterial activity against *E. coli* and *S. aureus* and improved killing kinetics against *S. enteritidis* cells observed for the C-terminally amidated C1-26 analog (C1-26*) are most likely a consequence of increased cationicity. The C-terminal glycine residue may be transformed into a carboxyamide through hydroxylation and oxidative cleavage by alpha-amidating enzymes, a phenomenon common among cathelicidins[189]. This would elevate CATH-2 cationicity (from +9 to +10), which is linked to augmented antibacterial activity[107]. In addition, this amide group is thought to render the peptide C-terminus less susceptible to exopeptidases[189]. Interestingly, C1-15, the truncated CATH-2 peptide lacking the complete C-terminal helical segment, displayed the highest overall antibacterial activity and fast killing kinetics. This N-terminal helical segment contains the bulk of the positive charges (+8) and, compared to mature CATH-2 and other CATH-2 derived

peptides, a lower mean hydrophobicity, but is still highly amphipathic (Table 1). The amidated C-terminal helical segment (C12-26*) was least bactericidal of all CATH-2 derived peptides although it possesses moderate cationicity (+5), considered to be sufficient for maximal lytic activity[190]. The predicted low amphipathicity and observed slow killing kinetics for this fragment suggest a lower efficacy in membrane permeabilization for the C-terminal segment alone. The lower antibacterial activity and slower killing kinetics observed for C1-21, a peptide corresponding to CATH-2 with a partially truncated C-terminal hydrophobic tail, indicate that in mature CATH-2, hydrophobic tail length is important for bacterial membrane penetration. Thus, the antibacterial activity in mature CATH-2 is primarily linked to the amphipathic, highly cationic, N-terminal region.

Because antibacterial activity and cytotoxicity against host cells are intricately linked we also investigated the toxic effects of truncated and substituted peptides against eukaryotic cells. Interestingly, the highly antibacterial C1-15 segment exhibited only low cytotoxicity towards chicken erythrocytes and was non-toxic towards PBMCs. This might be explained by the excess charge density present in the polar sector of the C1-15 segment, which renders the peptide less prone to structure (Figure 1A)[191]. This may significantly decrease cytotoxicity without affecting antibacterial activity[192]. The low toxicity of the C-terminal helical segment C12-26*, despite its similar size and having a higher propensity for helix formation (Figure 1A), is probably related to a lower mean hydrophobicity and amphipathicity (Tabel 1), a feature known to affect cytotoxicity more than antibacterial activity[193]. Compared to the parent peptide CATH-2, absence of the 5 C-terminal amino acid residues (C1-21) only slightly reduced cytotoxicity. These findings suggest that the short hydrophobic stretch (VTITIQ) between position 15 and 22 greatly contributes to CATH-2 cytotoxicity.

LPS-induced cytokine production, in particular TNFα and interleukins 1β, -6 and -8, plays an important role in the initiation and perpetuation of sepsis syndrome, which is associated with organ dysfunction and a high mortality rate[194]. In earlier studies, mature chicken cathelicidins have demonstrated LPS-binding properties as well as an ability to inhibit LPS-induced cytokine production[116,180] and are of potential interest for the development of novel anti-sepsis therapies. Therefore, we investigated the ability of different CATH-2 derived peptides to neutralize LPS-induced cytokine production by peripheral blood mononuclear cells (PBMCs). Our results show that both helical segments of mature CATH-2 are involved in LPS-neutralization, most potently by the N-terminal domain (C1-15). Similarly, SMAP-29 has been reported to contain two high affinity LPS-binding domains at the end of each terminus, the highest affinity LPS-binding site located in the cationic N-terminal segment[182]. The fact that LPS-induced TNFα production was most efficiently blocked by mature CATH-2 and its amidated 26 amino acid analog, indicates that the working mechanisms of the two helical segments are cooperative. Inhibition of LPS-induced cytokine expression by CATH-2 derived peptides was similar for TNFα, IL-6 and IL-10 secretion and implicates that prevention of LPS-mediated signaling through TLRs is a major mechanism. This is supported by the observation that several host defense peptides have shown to block the interaction between LPS and LPS-binding proteins, such as LBP and CD14, and thus preventing LPS-recognition and subsequent cell signaling[73,139,195]. However, it should be noted that cytokine production may be influenced via membrane receptors other than TLRs in a cell-specific and species-specific way. For

instance, LL-37 stimulates human monocytes to produce IL-1β via the purinergic P2X7 receptor[196] and keratinocyte production of IL-8 involves both Gi-protein signaling and epidermal growth factor receptor (EGFR) activation[108]. Moreover, even after internalization of LPS, cationic peptides may still be able to significantly block endotoxin activity[76]. Despite the ability of several CATH-2 derived peptides to block LPS-induced cytokine production, chemokine production was only moderately inhibited. This is in agreement with other studies in which LPS-induced gene expression of TNFα and nuclear factor kappa beta 1 (NF-κB1) in human monocytes was significantly downregulated by LL-37, while IL-8 gene expression levels were reduced but remained at a base level[76].

In addition to prevention of LPS binding to receptors, CATH-2 derived peptides may directly stimulate immune cells. For example, cationic peptides CEMA, LL-37 and IDR-1 induce expression of monocyte chemokines, including MCP-1[69,76,197,198]. MCP-1 is a chemoattractant for monocytes[199], memory T cells[200] and NK cells[201] and can protect against infection[202]. In our study, mature CATH-2, C1-26, C1-26* and C1-21 all significantly induced MCP-1 production in human PBMCs, while no effect on MCP-1 production was observed for the C1-15 peptide. Importantly, direct stimulation of human PBMCs with CATH-2 derived peptides did not lead to significant induction of TNFα, IL-6 or IL-8 production (data not shown). The mechanisms responsible for direct stimulation of cytokine and chemokine production are not known in detail. Cationic peptides have demonstrated to directly influence the gene expression repertoire of macrophages, affecting genes involved in cell cycle regulation, cell adhesion and apoptosis[197]. In addition, internalized peptides may interfere in cell signaling by direct binding to signaling cascade adaptor proteins. In conclusion, our findings suggest that some CATH-2 derived peptides may dampen pro-inflammatory responses, while stimulating the recruitment of monocytes and macrophages and so shift the balance from inflammation towards resolving of infection.

During the preparation of this manuscript, a structure-activity study has been reported independently, describing antibacterial and immunomodulatory properties of fowlicidin-2, the product of a putative second elastase cleavage site in the chicken CATH-2 precursor, and fowlicidin-2 derived peptides[75]. Although none of the peptides used in their study was identical to those described here and site-specific amino acid substitutions were not used, their observations indirectly support the importance of the hinge proline for bacterial killing and LPS neutralization. In our study, a comparison between CATH-2 (C1-27) peptide and the predicted complete fowlicidin-2 peptide (32 amino acids) showed no significant differences (data not shown), implicating that the presence of these five additional amino acid residues at the N-terminus (LVQRG) is of marginal importance for the biological activities investigated here.

The results presented here shows that core elements within mature CATH-2 can be identified that are linked to antibacterial and/or immunomodulatory activities. In this respect, the C1-15 segment is of particular interest to use as a template for further development, because it already combines high antibacterial activity with low cytotoxicity and does not appear to significantly stimulate MCP-1 production. Modification of peptides corresponding to these core elements by specific amino acid substitutions and N- and C-terminal end modifications can be applied to amplify desired biological activities. This approach may lead to development of host-specific peptide antibiotics that can be used clinically for prophylactic and/or therapeutic use.

CHAPTER 4

Chicken cathelicidin-2 derived peptides with enhanced immunomodulatory and antibacterial activities against biological warfare agents

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ABSTRACT

Host defense peptides are considered to be excellent candidates for the development of novel therapeutic agents. Recently, it was demonstrated that the peptide C1-15, an N-terminal segment of chicken host defense peptide cahthelicidin 2, exhibits potent antibacterial activity while lacking cytotoxicity towards eukaryotic cells. In the present study, we report that C1-15 is active against bacteria, such as *Bacillus anthracis* and *Yersinia pestis* that may potentially be used by bioterrorists. Substitution of single and multiple phenylalanine (Phe) residues to tryptophan (Trp) in C1-15 resulted in variants with improved antibacterial activity against *B. anthracis* and *Y. pestis* and decreased the salt-sensitivity. In addition, these peptides exhibited enhanced neutralization of lipopolysaccharide (LPS)-induced release of pro-inflammatory cytokines in human peripheral blood mononuclear cells (PBMCs). The antibacterial and LPS neutralizing activities of these C1-15 derived peptides are exerted at concentrations far below the concentrations that are toxic to human PBMCs. Taken together, we show that Phe to Trp substitutions in C1-15 variants enhance the antibacterial and LPS neutralizing activities against pathogenic bacteria including those that may potentially be used as biological warfare agents.

Based on
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INTRODUCTION

The current strategies to control disease outbreaks caused by intentional release of bacteria, such as biological warfare agents (BWA) are limited in their treatment regimes. Immediate immunity can be provided by passive antibody therapy[15], but requires identification of the target agent[16]. In case of vaccination, a sufficient protective immune response can often only be achieved by repeated vaccination during several months, while significant side effects may occur[16,17,18]. Commonly used antibiotic therapies to treat *Bacillus anthracis, i.g.* doxicycline and penicillin, may be effective only when applied within 24 hour post-exposure. Exposure to *B. anthracis* generally causes a highly progressive, acute infection which may be lethal in some forms. So, in case of acute exposure to an unknown micro-organism, broad-spectrum antibiotics might be life saving.

Host defense peptides (HDPs) are widely considered to be excellent candidates for the development of novel broad-spectrum antibiotics[28,30,31,32]. In general, HDPs target microbial membranes through a non-receptor mediated mechanism and therefore tend to have a lower frequency in selecting resistant strains compared to conventional antibiotics[28]. Furthermore, HDPs have shown to be effective against multi-drug resistant strains either occurring naturally or bio-engineered[16,20,203]. HDPs are easily produced by organic synthesis. Besides, by amino acid substitutions in natural occurring HDPs, novel structures with improved antimicrobial activity can be created[191,204,205,206,207,208].

Bacterial killing by antimicrobial compounds may cause a release of endotoxin from Gram-negative bacteria, which subsequently might lead to an exaggerated immune response. This can result in early sepsis, in which high levels of cytokines and inflammatory mediators become destructive, causing organ failure, cardiovascular shock and may even result in death. Some HDPs are found to counteract the development of septic shock by binding the endotoxin or by blocking the binding of endotoxin to lipopolysaccharide-binding protein (LBP), thereby providing a mechanism to overcome development of septic shock[73,80,102, 157,171,209,210].

Recently, four chicken cathelicidins (chicken CATH-1 to -3 and -B1) have been identified[46,116,118,119]. Previously, we demonstrated that CATH-2 displays potent antibacterial activity and is able to reduce LPS-induced cytokine release, but is also relatively cytotoxic to eukaryotic cells[51]. In addition, we have shown that a truncated variant of CATH-2, peptide C1-15, possesses improved broad antibacterial activity against both Gram-negative and Gram-positive bacteria, while being non hemolytic against erythrocytes and is therefore a promising lead for the development of novel antimicrobial drugs with a broad-spectrum activity.

We explored the use of HDPs *i.e.* C1-15 as potential novel lead compound to combat biowarfare agents. In doing so we aimed at broadening the antibacterial activity of C1-15 by substitution of phenylalanine (Phe) residues on the non polar face by tryptophan (Trp) residues. The antibacterial activity against Gram-negative and Gram-positive bacteria, including those potentially involved in bioterrorist attacks and toxicity against human peripheral blood mononuclear cells (PBMCs) of C1-15 variants were evaluated. Furthermore, the anti-inflammatory activity of C1-15 variants was examined by measuring inhibition of pro-inflammatory cytokine release in LPS-stimulated human PBMCs.

MATERIAL AND METHODS

Peptide synthesis

C1-15 (RFGRFLRKIRRFRPK) and C1-15 variants F_2W (RWGRFLRKIRRFRPK), F_5W (RFGRWLRKIRRFRPK), $F_{12}W$ (RFGRFLRKIRRWRPK) and $F_{2,5,12}W$ (RWGRWLRKIRRWRPK) were synthesized by Fmoc chemistry using a Syro peptide synthesizer (MultySyntech, Bochum, Germany) as described by Bikker and co-workers[211].

Colony count assays

Antimicrobial activity of the peptides was evaluated against Gram-positive *Bacillus anthracis* Vollum strain *(*ATCC14578, American Type Culture Collection, Manassas, VA, USA) and Methicillin-resistant *Staphylococcus aureus* (*MRSA*, ATCC 43300). In addition, the antibacterial activity of the peptides was evaluated against the Gram-negative virulent *Yersinia pestis* (NCTC08775, National Collection of Type Cultures, London, United Kingdom) and *Vibrio cholera* (clinical isolate, Slotervaart Hospital, Amsterdam, The Netherlands). All bacteria were grown and maintained in tryptic soy broth (TSB, Biotrading, Mijdrecht, The Netherlands) under aerobic conditions at 35°C (*B. anthracis, MRSA*, *V. cholera*) or 26°C (*Y. pestis*). Bacteria were cultured to mid-logarithmic phase by transferring 500 µl of stationary phase suspension into 5 ml TSB medium, followed by incubation for 2 h at 35°C. Mid-logarithmic phase cultures were centrifuged for 10 min at 4500 × g and the bacterial pellets were suspended in 10 mM potassium phosphate buffer containing 1/100 TSB (pH 7.0) or in 10 mM potassium phosphate buffer containing 1/100 TSB and 200 mM NaCl (pH 7.0) and diluted to ~ 1×10^5 CFU/ml. Initial concentrations of bacteria were determined by transferring 10-fold serial dilutions of bacteria on tryptic soy agar plates (TSA, Biotrading, Mijdrecht, The Netherlands), after which colonies were counted after 24 h incubation at 35°C, or 72 h incubation at 26°C in case of *Y. pestis.* The antimicrobial activity of the peptides was determined using colony count assays. Twenty-five microliters of bacterial culture was mixed with an equal volume of peptide in polypropylene 96-well microtiter plates (Costar, Corning, NY, USA) and incubated for 3 h at 35°C. After the incubation period, 200 µl of TSB medium was added, further diluted 10- to 100-fold in TSB and transferred onto TSA plates after which colonies were counted after 24 h incubation at 35°C (*B. anthracis, MRSA, V. cholera*) or 72 h incubation at 26°C (*Y. pestis)*.

Isolation and culture of peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin, Rotterdam, The Netherlands) within 24 h after venipuncture by density centrifugation on Ficoll Paque Plus (Amersham Pharmacia, Upsala, Sweden). PBMCs were washed twice, counted by trypan blue exclusion, resuspended in RPMI-1640 (Lonza BioWhittaker, Basel, Switzerland), supplemented with 10% heat inactivated fetal calf serum (FCS, Gold, PAA, Pasching, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza BioWhittaker, Basel, Switzerland) and seeded at 0.5×10^6 cells per well into 96-well flat bottom culture plate (Costar, Corning Inc., Corning, NY, USA) for stimulation experiments.

Cytotoxicity assay

The toxic effect of C1-15 variants on PBMCs was evaluated using WST-1 reagents. This method is based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells to a water-soluble formazan dye (Roche Diagnostics, Mannheim, Germany). Cells were incubated for 24 h with culture medium, or peptides at 37°C and 5% CO_2. Following centrifugation, supernatants were taken and 100 µl fresh RPMI-1640 supplemented with 10% FCS and 10 µl WST-1 reagents was added to the cells. After 60 min incubation time, absorbance was determined at 450 nm, with a reference wavelength at 650 nm.

Neutralization of the LPS-induced cytokine response by C1-15 variants

PBMCs were used to study the modulation of lipopolysaccharide (LPS)-induced cytokine secretion by C1-15 variants. PBMCs were stimulated with 1 ng/ml ultrapure LPS from *E. coli* O111:B4 (InvivoGen, San Diego, CA, USA) for 5 or 24 h in absence or presence of various concentrations of peptides. Following incubation, cell culture supernatants were collected and stored at -20°C until further analysis.

Stimulation of MCP-1 production

PBMCs were incubated for 24 h in the absence or presence of 15 µM C1-15 variants. CATH-2 was used as a positive control[63]. Following incubation, cell culture supernatants were collected and stored at -20 °C until further analysis.

Cytokine measurements

Levels of TNFα and IL-6 were determined by ELISA using the commercial PeliKine Compact™ human ELISA kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations. Levels of MCP-1 were determined by ELISA using the commercial Ready SET go ELISA kit of eBioscience (San Diego, CA, USA) following the manufacturer's recommendations.

LPS binding by C1-15 variants

The synthetic peptides were examined for LPS neutralizing activity in a limulus amoebocyte lysate (LAL) assay and a LPS - lipopolysaccharide binding protein (LBP) interaction assay. For the LAL assay, 25 µl peptide in concentrations from 0 to 20 µM diluted in RPMI-1640 (Lonza BioWhittaker, Basel, Switzerland) were incubated with 25 µl 2 ng/ml ultrapure LPS from *E. coli* O111:B4 (InvivoGen, San Diego, CA, USA) for 60 min at 37°C in a 96-well endotoxin free plate (Hycult biotechnology, Uden, The Netherlands). Residual LPS activity was detected using the LAL assay according to the manufacturer's recommendations (Hycult biotechnology, Uden, The Netherlands). The LPS-LBP interaction assay is based on a method described by Nagaoka and co-workers[171]. In this method, the inhibitory effect of the HDP on the LPS-LBP interaction reflects LPS binding. Microtiter 96-well plates were coated with ultrapure LPS from *E. coli* O111:B4 (100 ng/well, Invivogen, San Diego, CA, USA) by incubating 50 µl of 2 µg/ml LPS in 0.1 M Na_2CO_3, pH 9.6, overnight at room temperature. The LPS solution was flicked out, and plates were washed with PBS. Excess binding sites were blocked with 100 µl/well PBS containing 1% bovine serum albumin (BSA) for 1 h. Next, peptides were incubated for 1 h in 50 µl/well at 37°C. After

washing, 50 µl/well RPMI-1640 containing 10% FCS (which contains LBP) was incubated for 2 h at 37°C. Subsequently the plates were washed, and 50 µl/well 25 nM anti-LBP mAb6G3 in PBS containing 1% BSA (mouse-anti-human, Hycult Biotechnology, Uden, The Netherlands) was incubated for 1 h at 37°C. The mAb solution was rinsed out and replaced with HRP-conjugated rabbit anti-mouse IgG (diluted 1000-fold in PBS containing 0.1% BSA, DAKO, Glostrup, Denmark) for 1 h at room temperature. Next, plates were washed and 50 µl/well 3, 3', 5, 5' tetramethyl benzidine (TMB, Sigma, St. Louis, MO, USA) was incubated as substrate. After color development, 50 µl/well 1.8 M H_2SO_4 was added to stop the reaction and absorbance was read at 450 nm. Both assays were repeated three times.

Statistical analysis

Statistical significance was determined by two-way ANOVA followed by *post hoc* testing by the Bonferroni method. $P<0.05$ was considered statistically significant. Values shown are expressed as mean + SEM of the mean.

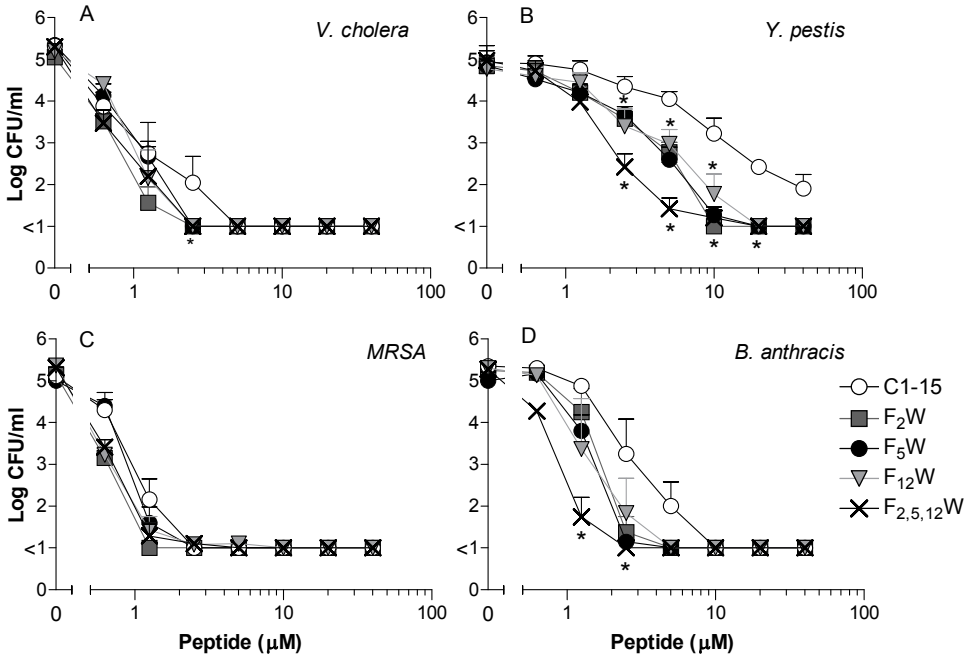

Figure 1. *Bactericidal activity of peptides against V. cholera (A), Y. pestis (B) , MRSA (C) and, B. anthracis (D) determined by colony count assays. Bacteria were incubated 3 h with various concentrations of peptide, serially diluted and plated on TSA. Bacterial survival was evaluated after 24 or 72 h of incubation. Each point represents the mean + SEM of at least 4 experiments. * Significantly different compared to C1-15 (p<0.05).*

RESULTS

Antibacterial activity of C1-15

Colony count assays were used to evaluate the antibacterial activity of the N-terminal segment of CATH-2, C1-15 against two Gram-positive (*MRSA*, *B. anthracis*) and two Gram-negative bacteria (*V. cholera, Y. pestis*). C1-15 affected the survival of all tested bacteria (Figure 1). The most susceptible bacteria were *MRSA* and *V. cholera;* a concentration of 2.5 and 5 μM respectively led to the decline of surviving bacteria below the detection limit. A higher concentration, 10 μM, was needed to reduce survival of *B. anthracis* below the detection limit. *Y. pestis* survival was least affected by C1-15: 40 μM peptide reduced the survival 3 log units, but no reduction below the detection limit was observed.

Antibacterial activity of C1-15 variants

In order to enhance the antimicrobial activity of C1-15, we designed a panel of C1-15 variants with phenylalanine (Phe) to tryptophan (Trp) substitutions. In comparison to Phe, Trp contains a more bulky hydrophobic side chain consisting of an indole chain. Since there are three Phe residues on the non polar face of C1-15, variants were synthesized with Phe to Trp substitutions at positions 2 (F_2W), 5 (F_5W) and 12 ($F_{12}W$) or a combination of these ($F_{2,5,12}W$). Colony count assays were used to test the antibacterial activity of the developed C1-15 variants (Figure 1). Compared to C1-15 single Phe to Trp substitution (F_2W, F_5W and $F_{12}W$) improved the antibacterial activity against *Y. pestis* and *B. anthracis*. The survival of *Y. pestis* was reduced with 50% by using 5 μM F_2W, F_5W or $F_{12}W$ compared to 20 μM for C1-15. Moreover, 2 μM of F_2W, F_5W or $F_{12}W$ led to 50% decline in *B. anthracis* survival compared to 4 μM for C1-15. The antibacterial activity against *Y. pestis* and *B. anthracis* was even further improved by multiple substitutions, respectively 2.5 and 1 μM of $F_{2,5,12}W$ was needed to reduce the survival of the bacteria by 50%.

Antibacterial activity in the presence of salts

The effect of salt on the antibacterial activity was evaluated by a colony count assay. The antibacterial activity of CATH-2 against MRSA was not affected by 100 mM NaCl (data not shown). However, C1-15 lost its antibacterial activity in the presence of 100 mM NaCl. Even the highest peptide concentration used, 40 μM, could only reduce the survival 1 log unit. Yet, Trp substitutions resulting in $F_{2,5,12}W$ partly preserved the antibacterial activity in the presence of NaCl (Figure 2).

Cytotoxicity of C1-15 variants

C1-15 variants were evaluated for their toxicity to human PBMCs with and without the presence of 10% FCS. Cytotoxicity of C1-15 to PBMCs in the presence of FCS was negligible. Substitution of Phe by Trp residues increased the cytotoxicity to PBMCs up to 75% at 40 μM after multiple substitutions ($F_{2,5,12}W$) (Figure 3). Similar experiments in the absence of FCS showed that FCS only had a marginal protective effect against peptide cytotoxicity (data not shown).

Neutralization of LPS-induced cytokine response

PBMCs were stimulated in RPMI-1640 containing 10% FCS with 1 ng/ml LPS in the presence of various concentrations of peptides. The concentration of the pro-inflammatory mediators TNFα and IL-6 were measured in culture supernatants (Figure 4). Substitution of Phe residues in C1-15 by Trp residues improved the LPS neutralizing capacity by improving the inhibition of the LPS-induced release of TNFα and IL-6. Multiple Phe to Trp substitutions in C1-15 resulted in even higher LPS neutralizing activity; 10 µM of $F_{2,5,12}W$ significantly inhibited 94% and 85% of the TNFα and IL-6 LPS response respectively.

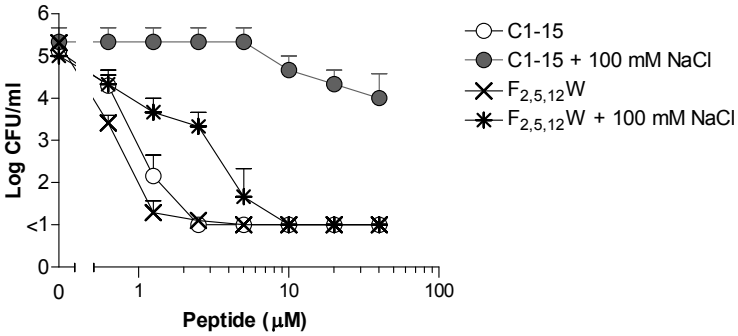

***Figure 2**. Salt sensitivity of C1-15 and $F_{2,5,12}W$ In colony count assays, MRSA was incubated 3 h with various concentrations of peptide in the presence of or without 100 mM NaCl added to the incubation buffer. Next, the bacteria were serially diluted and plated on TSA. Bacterial survival was evaluated after 24 h of incubation. Each point represents the mean + SEM of at least 4 experiments.*

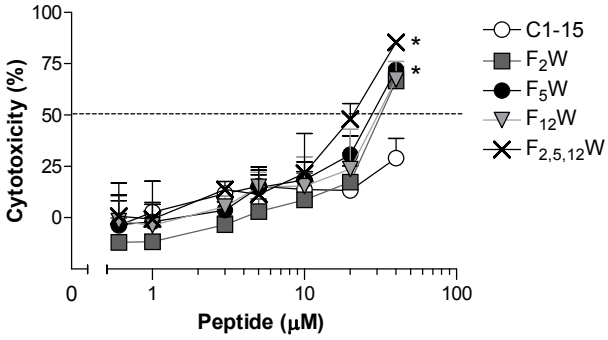

***Figure 3**. Cytotoxicity of peptides towards human peripheral blood mononuclear cells determined by the WST-1 method. Each point represents the mean + SEM of at least 3 experiments. * Significantly different compared to C1-15 ($p<0.05$).*

LPS binding by C1-15 variants

To verify whether the improved LPS neutralizing activities of C1-15 variants could be contributed to improved LPS binding, C1-15 variants were evaluated for their ability to bind LPS in a limulus amoebocyte lysate (LAL) assay (Figure 5A) and an LPS-LBP binding assay (Figure 5B). In the LPS-LBP binding method, the inhibitory effect of the HDP on the LPS-LBP interaction reflects LPS binding[171]. Both assays showed comparable results. Phe to Trp substitution in C1-15 enhanced LPS binding capacity of the peptides.

Immune stimulation of C1-15 variants

In contrast to CATH-2, the truncated form C1-15 has no MCP-1 stimulating properties. We evaluated the effect of C1-15 Phe to Trp substitutions on MCP-1 production in PBMCs. Phe to Trp substitutions in C1-15 had no effect on the capability of the peptide to induce MCP-1 production (Figure 6).

DISCUSSION

During military operations or bioterrorist attacks immediate diagnosis or identification of the bacterial agent is not always possible. In case of an exposure from an unknown source the availability and usability of broad-spectrum antimicrobials can be vital. Furthermore, due to the use and misuse of current antimicrobials, growing numbers of multi-resistant bacteria arose, including those which potentially might be used for offensive purposes. This implicates that there is a need for new broad-spectrum antimicrobials. Recently, it was reported that the host defense peptide (HDP) C1-15, a truncated variant of chicken cathelicidin-2 (CATH-2), possesses strong antibacterial activity against a number of Gram-negative and Gram-positive bacteria, including *Baccilus globigii* and *Staphylococcus aureus*. In addition, this peptide is not hemolytic against erythrocytes[63]. In this study we extended the earlier reported broad antibacterial activity with more bacteria including bacteria often related to bioterrorist attacks (*Bacillus anthracis*, *Vibrio cholera*, *Yersinia pestis).* In addition, we aimed to enhance the antibacterial activity of C1-15 by amino acid substitutions of phenylalanine (Phe) by tryptophan (Trp) residues.

Substitutions of C1-15 Phe to Trp residues resulted in a peptide containing a large number of arganine (Arg) and Trp residues. Previously it was reported by others that Arg and Trp complement each other well for the purpose of antimicrobial activity[212]. The cationic charge of Arg provides an effective means of attraction of the peptides to the target membranes and hydrogen bonding facilitates the interaction with the negatively charged surfaces of bacterial membranes. The hydrophobic bulk of Trp residues play a role in membrane association because it preferably interacts with the interfacial region of membranes[213]. In combination, Arg and Trp residues are capable of participating in stronger cation-π interactons compared to Arg and Phe residues. Cation-π interactions are non-covalent molecular interactions between the face of an electron-rich π system (*e.g.* benzene, ethylene, indole) with an adjacent cation (*e.g*. guanidinum group of Arg). Due to the formation of energetically favorable cation-π interactions, the positively charged Arg residue is shielded by the Trp indole ring[212,214,215]. Such interactions would facilitate deeper embedding of the peptides in the membrane and disrupt the membrane

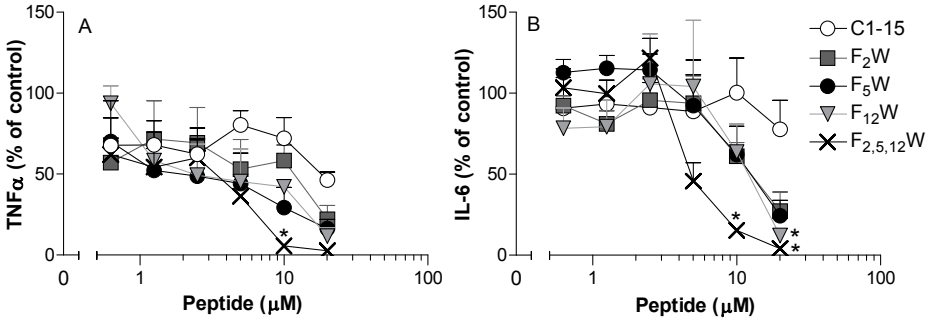

Figure 4. *Inhibition of the endotoxin-induced cytokine response of PBMCs by the peptides. Human PBMCs were stimulated with 1 ng/ml LPS with or without different concentrations of peptide. TNFα and IL-6 concentrations in cell supernatants were determined by ELISA. The amount of TNF-α and IL-6 resulting from cells incubated with LPS alone was set to 100% (52.4±5.4 ng/ml). Each point represents mean + SEM of 3 different donors. *Significantly different compared to C1-15 (p<0.05).*

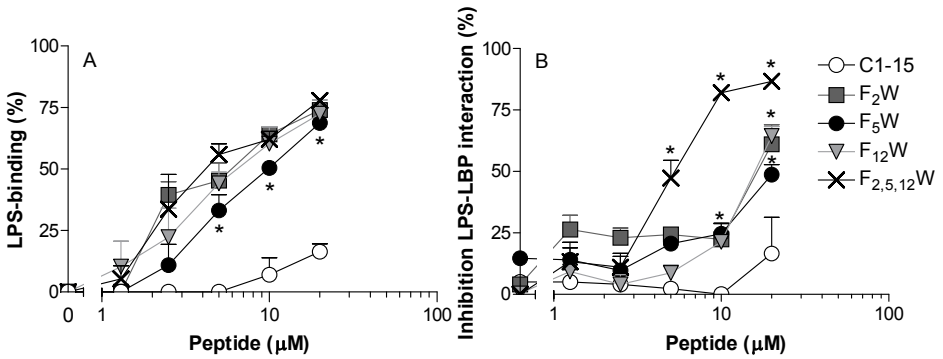

Figure 5*. Comparison of peptides for LPS neutralizing determined by Limulus amoebocyte lysate assay (A) and inhibition of the LPS-LBP interaction (B). Each point represents mean + SEM of 3 independent experiments. *Significantly different compared to C1-15 (p<0.05).*

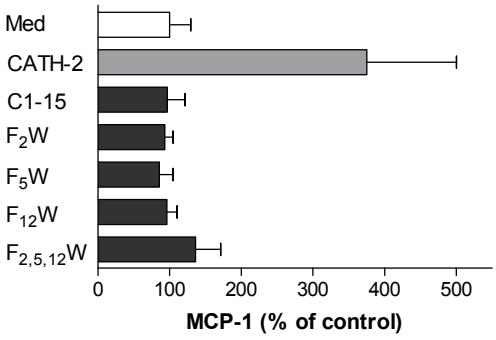

Figure 6. *Peptide induced MCP-1 production. Human PBMCs were stimulated for 24 h with 15 μM peptide. The level of MCP-1 was measured by ELISA. CATH-2 was used as a positive control. Medium alone (med) was used as a negative control. Data represents mean + SEM of 3 different donors*

structure[212,214,215]. Analysis of high resolution protein structures indicated that such interactions occur frequently[216]. In line with these data we observed that single Phe substitutions (F_2W, F_5W, $F_{12}W$) augmented the antibacterial activity with a factor 4 against *Y. pestis* and a factor 2 against *B. anthracis* respectively. Multiple substitutions ($F_{2,5,12}W$) further enhanced antibacterial activity: the peptide was 8 and 4 times more potent compared to C1-15 in reducing the survival of *Y. pestis* and *B. anthracis*, respectively. The ability to resist salt is highly relevant for HDPs in order to function under physiological conditions. Therefore the antibacterial activity of the peptides was evaluated in the presence of 100 mM NaCl. As reported earlier by Xiao and co-workers[75] and confirmed by us, CATH-2 is resistant to salt. However, the N-terminal of CATH-2, C1-15, which has broad-spectrum antibacterial activity, almost completely lost its antibacterial activity in the presence of salt. Moreover, we could preserve the antibacterial activity in the presence of salt by Phe to Trp substitutions. Overall, these findings show that the inclusion of Trp residues can make peptides less salt-sensitive and more potent in bacterial killing.

To determine the effect of the applied amino acid substitutions on LPS neutralizing activity, the inhibition of endotoxin (LPS) induced TNFα and IL-6 production by C1-15 and its variants was evaluated. C1-15 showed moderate LPS neutralizing activities below a concentration of 20 μM[63]. Increased hydrophobicity of peptide $F_{2,5,12}W$ by multiple Phe to Trp substitution resulted in a significant increase of LPS-neutralizing capacity. Recently, we[82] and others[217,171] reported the importance of hydrophobicity in neutralizing LPS cytokine responses. In line with our observations, Nan and co-workers[217] showed that substitution of Trp by Lys residues in the Trp-rich peptide indolicidin resulted in decreased hydrophobicity and loss of LPS neutralizing activity. Previously, it was shown that the inhibition of the pro-inflammatory response is mainly established by direct binding of cationic HDPs to the LPS[73,74,82]. In line with these observations, we found a correlation between LPS neutralizing activities and LPS binding.

It has been reported that increasing amounts of hydrophobic residues in cathelicidins may increase the hemolytic activity of the peptide[90,107,204]. In addition, it has been demonstrated that high concentrations of cationic HDPs containing a high amount of Trp residues may be toxic to the host[218]. We observed in the present study, that increasing the number of Trp results in increased cytotoxicity against mammalian cells like PBMCs. However, the Trp substitutes exerted broad antibacterial and LPS neutralizing activities at much lower concentrations than toxic values to human PBMCs.

In summary, we successfully increased the antibacterial activity of C1-15 and made it less salt sensitive by replacements of Phe by Trp residues. In addition, $F_{2,5,12}W$ showed good neutralizing activities of LPS cytokine responses.

CHAPTER 5

Improved stability of chicken cathelicidin-2 derived peptides by D-amino acid substitutions and cyclization

E. Margo Molhoek, Albert van Dijk, Edwin J.A. Veldhuizen, Henk P. Haagsman, Floris J. Bikker

ABSTRACT

A truncated version of host defense peptide chicken cathelicidin-2, C1-15, possesses potent, broad-spectrum antibacterial activity. A variant of this peptide, $F_{2,5,12}W$, which contains 3 phenylalanine to tryptophan substitutions, possesses improved antibacterial activity and lipopolysaccharide (LPS) neutralizing activity compared to C1-15. In order to improve the proteolytic resistance of both peptides we engineered novel chicken cathelicidin-2 analogs by substitution of L- with D-amino acids and head-to-tail cyclization. Both, cyclic and D-amino acid variants, showed enhanced stability in human serum compared to C1-15 and $F_{2,5,12}W$. The D-amino acid variants were fully resistant to proteolysis by trypsin and bacterial proteases. Head-to-tail cyclization of peptide $F_{2,5,12}W$ resulted in a 3.5-fold lower cytotoxicity towards peripheral blood mononuclear cells. In general, these modifications did not influence antibacterial and LPS neutralization activities. It is concluded that for the development of novel therapeutic compounds based on chicken cathelicidin-2, D-amino acid substitutions and cyclization must be considered. These modifications increase the stability and lower cytotoxicity of the peptides without altering their antimicrobial potency.

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INTRODUCTION

The increasing prevalence of antibiotic-resistant micro-organisms in humans has elicited a worldwide ongoing search for novel antibiotics. Host defense peptides (HDPs) comprise an alternative class of antibiotic compounds*, i.e.* naturally occurring peptides that exhibit broad antibacterial and/or immunomodulatory properties. Resistance is less likely to occur if peptides or variants thereof are used therapeutically[28]. These characteristics make HDPs interesting to study for their potential application as future antibiotics.

Recently, four chicken cathelicidins (chicken CATH-1 to -3 and -B1) have been identified[46,116,118,119]. We have demonstrated that CATH-2 displays potent antibacterial activity against a variety of bacteria, including those that could potentially be used for bioterrorist attacks. Besides, it was found that CATH-2 inhibits lipopolysaccharide (LPS)-induced cytokine release of peripheral blood mononuclear cells (PBMCs)[51]. The N-terminal domain of CATH-2 comprising the first 15 amino acids (C1-15) has a high antibacterial activity. We succeeded to improve the antibacterial and LPS neutralizing activity of C1-15 by 3 phenylalanine to tryptophan substitutions, resulting in peptide $F_{2,5,12}W$[63,142].

Despite the promising antimicrobial potency of C1-15 and $F_{2,5,12}W$, multiple challenges towards clinical applications are ahead of us[219,138]. For instance, it has been reported that HDPs are highly active in non-physiological conditions (*e.g*. phosphate buffer), but a significant reduction in their antibacterial potency occurs in the presence of complex fluids such as plasma, serum, saliva and sputum[138,139, 140]. The proposed inhibitory mechanisms exerted by biological fluids are numerous. For example, cations in serum, saliva or other fluids may compete with HDPs for binding to the surface of bacterial cells[139,142]. Besides, anionic proteins, such as albumin, which are abundantly present in biological fluids may scavenge HDPs and reduce their availability[139,140,145]. Furthermore, host proteases present in biological fluids may degrade HDPs which renders them inactive. Therefore, with regard to future *in vivo* application of HDPs, it is important to study their biological functions under conditions that are closely related to those encountered *in vivo*.

To date, several design strategies have been employed to optimize the biological activity of HDPs. Through peptide engineering, HDPs can be made less susceptible to serum proteases. Substitution of L- by D-amino acids[128,129,130,131,132] and peptide cyclization[136,137] are well-known strategies to improve peptide stability. Human serum proteases exclusively recognize peptide substrates composed of L-amino acids; substitution of L- by D-amino acids improves the stability to proteolysis by human proteases. On the other hand, cyclization may enhance protease stability by fixing the mobile ends of the molecule which result in conformational constraints thereby making recognition by the protease and subsequent hydrolysis, more difficult.

The aim of this study was to improve the resistance of C1-15 and $F_{2,5,12}W$ against proteolytic degradation. Here, we report the effects of D-amino acid substitutions and head-to-tail cyclization of peptide C1-15 and $F_{2,5,12}W$ on biological activity and stability in serum. Furthermore, the susceptibility of the peptides to mammalian and bacterial proteases was evaluated. We conclude that D-amino acid substitutions and head-to-tail cyclization increase the stability and lower cytotoxicity of the peptides without altering their antimicrobial potency.

MATERIAL AND METHODS

Peptide synthesis

Peptides were synthesized, purified to >90% purity by reversed phase-HPLC, and confirmed by mass spectroscopic analysis by Pepscan Pro (Table 1, Lelystad, the Netherlands).

***Table 1**. Amino acid sequence of CATH-2 derived peptides.*

Peptides	Sequence*
C1-15	**RFGRFLRKIRRFRPK**
D-C1-15	**rfgrflrkirrfrpk**
Cyclic-C1-15	**Cyclo(-RFGRFLRKIRRFRPK-)**
$F_{2,5,12}W$	**RWGRWLRKIRRWRPK**
D- $F_{2,5,12}W$	**rwgrwlrkirrwrpk**
Cyclic-$F_{2,5,12}W$	**Cyclo(-RWGRWLRKIRRWRPK-)**

** Lower case letters are D-amino acids*

Colony count assays

The antimicrobial activity of the peptides was evaluated against Gram-positive *Bacillus anthracis* (*B. anthracis*) Vollum strain (ATCC14578, American Type Culture Collection, Manassas, VA, USA) and Methicillin-Sensitive *Staphylococcus aureus* (MSSA, ATCC 25923, American Type Culture Collection, Manassas, VA, USA). In addition, the antibacterial activity of the peptides was evaluated against the Gram-negative virulent *Yersinia pestis* (*Y. pestis*, NCTC08775, National Collection of Type Cultures, London, United Kingdom) and *Pseudomonas aeruginosa* (*P. aeruginosa*, clinical isolate). All bacteria were grown and maintained in tryptic soy broth (TSB, Biotrading, Mijdrecht, The Netherlands) under aerobic conditions at 35°C (*B. anthracis, MSSA, P. aeruginosa*) or 26°C (*Y. pestis*). Bacteria were cultured to mid-logarithmic phase by transferring 500 µl of stationary phase suspension into 5 ml TSB medium, followed by incubation for 2-2.5 h at 35°C. Mid-logarithmic phase cultures were centrifuged for 10 min at 4500 × g and the bacterial pellets were suspended in 2 × assay medium (10 mM phosphate, pH 7.0; 1/100 TSB) and diluted to ~ 1.10^5 CFU/ml. The antimicrobial activity of the peptides was determined by using colony count assays. Twenty-five microliters of bacterial culture was mixed with an equal volume of peptide in polypropylene 96-well microtiter plates (Costar, Corning Inc., Corning, NY, USA) and incubated for 3 h at 35°C. After the incubation period, 200 µl of TSB medium was added, further diluted 10- to 100-fold in TSB and transferred onto TSA plates after which colonies were counted after 24 h incubation at 35°C (*B. anthracis, MSSA, P. aeruginosa*) or 72 h incubation at 26°C (*Y. pestis*).

Isolation and culture of peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin, Rotterdam, The Netherlands) within 24 h after venipuncture by density centrifugation on Ficoll Paque Plus (Amersham Pharmacia, Uppsala, Sweden). PBMCs were washed twice, counted by trypan blue exclusion, resuspended in RPMI-1640 (Lonza BioWhittaker, Basel, Switzerland), supplemented with 10% heat inactivated fetal calf serum (FCS, Gold, PAA, Pasching, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin

(Lonza BioWhittaker, Basel, Switzerland) and seeded at $0.5.10^6$ cells per well into 96-well flat bottom culture plates (Costar, Corning Inc., Corning, NY, USA) for stimulation experiments.

Cytotoxicity assay

The toxic effect of the peptides on PBMCs was evaluated using WST-1 reagents. This method is based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells to a water-soluble formazan dye (Roche Diagnostics, Mannheim, Germany). Cells were incubated for 24 h with culture medium alone or in the presence of different amounts of peptides at 37°C and 5% CO_2. Following centrifugation, supernatants were taken and 100 µl fresh culture medium, supplemented with 10 µl WST-1 reagents, was added and the cells were further incubated. After 60 min incubation time, absorbance was determined at 450 nm, with a reference wavelength at 650 nm.

LPS neutralizing activities

PBMCs were used to study peptide mediated modulation of LPS-induced cytokine secretion. PBMCs were stimulated with 1 ng/ml ultrapure LPS from *E. coli* O111:B4 (InvivoGen, San Diego, CA, USA) for 24 h in the absence or presence of various concentrations of peptide. Following incubation, cell culture supernatants were collected and stored at -20 ºC until further analysis.

Cytokine measurement

Levels of IL-6 were determined by ELISA using the commercial PeliKine Compact™ human ELISA kit (Sanquin, Amsterdam, The Netherlands) following the manufacturers recommendations.

Stability measurements by reverse-phase HPLC.

For trypsin stability experiments, peptides were pretreated with n-p-L-phenylanaline chloromethyl ketone (TPCK)-trypsin (Sigma, St. Louis, Mo., USA) at a concentration of 50 µg/ml in water for 2 h at 37°C followed by treatment with protease inhibitor cocktail (complete mini, Roche Diagnostics GmbH, Mannheim, Germany) for 30 min at 37°C. For bacterial protease stability experiments, peptides at a concentration of 80 µM were pretreated with the *S. aureus* derived proteases aureolysin (Enzo Life Sciences, Raamsdonksveer, the Netherlands), V8 protease (Sigma, St. Louis, Mo., USA) or *P. aeruginosa* derived protease elastase (Calbiochem, San Diego, USA) at a concentration of 20 µg/ml in 50 mM Tris-5 mM $CaCl_2$, pH 7.8 for 2 h at room temperature, followed by treatment with protease inhibitor cocktail for 30 min at 37°C. Peptides at a concentration of 80 µM (25 µl) were loaded onto a C18 reverse-phase column for HPLC analysis (4.6 mm, 5µ, Alltech, Altima, Deerfield, IL). Peptides were eluted by a 20 min (1 ml/min) linear gradient of 5 to 80% aqueous acetonitril in a 0.1% trifluoracetic acid constant. Eluted peptides were detected by absorbance at 214 nm with a UV monitor.

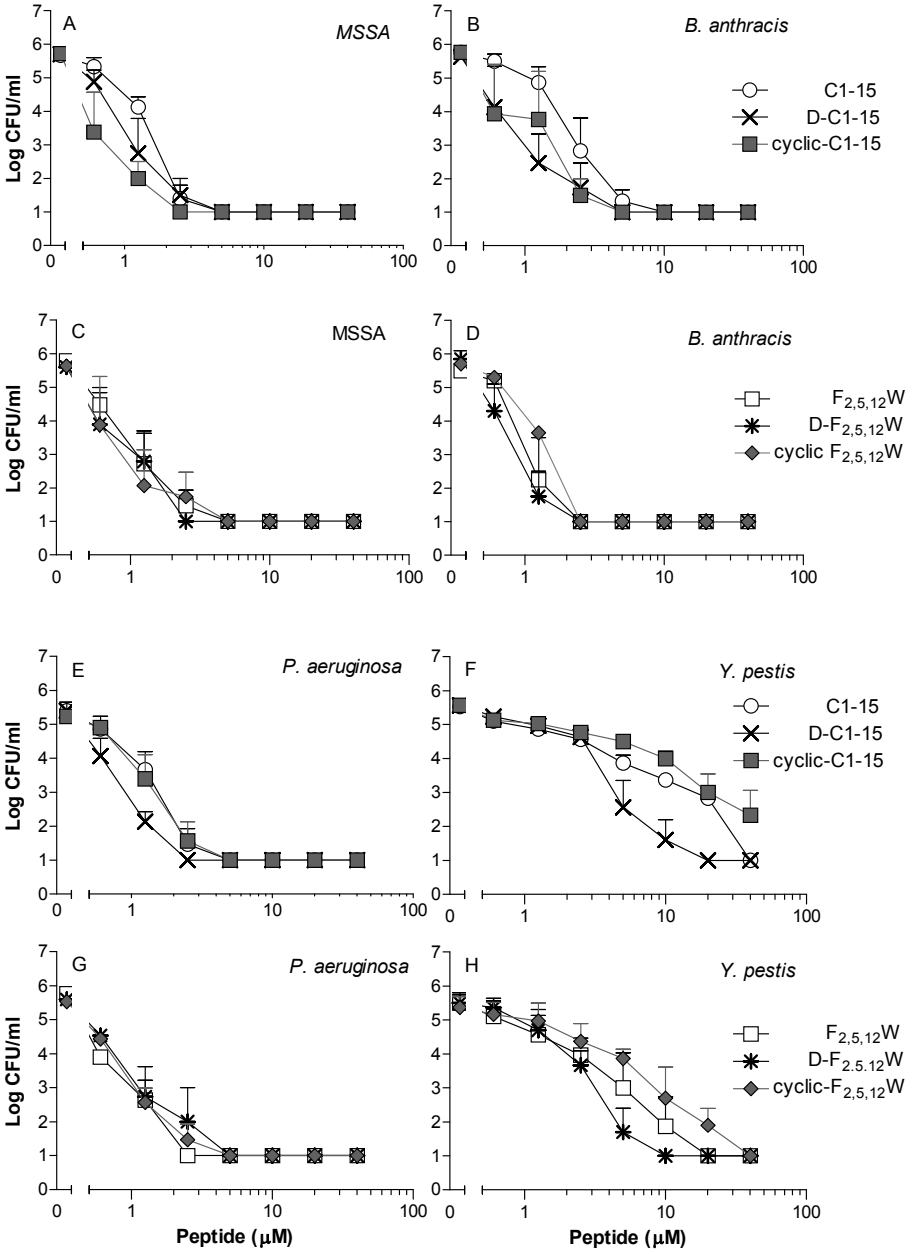

Figure 1. *Bactericidal activity of peptides against Gram-positive MSSA (A and C) and B. anthracis (B and D) and Gram-negative P. aeruginosa (E and G) and Y. pestis (F and H) in colony count assays. Each point represents the mean + SEM of 3 experiments.*

Serum stability measurements by the radial diffusion assay

The serum stability of the peptides was evaluated using radial diffusion assays as described by Lehrer and co-workers[220] with minor modifications. Briefly, MSSA was cultured to mid-logarithmic phase by transferring 500 µl of stationary phase suspension into 5 ml TSB medium, followed by incubation for 2.5 h at 35°C. Mid-logarithmic phase cultures were centrifuged washed and diluted to ~5.10^5 CFU/ml in the underlay gel containing 10 mM sodium phosphate buffer, 1/100 TSB and 1% (w/v) low melting point agarose (low EEO type 1, Sigma, St. Louis, Mo., USA). Sample wells of 5 mm were punched in the underlay gel. Peptides (160 µM) diluted in water, 50% human serum or 50% human serum pre-treated with protease inhibitor cocktail for 1 h at 37°C, were incubated up to 24 h at 37°C and subsequently 10 µl sample was added to each well. After 3 h of incubation at 35°C, the overlay gel containing 6% TSB powder, 1% low melting point agarose (low EEO type 1, Sigma, St. Louis, Mo., USA) was poured onto the underlay gel and incubated overnight at 37°C to visualize the bacterial clearance zones. The activity was represented as (diameter of clear zone in mm – 5 mm).

Statistical analysis

Significance differences between mean values of groups were evaluated using a one-way analysis of variance (ANOVA) and Dunnet's posthoc test and were indicated as * ($P<0.05$).

RESULTS

Antibacterial activity

In order to determine if the novel modified peptides were biologically active, the antibacterial activity of the peptides was evaluated *in vitro* against the Gram-positive bacteria MSSA and *B. anthracis* and the Gram-negative bacteria *P. aeruginosa*, and *Y. pestis*. Modifications of C1-15 and $F_{2,5,12}W$ did not affect the antibacterial activity very much (Figure 1). Cyclization of both peptides slightly reduced the activity against *Y. pestis*. D-amino acid substitutions slightly increased the antibacterial activity of the peptides.

LPS neutralizing properties

In addition to the effect of the modifications on antibacterial activity, the effect on the LPS neutralizing property was investigated. To this end, PBMCs were stimulated with 1 ng/ml LPS in the presence of various concentrations of peptides. The concentration of the pro-inflammatory mediator IL-6 was measured in culture supernatants. Peptide C1-15 showed no LPS neutralizing activity at concentrations up to 20 µM (Figure 2). Modification of the all L-C1-15 the all D-peptide (D-C1-15) increased its LPS neutralization activity up to 44%. Peptide $F_{2,5,12}W$ neutralized the LPS-induced cytokine response. The D-variant of this peptide showed an improved LPS neutralizing activity, while, the cyclic-$F_{2,5,12}W$ variant showed a comparable LPS neutralizing activity as $F_{2,5,12}W$.

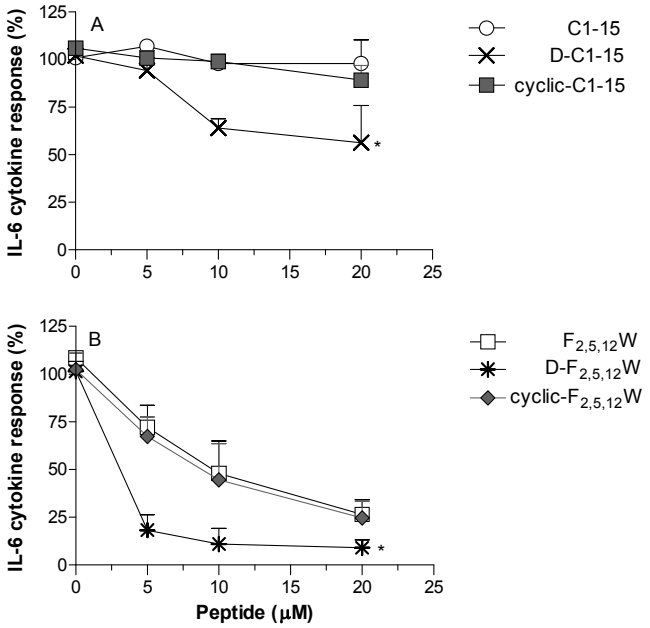

***Figure 2**. Inhibition of the endotoxin-induced IL-6 release in PBMCs by C1-15 (A) or $F_{2,5,12}W$ (B) modified peptides. The IL-6 production of cells incubated with LPS alone was set to 100%. Each point represents mean + SEM of 3 different donors. * Significantly different from C1-15 (A) or $F_{2,5,12}W$ (B).*

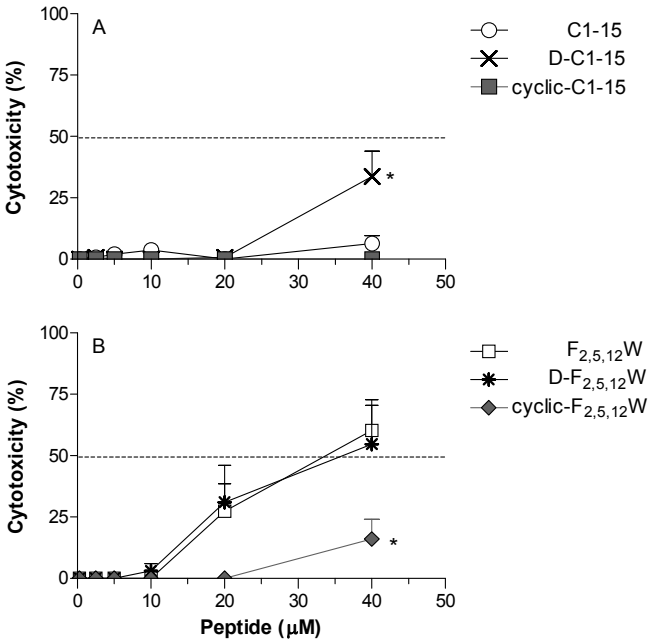

***Figure 3.** Cytotoxicity of C1-15 (A) and $F_{2,5,12}W$ (B) modified peptides towards PBMCs determined by the WST-1 method. Each point represents the mean + SEM of 3 experiments. * Significantly different compared to C1-15 (A) or $F_{2,5,12}W$ (B).*

Cytotoxicity

In order to determine their potential for applications in humans, peptides were evaluated for their toxicity to human PBMCs (Figure 3). Peptide C1-15 and its cyclic-variant are not toxic to human PBMCs while the D-variant showed ~35% toxicity to PBMCs at a concentration of 40 µM. On the other hand, peptide $F_{2,5,12}W$ and D-$F_{2,5,12}W$ showed a toxicity of ~55% against PBMCs. Cyclization of $F_{2,5,12}W$ reduced the toxicity to 16%.

Stability of peptides in human serum

To examine the effect of the applied peptide modifications on the antibacterial activity of the peptides against MSSA in the presence of 50% human serum (HS), a radial diffusion assay was performed. Peptides were pretreated with HS for different times at 37°C and subsequently their activity against MSSA was measured by the radial diffusion assay. The antibacterial activity of C1-15 decreased substantially after incubation for 1 h in serum, with a complete abolishment after 2 h. The antimicrobial activity of cyclic-C1-15 was lost after 3 h and the antibacterial activity of D-C1-15 was maintained up to 24 h incubation in serum (Figure 4A). Compared to C1-15, peptide $F_{2,5,12}W$ showed a 2.5 times improved stability of antibacterial activity in the presence of serum (Figure 4A and B). Cyclization of $F_{2,5,12}W$ further improved the stability in serum; in the presence of serum the peptide maintained its antibacterial activity up to 6 h. Sustained antibacterial activity up to 24 h was observed for peptide D-$F_{2,5,12}W$.

A wide variety of proteases exists in human serum. We hypothesized that some of these proteases may destroy the antibacterial activity of C1-15 and $F_{2,5,12}W$ by peptide cleavage. We evaluated whether the treatment of serum with protease inhibitors prevented the loss of activity of both peptides. Therefore, serum was pre-incubated with a cocktail of protease inhibitors. Subsequently, peptides were incubated in the pre-treated serum and evaluated for their antibacterial activity. Peptide C1-15 (Figure 4A) and $F_{2,5,12}W$ (Figure 4B) showed a sustained antibacterial activity in protease inhibitor treated serum.

HPLC analysis of peptide susceptibility to proteases

As described above, the incubation of serum with C1-15 and $F_{2,5,12}W$ abrogated their antibacterial activity, whereas the activities of D-variants remained unaffected up to 24 h after treatment. HPLC analysis was used to analyze the susceptibility of these peptides to trypsin, by comparing the HPLC profile of untreated peptides to the HPLC profile of the trypsin treated peptides (Table 2). It was found that peptide C1-15 and $F_{2,5,12}W$ were susceptible to trypsin, whereas the all D-variants were fully protected against trypsin proteolysis. Peptide cyclization did not protect the peptide against trypsin proteolysis.

HPLC analysis was also used to evaluate the stability of the peptides to the bacterial proteases from *S. aureus*, V8 protease, aureolysin, and *P. aeruginosa* elastase, LL-37 was included as control (Table 2)[122,123,124]. $F_{2,5,12}W$ was susceptible to elastase treatment, which resulted in a change in HPLC profile (Table 2). Substitution of all L- by D-mino acids resulted in a peptide resistant to elastase proteolysis (Table 2). Cyclization of $F_{2,5,12}W$ resulted in a peptide which was less susceptible to elastase proteolysis compared to the linear peptide. Two hour treatment partly degraded the peptide. With regard to susceptibility towards bacterial proteases, both C1-15 ass well as $F_{2,5,12}W$ were

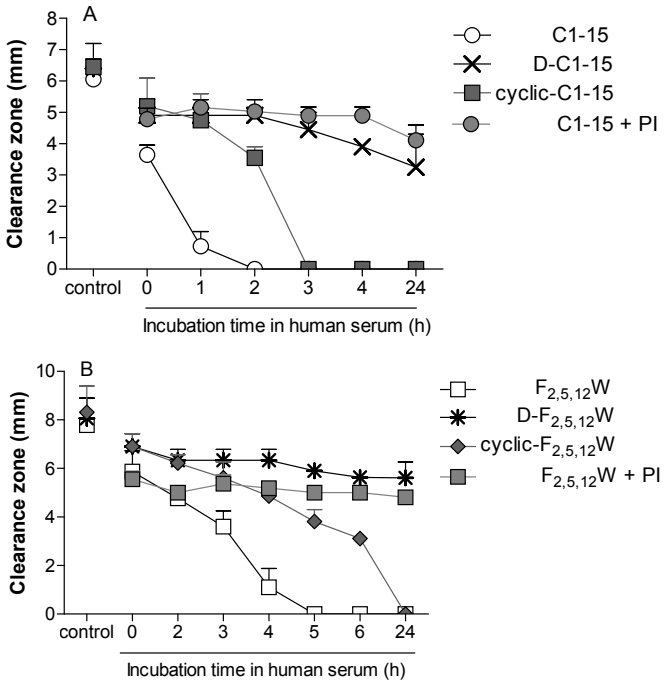

***Figure 4**. Antibacterial stability of C1-15 (A) and $F_{2,5,12}W$ (B) modified peptides in 50% human serum. Peptides were incubated in 50% human serum pre-treated with or without protease inhibitor cocktail (PI) for different times. Next, the antibacterial activity was evaluated in the radial diffusion assay. Peptides in water were used as positive control.*

***Table 2**. Stability of C1-15 and $F_{2,5,12}W$ modified peptides to proteases measured by HPLC. + Stable, - non stable.*

	LL-37	C1-15 variants			$F_{2,5,12}W$ variants		
		C1-15	D	cyclic	$F_{2,5,12}W$	D	cyclic
Trypsin	-	-	+	-	-	+	-
P. aeruginosa elastase	-	-	+	+/-	-	+	+/-
Aureolysin	-	-	+	-	-	+	-
V8 protease	-	+	+	+	+	+	+

susceptible to aureolysin and elastase, whereas both peptides were stable to V8 protease treatment (Table 2). Modifications resulting in D-C1-15 and D-$F_{2,5,12}$W fully protected the peptides against aureolysin and elastase proteolysis, whereas cyclization of the peptides did not (Table 2).

DISCUSSION

Cationic host defense peptides (HDPs) are regarded as promising lead structures in the development of novel broad-spectrum antibiotics. However, due to their predisposition to enzymatic degradation, peptides in general do not circulate in the blood for more than a few minutes[219,138]. Moreover, several pathogenic bacteria secrete proteases as a virulence mechanism to cleave and thereby inactivate HDPs[121,122]. For example, the human HDP LL-37 is inactivated by cleavage by *S. aureus* aureolysin and V8 protease[122,123], by *P. aeruginosa* elastase[122,124] and by *T. forsythia* produced karilysin[125]. The aim of this study was to analyze and improve the stability of C1-15 and $F_{2,5,12}$W, peptide variants of CATH-2. *In vitro* studies revealed that C1-15 and $F_{2,5,12}$W are promising lead structures for the development of anti-infectives to combat bacterial infection[63,142] . We modified the lead structures in several ways in order to enhance the enzymatic stability including substitution of L- by D-amino acids and cyclization.

In order to investigate if the modified peptides were biologically active, the antibacterial activity of the peptides was examined *in vitro* against the human pathogenic bacteria MSSA, *P. aeruginosa*, *B. anthracis* and *Y. pestis*. C1-15 and $F_{2,5,12}$W were antibacterial against all tested bacteria. In general, the modifications applied to C1-15 and $F_{2,5,12}$W did not negatively affect the antibacterial activity and LPS neutralizing activities. D-peptides even showed a slightly improved antibacterial activity and, in some cases, an improved LPS neutralizing activity. Treatment of C1-15 and $F_{2,5,12}$W with human serum abrogated their antibacterial activity within 2 or 4 h respectively. The stability could be improved by cyclization and the activity of D-amino acid variants maintained almost constant up to 24 h of human serum treatment. This corresponds to earlier studies by others applied to HDPs cecropin[221] granulysin[129], MUC7 12-mer[222] and pleurocidin, where D-variants retained their antibacterial and/or LPS neutralizing properties but were protected against proteolytic degradation. Proteases are present in mammalian serum, indicating that such proteases might degrade biologically active peptides *in vivo*. Human serum treatment decreased the antibacterial activity of C1-15 and $F_{2,5,12}$W over time, however the addition of a protease inhibitor cocktail, which inhibits the activity of serine, cysteine, aspartic and metalloproteases, prevented the loss of antibacterial activity of the peptides in human serum (Figure 4). However, the antibacterial activities of both peptides could not be restored to control levels. An explanation might be found in other factors present in human serum affecting peptide activity. For instance, it has been demonstrated that cations, found in serum, saliva or other fluids may compete with cathelicidins for binding to the surface of bacterial cells[139,142] . In addition, anionic proteins, like APO-A1 and albumin, which are present in biological fluids may scavenge cathelicidins and reduce their bio-availability[139,140,145]. For example, LL-37 is bound to APO-A1 in serum and thereby its antibacterial activity is inhibited[143]. Furthermore, the addition of human serum albumin (HSA) to human β-defensin-3 reduced its antibacterial activity[139].

C1-15 and its cyclic-variant are non-toxic to human PBMCs while the D-peptide showed ~35% toxicity to PBMCs at a concentration of 40 µM. On the other hand, $F_{2,5,12}W$ and D-$F_{2,5,12}W$ showed a toxicity of ~55% against PBMCs when used at a high concentration (40 µM), head-to-tail cyclization resulted in a 3.5 fold lower cytotoxicity. A relationship between peptide cyclization and improved selective toxicity has been described before. For example, the cyclic peptide CKLLLKWLLKLLKC[223] and cyclic mellitin analogues[224] exhibited strong antibacterial activity and significantly weaker host cell toxicity than the linear peptide[62,223].

Based on the peptide degradation study, both D-peptides were totally resistant to enzyme digestion. Hence this may explain the slightly improved antimicrobial activity, LPS neutralizing activity and enhanced toxicity of D-amino acid variants compared to their L-peptide against the bacteria. In addition to the susceptibility of peptides to human proteases, peptides can also be targeted by bacterial proteases[121,122]. For example, LL-37 was inactivated by *S. aureus* aureolysin and *P. aeruginosa* elastase[122,124]. Besides, LL-37 was cleaved by *S. aureus* V8 protease, though without loss of antibacterial activity[122,123]. By HPLC analysis we confirmed that LL-37 is cleaved by *S. aureus* aureolysin and V8 protease and *P. aeruginosa* elastase. We report that C1-15 and $F_{2,5,12}W$ are susceptible to *S. aureus* aureolysin and *P. aeruginosa* elastase. Strikingly C1-15 and $F_{2,5,12}W$ were fully resistant to V8 protease treatment. It has been demonstrated that *S. aureus* aureolysin cleaves peptide bonds on the N-terminal side of bulky, aliphatic, hydrophobic residues like isoleucine (Ile) and valine (Val), whereas *S. aureus* V8 protease cleaves peptide bonds exclusively on the carbonyl side of aspartate (Asp) and glutamate (Glu) residues[123]. C1-15 and $F_{2,5,12}W$ both have an Ile residue at position 9 whereas they do not contain Asp or Glu residues, possibly explaining the proteolysis of both peptides by aureolysin but their stability to V8 protease. *P. aeruginosa* elastase is known to cleave bulky hydrophobic residues such as phenylalanine (Phe) and tryptophan (Trp) which are abundant in C1-15 and $F_{2,5,12}W$. Modification of both peptides to their D-variants protected the peptides to aureolysin and elastase proteolysis.

In summary, the substitution of all L- to D-amino acids fully protected peptide C1-15 and $F_{2,5,12}W$ against proteases. These modifications did not influence both antibacterial and LPS neutralization activities for $F_{2,5,12}W$. In addition, we report that cyclization of linear peptide $F_{2,5,12}W$ resulted in a peptide with antibacterial activity against Gram-negative and Gram-positive bacteria, LPS neutralizing activity, enhanced stability in human serum and improved selective toxicity. So, for the development of novel therapeutic compounds based on chicken cathelicidin-2, D-amino acid substitutions and cyclization should be considered.

CHAPTER 6

A cathelicidin-2 derived peptide effectively impairs *S. epidermidis* biofilms

E. Margo Molhoek, Albert van Dijk, Edwin J.A. Veldhuizen, Henk P. Haagsman, Floris J. Bikker

ABSTRACT

Staphylococcus epidermidis (*S. epidermidis*) is a major cause of nosocomial infections due to its ability to form biofilms on the surface of medical devices. Biofilms are surface-adhered bacterial communities. In mature biofilms these communities are encased in an extracellular matrix which is composed of bacterial polysaccharides, proteins and DNA. The antibiotic resistance of bacteria present in biofilms can be up to 1000-fold higher compared to the planktonic phenotype. Host defense peptides are considered to be excellent candidates for the development of novel antibiotics. Recently, we demonstrated that a short variant of host defense peptide chicken cathelicidin-2 (CATH-2), peptide $F_{2,5,12}W$, has potent antibacterial and lipopolysaccharide (LPS) neutralizing activity. In the present study we report anti-biofilm activity of peptide $F_{2,5,12}W$ against two strains of *S. epidermidis* including a multi-resistant strain. Peptide $F_{2,5,12}W$ potently inhibited the formation of bacterial biofilms *in vitro*, at low concentrations (2.5 μM), which is below the concentration required to kill or inhibit growth (MIC = 10 μM). Moreover, peptide $F_{2,5,12}W$ also impaired existing *S. epidermidis* biofilms. A four hour challenge of pre-grown biofilms with 40 μM $F_{2,5,12}W$ reduced the metabolic activity of the wildtype strain biofilm completely and reduced the metabolic activity of the multi-resistant strain biofilm by more then 50%. It is concluded that $F_{2,5,12}W$ prevents biofilm formation and impairs mature *S. epidermidis* biofilms.

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INTRODUCTION

Staphylococcus epidermidis is a major cause of infections related to indwelling medical devices such as intravascular catheters, cerebrospinal fluid shunts, peritoneal dialysis catheters, intraocular lenses, cardiac pacemakers and prosthetic joints[226]. *S. epidermidis* is an important member of the human skin and mucous membrane microflora and can easily be transmitted to the surfaces of these devices when they are implanted or manipulated. *S. epidermidis* adheres to and grows on medical devices which may lead to a mature biofilm consisting of cells embedded in a sticky extracellular slime composed of bacterial polysaccharides, proteins and DNA. This matrix renders the *S. epidermidis* biofilms resistant to antibiotics and host defense systems and makes it difficult or almost impossible to eradicate. Bacteria in biofilms can be up to 1000-fold more resistant to antibiotic treatment than the planktonic phenotype[227].

The rising number of infections caused by bacterial isolates resistant to conventional antibiotics has lead to an intense search for novel antimicrobials and chemotherapeutics, including the use of host defense peptides (HDPs). HDPs comprise a class of antibiotic compounds, *i.e.* naturally occurring peptides that exhibit broad antibacterial and/or immunomodulatory properties[28]. Recently, the chicken HDP cathelicidin-2 (CATH-2) has been identified[46]. We demonstrated that CATH-2 displays potent antibacterial activity against a variety of bacteria, including biowarfare agents, and is able to inhibit lipopolysaccharide (LPS)-induced cytokine release of peripheral blood mononuclear cells (PBMCs)[51]. Furthermore, we found that the peptide C1-15, comprising the first 15 N-terminal amino acids of CATH-2, is the core element of CATH-2 required for the antibacterial activity. The antibacterial and LPS-neutralizing activity of C1-15 was subsequently improved by tryptophan substitutions of all three phenylalanine residues, resulting in peptide $F_{2,5,12}W$[63,142]. Here we report the inhibitory effect of $F_{2,5,12}W$ on *S. epidermidis* biofilm formation and on existing biofilms.

MATERIAL AND METHODS

Peptide

$F_{2,5,12}W$ (RWGRWLRKIRRWRPK) was purchased from Pepscan (Lelystad, the Netherlands). The purity was determined using HPLC and was >90%.

Micro-organisms

The strains used were the *S. epidermidis* clinical isolate strain BM185 and the *S. epidermidis* multi-resistant strain BM492 (TNO collection, Rijswijk, The Netherlands).

Bacterial susceptibility assay

Antimicrobial susceptibility of *S. epidermidis* was performed with the broth microdilution method. Briefly, 2-fold serial dilutions of $F_{2,5,12}W$ were prepared in mueller hinton broth (MHB) containing 0.5% glucose at a volume of 50 µl per well in 96-well flat-bottom tissue culture plates (Costar, Corning Inc., Corning, NY, USA). Overnight cultures of *S. epidermidis* were diluted 1:50 in MHB containing 0.5% glucose and 50 µl of this diluted culture was

added to each well and plates were incubated for 24 h at 35°C. The final concentration of $F_{2,5,12}W$ ranged between 0.6 and 40 µM. The minimal inhibitory concentration (MIC) was defined as the lowest peptide concentration that yielded no visible growth.

Biofilm formation and quantification of activity against biofilms

Biofilm formation was induced in 96-well flat-bottom tissue culture plates (Costar, Corning Inc., Corning, NY, USA). First, overnight cultures of *S. epidermidis* strains were diluted 1:100 in fresh MHB containing 0.5% glucose and 100 µl aliquots of the diluted cultures were added to each well. After incubation at 35°C for 24 h without shaking, each well was washed twice with phosphate buffered saline (PBS) to remove the planktonic bacteria. The biomass of the biofilm was quantified by the semi-quantitative crystal violet (CV) staining. After removal of the planktonic bacteria, wells were stained with 0.4% crystal violet for 15 min. Excess crystal violet was removed by washing each well 3 times with PBS. Bound crystal violet was solubilized in 100 µl 33% acetic acid and optical densities were measured at 630 nm. The metabolic activity of the biofilm was determined with alamar blue (AB, Invitrogen, Carlstad, CA, USA). After removal of the planktonic bacteria, 100 µl of MHB containing 0.5% glucose and 5% alamar blue was added to each well. Alamar blue is a redox indicator that shows both fluorescence and changes in color in response to chemical reduction. The extent of reduction is a reflection of bacterial viability[195]. After 1 h of incubation at 35°C, fluorescence was measured at 550_{ex} and 590_{em} nm. The effects of $F_{2,5,12}W$ on biofilm formation were determined by the addition of different concentrations of peptide (0-40 µM) during the 24 h biofilm forming period. Cell growth was determined by measuring the absorbance at 595 nm. Similarly, the effects of $F_{2,5,12}W$ on mature biofilm were determined by incubating the washed 24 h biofilms with different concentrations of peptides (0-40 µM) for 4 h. Positive controls were untreated biofilms with only MHB + 0.5% glucose. All experiments were performed in triplicate.

Statistical analysis

Significance differences between mean values of groups were evaluated using a one-way analysis of variance (ANOVA) and Dunnet's posthoc test and were indicated as * ($P<0.05$).

RESULTS

Minimal inhibitory concentration

The concentration of $F_{2,5,12}W$ required to inhibit the growth of planktonic bacteria (MIC) was 10 µM (data not shown). No differences in the MIC values were found between an antibiotic sensitive and a multi-resistant strain. Both the clinical isolate as well as the multi-resistant strain showed no visible growth at a concentration of 10 µM.

Biofilm formation of 2 S. epidermidis strains

Both strains formed biofilms *in vitro.* Twenty-four hours of culturing resulted in a significantly larger biomass and metabolic activity for the multi-resistant strain (BM492) compared to the wildtype strain (BM185). Furthermore, a strong correlation was observed

between biomass quantified by CV stain (Figure. 1A) and biofilm metabolic activity (Figure. 1B) quantified by alamar blue reduction in the 24 h old biofilm.

Peptide $F_{2,5,12}W$ impairs S. epidermidis biofilm formation.

An *in vitro* assay was performed to investigate the effect of $F_{2,5,12}W$ on the biofilm formation of S. *epidermidis*. As shown in Figure 2, all tested concentrations of the peptide inhibited biofilm formation. No differences were found between the two tested strains. The amount of biofilm biomass as well as the metabolic activity of the biofilm was significantly inhibited at 2.5 µM peptide, a concentration 4 times below the MIC. This inhibitory effect was increased to control levels at 10 µM, a concentration equal to the MIC.

$F_{2,5,12}W$ impairs the metabolic activity of existing biofilms.

Mature biofilms are known to be more resistant to eradication if compared to inhibition of the *novo* biofilm formation. Therefore, the activity of peptide $F_{2,5,12}W$ against mature biofilms was evaluated. Figure 3 show the amount of biomass quantified by a CV stain and the metabolic activity quantified by the alamar blue assay in treated and untreated biofilms. The metabolic activity of the *S. epidermidis* strain BM185, was significantly suppressed at 5 µM, a concentration 2 times below the MIC. Higher concentrations further suppressed the metabolic activity; at 40 µM the metabolic activity was similar to the negative control levels. The effect of $F_{2,5,12}W$ treatment on the biomass of strain BM185 was also dose-dependent, CV staining method is less reproducible than the alamar blue assay and therefore only significant at a concentration of 20 µM. The mature biofilms of the multi-resistant strain of *S. epidermidis* BM192, were less susceptible to $F_{2,5,12}W$ treatment. However, a significant suppression of the metabolic activity of the biofilm was found after treatment with 40 µM $F_{2,5,12}W$.

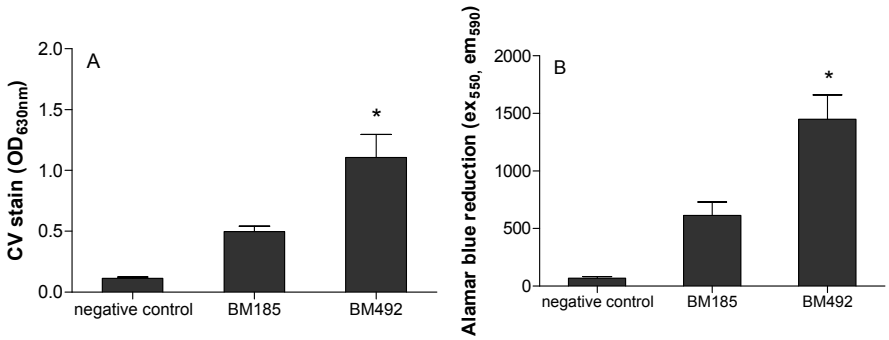


Figure 1. *Biomass of a 24 h biofilm of 2 different S. epidermidis strains. Quantification of biomass with crystal violet (A) and metabolic activity with alamar blue (B). Values represent means of 3 experiments + SEM *Significantly different compared to BM185.*

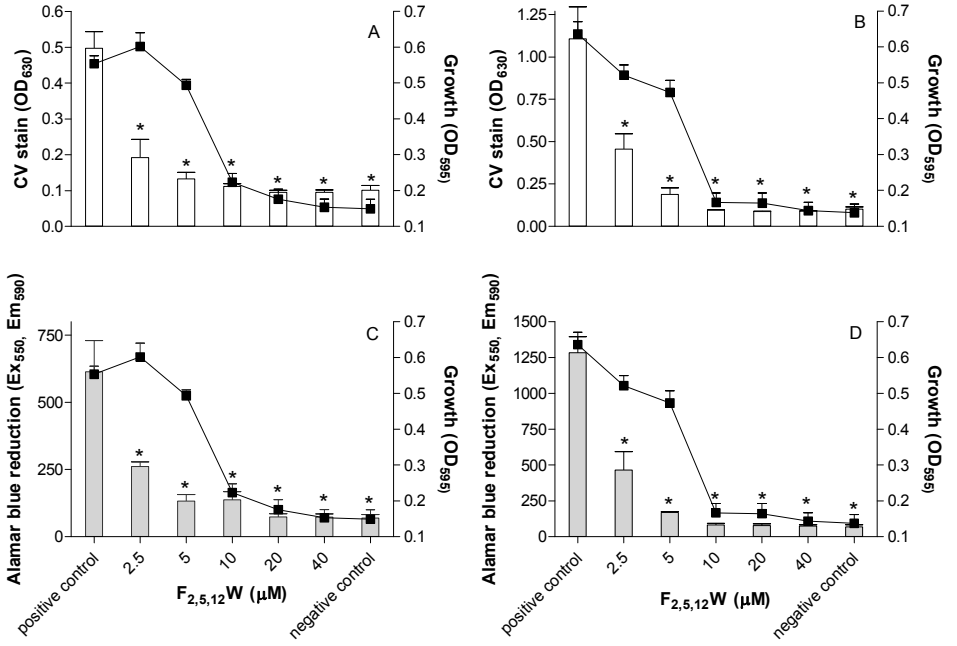


***Figure 2**. Effect of $F_{2,5,12}W$ on S. epidermidis biofilm formation from strain BM185 (A and C) and BM492 (B and D). The bacterial cells were inoculated in a 96-well plate containing different concentrations of peptides. Culture without $F_{2,5,12}W$ was used as positive control and media without culture was used as a negative control. After incubation at 35°C for 24 h absorbance at 595 nm was recorded to assess the cell growth (line graph). Biofilm biomass was determined by a crystal violet stain (white bars). The metabolic activity of the biofilm was determined by the alamar blue reduction assay (grey bars). Data represent mean + SEM of at least 3 experiments. * Significantly different compared to controls without peptide*

DISCUSSION

The involvement of *S. epidermidis* in infections associated with indwelling medical devices and the rapid development of multiple antibiotic resistances has continued to receive significant attention[228]. These types of infections are mediated by the propensity of *S. epidermidis* to adhere to surfaces and form biofilms. In general, bacteria grown as a biofilm are protected from host defenses and often exhibit reduced susceptibility to antibiotics, contributing to the persistence of the biofilm[227].

$F_{2,5,12}W$, a short peptide based on chicken cathelicidin-2, has been reported to have broad antibacterial activity[142], but so far its effects on bacterial biofilms had not been evaluated. The primary aim of this study was to investigate the effect of $F_{2,5,12}W$ on *S. epidermidis* biofilms using a simple screening method to quantify biofilms *in vitro*. We considered the viability of biofilm cells as most important aspect when evaluating the effect of antimicrobial agents. Crystal violet (CV) stains polysaccharides in the extracellular matrix but does not distinguish between living and dead cells. Therefore, we also included a quantification model based on the reduction of alamar blue by metabolic active cells.

First, we determined the antibacterial activity of $F_{2,5,12}W$ against two *S. epidermidis* strains, including a multi-resistant strain. The results, demonstrated that $F_{2,5,12}W$ exhibits activity with a MIC value of 10 µM for both strains. In addition, we demonstrated that $F_{2,5,12}W$ suppressed biofilm formation of both strains at a concentration 4 times below the MIC. The inhibition of $F_{2,5,12}W$ on the formation on the biofilm may be the result of the inhibition of the bacterial growth. However, the inhibition of biofilm formation was observed at concentrations that did not affect bacterial growth. Therefore the inhibiting effect of $F_{2,5,12}W$ on biofilm formation likely reflects decreased initial adhesion of bacteria to the surface and subsequent development of the biofilm.

Mature biofilms are known to be more refractory to eradication relative to inhibition of the *novo* biofilm formation. Therefore, the effect of peptide $F_{2,5,12}W$ on mature biofilms was evaluated. Low concentrations of $F_{2,5,12}W$ had a better antimicrobial effect on the weak biofilm producing *S. epidermidis* strain BM185 than on the strong biofilm forming strain BM492. Both the architecture of a thinner biofilm and the total number of bacteria in such a biofilm may explain this finding. Several other groups have investigated the effect of natural HDPs or its synthetic analogues on different types of biofilms[229,230,231,232,233,234]. It was reported that various peptides could be used to prevent

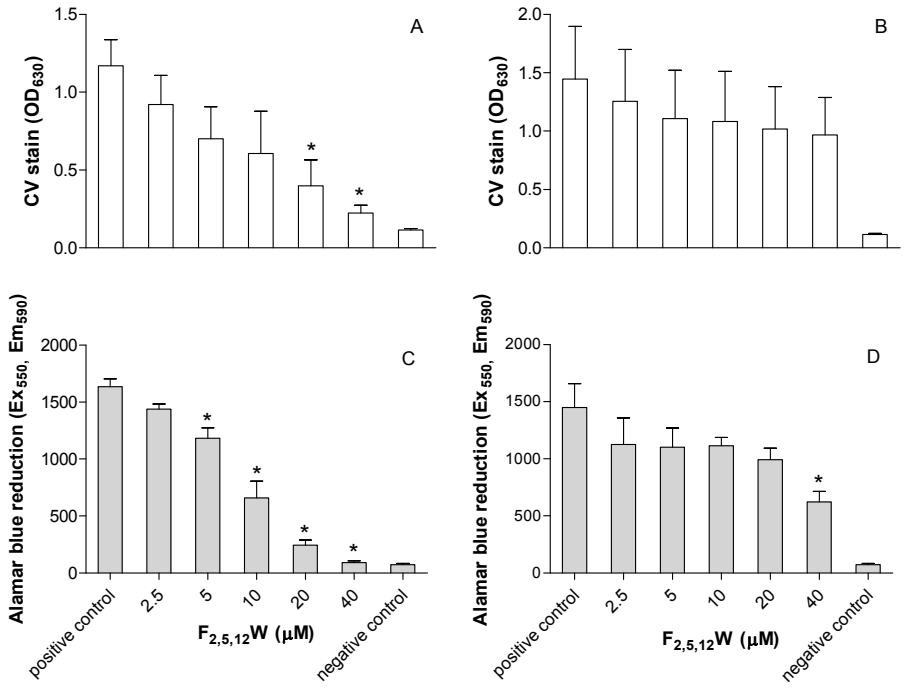


***Figure 3**. Effect of $F_{2,5,12}W$ on the S. epidermidis preformed biofilm from strain BM185 (A and C) and BM492 (B and D). The bacterial cells were inoculated in a 96-well plate and incubated for 24 h to develop a mature biofilm. Biofilms were rinsed and different concentrations of $F_{2,5,12}W$ were transferred onto the biofilm. After 4 h incubation at 35°C biofilm biomass was determined by a crystal violet stain (white bars) and the metabolic activity was determined by the alamar blue reduction assay (grey bars).*

biofilm formations. However, only three groups reported that peptides were effective in killing bacteria in a 24 hour biofilm[231,233,234] .

In summary, we demonstrate that, at low concentrations, below those that kill or inhibit growth, $F_{2,5,12}W$ strongly prevents bacterial biofilm formation *in vitro*. Moreover, $F_{2,5,12}W$ also impairs existing *S. epidermidis* biofilms.

CHAPTER 7

Summarizing discussion

INTRODUCTION

There exists a major concern that biological pathogens will be used as instruments of terror. The Centers for Disease Control and Prevention (CDC) formulated a list of biological agents most likely to be used for biological warfare or terroristic purposes. These include the bacteria *Bacillus anthracis* and *Yesinia pestis*, both causing highly progressive, acute infections which may be lethal in some forms. An attack involving biological warfare agents may go unnoticed until people start to exhibit clinical symptoms. Timely diagnosis is not always feasible. In addition, the initial clinical signs and symptoms are usually aspecific and flu-like which makes an accurate diagnosis even more difficult. Therefore, in case of an exposure from an unknown source and bacterial identity the availability and usability of broad-spectrum antimicrobials may be life-saving, serving as a robust first line of added molecular defense. Furthermore, the development, persistence, and rapidly increasing incidence of bacterial resistance to current antibiotic drugs underline the motivation for the search for new, alternative broad antimicrobial compounds.

The innate immune system produces a wide array of small cationic peptides to combat pathogenic micro-organisms. These so called host defense peptides (HDPs) are multifunctional peptides that have broad-spectrum activity against a variety of Gram-positive and Gram-negative bacteria. HDPs use multiple mechanisms to kill bacteria, *e.g*. cell wall permeabilization, inhibition of DNA replication and protein synthesis. However, besides direct antimicrobial activity HDPs exhibit additional immunomodulatory activities essential for the orchestration of the innate immune and inflammatory response. These regulatory functions include the recruitment and activation of immune cells and the boosting of immune functions. On the other side, HDPs can selectively block the LPS-induced pro-inflammatory response. The multifunctionality of these peptides makes it particularly difficult for bacteria to become resistant against HDPs[28]. The combination of broad antibacterial activity and the ability to block the LPS-induced pro-inflammatory response make them interesting candidates for the development as new antimicrobial and/or anti-septic agents. Bacterial killing by antimicrobial compounds causes a release of endotoxin from Gram-negative bacteria. This can result in early sepsis, in which high levels of cytokines and inflammatory mediators become destructive, causing organ failure, cardiovascular shock and may even cause death. Nevertheless, some HDPs are found to counteract the development of septic shock by binding endotoxin or by blocking the binding of endotoxin to lipopolysaccharide-binding protein (LBP), thereby preventing phagocyte activation and pro-inflammatory cytokine secretion, providing a mechanism to overcome development of septic shock[73, 80,102,157,171,209,210].

For the development of artificial variants of HDPs with optimized activity for therapeutic application an in depth understanding of the structure-activity relationship (SAR) is crucial. This thesis aims to determine the structure-function relationship of two HDPs: human LL-37 and chicken cathelicidin-2 (CATH-2). In addition, CATH-2 was used as a paradigm to develop very small, stable peptides with improved antibacterial and LPS-neutralizing activities.

LL-37

Structure-function relationship of LL-37

The sole human cathelicidin LL-37 is one of the most studied cathelicidins[83,105,108]. LL-37 exhibits, in addition to its antimicrobial activity, immunomodulatory activity such as inhibition of pro-inflammatory responses to bacterial LPS and therefore has potential in the treatment of sepsis.

An in depth understanding of the structure-activity relationship (SAR) is crucial for the development of artificial variants with optimized activity for therapeutic application. SARs for especially antibacterial and cytotoxic activities have revealed that the activities of amphipathic α-helical antimicrobial peptides are not determined by the precise primary structure. Rather, activity was found to be determined by the subtle interplay of structural and physico-chemical parameters such as cationicity, hydrophobicity and amphipathicity[107].

By using structure-function studies different groups gathered evidence that the antibacterial activity of LL-37 is not located in the N-terminal of the peptide[64,108,109,110,111,112,113] but can be ascribed to the mid-region of LL-37[108,110,113]. Li and co workers were able to use this approach to identify the minimal antibacterial domain of LL-37, residues 17-33, active against Gram-positive and Gram-negative bacteria[110] (Figure 1). This domain consists of an amphipathic α-helix and 5 cationic residues. Although hydrophobicity and amphipathicity contributed to the antibacterial activity, the cationic side chains on the hydrophilic surface of the peptide were most accountable[110]. This implied that the electrostatic interaction of the cationic residues of LL-37 with anionic phospholipids, such as phosphatidylglycerol and negatively charged liposaccharides, which are abundant in the bacterial cell membrane, are important for the antibacterial activity.

A similar approach was used to determine the core domain of LL-37 in LPS neutralization. Corresponding to the antibacterial activity, the LPS-neutralizing activity is not located in the N-terminal of LL-37[80,82,88,102,111,114] but can be ascribed to the mid-region of LL-37. In **Chapter 2** we identified the minimal LPS neutralizing domain, residues 13-31[82] (Figure 1). This domain consists of the glycine residue responsible for the bend in LL-37, an amphipathic α-helix and 5 cationic residues. Although α-helicity and cationicity contributed moderately, hydrophobicity played a significant role with respect to the LPS neutralizing activity. This view is supported by a study of Nagaoka and co-workers, in

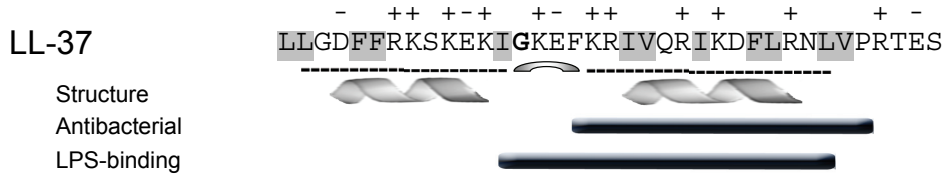


***Figure 1**. Distribution of cationic (+), anionic (-), hydrophobic (gray) and hinge region glycine residue (**bold**). Helical segments are underlined. Bars indicate the segments that involved in each biological activity. Note that the C-terminal helix is indispensable for antibacterial and LPS-neutralizing activities.*

which LPS neutralizing activities of LL-37 were enhanced by increasing the hydrophobicity[171]. Moreover, we confirmed that the inhibition of the pro-inflammatory response is mainly established by direct binding of LL-37 to LPS[73,74,171]. This implies that LL-37–LPS interaction is mediated by electrostatic attraction between the cationic residues of LL-37 and anionic residues of LPS. In addition, hydrophobic interactions seem to be essential for a proper LL-37-LPS binding. However, for a better understanding of LL-37 derived peptides in complex with LPS three-dimensional structures need to be determined[235].

In contrast to the antibacterial and LPS neutralizing activity, discrepancies exist between studies that aimed to define the core domain of LL-37 responsible for cytotoxic effects to eukaryotic cells. However, it is clear that hydrophobic residues are essential for the association of LL-37 with phospholipids, including zwitterionic phospholipids[64,110,109,111].

CHICKEN CATHELICIDIN-2 (CATH-2)

Structure-function relationship of CATH-2

Chicken cathelicidin-2 (CATH-2), is a recently discovered cathelicidin that exhibits antimicrobial activity against Gram-positive and Gram-negative bacteria[46,116]. Moreover, CATH-2 is potent in neutralizing LPS-induced cytokine responses[116].

It has been demonstrated by NMR spectroscopy analysis that CATH-2 consists of two well defined α-helices separated by a proline residue induced extensive hinge and a flexible region at the N-terminus. In contrast to most α-helical cathelicidins with a limited degree of hinge near the center, the hinge of CATH-2 in the central region is extensive due to the presence of the proline residue. The N-terminal α-helix adopts a typical amphipathic structure, while the C-terminal helix is highly hydrophobic. It is noted that the central kink region of CATH-2 is strongly positively charged and contains 5 cationic residues[63,116].

Using amino acid substitutions, it was shown that the central proline residue, inducing a kink by destabilizing α-helix formation, is of great importance for the biological activities of CATH-2 (**Chapter 3**). Stabilizing the α-helix formation in CATH-2 by proline to leucine substitution, greatly affected its biological activity; antibacterial activity, hemolytic activity, LPS neutralization and the ability to significantly induce MCP-1 production by PBMCs[63]. In addition, Xiao and co-workers reported that other amino acids present in the hinge, are of great importance for the biological activity[75]. Based on these observations, we hypothesize that, after initial electrostatic interaction of the N-terminal segment with the bacterial membrane, due to the proline induced kinked conformation the C-terminal will be inserted more rapidly and deeper into the lipid bilayer.

By using truncated variants of CATH-2, the core domains of antibacterial activity, LPS neutralizing activity, immune-stimulating activity and toxicity were elucidated (Figure 2). The N-terminal part of CATH-2, containing residues 1-15, displayed the highest overall antibacterial activity and fastest killing kinetics. This N-terminal helical segment contains the bulk of the cationic charges (+8) and an amphipathic α-helix. This implies, corresponding to most cathelicidins, that cationic residues facilitate the initial attraction of

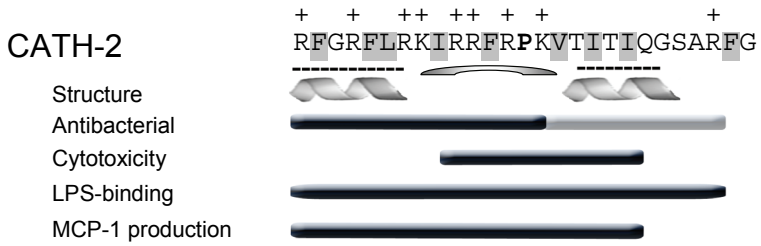


***Figure 2**. Distribution of cationic (+), hydrophobic (gray) and hinge region proline residue (**bold**). Helical segments are underlined. Bars indicate the segments that are moderately (gray) or strongly (black) involved in each biological activity. Note that the stretch IRRFRP, the hinge region of the peptide, is essential for antibacterial, cytolytic, lipopolysaccharide (LPS) neutralization, and MCP-1 inducing activities. The hinge region in combination with the N-terminal, amphipathic, helix is critically involved in antibacterial activity. The hinge region in combination with the C-terminal is involved in toxicity to mammalian cells, but less critically involved in the antibacterial activity. Both helices and the hinge region are involved in immunomodulation; LPS neutralization and MCP-1 induction [63,75].*

the peptides to the anionic phospholipids in bacterial membranes. Subsequently, hydrophobic interactions facilitate insertion of the peptides in the lipid bilayer. On the other hand, structure-function analysis showed that both helical segments of CATH-2 are involved in LPS-neutralization (Figure 2). Similarly, HDPs SMAP-29[182] and CATH-1[120] have been reported to contain two high affinity LPS-binding domains at the end of each terminus.

In addition to hinder LPS binding to receptors, CATH-2 derived peptides may directly stimulate immune cells. For example, cationic peptides CEMA, LL-37 and IDR-1 induce expression of monocyte chemokines, including MCP-1[69,76,197,198]. MCP-1 is a chemoattractant for monocytes[199], memory T cells[200] and NK cells[201] and can protect against bacterial infection[202]. Both helical segments of CATH-2 are essential in MCP-1 production in human PBMCs. For this reason, the core antibacterial peptide C1-15 was not able to stimulate MCP-1 production, which may be an advantage if C1-15 derived peptides are used for therapy. Recruitment of inflammatory cells by MCP-1 to local sites of microbial invasion is beneficial during acute infections but for treatment of chronic infections it may be more favorable to dampen the immune response *e.g.* inhibition of LPS-induced pro-inflammatory response, and not to attract inflammatory cells.

Safety is a very important parameter for the clinical use of HDPs. Despite the fact that most cathelicidins are selective for bacterial cells, some cathelicidins are toxic to host cells if used in higher concentrations. Compared to the parent peptide CATH-2, the absence of the 5 C-terminal amino acid residues only slightly reduced cytotoxicity. These findings suggest that the short hydrophobic stretch (VTITIQ) between position 16 and 22 greatly contributes to CATH-2 cytotoxicity. In a corresponding study, Xiao and co workers reported that the stretch IRRFR (residue 14 to 18) present in the hinge region is most accountable for its toxicity to eukaryotic cells[75]. Interestingly, the highly antibacterial peptide C1-15 segment exhibited only low cytotoxicity towards chicken erythrocytes and was non-toxic towards PBMCs. This might be explained by the excess charge density present in the polar sector of the C1-15 segment, which renders the peptide less prone to

structure in an α-helix[191]. This may significantly decrease cytotoxicity without affecting antibacterial activity[192].

In **Chapter 3** the core elements within the mature CATH-2 that are linked to antibacterial and/or immunomodulatory activities were identified. In this respect, the C1-15 segment is of particular interest to use as a template for further development of a sole antibacterial compound, because it combines a high antibacterial activity with a low cytotoxicity and does not appear to significantly stimulate MCP-1 production. Therefore, modification of peptides corresponding to these core elements by specific amino acid substitutions can be applied to amplify desired biological activities. This approach may lead to development of peptide antibiotics that can be used clinically for prophylactic and/or therapeutic use.

Optimization of the antibacterial and LPS activity of CATH-2 derived peptides

Amino acid substitutions in HDPs are frequently used to create a new structure with altered biological activity *i.e*. high antibacterial activity, high LPS neutralizing activity and low cytotoxicity to host cells[38,134,191,204,205,206,207,208].

In an attempt to improve the antibacterial and LPS neutralizing properties of CATH-2 derived peptide C-15, phenylalanine residues were substituted by tryptophan residues (**Chapter 4**). In comparison to phenylalanine, tryptophan has a more pronounced hydrophobic bulk. The hydrophobic bulk of tryptophan residues play a role in membrane association because it preferably interacts with the interfacial region of membranes[213]. In addition, hydrophobicity is reported to be an important factor in the LPS neutralizing activity of HDPs[82,171,217,221]. Moreover, it is reported for CATH-1 that the tryptophan residue in CATH-1 plays a role in close packing of the peptide and the of acyl chains of LPS, the indole chain was found to be positioned at the interface between the polar and hydrophobic layer of LPS[235]. In line with these observations, the antibacterial activity could be improved, specifically against the biological warfare agents *Bacillus anthracis* and *Yersinia pestis,* and the LPS neutralizing properties of peptide C1-15 were improved by phenylalanine to tryptophan substitutions.

Although inclusion of tryptophan residues in peptide C1-15 is associated with improved antibacterial and LPS neutralizing properties, it has been demonstrated that high concentrations of cationic HDPs containing a high amount of tryptophan residues may be toxic to mammalian cells[218]. If used therapeutically, low toxicity to mammalian cells is of great importance regarding to safety. Substitution of phenylalanine by tryptophan residues increased to toxicity to mammalian cells like human PBMCs. However, the tryptophan substitutes exerted broad antibacterial and LPS neutralizing activities at much lower concentrations than toxic values to human PBMCs**.**

Improving the stability of CATH-2 derived peptides to proteases and serum components.

HDPs are highly active in non-physiological conditions (*e.g.* phosphate buffer), but a significant reduction in their antibacterial potency occurs in the presence of complex fluids such as plasma, serum, saliva and sputum[138,139,140]. One proposed inhibitory mechanism exerted by biological fluids is the presence of cations; monovalent cations such as Na^+ and K^+ (100 mM) and divalent cations like Mg^{2+} and Ca^{2+} (1-2 mM)[141], present in serum and other biological fluids that may compete with cathelicidins for binding to the anionic

surface of bacterial cells and thereby inhibit their antibacterial activity[28,139,142]. Furthermore, host proteases present in biological fluids may degrade HDPs which renders them inactive. Therefore, with respect to future *in vivo* application of HDPs, it is important to study their biological functions under conditions that are closely related to those encountered *in vivo*.

The antibacterial activity of peptide C1-15 was completely lost in the presence of salt while its phenylalanine to tryptophan substituted variant, peptide $F_{2,5,12}W$, lost some activity, but was still strongly antibacterial at 100 mM NaCl (**Chapter 4**). In addition, peptide C1-15 and $F_{2,5,12}W$ were degraded in human serum. However, compared to peptide C1-15, the variant peptide was less susceptible to degradation (**Chapter 5**). Because peptide $F_{2,5,12}W$ shows improved antibacterial and LPS neutralizing activity, is less affected by cations and to degradation compared to peptide C1-15, it could be a better candidate for use as a therapeutic in the treatment of bacterial infections.

Through peptide engineering, HDPs can be made less susceptible to proteases. Substitution of L- by D-amino acids[128,129,130,131,132] and peptide cyclization[136,137] are well known strategies to improve peptide stability. Human serum proteases exclusively recognize peptide substrates composed of L-amino acids; substitution of L- by D-amino acids improves the stability to proteolysis by human proteases. On the other hand, cyclization may enhance protease stability by fixing the mobile ends of the molecule which result in conformational constraints thereby making recognition by the protease and subsequent hydrolysis, more difficult. In **Chapter 5**, both strategies were used to improve the stability of CATH-2 derived peptides against proteolysis. Both cyclic and D-amino acid variants of CATH-2 variants showed enhanced stability in human serum and still exerted potent LPS neutralizing and/or antibacterial activity. In addition, cyclization of peptide $F_{2,5,12}W$ resulted in a 3.5-fold lower toxicity to mammalian cells. A relationship between peptide cyclization and improved selective toxicity has been described before. For example, the cyclic peptide CKLLLKWLLKLLKC[66] and cyclic mellitin analogues[43] exhibited strong antibacterial activity and significantly weaker host cell toxicity than the linear peptide[66,88]. However, in contrast; cyclization of the hexapeptide RRWWRF increased both antibacterial and hemolytic activity[236]. Taken together, we conclude that D-amino acid substitutions and head-to-tail cyclization increase the stability in serum and decrease the toxic effects of the peptides without altering their antimicrobial and LPS neutralizing potency and therefore modified peptides may be more suitable for use as therapeutics.

Activity of CATH-2 derived peptides against biofilms

In different infections, pathogenic bacteria are imbedded in biofilms. Biofilms on indwelling medical devices cause serious problems in hospitals. Microbial biofilms develop on the surfaces of medical devices and proceed to cause bacterial infections and sepsis. In a patient with urinary catheters, infection rates increase with the duration of catheterization at rates of 5-10% per day with virtually all patients who undergo long-term catheterization (>28 days) becoming infected. *Staphylococcus epidermidis* is commonly isolated from biofilms in medical devises[226]. Biofilms are surface-adhered bacterial communities. In mature biofilms these communities are encased in an extracellular matrix which is composed of bacterial polysaccharides, proteins and DNA. Bacteria present in biofilms show up to 1000-fold higher antibiotic resistance compared to the planktonic phenotype[227].

The effect of the antibacterial and LPS neutralizing CATH-2 variants $F_{2,5,12}W$ on *S. epidermidis* biofilm formation and preformed biofilms was evaluated in **Chapter 6**. We report that peptide $F_{2,5,12}W$ prevented biofilm formation of two tested *S. epidermidis* strains, including a multi-resistant strain, below concentrations that affect the bacterial growth. $F_{2,5,12}W$ was only moderately able to degrade the pre-grown *S. epidermidis* mature biofilm. However, $F_{2,5,12}W$ significantly affected the bioavailability of bacteria in the biofilm by killing bacteria in the biofilm. Several other groups have investigated the effect of natural HDPs or its synthetic analogues on different types of biofilms[229,230,231,232,233,234]. It was reported that various peptides could be used to prevent biofilm formation. However, only three groups reported that peptides were effective in killing bacteria in a 24 hour biofilm[231,233,234] . Taken together, the study suggests that CATH-2 derived peptide $F_{2,5,12}W$ has potential activity against biofilms.

CONCLUDING REMARKS

This thesis presents the *in vitro* antibacterial and LPS neutralizing activities of LL-37 and CATH-2 derived peptides, including studies which determine the effects of physiological factors on biological activity as well as possible strategies to enhance activity under physiological activities. We can conclude that CATH-2 derived peptides are effective antibacterial and LPS neutralizing agents *in vitro* and can potentially be used as a promising agent for the development of novel drugs for the control of infectious diseases. In addition, D-isomers and head-to-tail cyclic variants of these peptides may potentially enhance their therapeutic efficacy. However, despite these encouraging results, the applicability of these peptides depends on other factors besides *in vitro* potency; a new drug must meet many criteria such as efficacy, safety, stability and preferably low production costs before it can be used clinically. Thus, from here novel interesting challenges appear towards the development of antimicrobial drugs for *in vivo* applicability.

Nederlandse samenvatting

Micro-organismen, zoals bacteriën, schimmels en virussen, kunnen bij mensen, planten en dieren ziektes veroorzaken. Ziekteverwekkende micro-organismen worden pathogenen genoemd. Een flink aantal pathogenen is in het verleden gebruikt, en zou ook in de toekomst gebruikt kunnen worden voor biologische oorlogsvoering. Een pathogeen kan beschouwd worden als een biologisch strijdmiddel als het gebruik ervan het doel heeft mensen of dieren te beschadigen, uit te schakelen of te doden. De Amerikaanse 'Centers for Disease Control and Prevention' hebben zes pathogenen aangewezen als de meest gevaarlijke. Deze selectie is gebaseerd op (1) het gemak waarmee de pathogenen geproduceerd en verspreid kunnen worden en (2) de ernst van de ziekte en daarmee gepaarde sterfte die ze kunnen veroorzaken. Hieronder vallen de bacteriën *Bacillus anthracis* en *Yersinia pestis*.

De ondertekening van de Biologische wapen conventie in 1972, een verbod op de ontwikkeling en opslag van biologische wapens door de meeste landen, bleek onvoldoende om misbruik van dergelijke organismen door terroristen te voorkomen. Dit werd onder meer duidelijk, kort na de aanslag op september 2001 in de Verenigde Staten, toen een serie brieven werd gepost naar verschillende media en politici die de sporen van *B. anthracis* bevatten en waarbij 5 mensen overleden door een infectie van *B. antracis* na inademing van de sporen.

Na de blootstelling aan een biologisch strijdmiddel is een snelle en accurate diagnose niet altijd mogelijk, hierdoor blijft een terroristische aanval mogelijk onopgemerkt totdat de ziekteverschijnselen (klinische symptomen) optreden. Doordat de vroege klinische symptomen van veel biologische strijdmiddelen algemeen en griepachtig van aard zijn, is het stellen van een juiste diagnose erg moeilijk. Een generieke aanpak die de effecten van een breed scala aan bacteriën kan tegen gaan, kan na blootstelling aan een biologisch strijdmiddel van bacteriële aard van levensbelang zijn. Door spontane mutaties kan het echter voorkomen dat een bacteriestam ongevoelig (resistent) is geworden voor de huidig gebruikte antibiotica. Het aantal stammen dat ongevoelig is voor de huidig gebruikte antibiotica neemt toe. Daarnaast is het moedwillig resistent maken van bacteriën met behulp van genetische modificatie voor velen steeds toegankelijker geworden. Beide ontwikkelingen benadrukken de behoefte aan nieuwe breedspectrum antibiotica.

Potentieel geschikte kandidaten voor de ontwikkeling van nieuwe antibiotica zijn kleine kationische (positief geladen) peptiden (KAPs) die onder andere door het aangeboren immuunsysteem in mensen en dieren worden gemaakt. KAPs vertonen antimicrobiële activiteit tegen verschillende Gram-positieve en Gram-negatieve bacteriën, waardoor ze functioneren als een natuurlijk- breed spectrum antibioticum. KAPs hebben over het algemeen meerdere manieren om bacteriën te doden, waaronder het permeabiliseren van de bacteriële membraan, de remming van de DNA replicatie en de remming van de eiwitsynthese. Naast de directe antimicrobiële werking hebben sommige KAPs ook een regulerende werking op het immuunsysteem. KAPS kunnen bijvoorbeeld immuuncellen aantrekken en stimuleren, waardoor de imuunrespons versterkt wordt. Naast deze immuunstimulerende activiteit kunnen KAPs selectief de acute immuunrespons, die veroorzaakt wordt door het bacterieel endotoxine LPS, blokkeren. Het endotoxine LPS, een onderdeel van het buitenmembraan van Gram-negatieve bacteriën, werkt als 'gevaar' signaal voor immuuncellen, waardoor het immuunsysteem geactiveerd wordt om de infectie op te ruimen. Als een infectie uit de hand loopt kunnen

bacteriën de bloedbaan binnentreden. Een te hoge concentratie LPS in het bloed kan leiden tot een ongeremde productie van ontstekingsbevorderende boodschapperstoffen (inflammatoire cytokines) waardoor de immuunrespons destructief wordt en leidt tot sepsis. Dit veroorzaakt schade aan de organen, cardiovasculaire shock (septische shock) en kan in het ergste geval leiden tot de dood. Sommigen KAPs kunnen de ontwikkeling van sepsis tegen gaan door LPS te binden of door de binding van LPS aan transporteiwitten te verhinderen. Hierdoor wordt voorkomen dat LPS wordt herkend door immuuncellen, waardoor de productie van de acute inflammatoire cytokines voorkomen of geremd wordt. De antibacteriële activiteit, in combinatie met de capaciteit om endtoxines te binden, maakt deze peptiden interessante kandidaten voor de ontwikkeling van therapeutica voor de behandeling van bacteriële infecties en sepsis.

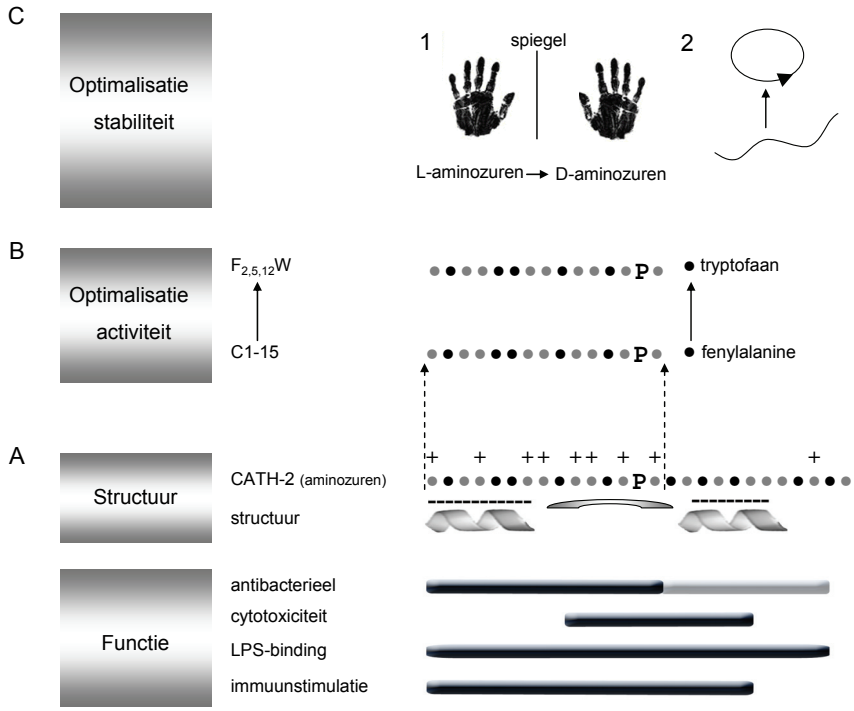
Om goed te kunnen begrijpen hoe KAPs werken is het van essentieel belang om te weten welke structuren van KAPs bij bepaalde, specifieke functies betrokken zijn. Met andere woorden hoe verhouden de structuren van KAPs zich ten opzicht van hun functies. Een goede manier om dit te bestuderen is om varianten te maken die op bepaalde punten afwijken van de natuurlijk voorkomende KAPs. Als een (kleine) structurele afwijking ten opzichte van het origineel een effect heeft op de functie, dan is het aannemelijk dat dat specifieke onderdeel van de KAP een rol speelt in het tot stand komen van die functie. Eerdere studies naar het verband tussen structuur en functie hebben aangetoond dat de activiteit van KAPs over het algemeen bepaald wordt door structurele en chemische eigenschappen zoals de mogelijkheid om een alfahelix te vormen, de aanwezigheid van een positieve lading, de mate van hydrofobiciteit ('water-afstotendheid') en de aanwezigheid van een amfipatisch karakter (scheiding van geladen en hydrofobe aminozuren). Het in dit proefschrift beschreven onderzoek evalueert het verband tussen structuur en functie van twee KAPs: het in de mens voorkomende LL-37 en het kippen cathelicidine-2 (CATH-2). Daarnaast is CATH-2 gebruikt als basis-structuur om zeer korte, stabiele peptide varianten met goede antibacteriële en LPS neutraliserende activiteit maar zonder immuun-stimulerende activiteit te ontwikkelen.

LL-37 is een van de meest bestudeerde KAPs. LL-37 heeft een goede antibacteriële activiteit en een LPS neutraliserende werking. De LPS neutraliserende activiteit van LL-37, maakt dit peptide potentieel geschikt voor de behandeling van sepsis. In **Hoofdstuk 2** is onderzocht welke parameters belangrijk zijn voor de LPS-neutraliserende werking van LL-37. Om het minimale domein te bepalen dat nodig is voor een goede LPS neutraliserende werking zijn van LL-37 verschillende, overlappende fragmenten (varianten) van verschillende lengte gebruikt. Door de LPS-neutraliserende activiteit van de fragmenten met elkaar te vergelijken werd duidelijk dat het centrale deel van LL-37, bestaande uit de aminozuren (bouwstenen van peptiden en eiwitten) 13 tot en met 31, essentieel is voor de remming van de LPS-geïnduceerde acute ontstekingsreactie. Vervolgens zijn de structurele en chemische eigenschappen van de peptide fragmenten gerelateerd aan hun LPS neutraliserende activiteit. Hieruit bleek dat de mate van hydrofobiciteit essentieel is voor de LPS-neutraliserende werking. De alfaheliciteit en de hoeveelheid positieve ladingen blijken in dit geval van minder van belang.

Kippen cathelicidine-2 (CATH-2), is een recentelijk ontdekt KAP. CATH-2 bestaat uit twee alfahelices die van elkaar gescheiden worden door een proline-geïnduceerde knik. CATH-2 heeft een brede antibacteriële werking en LPS-neutraliserende activiteit. In **Hoofdstuk 3** is de structuur functie relatie van CATH-2 beschreven (Figuur 1A). Hieruit blijkt dat de door de proline (P)-geïnduceerde knik essentieel is voor de activiteit van het peptide. Vervanging van de proline door een leucine (L), hetgeen leidt tot een meer lineair peptide, resulteerde in een verlies in antibacteriële, LPS-neutraliserende en imuuunstimulerende werking. Daarnaast werd vastgesteld dat de N-terminale helix van CATH-2, de hoogste antibacteriële activiteit heeft. Er werd een duidelijk verband tussen de antibacteriële activiteit, de positieve lading en het amfipatisch karakter gevonden. Dit wijst erop dat de positieve lading van het peptide, die verantwoordelijk is voor de elektrostatische aantrekkingskracht van het peptide, met het negatief geladen bacteriële membraan een belangrijke eigenschap is voor de interactie met bacteriën. Vervolgens kan het peptide het bacteriële membraan binnendringen waarbij de proline-geïnduceerde knik en de hydrofobe aminozuren een belangrijke rol spelen. Voor een goede imuunmodulerende activiteit van CATH-2 (LPS-neutralisatie en immuunstimulatie) blijken beiden helices van belang. Daarnaast konden we concluderen dat in tegenstelling tot het gehele CATH-2 peptide, de N-terminale helix inclusief de knik niet toxisch is voor witte en rode bloedcellen. Een lage toxiciteit tegen cellen van de gastheer is van groot belang voor de toepassing als therapeuticum. De resultaten beschreven in **Hoofdstuk 3** identificeren het N-terminale deel van CATH-2, ofwel peptide C1-15, als een goede basisstructuur voor verdere ontwikkeling als antibacterieel en anti-sepsis medicijn.

In **Hoofdstuk 4** is met behulp van aminozuursubstitutie getracht de antibacteriële en LPS-neutraliserende activiteit van peptide C1-15 te verhogen (Figuur 1B). Hiervoor werden de fenylalanines (F) van peptide C1-15 vervangen door tryptofanen (W), *o.a*. resulterend in peptide $F_{2,5,12}W$, die de eigenschap hebben dat ze gemakkelijk membranen kan binnendringen. Dit leidde tot een verhoogde antibacteriële activiteit, tegen *B. anthracis* en *Y. pestis* en een verbeterde LPS-neutraliserende activiteit. De toxiciteit van deze C1-15 varianten tegen gastheercellen was iets verhoogd, maar bleef beperkt.

KAPs zijn gevoelig voor afbraak door enzymen die aanwezig zijn in biologische vloeistoffen zoals serum en speeksel waardoor de werkzame stof niet lang genoeg in het lichaam blijft om zijn werk te doen. Dit kan het gebruik als medicijn beperken. In **Hoofdstuk 5** laten we zien dat de stabiliteit van de op CATH-2 gebaseerde peptiden verhoogd kan worden door de vervanging van L- door D-aminozuren en door het circulair maken van de peptiden zonder verlies van de antibacteriële en LPS-neutraliserende activiteit (Figuur 1C). Door de vervanging van L- naar hun gespiegelde vorm, D-aminozuren, kunnen gastheerenzymen de aminozuren niet meer herkennen waardoor afbraak voorkomen wordt. Het circulair maken van peptides zorgt voor een conformatie verandering waardoor de toegang tot de peptidebindingen voor de enzymen minder toegankelijk wordt en de peptiden hierdoor minder gemakkelijk afgebroken worden. De circulaire variant van peptide $F_{2,5,12}W$ vertoonde naast een verbeterde stabiliteit ook een verlaagde toxiciteit tegen gastheercellen ten opzichte van de lineaire variant. Deze resultaten duiden erop dat de vervanging van L- door D-aminozuren en het circulair maken van peptides geschikte strategieën zijn om op CATH-2 gebaseerde KAPs voor therapeutisch gebruik te ontwikkelen door de verhoogde stabiliteit en verlaagde toxiciteit tegen gastheercellen.



Figuur 1. *Structuur - functie relatie van CATH-2 en optimalisatie van op CATH-2 gebaseerde peptiden. (A) Verdeling van kationische (+), hydrofobe (grijs symbool) en knik regio (proline,P). De helices zijn onderstreept. De balken geven de segmenten weer die een matige (grijs) of een sterke (zwart) bijdrage leveren aan de verschillende biologische activiteiten (B). Optimalisatie van de antibacteriële en LPS-neutraliserende activiteit door vervanging van de fenylalanines door tryptofaan. (C) Optimalisatie van de stabiliteit door de substitutie van alle L-aminozuren door D-aminozuren (1) en het circulair maken van de peptiden (2).*

Bij verschillende infecties komen bacteriën voor in een biofilm. Een biofilm bestaat uit een verzameling bacteriën die zich hebben gehecht aan een oppervlak. In de biofilm zijn de bacteriën omgeven door een extracellulaire substantie die is samengesteld uit polysacchariden, eiwitten en DNA. Bacteriën die zich hechten aan ingebrachte medische hulpmiddelen, zoals katheters en hartkleppen en hierop een biofilm vormen, zorgen voor medische problemen doordat de bacteriën in een biofilm moeilijk te behandelen zijn. Dit komt doordat bacteriën aanwezig in een biofilm soms tot 1000 maal meer resistent zijn tegen de huidig gebruikte antibiotica in vergelijking tot bacteriën buiten de biofilm. Een veel voorkomende bacterie die in biofilms aanwezig is op ingebrachte medische hulpmiddelen, is *Staphylococcus epidermides*. In **Hoofdstuk 7** beschrijven we dat het, op CATH-2 gebaseerde, peptide $F_{2,5,12}W$ de vorming van *S. epidermidis* biofilms kan voorkomen bij concentraties die de bacteriële groei niet beïnvloeden. Hoewel peptide $F_{2,5,12}W$ de extracellulaire matrix van een voorgevormde biofilm slechts matig kon afbreken, lijkt het wel de levensvatbaarheid van de biofilm te verlagen. Samenvattend, de *in vitro* studies wijzen erop dat op CATH-2 gebaseerde peptiden gebruikt kunnen worden bij de bestrijding van biofilms.

Dit proefschrift presenteert een studie naar het verband tussen structuur en functie van de KAPs LL-37 en CATH-2 ten aanzien van de antibacteriële en LPS-neutraliserende activiteiten *in vitro*. Hierdoor werd duidelijk welke delen (structuur) van het peptide essentieel zijn voor de biologische activiteit (functie). Daarnaast werden mogelijke strategieën bepaald die gebruikt kunnen worden om de activiteit in fysiologische omstandigheden te verbeteren. Het onderzoek laat zien dat de ontwikkelde, van CATH-2 afgeleide, peptiden, een zeer effectieve LPS-neutraliserende en antibacteriële activiteit hebben *in vitro.* Het gebruik van D-isomeren en het circulair maken van de peptiden kunnen een significante bijdrage leveren aan het verbeteren van het therapeutisch potentieel van deze peptiden. Peptiden die op CATH-2 gebaseerd zijn hebben de potentie om uit te groeien tot een nieuwe klasse van antibiotica.

Abbreviations

AB assay	Alamar blue assay
Apo-A1	Apolipoprotein-A1
B. anthracis	*Bacillus anthracis*
B. globigii	*Bacillus globogii*
BSA	Bovine serum albumin
BW	Biological warfare
CATH-2	Chicken cathelicidin-2
CD spectroscopy	Circular dichroism spectroscopy
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CV stain	Crystal violet stain
DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium
E. coli	*Escherichia coli*
ERK	Extracellular signal-regulated kinase
ESBL	Extended-spectrum β-lactamases
FCS	Fetal calf serum
Glu	Glutamate
Gly	Glycine
GRAVY	Grand average of hydropathicity
HDP	Host defense peptide
HEK cell	Human embryonic kidney cell
IL	Interleukin
Ile	Isoleucine
iNOS	inducible NO synthase
KAP	Kationisch peptide
LAL assay	Limulus amoebocyte lysate
Leu	Leucine
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Lys-PG	lys-Phosphatidyglycerol
mAb	Monoclonal antibody
MAP kinase	Mitogen-activated protein
MCP-1	Monocyte chemotactic protein-1
MHB	Mueller hinton broth
MIC	Minimal inhibitory concentration
MBC	Minimal bactericidal concentration
MDR	Multi-drug-resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NF-κβ	Nuclear factor kappa beta
PAMP	Pathogen –associated molecular patterns
P. aeruginosa	*Pseudomonas aeruginosa*
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

Phe	Phenylalanine
Pro	Proline
PRR	Pattern recognition receptors
RBC	Red blood cell
RP-HPLC	Reverse-phase HPLC
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. enteritidis	*Salmonella enterica* serovar *enteritidis*
TFE	Trifluorethanol
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TMB	3, 3', 5, 5' Tetramethyl benzidine
Trp	Tryptophan
TSA	Tryptic soy broth
TSB	Tryptic soy agar
Val	Valine
V. cholera	*Vibrio cholera*
Y. pestis	*Yersinia pestis*

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Curriculum Vitae

Elisabeth Margaretha (Margo) Molhoek werd geboren op 9 januari 1981 te Rotterdam. Vanaf 1993 volgde ze middelbaar onderwijs aan het Libanon Lyceum te Rotterdam, waar ze in 1999 het VWO diploma behaalde. Hetzelfde jaar begon ze aan de studie Psychologie aan de Rijksuniversiteit Groningen. In 2001 besloot ze de overstap te maken naar de studie Biologie aan dezelfde universiteit. Haar eerste onderzoekstage volgde ze onder begeleiding van Dr. Dirk Fokkema bij de vakgroep Biologische Psychiatrie aan de Rijksuniversiteit Groningen en was gericht op onderzoek naar de ontwikkeling van postnatale depressie. Haar tweede onderzoekstage voerde ze uit bij de afdeling Target Discovery van het farmaceutisch bedrijf Organon te Oss onder begeleiding van Dr. Dianne Delsing waar ze zocht naar nieuwe aangrijppunten voor therapeutica voor de behandeling van arteriosclerose. In maart 2007 studeerde ze af met als specialisatie Medische Biologie. In april van hetzelfde jaar startte ze haar promotieonderzoek bij het kerngebied Defensie en Veiligheid van TNO te Rijswijk.

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