Antimicrobial peptides in the treatment of infectious and inflammatory conditions

Preclinical studies of mechanism of action, efficacy, and safety

Camilla Björn

Department of Molecular
and Clinical Medicine,
Institute of Medicine at
Sahlgrenska Academy
University of Gothenburg

Gothenburg, Sweden, 2016

UNIVERSITY OF
GOTHENBURG

Cover photo: Blood agar plates by Patrik Stenlund

Antimicrobial peptides in the treatment of infectious and inflammatory conditions
– Preclinical studies of mechanism of action, efficacy, and safety

camilla.bjorn@sp.se

ISBN 978-91-628-9925-7

Printed in Gothenburg, Sweden 2016
Ineko AB

Till Ove,
Linnea, Maja och Stina

Kom närmre

Abstract

The rapid emergence of antibiotic-resistant microbes worldwide and the urgent need of new antimicrobial agents have stimulated interest in antimicrobial peptides (AMPs) as new therapeutics for treatment of infectious diseases. AMPs are present in all living species and constitute an important part of the innate immune system in multicellular organisms, including humans. AMPs display a remarkably broad spectrum of antimicrobial activity covering both Gram-positive and Gram-negative bacteria, including many antibiotic-resistant strains, as well as fungi, viruses, and protozoa. Further, in contrast to many conventional antibiotics, AMPs rapidly kill bacteria instead of just inhibiting bacterial growth. In addition, AMPs act as modulators of the innate immune system and, importantly, bacteria seem less efficient in developing resistance towards AMPs than towards conventional antibiotics. Together these properties make AMPs highly attractive as a new class of antimicrobials, with clinical potential also extending to diseases where inflammation is part of the pathology.

The aim of this thesis was to study novel AMPs with respect to their mechanism of action (MOA), antimicrobial spectrum, propensity to select for resistance, and *in vivo* efficacy and safety. To achieve this, we used a number of *in vitro* and *in vivo* assays, together generating a comprehensive preclinical evaluation of the peptides. The hypothesis was that the AMPs in this thesis have potential to be developed as therapeutic agents for several infectious and inflammatory conditions, including treatment of skin and soft tissue infections and prevention of postsurgical adhesion formation.

The results showed that all AMPs tested (i.e. PXL03, PXL150, HLR1r, and five variants of CEN1 HC-Br) had broad antimicrobial spectra *in vitro* with varying sensitivity to salt and serum. Furthermore, PXL150 caused a rapid permeabilization of bacterial membrane *in vitro*, indicating that this is at least one part of the MOA of this peptide. Under selection pressure *in vitro*, bacteria did not develop resistance to the peptides tested, i.e. PXL150 and CEN1 HC. Interestingly, all peptides showed anti-inflammatory activity by inhibiting the secretion of proinflammatory mediators from stimulated human cell lines. In addition, PXL01, PXL150, and HLR1r demonstrated fibrinolytic ability *in vitro* by suppressing the release of plasminogen activator inhibitor-1 (PAI-1). In *ex vivo* and *in vivo* skin/wound infection models, the peptides reduced the number of viable bacteria and yeast cells. Further, PXL01 decreased postsurgical adhesion formation *in vivo*. Notably, nonclinical safety studies showed that PXL150 was safe and well tolerated.

In conclusion, several of the peptides evaluated in this thesis demonstrated a promising preclinical efficacy and safety profile motivating further development as drug candidates for local treatment of infectious and inflammatory conditions.

Keywords

Antimicrobial peptides, AMPs, innate immunity, infection, inflammation, mechanism of action, efficacy, safety, antimicrobial resistance, antibiotic resistance

Sammanfattning på svenska

Bakterier som har utvecklat motståndskraft, resistens, mot antibiotika utgör ett stort och växande globalt problem. Fler och fler infektioner blir allt mer svårbehandlade då tillgängliga antibiotika blir mer och mer verkningslösa. Detta leder till längre sjukhusvistelser, högre medicinkostnader och ökad dödlighet. Det är därför extremt viktigt att det utvecklas nya läkemedel som är effektiva, både mot antibiotikakänsliga och mot antibiotikaresistenta bakterier. En grupp substanser som undersöks för detta ändamål är antimikrobiella peptider (AMPs). AMPs finns i alla levande arter, allt ifrån encelliga bakterier till människa, och utgör hos oss människor en viktig del av vårt immunförsvar mot infektionsframkallande mikroorganismer. AMPs har förmåga att döda många olika typer av bakterier, även antibiotikaresistenta stammar. De är även effektiva mot svampar, virus och encelliga organismer, så kallade protozoer. Utöver detta har AMPs förmåga att även reglera aktiviteten av kroppens egna immunceller. Av stor vikt är att trots att AMPs har funnits i flera miljoner år har ännu ingen större resistensutveckling uppstått och det verkar som bakterier har svårare att utveckla resistens mot AMPs jämfört med vanliga antibiotika.

Målet med den här avhandlingen var att studera viktiga egenskaper hos några utvalda nya AMPs för att se om de skulle kunna vara lämpliga att utveckla till nya läkemedel. För detta ändamål använde vi ett flertal experimentella metoder som tillsammans skulle generera en omfattande utvärdering av peptiderna. Resultaten visade att alla testade AMPs (PXL03, PXL150, HLR1r och fem varianter av CEN1 HC-Br) hade brett antimikrobiellt spektrum, d.v.s. de hade förmåga att döda många olika typer av bakterier (och även jästsvamp), med varierande känslighet för salt och serum i testmediet. Då bakterier odlades i närvaro av låga koncentrationer av CEN1 HC and PXL150, utvecklades under försöket ingen resistens hos bakterierna mot peptiderna. I tester på celler från människa, uppvisade alla testade peptider en inflammationsdämpande effekt. Vidare visade resultaten att dessa AMPs kunde döda bakterier och jästsvampar i olika typer av infekterade sår i djurmodeller. I en annan djurmodell visades att PXL01 kunde minska den ärrbildning som orsakar sammanväxningar av vävnader efter bukoperationer. Slutligen kunde säkerhetsstudier på djur inte påvisa någon skadlig effekt av behandling med PXL150.

Sammanfattningsvis uppvisade flera av de testade peptiderna goda förutsättningar för att kunna utvecklas vidare till effektiva och säkra läkemedel för behandling av infektioner och inflammationer hos människa.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Nilsson E.*, Björn C.*, Sjöstrand V., Lindgren K., Münnich M., Mattsby-Baltzer I., Ivarsson ML., Olmarker K., Mahlapuu M.
A novel polypeptide derived from human lactoferrin in sodium hyaluronate prevents postsurgical adhesion formation in the rat.
Annals of Surgery 2009; 250: 1021–1028. * Equal contribution.

II. Björn C., Håkansson J., Myhrman E., Sjöstrand V., Haug T., Lindgren K., Blencke HM., Stensvåg K., Mahlapuu M.
Anti-infectious and anti-inflammatory effects of peptide fragments sequentially derived from the antimicrobial peptide centrocin 1 isolated from the green sea urchin, Strongylocentrotus droebachiensis.
AMB Express 2012; 2: 67.

III. Myhrman E., Håkansson J., Lindgren K., Björn C., Sjöstrand V., Mahlapuu M.
The novel antimicrobial peptide PXL150 in the local treatment of skin and soft tissue infections.
Applied Microbiology and Biotechnology 2013; 97: 3085–3096.

IV. Håkansson J., Björn C., Lindgren K., Sjöström E., Sjöstrand V., Mahlapuu M.
Efficacy of the novel topical antimicrobial agent PXL150 in a mouse model of surgical site infections.
Antimicrobial Agents and Chemotherapy 2014; 58: 2982–2984.

V. Björn C., Noppa L., Näslund Salomonsson E., Johansson AL., Nilsson E., Mahlapuu M., Håkansson J.
Efficacy and safety profile of the novel antimicrobial peptide PXL150 in a mouse model of infected burn wounds.
International Journal of Antimicrobial Agents 2015; 45: 519–524.

VI. Björn C., Mahlapuu M., Mattsby-Baltzer I., Håkansson J.
Anti-infective efficacy of the lactoferrin-derived antimicrobial peptide HLR1r.
Peptides 2016; 81: 21–28.

Content

Abbreviations

AMPs	antimicrobial peptides
API	active pharmaceutical ingredient
BHI_{dil}	100 × diluted brain–heart infusion broth
BLI	bioluminescence imaging
CFU	colony forming units
$DiSC_3(5)$	3,3'-Dipropylthiadicarbocyanine iodide
EDTA	ethylene diamine tetra-acetic acid
FDA	Food and Drug Administration
GLP	good laboratory practice
HPC	hydroxypropyl cellulose
IL	interleukin
LBP	LPS binding protein
LPS	lipopolysaccharides
LTA	lipoteichoic acid
MBC	minimum bactericidal concentration
MCP-1	monocyte chemoattractant protein-1
MIC	minimum inhibitory concentration
MMC	minimum microbicidal concentration
MOA	mechanism of action
MRSA	methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor-κB
PAI-1	plasminogen activator inhibitor-1
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PRRs	pattern recognition receptors
SC	subcutaneous
SH	sodium hyaluronate
SPPS	solid phase peptide synthesis
SSIs	surgical site infections
SSTIs	skin and soft tissue infections
SWF_{dil}	2 × diluted simulated wound fluid
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor-α
tPA	tissue-type plasminogen activator

1. Introduction

Living organisms are constantly exposed to potentially harmful microorganisms via contact, ingestion, and inhalation [1]. The ability of the organism to protect itself from infection via its host defense mechanisms is crucial for its survival. In multicellular organisms, the first line of defense against pathogens is provided by the innate immunity [2], where antimicrobial peptides (AMPs) play an important role [3]. However, the presence of AMPs is not only limited to higher organisms, in fact, AMPs are found in all living species, from prokaryotes to humans [4]. Antimicrobial proteins and peptides were isolated from tissues and body fluids already during the first half of the 20th century; however, it was not until the 1980s that the research field of AMPs really started to expand [5, 6] owing to the discoveries of insect cecropins by Hans Boman [7], amphibian magainins by Michael Zasloff [8], and human defensins by Robert Lehrer [9]. Since then, more than 2000 AMPs have been discovered [5]. Due to the rapidly increasing antimicrobial resistance among microorganisms and the urgent need for new antibiotics, AMPs have recently received increasing attention as candidates for new therapeutics against infectious diseases [10, 11].

1.1. The innate immune system

1.1.1. Innate *versus* adaptive immunity

The human immune response is divided into innate and adaptive immunity. The innate immune system is the host's first line of defense against infections and is found in all multicellular organisms, unlike the adaptive immune system which is only found in vertebrates [2]. Thus, most organisms have to rely solely on innate immunity for survival against infections, which emphasizes its importance [12]. The adaptive immunity is very sophisticated due to its ability to remember previous encountered pathogens and destroy them when they attack again. However, the adaptive immunity is slow, requiring several days of clonal expansion of B and T lymphocytes after first exposure to a pathogen before an effective response is achieved [1, 12]. Notably, one single bacterium with a doubling time of 50 minutes can produce 5×10^8 bacteria, i.e. a full-blown infection, within 24 hours [4]. This is too fast

for the adaptive immunity and therefore, during the first critical hours/days after exposure to a new pathogen, the body instead depends on the innate immunity to prevent infection [1]. The innate immunity is, in contrast to the adaptive immune system, rapid (effective within minutes), not antigen-specific, and does not rely on memory [2, 4, 12].

Epithelial surfaces first encounter the infectious organisms and provide both physical and chemical barriers to invasion by several mechanisms, such as tight junctions, mucus, cilia, low pH, and antimicrobial secretions [1, 12]. In addition, epithelial cells also function as immune cells by producing signaling molecules [13]. When the epithelial barrier fails to prevent pathogens from entering the host, the (non-epithelial) cells and the humoral (i.e. extracellular) components of the innate immunity will help promoting pathogen clearance.

1.1.2. Cells of the innate immunity and pathogen recognition

The innate immunity is largely dependent upon several cell types, including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, mast cells, and natural killer cells, as well as epithelial cells [12, 13]. These cells express pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs). PAMPs are conserved microbial molecules shared by many microorganisms but absent in the host [1, 14]. Of the PRRs, the Toll-like receptors (TLRs) are the ones most well described [2, 14]. Humans express ten TLRs and these receptors recognize different ligands. For example, TLR2 recognizes the bacterial cell wall component lipoteichoic acid (LTA), TLR3 recognizes viral double-stranded RNA, and TLR4, together with associated proteins including membrane bound CD14, recognizes bacterial lipopolysaccharides (LPS) [2, 12, 15]. Recognition of a pathogen by PRRs on primarily macrophages and neutrophils leads to engulfment of the pathogen and subsequent killing by means of degradative enzymes, AMPs, and toxic reactive oxygen species [1, 16]. Dendritic cells also phagocytose microbes and by presenting antigens of the engulfed microbe to T lymphocytes, they initiate the adaptive immunity [16]. Activation of PRRs by PAMPs does not only lead to phagocytosis, but also stimulates the immune cells to secrete a variety of signaling molecules that induce a local inflammatory response at the site of infection [1].

1.1.3. Inflammation

Pathogen recognition initiates an inflammatory response, clinically characterized by redness, heat, swelling, and local pain. In this process, dilation and permeabilization of blood vessels occur and the endothelial cells lining the blood vessels start to express cell adhesion molecules, that facilitate attachment and extravasation of leukocytes, such as neutrophils and monocytes, from the blood to the site of infection [1]. The inflammatory response is mediated by numerous signaling molecules released from PRR-activated immune cells. Although different TLRs recognize different ligands, many of them use the nuclear factor-κB (NF-κB) signaling pathway. TLR-activation thus leads to translocation of the transcription factor NF-κB into the nucleus where it activates transcription of several genes resulting in the expression and release of inflammatory molecules, such as proinflammatory cytokines and chemokines [1, 13]. Proinflammatory cytokines, e.g. tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), induce local expression of chemokines and upregulate cell adhesion molecules on endothelial cells, thus stimulating further leukocyte recruitment [13]. Examples of chemokines are interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), which act as chemoattractants for neutrophils and monocytes, respectively [17]. Further, proinflammatory cytokines stimulate immune cells to express proteins, such as tissue factor (TF), that trigger the coagulation cascade to form fibrin clots in the local small vessels, thereby preventing the pathogen from entering the bloodstream and spreading [18]. This effect could be further enhanced by the release of plasminogen-activator inhibitor-1 (PAI-1) from endothelial cells in response to proinflammatory cytokines, leading to inhibition of fibrinolysis [18, 19]. Besides acting as a fibrinolysis inhibitor, PAI-1 is also a mediator of proinflammatory responses by other mechanisms, such as acting as an acute-phase protein during inflammation, by promoting secretion of proinflammatory cytokines from LPS-stimulated macrophages, and by acting as a chemoattractant [20-22].

1.1.4. Humoral components of the innate immunity

Besides the cellular components of the innate immunity, the humoral components are also important for its function. The humoral components are extracellular molecules whereof some have ability to recognize and bind PAMPs, such as mannose-binding protein (that activates the complement cascade), LPS binding protein (LBP), and soluble CD14, whereas other humoral components are capable of killing the microbes. To this latter group belong complement proteins and lysozyme, as well as the antimicrobial protein lactoferrin and AMPs [16].

1.2. Antimicrobial peptides (AMPs)

1.2.1. AMPs and their importance in innate immunity

While bacteria produce AMPs in order to kill other bacteria competing for the same specific ecological niche [23], in higher organisms, AMPs contribute to innate immunity by playing several important roles [4, 23]. AMPs are able to kill an exceptionally wide range of pathogens, including both Gram-positive and Gram-negative bacteria, and sometimes even fungi, viruses, and protozoa [4, 10, 24]. In addition to this direct antimicrobial activity, many AMPs also have ability to modulate the innate immune responses of the host. These dual activities of AMPs give them ability to both promote pathogen clearance while also preventing excessive and potentially harmful proinflammatory responses [25, 26]. To capture this broad function in innate immunity, AMPs are often referred to as host defense peptides (HDPs) [26-28]. The critical role of AMPs in innate immunity is supported by their widespread distribution and abundance in all multicellular organisms [10, 15]. Their importance is further demonstrated by the increased infection susceptibility of mice genetically modified to lack the gene coding for the mouse analogue of the human AMP LL-37 [29] and by the increased risk of infection affecting humans with conditions associated with reduced production of AMPs, such as atopic dermatitis [30].

1.2.2. Biosynthesis and expression

AMPs in nature are produced either by ribosomal translation of mRNA or by nonribosomal peptide synthesis [31]. Nonribosomally synthesized peptides are mainly produced by bacteria, where the AMPs are assembled by large enzyme complexes called peptide synthetases and the resulting AMPs contain drastically modified amino acid residues [26, 31]. In contrast, ribosomally synthesized AMPs are genetically encoded and produced by all species of life, bacteria included [31]. Compared to peptides of nonribosomal origin that have been known for several decades and whereof many are used as antibiotics (e.g. polymyxins and gramicidin S), the ribosomally synthesized AMPs started to become recognized for their role in innate immunity and for their clinical potential during the early 1990s [31, 32]. Thus, the AMP research during the recent years has mostly focused on these genetically encoded peptides, which are also the focus of this thesis.

In mammals, AMPs are primarily found within granules of neutrophils, in secretions from epithelial cells covering skin and mucosal surfaces, or as degradation products of proteins [31, 33]. Expression of AMPs differs depending on the peptide,

cell, and tissue, but in many cases AMPs are encoded in clusters in the genome and co-expressed, resulting in multiple AMPs accumulating at a single site [34]. Notably, AMPs are produced as inactive precursors, often in the form of a prepropeptide consisting of a signal sequence, an anionic proregion, and the cationic peptide, requiring proteolytic cleavage to become active [35]. Their regulation is therefore not only dependent on their own expression but also on the abundance of appropriate proteases [34]. In multicellular organisms, some AMPs are constitutively expressed, stored at high concentrations as inactive precursors in granules and released locally at sites of infection and inflammation, whereas the expression of others is induced in response to PAMPs or cytokines [4, 34].

1.2.3. Characteristics and classification

Several databases exist trying to catalogue natural AMPs, today covering more than 2000 peptides [36]. Most of these AMPs share certain common features. They are relatively short, commonly consisting of 10-50 amino acids, they display an overall positive charge ranging from +2 to +11, and contain a substantial proportion (typically 50%) of hydrophobic residues. Importantly, upon interaction with a biological membrane, AMPs adopt an amphipathic tertiary structure with one positively charged face and one hydrophobic face [26, 37, 38].

Since AMPs have diverse amino acid sequences, classification based on sequence similarities is difficult. Instead, AMPs are commonly classified based on their secondary structure upon interaction with a biological membrane or membrane mimetic [3, 39]. Classically, most AMPs are divided into α-helical peptides, β-sheet peptides, and peptides with extended/random coil structures [37, 39, 40], with the two former groups most common in nature (**Fig. 1**).

***Figure 1**. Representative peptides of the major structural classes of antimicrobial peptides (AMPs); α-helical peptides (LL-37), β-sheet peptides (human β-defensin 1), and extended/random coil peptides (indolicidin). Adapted from Protein Data Bank in Europe [41] using PDB id codes 2k6o, 1kj5, and 1g89, respectively.*

Of all known secondary structures of natural AMPs, 30–50% are α-helical [37, 40]. These peptides are often unstructured in aqueous solution, but due to their arrangement of hydrophobic residues in a regular pattern they adopt an amphipathic helical structure in contact with a biological membrane [37, 38]. Although the ability to form an amphipathic α-helix is critical for their antibacterial activity, a very high propensity for helix formation has been shown to increase the risk for toxicity to host cells [39, 40]. One of the most studied AMPs in this group is human LL-37 [37, 42], which is produced as the 18-kDa inactive precursor human cathelicidin antimicrobial protein (hCAP18) in neutrophils and epithelial cells [34]. Furthermore, human lactoferricin, derived from proteolytic cleavage of lactoferrin and used as a template for some of the AMPs studied in this thesis, also belongs to this class [43].

Half of all known natural AMPs belong to the class of β-sheet peptides [40]. These cysteine-containing peptides form β-strands, which are stabilized by disulphide bonds and organized to create an amphipathic molecule [38, 44, 45]. Due to their rigid structure, the β-sheet peptides are more ordered in aqueous solution and do not undergo such a drastic conformational change as helical peptides do upon membrane interaction [38]. The best studied β-sheet peptides are the defensins, a large group of AMPs which are produced as inactive precursors in neutrophils, macrophages, and epithelial cells [34, 37]. In mammals, more than 140 different defensins have been identified and classified either as α-, β-, or θ-defensins [34].

A small portion of the natural AMPs belong to the class of extended/random coil peptides [40]. These peptides do not form regular secondary structure elements and they often contain a high content of arginine, proline, tryptophan, or histidine residues [39, 40]. Like other AMPs, many of extended peptides adopt amphipathic structures in the presence of a membrane [39]. One of the best studied peptides in this group is indolicidin, which is produced by bovine leukocytes [45, 46].

1.2.4. Mechanisms of action (MOA): Direct antimicrobial effect

Many AMPs display direct and rapid microbial killing activities by causing disruption of the physical integrity of the microbial membrane and/or by translocating across the membrane into the cytoplasm of microorganisms to act on intracellular targets essential for the organism [26] (**Fig. 2**). It is widely accepted that membrane interaction is a key factor for the direct antimicrobial activity of AMPs, both when the membrane itself is the target as well as when intracellular targets must be reached [25, 39, 47]. It is also recognized that the cationicity, hydrophobicity, and amphipathicity of the AMPs are of importance for this action [3, 48].

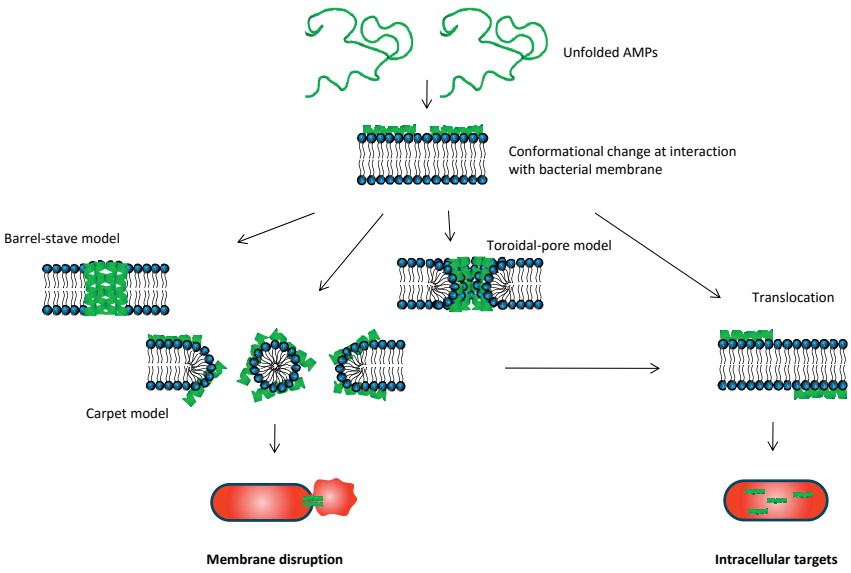

Figure 2. *Schematic illustration of the mechanisms of direct bacterial killing by antimicrobial peptides (AMPs).*

1.2.4.1. Membrane target

AMPs need to interact with biological membranes to execute their action, and electrostatic forces between the cationic peptides and the negatively charged bacterial surface are critical determinants for this interaction [25, 38, 49, 50]. Bacteria are commonly divided into two families, Gram-positive, and Gram-negative, based on their differences in cell envelope structures (**Fig. 3**). In Gram-positive bacteria, the cytoplasmic membrane is surrounded by a thick peptidoglycan layer, whereas the cytoplasmic membrane in Gram-negative bacteria is surrounded by a thin peptidoglycan layer as well as an outer membrane [51].

The cytoplasmic membranes of both Gram-positive and Gram-negative bacteria are rich in the phospholipids phosphatidylglycerol, cardiolipin, and phosphatidylserine, which have negatively charged head groups, highly attractive for positively charged AMPs [38, 50]. The presence of teichoic acids (including membrane anchored LTA) in the cell wall of Gram-positive bacteria and LPS in the outer membrane of Gram-negative bacteria provide additional electronegative charge to the bacterial surfaces [34, 50].

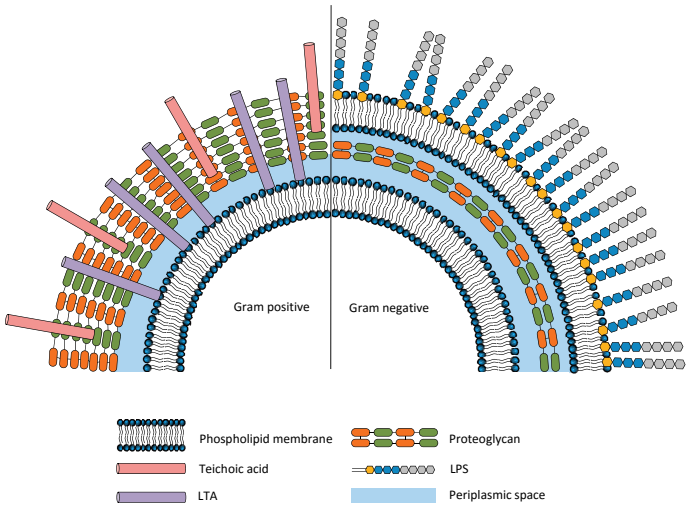

Figure 3. *Simplified illustration of the cell envelopes of Gram-positive and Gram-negative bacteria. LTA, lipoteichoic acid; LPS, lipopolysaccharides.*

Fundamental differences exist between bacterial and mammalian cell membranes, protecting mammalian cells against AMPs and enable selective action of AMPs [38]. In contrast to bacteria, the mammalian cell membrane is rich in the zwitterionic phospholipids phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin, providing the membrane with neutral net charge [38, 50]. There is also an asymmetric distribution of phospholipids in mammalian cell membranes, with the zwitterionic phospholipids being present in the outer leaflet, while phospholipids with negatively charged head groups, when present, are localized in the inner leaflet facing the cytoplasm [10, 34, 38]. Therefore, interactions between AMPs and the mammalian cell membrane occur mainly via hydrophobic interactions, which are relatively weak compared to the electrostatic interactions between AMPs and bacterial membranes (**Fig. 4**). Further, mammalian cell membranes, unlike those of microbes, have a high content of cholesterol [34, 38]. The cholesterol content is proposed to reduce the activity of AMPs towards mammalian cells, either via stabilization of the phospholipid bilayer or via interactions between cholesterol and the peptides [10]. In addition, there is a difference between bacterial and mammalian cells in the transmembrane potential, i.e. the difference in electric charge between the internal and external environment of the cell. Bacteria typically have an inside-negative transmembrane potential between −130 mV and −150 mV, whereas the transmembrane potential of mammalian cells is between −90 mV and −110 mV [38, 50, 52]. This stronger negative membrane potential in bacteria compared to mammalian cells may also contribute to the selectivity of AMPs between bacterial *versus* mammalian cells [38].

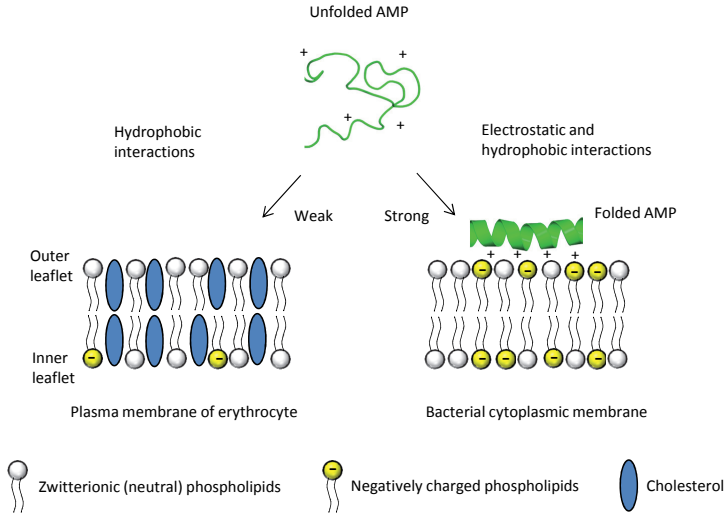

Figure 4. *Membrane interaction of antimicrobial peptides (AMPs) with bacteria and mammalian cells, and the basis of selectivity. Adapted with permission from Macmillan Publishers Ltd: Nature [10], copyright 2002.*

Similar to bacteria, fungal cells also have a higher content of negatively charged phospholipids (phosphatidylinositol and diphosphatidylglycerol) in comparison to mammalian cells, thereby providing a membrane more attractive for cationic AMPs [53]. However, similar to the cholesterol in mammalian cell membranes, fungal membranes contain ergosterol. Moreover, the fungal cytoplasmic membrane is surrounded by a cell wall consisting of chitins, glucans, mannans, and glycoproteins, which potentially could constitute a barrier towards AMPs [53, 54].

1.2.4.2. Membrane-disruptive mechanisms

In order to reach the inner cytoplasmic membrane of Gram-negative bacteria, AMPs have to translocate through the outer membrane. This outer membrane constitutes a permeability barrier for many macromolecules, partly due to the divalent cations Ca^{2+} and Mg^{2+} that bind to the phosphate groups of the inner core of LPS and thereby provide stabilization of the outer leaflet [55]. AMPs are proposed to be translocated through the outer membrane via so called self-promoted uptake [31, 49, 56]. This model suggests that due to greater affinity for LPS, AMPs displace the stabilizing divalent cations and bind to the LPS. By being bulky, the AMPs cause transient cracks and permeabilize the outer membrane, thereby permitting passage of the peptide itself across the membrane.

In contact with the cytoplasmic membrane, the AMPs form an amphipathic secondary structure (if not already present) [50]. The charged domains of the peptides

allow for interaction with the hydrophilic head groups of the phospholipids, while the hydrophobic domains of the peptides interact with the hydrophobic core of the lipid bilayer, thereby driving the AMP deeper into the membrane [50]. Although the cationic, hydrophobic, and amphipathic properties of AMPs are essential for this interaction and the subsequent antimicrobial effect, a very high positive net charge, an excessive hydrophobicity, or a highly segregated amphipathicity lead to decreased antimicrobial activity and/or make the AMPs more toxic towards mammalian cells [38, 57-59].

Several models have been proposed describing the next events occurring at the bacterial cytoplasmic membrane that ultimately lead to membrane permeabilization and disruption [39]. In the three classical models described below (also see **Fig. 2**), peptides are proposed to bind to the lipid bilayer in a parallel fashion and as more peptides accumulate at the membrane, a threshold concentration is eventually reached when the peptides begin to insert into the bilayer [6, 38, 39, 60, 61]. In the barrel-stave model, the peptides insert perpendicularly into the bilayer and subsequent recruitment of additional peptides results in formation of a peptide-lined transmembrane pore. In this pore, the peptides are aligned with the hydrophobic side facing the lipid core of the membrane and the hydrophilic regions facing the interior region of the pore [6, 60]. In contrast, according to the toroidal-pore model, insertion of peptides forces the phospholipid to bend continuously from one leaflet to the other, resulting in a pore lined by both peptides and the head groups of the phospholipids. Finally, in the carpet model, accumulation of peptides on the membrane surface causes tension in the bilayer that ultimately leads to disruption of the membrane and formation of micelles [6, 60].

Membrane permeabilization by AMPs is suggested to initially lead to leakage of ions and metabolites, depolarization of the transmembrane potential with subsequent membrane dysfunction (e.g. impaired osmotic regulation and inhibition of respiration), and ultimately membrane rupture and lysis of microbial cells [6, 38, 62]. Since many AMPs kill the target bacteria very fast, it is difficult to monitor and characterize the exact stages of killing [31]. Notably, membrane permeabilization does not always lead to microbial killing *per se* and sometimes AMPs kill microbial cells without lysis [34, 38, 60, 63].

1.2.4.3. Intracellular mechanisms

Besides leading to membrane dysfunction and disruption, membrane permeabilization is important for translocation of certain AMPs into the cytoplasm. Inside the microbial cell, the AMPs bind to different intracellular targets thereby affecting key cellular processes, including DNA/RNA synthesis, protein synthesis, protein folding,

enzymatic activity, and cell wall synthesis [6, 38, 39, 44]. Notably, it is suggested that cell death caused by AMPs could be a result of several and complementary actions and targets, referred to as multi-hit mechanism. This strategy may help to increase their efficiency and to evade resistance development [38, 39, 64, 65]. It is also likely that the MOA of individual peptides varies depending on parameters such as peptide concentration, target bacterial species, as well as tissue localization and growth phase of the bacteria [38, 47].

1.2.4.4. Activity on fungus, virus, and protozoa

The MOA of antifungal peptides is far from being fully characterized. As mentioned above, the cytoplasmic membrane of fungal cells is more negatively charged than the membrane of mammalian cells, possibly allowing for selectivity of AMPs for fungal cells [53]. In addition, the negatively charged mannosylated glycoproteins in the fungal cell wall have shown to be important to the interaction with cationic peptides [66]. Besides non-specific membrane permeabilization, several other mechanisms have been proposed [67]. Some peptides, e.g. β-defensins, have shown to exert their effect by specific interactions to proteins on the fungal surface [68]. In addition, internal targets have been suggested for several peptides [67]. In particular, the mitochondrial membrane is interesting due to structural and functional similarities between the bacterial cell membrane and the mitochondrial membrane [44]. For example, histatin-5 has been demonstrated to internalize into *Candida albicans* and target the mitochondrion [69].

Antiviral peptides can exert their action using several mechanisms [48]. Some AMPs, e.g. indolicidin, have demonstrated a disruptive mechanism on the viral envelopes [70]. Other peptides have ability to bind glycoproteins on the viral surface, thereby preventing the virus from binding to heparan sulphate receptors on host cells and entering the cells [71]. Moreover, some AMPs can inhibit viral gene expression in host cells via other mechanisms than competitively inhibit viral binding [72].

Regarding the action of AMPs on protozoan parasites, the mechanisms have been described to include disruption of the cytoplasmic membranes, which are more anionic in their nature compared to mammalian cells, as well as interfering with key processes in the parasite metabolism [73].

1.2.5. Mechanism of action (MOA): Immunomodulatory activities

Many AMPs have shown ability to profoundly modulate the innate immune response [25] (**Fig. 5**). The broad range of immunomodulatory activities exerted by AMPs

include stimulation of chemotaxis, modulation of immune cell differentiation including dendritic cell maturation and hence initiation of adaptive immunity, together contributing to the bacterial clearance of the host. The immunomodulatory activities further include suppression of TLR- and/or cytokine-mediated production of proinflammatory cytokines and anti-endotoxin activity, together preventing excessive and harmful proinflammatory responses, including sepsis [25, 34, 74-77]. In addition, other immunomodulatory activities have been described, including ability to promote wound healing and angiogenesis [25, 34].

1.2.5.1. Chemotactic activity

Upon release at sites of infection and inflammation, AMPs are able to recruit immune cells to the site either directly by acting as chemotactic agents or indirectly by inducing secretion of chemokines by immune cells [34, 78]. For example, human defensins and LL-37 display direct chemotactic activity on immune cells, e.g. monocytes, neutrophils, and lymphocytes. This is suggested to occur via the so called alternate ligand model, in which the AMPs bind directly to specific cell surface receptors, in this case the G-protein coupled receptors chemokine receptor 6 (CCR6) and formyl peptide receptor-like 1 (FPRL-1), on the immune cells and thereby inducing receptor signaling [34, 79]. In addition, these AMPs induce secretion of chemokines, such as IL-8 and MCP-1, from e.g. epithelial cells, tentatively also via receptor dependent mechanisms [34, 80, 81].

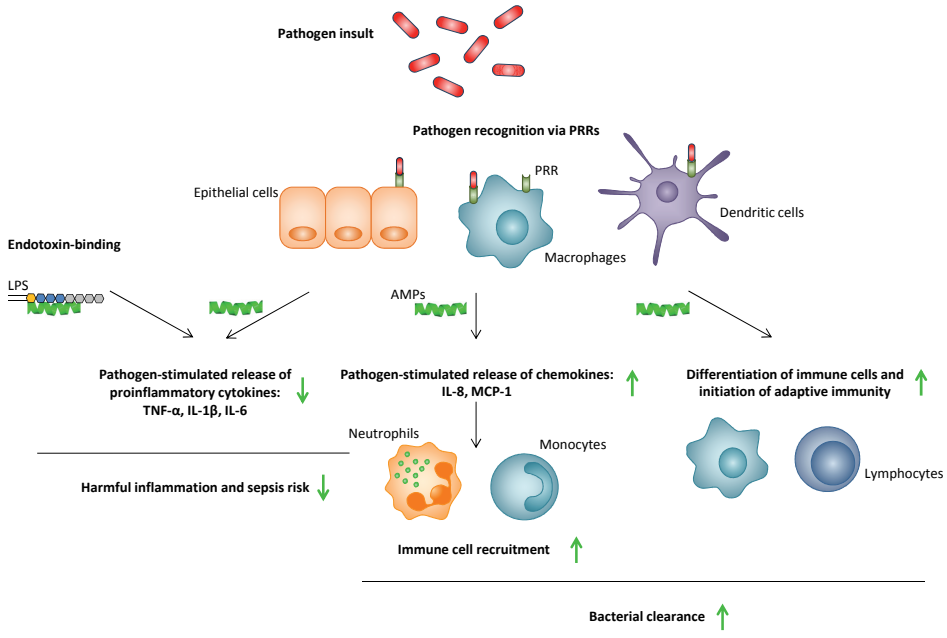

Figure 5. *Schematic illustration of the mechanisms of immunomodulatory activities of antimicrobial peptides (AMPs). Besides phagocytosis, pathogen recognition via pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) by innate immune cells, leads to release of proinflammatory cytokines and chemokines, inducing an inflammatory response and stimulating recruitment of additional immune cells to the site of infection, respectively. In addition, pathogen insult leads to differentiation of immune cells including maturation of dendritic cells and hence initiation of adaptive immunity. AMPs indirectly promote pathogen clearance by stimulating chemotaxis and immune cell differentiation, while also preventing harmful inflammation and sepsis by suppressing the release of proinflammatory cytokines and by scavenging bacterial endotoxins, such as lipopolysaccharides (LPS).*

1.2.5.2. Suppression of proinflammatory cytokine production

Several AMPs have shown ability to suppress the TLR-induced production of proinflammatory cytokines. For example, bovine lactoferricin has been reported to inhibit the secretion of TNF-α from LPS-stimulated cell line monocytes [82]. In addition, LL-37 has been shown to suppress the LTA and LPS-induced release of TNF-α, IL-1β, IL-6, and IL-8 from primary monocytes [74]. One suggested mechanism for this anti-inflammatory effect of AMPs is via the membrane disruption model, in which the AMPs locally modify the part of the membrane that contains the receptor (e.g. TLR4) and thereby indirectly alter its activation state and function [34]. Another proposed mechanism is via multiple points of intervention directly interfering with the TLR to NF-κB signalling pathway, although the exact details of this mechanism remain to be elucidated [74]. In addition, AMPs have ability to directly bind and neu-

tralize LPS, i.e. prevent LPS from binding the TLR4 receptor complex and triggering inflammation [34, 83].

1.2.5.3. Promotion of wound healing

AMPs are suggested to promote wound healing via several activities [25, 34], including stimulation of endothelial cell proliferation and angiogenesis [84], stimulation of keratinocyte migration [80], and prevention of fibroblast collagen expression leading to an anti-fibrotic effect [85]. Some of these activities are suggested to occur via the trans-activation model, in which the AMPs cause release of a membrane-bound growth factor (e.g. epidermal growth factor), which could then bind to its receptor [34].

1.3. AMPs as pharmaceutical agents

AMPs possess features that make them highly interesting to be developed as new anti-infectious agents. These properties include a rapid killing activity on a wide spectrum of microorganisms, including drug-resistant strains, with potentially low risk for resistance development, in combination with immunomodulatory effects [24, 26]. Notably, the ability to affect the host's immune responses gives AMPs potential to be used in indications beyond treatment of infections, e.g. to promote wound healing, as cancer treatment, and as vaccine adjuvants [86]. To date, only nonribosomally synthesized peptides, such as polymyxins and gramicidin S, are approved for clinical use for treatment of infections [24, 28, 62]. In addition, the ribosomally synthesized lantibiotic nisin, produced by certain bacterial strains of e.g. *Lactococcus lactis* [87], has been used as a food preservative for decades [88].

There are numerous AMPs derived from genetically encoded peptides currently under clinical development as anti-infectious and immunomodulatory agents [28]. A few of these have been evaluated in phase III clinical trials, including the magainin-analogue pexiganan as topical treatment of infected diabetic foot ulcers [89], the indolicidin-analogue omiganan as topical treatment for prevention of catheter-associated infections and treatment of rosacea, and the protegrin-analogue iseganan for local prevention of oral mucositis [90]. Pexiganan and omiganan, but not iseganan, demonstrated efficacy in these trials, but have not yet been approved by the Food and Drug Administration (FDA) due to non-superiority to existing therapy (pexiganan) or failure to meet the primary therapeutic endpoint (omiganan) [26, 28, 62]. The decline in the approval of new anti-infectious agents, in combination with the alarming rise in resistance toward conventional antibiotics, have resulted in re-

cent initiatives by the FDA and the European Medicines Agency (EMA) to facilitate the development of novel anti-infectious agents, including a more flexible clinical trial design e.g. without superiority requirement, as well as additional years of market exclusivity [91]. However, besides the regulatory hurdles, the major obstacles in developing AMPs as therapeutic agents have been their susceptibility to proteolytic degradation, their potential risk for toxicity, and high cost of manufacturing peptides [24, 26, 28, 62].

Besides direct administration of AMPs, there are several attempts ongoing to use agents to increase the body's endogenous production of AMPs in order to boost the innate immune system and thereby combat infections. As one example, vitamin D3 has shown to directly induce expression of several AMPs [92, 93] and vitamin D supplements are now evaluated in several clinical trials for their potential as treatment of infectious diseases [94].

1.3.1. AMPs *versus* conventional antibiotics

1.3.1.1. Different targets and mechanisms

As mentioned above, AMPs exert their antimicrobial effect via direct mechanisms, including membrane disruption/dysfunction and/or interference with intracellular targets, as well as via indirect mechanisms by modulating the immune response of the host. In contrast, conventional antibiotics act via one target only, interfering with cell-wall biosynthesis (e.g. β-lactam-containing penicillins and cephalosporins), bacterial protein synthesis (e.g. aminoglycosides, tetracyclines, mupirocin, and fusidic acid), or nuclear acid replication and repair (e.g. rifampicin and fluoroquinolones) [95, 96]. The antibiotic action is categorized as either bacteriostatic or bactericidal depending on whether it only prevents bacterial growth or causes death of bacteria, respectively [95, 97]. However, bactericidal ability is not always an intrinsic property of the drug but can depend on target strain and/or concentration of the drug [95, 97]. Using the standard *in vitro* assays minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), the lowest concentration of an antimicrobial agent that inhibits visible growth of a microorganism [98] and the lowest concentration that kills the microbes [99], are determined, respectively. An antibacterial agent is usually regarded as bactericidal if the MBC is less than or equal to 4 × MIC [99]. In contrast to many conventional antibiotics, the action of AMPs is generally bactericidal, with MIC and MBC values often coinciding [24, 100]. Further, compared to conventional bactericidal antibiotics, AMPs kill bacteria more rapidly, sometimes

causing a > 99.9% reduction of viable bacteria within just a few minutes [63, 101-103]. There are many advantages of bactericidal activity as compared to bacteriostatic, such as rapid elimination of bacteria, lower risk of resistance development, and decreased infection recurrence [97]. However, in certain clinical situations the use of conventional bactericidal antibiotics should be avoided due to the risk of potentially harmful inflammation caused by high release of bacterial products (e.g. LPS) from lysed bacteria [97].

1.3.1.2. Differences in spectrum of activity

Antibiotics are classified as either broad-spectrum or narrow-spectrum depending on whether they are active against many different types of bacteria or just against a selected group of bacteria. Broad-spectrum antibiotics are used, for example, as an initial treatment of serious infections when the causative organisms are yet unknown and the treatment cannot be delayed [104]. However, the use of broad-spectrum antibiotics is more associated with resistance development compared to narrow-spectrum antibiotics [95]. In addition, broad-spectrum antibiotics will not discriminate between pathological and beneficial bacteria and treatment could therefore result in worsened and/or prolonged infections due to opportunistic pathogens (e.g. *C. albicans*, *Clostridium difficile*, and *Pseudomonas aeruginosa*) being able to grow when unhindered by a weakened normal microflora [62].

As mentioned above, many AMPs have a remarkable broad spectrum of activity covering both Gram-positive and Gram-negative bacteria, and sometimes even fungi, viruses, and protozoa [4, 10, 24]. The broad-spectrum property is especially attractive in that several pathogens could be potentially eliminated using only one treatment with combined antibacterial, antiviral, or antifungal activity [47]. Most importantly, AMPs are not affected by resistance mechanisms towards conventional antibiotics, i.e. they can be equally active against drug- and multidrug-resistant bacteria as against sensitive strains [24, 100, 105]. Further, due to the restricted permeability of conventional antibiotics through the outer membrane of Gram-negative bacteria in combination with effective intrinsic resistance mechanisms, such as active antibiotic efflux and production of periplasmic β-lactamase, Gram-negative bacteria are more insensitive to antibiotics, which also makes it more difficult to develop new antibiotics against these bacteria [63]. In contrast, due to the usage of self-promoted uptake, AMPs often work well against Gram-negative bacteria [63]. This is of great importance since infections caused by multidrug-resistant Gram-negative bacteria have become a severe problem for healthcare, with many infections being virtually untreatable and with a very limited number of new drug candidates in clinical pipeline [106, 107].

1.3.2. Challenges for developing AMPs as pharmaceuticals

1.3.2.1. Efficacy in physiological environment

One feature of many AMPs that causes much controversy and complicates the drug development, is that the antimicrobial activity of the AMPs is highly sensitive to environmental conditions. Many AMPs have pronounced antimicrobial effect *in vitro* under low-ionic strength conditions, but under physiological concentrations of Na^+ (150 mM) or divalent cations like Mg^{2+} and Ca^{2+} (1-2 mM) *in vitro,* the activity of the peptides is weaker or even fully lost [36, 108-112]. This salt sensitivity is suggested to explain why endogenous β-defensins are unable to sufficiently kill *P. aeruginosa* in the high-salt conditions in the lungs of cystic fibrosis patients [113], although other explanations also exist. Loss of activity of AMPs in high-ionic strength conditions is suggested to be mainly due to weakening of the electrostatic forces between the cationic AMPs and negatively charged bacterial surfaces [114, 115]. However, this is probably not the only explanation since peptides with similar net charge can vary in their salt sensitivity [114]. Other factors, besides low net charge, suggested to contribute to salt sensitivity of AMPs are imperfect amphipathicity, structural instability, and absence of large clusters of charged residues [114, 115], and thus many studies have been focusing on improving the salt tolerability of AMPs by implementing structure modifications affecting e.g. structural stability, hydrophobicity, and amphipathicity [101, 114-116]. Interestingly, there are AMPs that are naturally tolerant to high-ionic environments, such as plectasin, tachyplesins, clavanins, and protegrins [115, 117-119].

Besides salt, AMPs often also have weaker antimicrobial activity *in vitro* in the presence of serum [108, 111, 120]. This loss of activity in serum is explained by AMPs binding to serum proteins [34], such as albumin and lipoproteins [121, 122], which sequester the peptides from the bacterial cells and subsequently hinder their activity [123, 124]. For example, LL-37 has been shown to bind apolipoprotein A-I and B in plasma [122].

Notably, many AMPs with a weakened antimicrobial activity when evaluated *in vitro* in the presence of physiologic salt concentrations and/or serum, have demonstrated strong antimicrobial effect in relevant experimental animal models [113, 120, 125-128] (paper II, III, and VI). Furthermore, it has been shown that naturally occurring AMPs are often present in their natural environment at concentrations that do not kill bacteria *in vitro* [34, 110]. Several possible explanations have been proposed for this apparent inconsistency between *in vitro* and *in vivo* activities. It has been suggested that natural AMPs exert a direct antimicrobial effect at specific locations where AMPs are accumulated at high concentrations (e.g. in the phagosomes of in-

nate immune cells, in close proximity to degranulating neutrophils, and in intestinal crypts). In contrast, when present at lower concentrations, the antimicrobial mechanism has been suggested to be primarily mediated through their immunomodulatory activity, which is less affected by physiological salt concentrations [15, 78]. Furthermore, co-expression of different antimicrobial proteins and peptides, e.g. lactoferrin and LL-37, at infection sites enables the AMPs to act synergistically to exert optimal killing [34]. Importantly, it has been suggested that bacterial susceptibility to AMPs is significantly higher in the mammalian ionic environment compared to in the conditions commonly used in antimicrobial assays *in vitro*.This has been demonstrated by culturing bacteria in media containing carbonate (e.g. in the form of $NaHCO_3$) at levels similar to human blood, followed by exposure to AMPs. In these experiments, the bacterial gene expression was altered for more than 300 genes (of which some are involved in virulence, stress response, and cell wall maintenance), the thickness of the cell wall of Gram-positive bacteria decreased, and both Gram-positive and Gram-negative bacteria became more susceptible to permeabilization by cationic compounds. Most importantly, the susceptibility of the bacteria towards the AMPs was retained even under high-ionic strength conditions [110].

The poor correlation of the *in vitro* antimicrobial effect of AMPs and their *in vivo* efficacy highlights the importance to evaluate the antimicrobial action of AMPs in different culture media, and to confirm the *in vitro* findings in *in vivo* models that better replicate the clinical situation.

1.3.2.2. Resistance development

Bacteria have ability to rapidly develop resistance to conventional antibiotics. This is explained by antibiotics acting on one single high-affinity target and therefore the action of the antibiotic can be completely inhibited via a single resistance mechanism [34]. In contrast, although microorganisms have been exposed to AMPs for millions of years, any widespread resistance has not been reported and AMPs still continues to provide protection against infections [28, 34, 129], thus it is proposed that bacteria are less prone to develop high-level resistance to AMPs [34]. This is suggested to relate to the MOA of AMPs that, in contrast to conventional antibiotics, involves acting on multiple low-affinity, targets, which makes elimination of one such target due to mutations less effective, and thus it is more difficult for bacteria to develop mutants that are totally resistant to AMPs [24, 28, 34, 65]. In particular, given that the main target of AMPs is the bacterial cell membrane, it is considered to be too challenging for bacteria to acquire mutations altering the membrane and thereby causing resistance to AMPs, while keeping the functional and structural integrity of the membrane [34]. Nevertheless, there are many studies describing several coun-

termeasures already developed by bacteria to resist the action of AMPs and these intrinsic resistance mechanisms are described to be important for the ability of the bacteria to colonize and infect the host [130-132]. The intrinsic mechanisms include incorporation of positively charged molecules into the bacterial cell surface leading to reduced electrostatic interaction with AMPs, protease production leading to AMP degradation, increased activity of efflux pumps leading to active removal of AMPs, as well as suppression of the host's AMP production [65, 129, 130, 133].

There is relatively little information regarding the ability of microorganisms to acquire resistance to AMPs by genetic alterations as a result of prolonged exposure to therapy, as well as the mechanisms and risks of this acquired resistance [130, 132]. Studies of acquisition of resistance *in vitro* can be performed either by serial passage of bacteria in medium containing AMPs in subinhibitory and progressively increasing concentrations, or by direct selection on agar plates containing AMPs at concentrations above MIC [132]. However, the results from such studies vary. In some studies, low or no resistance was developed towards AMPs during several passages, while high levels of resistance were acquired towards conventional antibiotics [103, 134] (paper II and III). In another study, high-level resistance to AMPs was developed although a substantial number of serial passages were required [135]. In contrast, in some studies, high levels of resistance to AMPs were developed very rapidly [136-138]. The properties of the obtained mutants also differed between the studies. Some mutants were irreversibly resistant [135] whereas others lost their resistance when cultured in the absence of the AMP [134]. Furthermore, some mutations were associated with fitness costs [139, 140], typically observed as a reduced bacterial growth rate in the absence of the agent [141], whereas others had unaffected growth rates [137]. Importantly, while some mutants showed no or little cross-resistance to other AMPs [134, 137], a number of studies describe mutant strains with considerable cross-resistance [138, 139].

Currently, there are only a few studies reporting of the genetic identity of the acquired mutations and the mechanisms of the resulting resistance [132]. As one example, in a recent study *Salmonella enterica*, serially passaged in the presence of AMPs, developed resistance mutations in genes connected with LPS modifications and it was suggested that these mutations would confer resistance by e.g. reducing negative charges in the membrane and thus leading to decreased AMP interaction [140].

Based on these results, although perhaps to a lesser extent compared to conventional antibiotics and not as rapid, it could not be excluded that clinical administration of AMPs might lead to selection for resistance towards AMPs. Even more important is the concern that treatment with AMPs could potentially also select for cross-resistance to our endogenous AMPs which could result in microorganisms that are more capable of escaping our innate immunity [132, 142, 143]. Notably, there are

studies reporting of acquired resistance to colistin (i.e. polymyxin E) among clinical isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae* and even cross-resistance to LL-37 has been reported [144, 145]. It is therefore of great importance to thoroughly characterize the probability for resistance and cross-resistance development to AMPs before any widespread clinical use [130, 135].

1.3.2.3. In vivo stability

One of the principal limitations for clinical application of AMPs is the low *in vivo* stability [62]. Peptide drugs are generally characterized by low oral bioavailability due to proteolytic degradation in the digestive tract and poor penetration of the intestinal mucosa, which makes oral administration difficult [146]. Furthermore, systemic administration of peptides by, e.g., intravenous injection, is limited by a short half-life because of rapid degradation by proteolytic enzymes in blood plasma and rapid removal from the circulation by renal and hepatic clearance [146]. Moreover, proteolytic enzymes are especially abundant at sites of inflammation and infection [25], and thus even topically administered AMPs are subjected to degradation by proteases.

The lack of *in vivo* stability can be addressed by several means, including cyclization of the peptide sequence through disulphide bonds, exchanging the natural L-amino acids for D-residues or unnatural amino acids, changing the peptide into a peptide mimetic with a non-peptide backbone structure, or using protective formulations [26-28, 62, 146]. In addition, end-tagging by short, hydrophobic amino acid stretches has been shown to influence sensitivity of AMPs for proteolytic degradation [147] and blocking N- or C-terminal ends of the AMPs by modifications such as N-acetylation, N-pyroglutamate, or C-amidation are frequently used to increase resistance towards peptidases [148, 149].

1.3.2.4. Toxicity

Due to their complex MOA, potential risk for toxicity is another limitation of clinical development of AMPs [26]. So far, most clinical trials have studied topical administration of AMPs and systemic toxicity issues thus remain mainly uncharacterized [24, 25]. In addition, publications describing data from standardized nonclinical safety studies of AMPs, similar to paper V, are rare. Since AMPs are known to interact with cell membranes, the ability of AMPs to cause hemolysis of erythrocytes has often been used to study selectivity for bacterial *versus* host membranes, and thus toxicity [25]. However, the predictive value of this model has been questioned since

the AMPs rarely demonstrate a similar degree of cytotoxicity to erythrocytes in their natural milieu in the blood as compared to when suspended in phosphate buffered saline (PBS) [25, 147, 150]. Besides toxicity due to membrane interactions, potential toxicity could also result from translocation and uptake of AMPs into host cells, an issue that has not been fully characterized [25, 26]. However, compared to small molecule drugs, AMPs are considered more advantageous from a safety perspective, since the degradation products of AMPs are natural amino acids and since the peptide accumulation in tissues is low due to their short half-life, thus together reducing the risk of systemic toxicity and complications caused by metabolites [146].

One approach to address the risk of toxicity is to use formulations masking the peptides [28], while another approach is to modulate properties of the AMPs, such as hydrophobicity, helicity, and amphipathicity, making them less prone to act on mammalian cells [24]. Finally, topical administration of antimicrobials reduces the risk for systemic toxicity [151].

1.3.2.5. Costs of goods

One limiting issue for developing AMPs as pharmaceuticals is the high manufacturing costs [26]. Compared to the relatively low production costs of some antibiotics (e.g. 0.8 USD per gram for aminoglycosides), the costs for peptide synthesis is much more expensive, ranging from 50 to 600 USD per gram [24, 26]. Solid phase peptide synthesis (SPPS) is the standard method to produce peptides [152]. To reduce manufacturing costs, peptides could be made shorter and other manufacturing alternatives could be exploited, such as solution-phase or recombinant production using bacterial-, fungal-, or mammalian expression systems [24, 26, 27, 62]. In addition, for indications where the immunomodulatory effect rather than the direct antimicrobial effect of the AMPs is most important, lower doses of the peptide may be required and, similarly, local administration may require lower doses compared to systemic administration [26], which would then reduce the overall treatment costs.

1.4. Sources of the AMPs in this thesis

In this thesis, we have studied AMPs derived from two distinct sources; the human protein lactoferrin and centrocin extracted from the green sea urchin *Strongylocentrotus droebachiensis*.

1.4.1. Lactoferrin and lactoferrin-derived peptides

Lactoferrin is an iron-binding glycoprotein found in exocrine secretions, including tears, saliva, gastric fluids, and, in particular, milk, as well as in secondary granules of neutrophils [153]. Lactoferrin acts as a key element in the innate immunity of mammals, protecting the host against infection and excessive inflammation [154, 155]. Lactoferrin exerts a direct antimicrobial function by limiting the proliferation of microorganisms and/or by killing them [154]. This antimicrobial property relates to the ability of lactoferrin to, like AMPs, destabilize membranes as well as to its ability to sequester iron essential for bacterial growth [154, 156]. In addition, lactoferrin has immunomodulatory and anti-inflammatory properties exemplified by its ability to suppress LPS- and cytokine-induced production of proinflammatory cytokines [75] and its ability to down regulate over-production of toxic reactive oxygen species during inflammation [157]. Several mechanisms exist for these anti-inflammatory activities of lactoferrin. These include the ability of lactoferrin to bind iron, its ability to bind LPS and thereby preventing LPS from binding LBP and TLR4/CD14 receptor complex, as well as its ability to directly bind to immune cells via cell surface molecules, such as proteoglycans and/or specific receptors [154, 155, 158, 159]. Notably, lactoferrin can also become internalized into immune cells and translocated into the nucleus where it is proposed to directly interfere with NF-κB activation and thus the production of proinflammatory cytokines [75]. The two cationic sites at the N-terminal of human lactoferrin, ^{1}GRRRR^{5} and ^{28}RKVR^{31}, have been identified as important for the binding of lactoferrin to LPS and to glycosaminoglycans (e.g. on cell surface proteoglycans), as well as for its antimicrobial activity [160-163]. In the tertiary structure of lactoferrin, these two sites end up in close proximity, together forming the so called cationic cradle [160].

Proteolytic cleavage of human lactoferrin in the gastrointestinal tract and at sites of infection generates the peptide lactoferricin, consisting of residues 1–49 from the N-terminal domain [164, 165] (**Fig. 6**). This AMP adopts an α-helical structure in membrane mimetic solvents [43] and exhibits more potent antimicrobial properties than lactoferrin as well as immunomodulatory functions similar to the parent protein [164, 165]. In addition, shorter sequences of human lactoferricin have shown anti-infectious and anti-inflammatory activities using *in vivo* models [166, 167]. From the sequence of human lactoferricin, numerous different peptide variants were previously derived by modulating the peptide length, helix stability, amphipathicity, net charge, and hydrophobicity. In total, more than hundred peptides were screened *in vitro* with focus on antimicrobial and anti-inflammatory properties and selected peptides were evaluated for their efficacy in *in vivo* experiments (data mostly unpublished) [166-169]. Based on the results from these extensive screening programs,

three peptides were selected for evaluation as pharmaceutical agents in this thesis: PXL01, PXL150, and HLR1r.

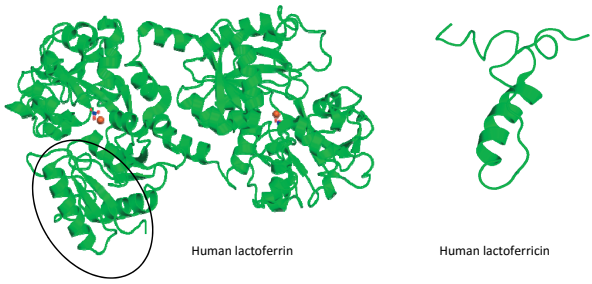

Figure 6. *Molecular structures of human lactoferrin (with two Fe^{3+} and two CO_3^{2-}) and human lactoferricin. The N-terminal sequence of lactoferrin corresponding to the lactoferricin fragment is circled. Adapted from Protein Data Bank in Europe [41] using PDB id codes 1b0l and 1z6v, respectively.*

1.4.2. Centrocin and centrocin 1-derived peptides

Invertebrates constitute a potentially rich source of effective AMPs since they only rely on the innate immunity to fight infections [170]. Further, AMPs isolated from marine organisms may have unique structures possibly reflecting their adaption to their natural environment of high salt (seawater salinity is on average 3.5%), low temperature, elevated pressure, and large amounts of microbes [170]. In addition, AMPs from marine organisms are often post-translationally modified to contain unusual amino acids, such as brominated tryptophans, suggested to make these peptides less susceptible to proteolytic degradation and/or enhance their antibacterial activity [170, 171]. Together, these properties, including the potentially low sensitivity to high-ionic strength [170, 172], make AMPs of marine origin highly interesting to be developed as pharmaceuticals.

Based on previous identification of the antimicrobial bromotryptophan-containing heavy chain of centrocin 1 (CEN1 HC-Br) [171] isolated from coelomocyte (i.e. blood cell) extracts of the green sea urchin *S. droebachiensis* [173], four variants of this AMP, as well as the original sequence itself, were studied in this thesis as potential pharmaceutical agents.

1.5. Proposed indications

In this thesis, the selected peptides were evaluated as pharmaceutical agents for two separate indications, prevention of postsurgical adhesion formation and treatment of different skin and soft tissue infections (SSTIs).

1.5.1. Prevention of postsurgical adhesions

Adhesions are bands of scar tissue that form between internal tissues and organs as a part of the body's healing process after e.g. surgery, trauma, infections, and inflammation [174]. Postsurgical adhesions can arise after in principle any surgical procedure, but are almost inevitable after open abdominal and gynecological pelvic surgery, where the incidence of intraperitoneal adhesions ranges from 60% to 97% [175-177]. Intraperitoneal adhesions severely affect the patients' health by causing small-bowel obstruction, female infertility, difficulties at reoperation, and chronic abdominal pain [176, 178].

Following peritoneal injury, formation and deposition of a fibrin-rich exudate at the wound site occur as a part of the normal hemostatic process [19]. The fibrin is sticky and attaches to adjacent surfaces and if not degraded by plasmin within the following few days, the fibrin provides a matrix for ingrowth of fibroblasts with subsequent collagen deposition and transformation into fibrous, permanent adhesions [19, 179, 180]. Inadequate fibrinolysis is therefore widely accepted to be the main cause of adhesion formation [19, 181]. Plasminogen is converted to active plasmin by tissue-type plasminogen activator (tPA), and the activity of tPA is mainly inhibited by PAI-1 [19] (**Fig. 7**). Elevated levels of PAI-1, which results in low activity of tPA and hence less formation plasmin, are therefore suggested to be a major factor causing adhesion formation by means of reducing fibrinolysis [182]. PAI-1 is produced by many cell types, including endothelial cells, macrophages, and mesothelial cells (the latter forming the mesothelium monolayer covering the surface of the serosal cavities in the body and the organs within these cavities [183]) in response to e.g. proinflammatory cytokines, and its production is increased during surgery and inflammation [19, 179, 184, 185]. Besides leading to anti-fibrinolytic activity, inflammation is also involved in adhesion formation by promoting coagulation and subsequently fibrin deposition [19]. Furthermore, the presence of bacteria has been shown to result in increased adhesion formation, possibly by affecting the immune system [186].

Numerous attempts have been made to prevent adhesion formation, including improving the surgical techniques, using fine and biocompatible sutures, avoiding foreign materials such as starch from gloves, and by using physical barriers and different pharmacological compounds. Nevertheless, adhesions still remain a major clinical problem and there is today no adhesion-preventing drug/device that is clearly effective [187, 188].

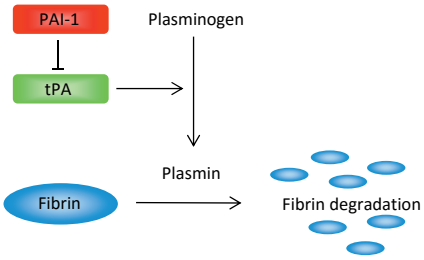

Figure 7. *Fibrinolysis pathway. Tissue-type plasminogen activator (tPA) converts plasminogen into active plasmin, which is responsible for the fibrin degradation. Plasminogen-activator inhibitor-1 (PAI-1) inhibits tPA and hence elevated levels of PAI-1 results in reduced fibrinolysis.*

1.5.2. Skin and soft tissue infections (SSTIs)

SSTIs are the most common of infections, affecting everyone at some point [189]. SSTIs are caused by a break in the skin barrier (e.g. ulcers, burn, surgical incisions, and trauma) allowing colonization and subsequent infection of the epidermis, dermis, and/or subcutaneous tissue [189]. The discrepancy between colonization and infection is vague, but a wound bioburden of 10^5 colony forming units (CFU)/g or cm^2 is generally accepted as an indication of an infected wound [190, 191]. SSTIs include a wide range of infections, from minor superficial infections to life-threatening infections, including surgical site infections (SSIs), impetigo, cellulitis, complex abscesses, infected burns, and necrotizing fasciitis [192]. The most common cause of SSTIs is the Gram-positive *S. aureus* (globally accounting for 34–45% of the isolates) while of the Gram-negative species *P. aeruginosa* and *Escherichia coli* are most frequently isolated [193]. The rapid increase in strains resistant to one or multiple antibiotics is of great concern. For example, globally 23–36% of the *S. aureus* isolates from SSTIs are methicillin-resistant (MRSA; resistant to all β-lactam antibiotics [194]) [193]. In some smaller studies, MRSA isolates even accounted for up to 77–88% of the isolates [195, 196]. Notably, 3–25% of the *P. aeruginosa* isolates have been reported to be multidrug-resistant [193].

1.5.2.1. Surgical site infections (SSIs)

SSIs are postoperative infections affecting either the skin and subcutaneous tissue of the incision (i.e. superficial incisional SSIs), the deeper soft tissues of the incision (i.e. deep incisional SSIs), or the other organs/spaces opened or manipulated during surgery (not classified as SSTIs) [192, 197]. SSIs are reported to affect between 1.5% and 20% of surgical patients, depending on type of surgical procedure and the body site [198]. Microorganisms infecting the surgical wounds are most often from

the patient's endogenous flora, but occasionally also from external environment [199]. Consistently with other SSTIs, *S. aureus, Staphylococcus epidermidis, E. coli,* and *P. aeruginosa* are commonly isolated from SSIs [198-200], although the causative organisms are largely dependent on the procedure/site [199]. As for other SSTIs, a significant increase in antibiotic-resistant strains has been reported for SSI isolates, both for Gram-positive and Gram-negative bacteria [200, 201], and, in addition, polymicrobial infections appear to increase [200].

1.5.2.2. Infected burn wounds and microbial biofilms

Burn wound infections constitute a very serious complication following thermal injury [202] accounting for up to 80% of all hospital acquired infections affecting patients admitted to specialized burn units [203-205]. Immediately after thermal injury, the sterile wound surface is rapidly colonized with Gram-positive bacteria, primarily *S. aureus* and coagulase-negative staphylococci, such as *S. epidermidis*, from the endogenous flora and external environment. These are followed by Gram-negative bacteria, in particular *P. aeruginosa*, and eventually fungus, such as *Candida* spp. and *C. albicans* in particular [192, 202, 206, 207]. Colonization can lead to burn wound infection characterized by a high concentration of bacteria ($> 10^5$ CFU per gram tissue) within the wound or wound eschar [208]. The infection can eventually become invasive, where microorganisms invade and destruct unburned tissue [208]. Furthermore, if invasion extends into deeper tissues, it may lead to serious and even life-threatening complications, such as bacteremia (i.e. presence of bacteria in the blood), sepsis (i.e. a systemic inflammatory response to an infection), and even multiple-organ dysfunction syndrome [202]. Burn wounds are particularly susceptible to infections due to destruction of the skin barrier, the moist and protein-rich environment in the burn wound, impaired migration of host immune cells due to destruction of blood vessels, and a generalized immunosuppression of the patient [202, 209]. Moreover, infected burn wounds can be difficult to treat, partly because the avascular burn tissue restricts the access of systemically administered antibiotics to the wound site [202, 209], partly because the emergence of antibiotic-resistant bacteria [202], and partly because bacteria in burns tend to form biofilms [202, 210].

Biofilms are surface-attached communities of microorganisms embedded in a self-produced polymeric matrix. The biofilm provide protection for the bacteria from the host's immune system by several mechanisms [211], e.g. the extracellular slime has shown ability to suppress the phagocytic activity of macrophages [212]. In addition, bacteria in biofilms are highly resistant to antibiotics; up to 1000 × higher doses of antibiotics may be required to eradicate bacteria in biofilms compared to their free-floating (i.e. planktonic) counterpart [210, 211, 213]. Biofilms are not only as-

sociated with burn wound infections. In fact, biofilms account for at least 65% of all human infections, being particularly associated with e.g. catheter- and medical-device related infections, chronic wounds, cystic fibrosis lung infections, and periodontitis [213-215]. Although the effect of AMPs on biofilms has been questioned due to the potential of negatively charged polymers in the biofilm matrix to bind and neutralize AMPs [216, 217], there are peptides with proven effect on biofilm *in vitro* and/or *in vivo*, including LL-37, human β-defensin 3, histatins, and tachyplesin [105, 125, 218-221].

1.5.3. Cutaneous *Candida* infections

Candida spp. are the most common cause of fungal infections in humans, leading to a range of diseases from superficial skin- and mucosal candidiasis to life-threatening invasive diseases [222, 223]. Among the Candida spp., *C. albicans* is the most common infectious agent [222, 224, 225]. *C. albicans* asymptomatically colonizes the skin, genital and/or intestinal mucosa in 30-70% of healthy individuals [224, 226, 227], but when the host defense of the individual is weakened, the fungus can cause infections [226]. Risk factors, such as neutropenia, corticosteroid treatment, systemic broad-spectrum antibiotic exposure, central venous catheters, surgery, hospital stay, and burns, predispose individuals to invasive and even life-threatening systemic candidiasis [225, 226]. Furthermore, in some settings, resistance to conventional antimycotics, such as fluconazole, has been observed in *Candida* spp. [222, 228], which complicates treatment of the infections.

2. Aims

The overall aim of this thesis was to study novel AMPs with respect to their MOA, antimicrobial spectrum, propensity to select for resistance, and *in vivo* efficacy and safety. To achieve this, we used a number of *in vitro* and *in vivo* assays, together generating a comprehensive preclinical evaluation of the peptides. The hypothesis was that the AMPs in this thesis have potential to be developed as therapeutic agents for several infectious and inflammatory conditions.

The specific aims of the papers included in this thesis were:

Paper I: To study the potential of the peptide PXL01 as an anti-adhesion agent for prevention of postsurgical adhesion formation.

Paper II: To study five peptides derived from centrocin-1 for their applicability as local anti-infective agents.

Paper III: To study the applicability of the peptide PXL150 as an anti-infective agent for local treatment of SSTIs.

Paper IV: To study the potential of PXL150 as a local treatment of SSIs.

Paper V: To study PXL150 for its applicability as a local therapeutic agent for infected burn wounds and to characterize its *in vivo* safety profile.

Paper VI: To study the potential of the peptide HLR1r as a therapeutic alternative for local treatment of SSTIs.

3. Materials and methods

3.1. Peptides and controls

3.1.1. Peptides

Human lactoferrin-derived peptides were studied in paper I (PXL01), paper III, IV, and V (PXL150), and paper VI (HLR1r). PXL01 corresponds to residues 16–40 of human lactoferricin, HLR1r corresponds to residues 21–32 of human lactoferricin with an additional arginine-rich tail attached to the C-terminal end, whereas PXL150 is as a modified version of amino acids 20–31 of human lactoferricin (**Table 1**). Both the N-terminal and C-terminal ends of PXL01 and HLR1r were modulated by acetylation (i.e. NH_2- is converted to CH_3CONH-) and amidation (i.e. –COOH is converted to -$CONH_2$), respectively, in order to become uncharged.

Table 1. *Amino acid sequences of the human lactoferrin-derived peptides studied in this thesis.*

Peptide	Sequence
PXL01	Ac-EATKCFQWQRNMRKVRGPPVSCIKR-NH_2 (disulphide bond)
PXL150	RRLWRRWMRKVL
HLR1r	Ac-FQWQRNMRKVRGSRRRRG-NH_2

Ac, acetyl group

Centrocin 1-derived peptides were studied in paper II (**Table 2**). CEN1 HC, without the brominated tryptophan present in the original sequence CEN1 HC-Br, was synthesized to elucidate if this unusual residue had any effect on the activity. C-terminal truncated versions, CEN1 HC-Br (1-20) and CEN1 HC (1-20), were also tested to map the peptide region important for the biological activity. Finally, CEN1 HC (Ser) was designed to investigate the possibility to replace the free cysteine residue with an isosteric serine since free cysteines might complicate the product development due to formation of intermolecular disulphide bonds.

The peptides were produced by SPPS using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. The purity of the peptides was $\geq 95\%$, as determined by the suppliers using high-performance liquid chromatography (HPLC) with UV detection. Counter ion was either trifluoroacetate or acetate for the lactoferrin-derived peptides, and trifluoroacetate for the centrocin 1-derived peptides. The peptides were supplied as lyophilized powder.

Table 2. *Amino acid sequences of the centrocin 1-derived peptides studied in this thesis.*

Peptide	Sequence
CEN1 HC-Br	GW(Br)FKKTFHKVSHAVKSGIHAGQRGCSALGF
CEN1 HC	GWFKKTFHKVSHAVKSGIHAGQRGCSALGF
CEN1 HC-Br (1-20)	GW(Br)FKKTFHKVSHAVKSGIHA
CEN1 HC (1-20)	GWFKKTFHKVSHAVKSGIHA
CEN1 HC (Ser)	GWFKKTFHKVSHAVKSGIHAGQRGSSALGF

3.1.2. Control antibiotics

As positive controls for antimicrobial activity, commercial antibiotic creams/ointments indicated for topical treatment of SSTIs, or their active pharmaceutical ingredient (API) only, were used including mupirocin (API or Bactroban; 2% w/w ointment), fusidic acid (API or Fucidin; 2% cream), and gentamicin sulphate (API or Garamycin; 0.1% w/w cream). Mupirocin has a spectrum of activity including some Gram-positive bacteria, in particular *Staphylococcus* spp. and *Streptococcus* spp., and a few Gram-negative bacteria [229, 230]. Fusidic acid has a spectrum of activity limited to some Gram-positive bacteria (in particular staphylococci, whereas streptococci are less susceptible) and a few Gram-negative species [231, 232]. Gentamicin has a broad-spectrum activity covering both Gram-positive and Gram-negative bacteria [233].

3.2. Carriers and release assay

The peptides were dissolved/formulated in ultrapure water, physiological saline (i.e. 9 mg/ml NaCl in water), or in a formulation intended for clinical application; sodium hyaluronate (SH) or hydroxypropyl cellulose (HPC).

3.2.1. Sodium hyaluronate (SH)

In paper I, PXL01 was formulated in SH (1.5% w/v in physiological saline) extracted from rooster combs. SH is the sodium salt of hyaluronic acid, a polysaccharide belonging to the group glycosaminoglycans. It is found in all tissues and body fluids of vertebrates, especially in the connective tissue, and constitutes the main component of extracellular matrix [234]. SH was used as formulation in order to extend the release of PXL01 at the site of administration, to protect the peptide from degradation, and to act as a physical barrier, preventing contact between surfaces in close proximity.

3.2.2. Hydroxypropyl cellulose (HPC)

In paper IV and V, PXL150 was formulated in 1.5% w/w HPC in 10 mM acetate buffer (pH 4.9), with or without 25% w/w propylene glycol and 0.1% w/w ethylene diamine tetra-acetic acid (EDTA). HPC is a nonionic, water soluble polymer widely used as pharmaceutical formulation [235]. The formulation and its supplements were chosen based on the *in vitro* and *in vivo* results from a previous study, where the stability and activity of PXL150 was evaluated using several formulations/supplements (data not published).

3.2.3. *In vitro* release assay (paper I)

In paper I, to study the release profile of PXL01 from SH gel, an *in vitro* release system was set up in which a thin film of the product (PXL01 in SH) was placed in a tissue culture plate and covered with PBS. The plate was incubated on a shaker and at certain time points, aliquots of release medium were collected and the peptide concentration was analyzed using UV-absorbance measurements (230 nm).

3.3. Microorganisms and microbiology assays

3.3.1. Microorganisms

The microorganisms used in this thesis are presented in **Table 3**. These are representative strains of common causative agents of infections, in particular SSTIs. Both extensively used American Type Culture Collection (ATCC) strains were included as well as more recently acquired clinical isolates. For most assays in this thesis, actively replicating microorganisms were used as is commonly done to assess microbicidal activity of conventional antibiotics [236] as well as AMPs [111, 237]. To reach log-phase growth, overnight cultures were diluted in fresh medium and incubated for an additional 2 hours [168, 169].

Table 3. *Microorganisms used in the thesis.*

Species/Strain	Growth under aerobic conditions	G+/G-/F	Resistance	Strain no.	In paper
S. aureus	Yes	G+		CCUG 1800 ATCC 12600 ATCC 29213	I II, III, VI III, IV
MRSA	Yes	G+	Methicillin*	ATCC 33591	II, III, VI
S. aureus	Yes	G+	Fusidic acid	Isolate	III
S. pyogenes	Yes	G+		ATCC 12344	II, III, VI
S. epidermidis	Yes	G+		ATCC 12228	II, III
P. aeruginosa	Yes	G-		ATCC 15442 ATCC 27853 PAOI- Lux1	I, II, III, VI V V
K. pneumoniae	Yes	G-	Penicillins†	CCUG 59413	II, III
A. baumannii	Yes	G-	Tobramycin‡	CCUG 58437	II, III
E. coli	Yes	G-		CCUG 31246 ATCC 11775	I, VI II, III
P. acnes	No	G+		ATCC 6919	II, III, VI
C. albicans	Yes	F		ATCC 64549	II, III, VI
C. parapsilosis	Yes	F		ATCC 22019	III
C. krusei	Yes	F		ATCC 6258	III
C. glabrata	Yes	F		CCUG 35267	III

*MRSA, methicillin-resistant Staphylococcus aureus; ATCC, American Type Culture Collection; CCUG, Culture Collection, University of Gothenburg; G+, Gram positive; G-, Gram negative; F, fungus. *Resistant to methicillin and other β-lactam antibiotics; † Resistant to penicillins, cephalosporins, aztreonam, and carbapenems; ‡ Resistant to tobramycin, trimsulfa, ciprofloxacin, cefotaxim, ceftazidim, meropenem, and piperacillin/tazobactam.*

3.3.2. *In vitro* minimum microbicidal concentration (MMC) assay (paper I, II, III, V, and VI)

To study the *in vitro* antimicrobial activity of the AMPs, an MMC assay was performed based on previous publications [168, 169]. Traditionally, a MIC assay is used to determine the susceptibilities of bacteria to antimicrobial agents. In this thesis; however, the MMC assay, in which the lowest microbicidal concentration is determined, was used instead of the MIC assay since this assay was considered more relevant for testing AMPs due to their ability to kill microorganisms and not just inhibit their growth. Microorganisms were incubated with serially diluted AMPs for

2 hours, followed by subculturing small aliquots (drops) onto agar plates. Viable cell counts were performed in the spots formed by the drops after 1–3 days of incubation. Less than 10 CFU/spot, corresponding to a > 99.6% reduction of the initial inoculum, was defined as the MMC_{99} value. A difference in MMC_{99} values by a factor of four (i.e. two titer steps) was regarded as significantly different [169]. The 2-hour incubation period was selected based on previous publications [111, 168, 169, 237, 238]. Similar to the MIC assay, only one value is obtained for each peptide in the MMC assay, making it ideal for screening purposes. In contrast, in another commonly used bactericidal assay each incubation mixture is serially diluted before plating, which makes it possible to evaluate the killing capacity of the AMPs in more detail but is also more labor intense [111, 237, 238].

The AMPs were tested under low salt/serum conditions to increase the level of sensitivity in an initial screening as frequently done [111, 112, 239, 240]. For this purpose, 100 × diluted brain–heart infusion broth (BHI_{dil}) was used as standard low-ionic medium [167-169]. To evaluate the AMPs in a wound-like environment, 2 × diluted simulated wound fluid (SWF_{dil}), i.e. a 2 × dilution of a 1:1 mixture of 0.1% peptone water in 150 mM NaCl and fetal bovine serum (FBS), was used. To elucidate the effect of salt on the activity of the peptides, the MMC assay was also performed in BHI_{dil} supplemented with 85 mM or 150 mM NaCl. In paper V, the bactericidal effect of PXL150 was assessed when formulated in HPC.

3.3.3. Cytoplasmic membrane depolarization assay (paper III)

To study the antimicrobial MOA of PXL150, a cytoplasmic membrane depolarization assay in combination with viable cell counts, was performed based on previous publication [64, 241]. Under the influence of an intact transmembrane potential, the fluorescent probe 3,3'-Dipropylthiadicarbocyanine iodide, $DiSC_3(5)$, accumulates in the membrane of intact bacterial cells, resulting in diminished fluorescence due to self-quenching [63, 64]. Permeabilization of the cytoplasmic membrane leads to loss of membrane potential, which causes release of $DiSC_3(5)$ with a subsequent increase in fluorescence [63]. $DiSC_3(5)$ was incubated with a suspension of *S. aureus* followed by addition of KCl to equilibrate the cytoplasmic and external potassium ion concentrations. Thereafter, peptide was added and the fluorescence intensity was monitored continuously for 15 minutes. Aliquots of the suspension were collected at specified time points and used for viable cell counts to be able to draw conclusions of the correlation between depolarization and killing.

3.3.4. Multistep resistance assay (paper II and III)

In order to study the ability of bacteria to develop resistance to AMPs, a multistep resistance assay was performed based on previous publications [242, 243]. Bacteria (*S. aureus*, MRSA, and *P. aeruginosa*) were incubated with serially diluted peptide (in 10 × diluted BHI) in a concentration range covering the MIC (or MMC) for 24 hours, after which the turbidity was measured. To create an optimal selection pressure, the overnight culture incubated with the highest peptide concentration with no marked reduction of bacterial growth was selected for further passaging. One part of this suspension was diluted to a start inoculum and incubated with a new dilution series of the peptide, and the remaining part was frozen until analysis using the MMC assay at the end of the study. The procedure was repeated for up to 21 days, after which the resistance development was evaluated and observed as an increase in killing concentration compared to the initial MMC value.

3.4. Cell based assays

3.4.1. *In vitro* anti-inflammatory assay using macrophages (paper I, II, III and VI)

To study the ability of the AMPs to suppress the release of proinflammatory cytokines/chemokines from LPS-stimulated macrophages, an *in vitro* anti-inflammatory assay using differentiated THP-1 cells was performed based on the previous publication [75]. THP-1 (ATCC TIB-202) is a cell line of monocytes obtained from peripheral blood of a person suffering from acute monocytic leukemia. The cell line has been extensively used to study immune responses in monocytes/macrophages [244]. Using phorbol-12-myristate-13-acetate (PMA), THP-1 cells in the monocyte state can be differentiated into a macrophage-like phenotype, characterized by adherent cells and a flat amoeboid shape [244, 245]. PMA-differentiated THP-1 cells were stimulated with LPS to induce expression and release of cytokines/chemokines, 30 minutes before addition of the peptides. After 6 hours of incubation, the cell culture supernatants were collected and the levels of TNF-α, IL-1β, IL-6, and/or IL-8 were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) with microplate-bound primary/capture antibodies and biotinylated secondary/detection antibodies obtained from R&D Systems (Minneapolis, MN, USA). Potential peptide-induced cytotoxicity was elucidated by determining the viability of the cells in each well (see below).

3.4.2. *In vitro* anti-inflammatory/fibrinolytic assay using mesothelial cells (paper I, III, and VI)

To study the ability of the AMPs to suppress the release of proinflammatory and fibrinolytic markers from mesothelial cells, an *in vitro* anti-inflammatory/fibrinolytic assay using MeT-5A cells was performed. MeT-5A (ATCC CRL-9444) is an immortalized cell line of mesothelial cells obtained from pleural fluids of non-cancerous individuals. Mesothelial cells play an important role in innate immunity and tissue repair by e.g. producing both proinflammatory and fibrinolytic mediators [179, 183, 246]. MeT-5A cells were stimulated with IL-1β to induce expression and release of cytokines/fibrinolytic markers, immediately before addition of the peptides. After 3–6 hours of incubation, the cell culture supernatants were collected and the levels of IL-6 and PAI-1 were quantified using the ELISA described above and the commercial ELISA kit TintElize PAI-1 (Trinity Biotech, Bray, Ireland), respectively. Peptide-induced cytotoxicity was elucidated by determining the viability of the cells in each well (see below).

3.4.3. Cell viability assays (paper I, II, III, and VI)

To elucidate the possibility that the suppression of the release of inflammatory/fibrinolytic markers by the peptides could be due to peptide-induced cytotoxicity, the cell viability at the end of the cell based assays described above, was determined using two different approaches; NucleoCounter system (ChemoMetec, Allerød, Denmark) and TACS MTT assay (R&D Systems).

The NucleoCounter system (paper I and VI) is based on propidium iodide, which binds to the DNA of non-viable cells forming a fluorescent stain but is excluded from viable cells due to impermeable plasma membrane. By measuring the fluorescence of an untreated as well as of a detergent-lysed sample, both a non-viable cell count and a total cell count are determined, respectively. The viable cell count is then calculated from the difference of the total cell count and the non-viable cell count. After removal of the cell culture supernatants described above, the cells were detached from the bottom of the wells by trypsinization and re-suspended in culture medium. Two samples were analyzed from each well, one untreated and one detergent-lysed, and the cell viability was determined.

The MTT assay (paper II and III) is based on the measurement of cell viability via their metabolic activity. The substance 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to purple formazan by metabolically active cells. The number of viable cells correlates with the color intensity measured spectrophotometrically after solubilization of the formazan. After removal of the cell

culture supernatant, MTT was added to the cells and after 2 hours of incubation, detergent was added and an absorbance measurement was performed the following day.

3.4.4. Hemolytic assay (paper III)

To determine the cytotoxicity against human cells and the selectivity of AMPs for microbial cells *versus* mammalian cells, the hemolytic activity against human erythrocytes was assessed based on the previous publication [247]. Blood samples from human healthy volunteers were collected in EDTA-containing tubes. The tubes were centrifuged, the plasma was removed, and the erythrocytes were suspended in PBS. Serially diluted peptide was added to the cell suspension, and after 1 hour of incubation and following centrifugation, absorbance measurement was performed on the supernatant to determine the release of hemoglobin from lysed erythrocytes.

3.5. *In vivo* efficacy and safety

3.5.1. Ethical considerations

All animal experiments were performed after prior approval from the local ethics committee for animal studies at the administrative court of appeals in Gothenburg, Sweden, the local ethics committee for animal studies at the court of appeals in Umeå, Sweden, or the CERB internal ethics committee in Baugy, France. All animals were cared for in accordance with national legislation and regulations for the protection of animals used for scientific purposes.

3.5.2. Efficacy and safety as anti-adhesion agent

3.5.2.1. Postsurgical adhesion model in rats (paper I)

To study the ability of PXL01 to prevent postsurgical adhesion formation, a sidewall defect-cecum abrasion model in rats was performed based on previous publications [248, 249]. PXL01 (in distilled water or in SH) was applied over a full-thickness excision wound (5 × 25 mm) created on the peritoneal wall in the rat abdomen, as well as over two abrasion wounds (10 × 15 mm), one on each side of the cecum. One group of animals received two additional doses of peptide by intraperitoneal injec-

tions 24 and 48 hours after surgery. After 6 days, the animals were euthanized and the extent of adhesion formation in the abdomen was evaluated. Studies have demonstrated that the peritoneum is only susceptible for adhesion formation during the first few hours/days after surgery [248, 250] and that adhesions that are present after one week after surgery are also there after six months [251, 252], thus supporting the choice of time for evaluation.

3.5.2.2. Intestinal anastomosis healing model in rats (paper I)

To study if treatment with PXL01 during abdominal surgery would have any negative effect on wound healing, a colonic/large bowel anastomosis healing model in rats was performed based on previous publications [253-257]. Although this model has often been used to study potential beneficial effects of different treatment regimens to promote healing and prevent leaking after colorectal anastomosis surgery [253-256], the model has also been used to evaluate the safety of anti-adhesion therapies [257]. PXL01 in SH was applied over an anastomosis site created by transecting the colon and performing an end-to-end anastomosis. After 7 days, the animals were euthanized and a segment of the colon with the anastomosis site in the middle was cut out. One end of the segment was ligated and into the other end, a tube connected to a pressure monitor was inserted. Stained saline was infused into the segment via the tube and the intraluminar pressure was measured until burst. The maximum pressure before burst was used as a measurement of the healing of the anastomosis.

3.5.3. Efficacy and safety as anti-infectious agents

3.5.3.1. Infection models

To our knowledge, no golden standard model for preclinical assessment of anti-infective activity of antimicrobials developed for treatment of SSTIs exists. However, different infection models, similar to the ones used in this thesis, have frequently been used for this purpose [147, 150, 169, 258-265].

3.5.3.2. *Ex vivo pig skin wound infection model (paper II, III, and VI)*

To study the ability of the peptides to reduce the bacterial load in infected wounds, an *ex vivo* pig skin wound infection model was performed based on previous publications [147, 150, 258]. Pig skin has long been considered as an appropriate model for human skin [258] and the pig skin infection model was used as a bridge between *in vitro* experiments and costly, ethically restrained, and time consuming *in vivo* experiments. Importantly, this model has been used to test AMPs by other research groups [147, 150] and topical agents showing efficacy in this model have later proved to be effective in *in vivo* studies in pigs and in clinic [150, 258]. Punch biopsy wounds were made on excised pig skin and separated from each other by gluing the top cylinder of a cut 1.5 ml tube around each wound (**Fig. 8**). *S. aureus* were inoculated onto the skin/wound area inside the cylinder and after 2 hours of incubation in a moist chamber, peptide (in sterile ultrapure water) was added to each wound/skin area. After an additional 4 hours of incubation, the liquid inside the cylinder was removed, the bacteria were harvested by adding Kligman buffer (0.1% Triton X-100 in 75 mM phosphate buffer) to the wound/skin area and gently rubbing, and viable cell counts were performed on dilutions of the obtained suspension (**Fig. 9a**).

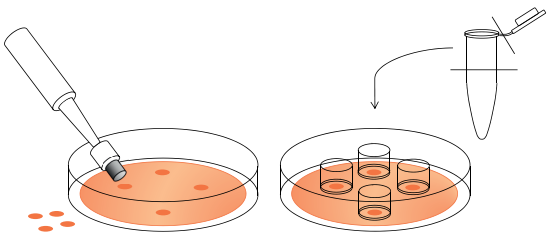

Figure 8. *Ex vivo pig skin wound infection model. Punch biopsy wounds were made on excised pig skin and separated by attaching a cut 1.5 ml tube around each wound.*

3.5.3.3. *Excision wound infection model in rats (paper II, III, VI)*

To study the ability of the AMPs to reduce the bacterial load in infected wounds, an excision wound infection model in rats was performed based on previous publication [266]. This model has been used in a number of studies assessing anti-infective and wound healing effects of topical agents [259-261, 266]. Bacteria (MRSA or *P. aeruginosa*) were inoculated onto full-thickness excision wounds (10 × 10 mm) made on the back of rats, anaesthetized during entire experiment. After 2 hours, peptide (in sterile ultrapure water) was applied onto each wound and after an additional 2 hours, the animals were euthanized. The entire wound area was excised, followed by vortexing and shaking in Kligman buffer. The suspension was diluted and viable

cell counts were performed (**Fig. 9b**). To study the kinetics of the antibacterial activity (paper III), samples were collected from the same wound at six time points up to 4 hours post-treatment by adding BHI_{dil} to each wound, gently scraping with a plastic loop, and removing a small sample for viable cell counts.

3.5.3.4. Surgical site infection model in mice (paper IV)

To study the ability of PXL150 to reduce the bacterial load in infected surgical wounds, a mouse model of SSIs was performed based on previous publications [262-264, 267]. This model is viewed as a valuable tool for predicting the effect of topical (and systemic) antimicrobial agents in humans. Silk sutures were soaked in a stationary phase suspension of *S. aureus* and dried. The suture was placed into a full-thickness incision wound (1 cm) made at the neck region of mice. To study the infection progress, at certain time points up to 96 hours after infection, the animals were euthanized and the entire wound area was excised and homogenized in BHI_{dil}. The homogenate was diluted and viable cell counts were performed. To study the effect of the peptide, 2 hours after inoculation of the wound with the suture, peptide (in sterile ultrapure water or HPC) was applied into the wounds and after an additional 2 hours, the animals were euthanized and bacteria were harvested by excision of the wound area as above (**Fig. 9c**).

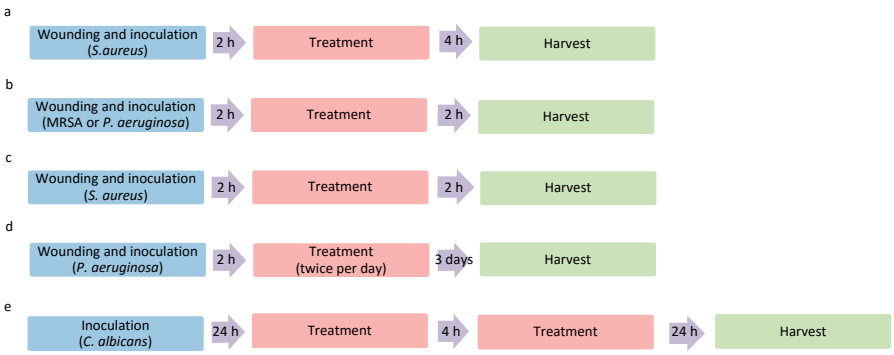

Figure 9. *Schematic presentation of the experimental setup of the ex vivo/in vivo infected skin/wound models used in this thesis. (a) Ex vivo pig skin wound infection model, (b) excision wound infection model in rats, (c) surgical site infection model in mice, (d) infected burn wound model in mice, and (e) cutaneous candidiasis model in mice. h, hours.*

3.5.3.5. Infected burn wound model in mice (paper V)

To study the ability of PXL150 to reduce the bacterial load in infected burn wounds, a mouse model of infected burn wounds was performed based on previous publications [265, 268]. *P. aeruginosa* was used, which is the bacterial species most common in burn wound infections [202, 207], and to be able to follow the infection in live animals, a bioluminescent strain was used [265, 269]. Bacteria were inoculated onto a third-degree burn wound (1 cm^2) created on the back of mice by placing the end of a metallic rod pre-heated in boiling water onto a shaved skin area for 30 seconds. After 2 hours, peptide (in HPC) was applied onto each wound. The treatment was repeated after an additional 4 hours and thereafter twice per day for 3 days. Prior to the treatments, the mice were anaesthetized and bioluminescence imaging (BLI) was performed. After 4 days, the animals were euthanized, BLI was performed, and the entire wound area was excised and homogenized in PBS. The homogenate was diluted and viable cell counts were performed (**Fig. 9d**).

3.5.3.6. Cutaneous candidiasis model in mice (paper VI)

To study the antifungal effect of HLR1r, a mouse model of cutaneous candidiasis was performed based on previous publications [169, 270]. In this model, *C. albicans* were inoculated onto each of four circled areas (1.7 cm^2) on intact shaved skin on the back of mice. After 1 day, peptide (in ultrapure water) was applied twice to the infected spots, with 4 hours between the treatments. The following day, the animals were euthanized and the fungal cells were harvested by placing a metal ring over the treated spot, adding phosphate buffer supplemented with 0.01% Triton X-100, and gently rubbing the area. The suspension was diluted and viable cell counts were performed (**Fig. 9e**).

3.5.4. *In vivo* safety

3.5.4.1. Preliminary study of single dose toxicity (paper III)

As a first step to evaluate the safety profile of PXL150, a preliminary study of acute systemic toxicity was performed, in which high doses (2.5 and 12.5 mg/kg body weight) of the peptide (in physiological saline) were administrated as a single subcutaneous (SC) injection into the neck region of rats. General behavior and clinical

signs were monitored for up to 48 hours after treatment to assess potential systemic effects.

3.5.4.2. In vivo nonclinical safety studies (paper V)

To support applications for clinical trials, a nonclinical safety program was conducted to determine systemic toxicity and local tolerance of PXL150 when administered repeatedly. Three consecutive studies were performed; (i) a 5-day pilot repeated dose toxicity study in rats (feasibility study), (ii) a 14-day pivotal repeated dose toxicity study in rats, and (iii) a 28-day local tolerance study in rabbits. The studies were performed by CERB (Baugy, France) in accordance with the ICH Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3(R2) [271]. Study (ii) and (iii) were performed according to good laboratory practice (GLP). In study (i), SC administrations of PXL150 in saline (0, 10, and 50 mg/kg body weight) were performed and in study (ii), SC administrations of PXL150 in saline (0, 1, and 5 mg/kg body weight) were performed. In study (iii), PXL150 (2 and 10 mg/g PXL150 in HPC) was dermally administered onto one healthy and one abraded skin area (5 ×5 cm each) per animal, 0.5 ml/area. To assess reversibility/delayed toxicity, the 28-day treatment period was, for some animals, followed by a recovery period of 14 days. To assess ocular tolerance, one ocular administration of PXL150 (0.1 ml of 0, 2 mg/g, and 10 mg/g PXL150 in HPC) was performed. During the studies, several tests were performed according to standard procedures, including morbidity/mortality, general observations, and necropsy with subsequent macroscopic and histopathological examinations.

4. Results

Below is a summary of the main results of the papers included in this thesis. For further details, see the full papers at the end of the thesis.

4.1. Paper I

In paper I, we evaluated the potential of PXL01 as an anti-adhesion agent for prevention of postsurgical adhesion formation by studying its effect on key processes in the wound healing cascade (i.e. infection reduction, inflammation resolving, and fibrinolysis) as well as its ability to safely reduce formation of postsurgical adhesions *in vivo*. This was achieved by using *in vitro* assays for microbicidal activity, anti-inflammatory effect, cytotoxicity, and fibrinolytic activity, as well as by using a postsurgical adhesion formation model in rats to study *in vivo* efficacy and an intestinal anastomosis healing model in rats to study *in vivo* safety. The peptide was formulated in either water or in SH.

The results showed that PXL01 was able to kill both Gram-positive and Gram-negative bacteria *in vitro* using standard assay medium (**Table 4**). The peptide possessed anti-inflammatory properties by inhibiting secretion of proinflammatory cytokines/chemokines from LPS-stimulated macrophages as well as anti-inflammatory/fibrinolytic properties by inhibiting secretion of PAI-1 from IL1-β-stimulated mesothelial cells, without showing any cytotoxicity. *In vivo*, PXL01 in SH showed a significant anti-adhesion effect, which was more pronounced compared to water solution of PXL01 or SH *per se*. Further, PXL01 in SH demonstrated no adverse effects on the healing potential of the anastomosis. In addition, *in vitro* release experiments demonstrated a rapid release of PXL01 from SH, with 70% released within 1 hour.

Table 4. *Compilation of the MMC_{99} values of PXL01, PXL150, HLR1r, CEN1 HC, and controls from paper I, II, III, V, and VI.*

Assay medium	Strain	MMC_{99} (µg/ml)					
		PXL01	**PXL150**	**HLR1r**	**CEN1 HC**	**Mup**	**Fus**
BHI_{dil}	*S. aureus*	12.5	3.1	6	12.5	3.1	6.3
	MRSA	-	3.1	6	6.3	3.1	6.3
	*S. aureus**	-	3.1	-	-	-	-
	S. pyogenes	-	3.1	3	3.1	6.3	>200
	S. epidermidis	-	3.1	-	3.1	3.1	6.3
	P. acnes	-	6.3	50	6.3	>200	-
	P. aeruginosa	25	6.3	6	12.5	>200	>200
	E. coli	12.5	6.3	3	6.3	>200	>200
	K. pneumoniae	-	6.3	-	3.1	>200	>200
	A. baumannii	-	3.1	-	3.1	>200	>200
	C. albicans	-	3.1	3	6.3	>200	>200
	C. parapsilosis	-	6.3	-	-	-	-
	C. krusei	-	6.3	-	-	-	-
	C. glabrata	-	12.5	-	-	-	-
SWF_{dil}	*S. aureus*	-	50	>200	25	3.1	6.3
	MRSA	-	50	>200	25	3.1	6.3
	S. pyogenes	-	-	>200	50	3.1	>200
	S. epidermidis	-	-	-	12.5	3.1	6.3
	P. acnes	-	-	>400	12.5	>200	-
	P. aeruginosa	-	-	>200	100	>200	>200
	E. coli	-	-	>200	25	>200	>200
	K. pneumoniae	-	-	-	12.5	>200	>200
	A. baumannii	-	-	-	6.3	>200	>200
	C. albicans	-	-	-	>200	>200	>200
BHI_{dil} + 85 mM NaCl	*S. aureus*	-	-	>200	25	-	-
	P. aeruginosa	-	-	25	12.5	-	-
	E. coli	-	-	25	-	-	-
BHI_{dil} + 150 mM NaCl	*S. aureus*	-	-	>200	50	-	-
	P. aeruginosa	-	-	>200	12.5	-	-
	E. coli	-	-	>200	-	-	-
0.01% HPC	*P. aeruginosa*	-	1.56	-	-	-	-
0.1% HPC	*P. aeruginosa*	-	0.78	-	-	-	-
0.5% HPC	*P. aeruginosa*	-	<0.01	-	-	-	-

*Data are presented as maximum values from at least two independent experiments. MMC, minimum microbicidal concentration; Mup, mupirocin; Fus, fusidic acid; BHI_{dil}, 100 × diluted brain–heart infusion broth; SWF_{dil}, 2 × diluted simulated wound fluid; HPC, hydroxypropyl cellulose; res., resistant; –, not determined. * Resistant to fusidic acid,*

4.2. Paper II

In paper II, we evaluated the potential of five AMPs derived from centrocin 1 for local treatment of infections. For this purpose, *in vitro* assays for microbicidal activity, anti-inflammatory effect, cytotoxicity, and resistance development were used, as well as an *ex vivo* pig skin wound infection model and an *in vivo* excision wound infection model in rats. The peptides were formulated in water.

The results showed that the AMPs had similar microbicidal activities against Gram-positive and Gram-negative bacteria *in vitro* using standard assay medium. The bactericidal effect was only slightly reduced for CEN1 HC and CEN1 HC-Br using high serum and/or salt conditions, while C-terminal truncation and cysteine to serine substitution resulted in a pronounced decrease in activity. The AMPs also displayed anti-inflammatory properties by inhibiting secretion of TNF-α and/or IL-6 from LPS-stimulated macrophages, and C-terminal truncation resulted in reduced efficacy. No cytotoxicity was observed for the debrominated peptides; however, CEN1 HC-Br showed cytotoxicity and thus the suppression of cytokine secretion observed for this peptide may, at least partly, be due to a decline in cell viability. Since CEN1 HC demonstrated the best efficacy and safety profile *in vitro*, it was selected for further studies.

The microbicidal action of CEN1 HC was investigated against a wide range of microorganisms. Using standard assay conditions, CEN1 HC showed pronounced effect against both Gram-positive and Gram-negative bacterial strains, including multidrug-resistant strains, and against the fungus *C. albicans*, with MMC values similar or better than mupirocin and fusidic acid (**Table 4**). Using SWF_{dil}, the bactericidal activity of CEN1 HC was slightly reduced against bacteria and impaired against *C. albicans* ($MMC_{99} > 200\mu g/ml$). Under selection pressure *in vitro* for 14 days, no resistance development to CEN1 HC was observed for *S. aureus* or MRSA, whereas resistance to mupirocin was developed. In the *ex vivo* model, CEN1 HC significantly reduced viable counts of *S. aureus* in a dose–response fashion, with the highest concentration (2 mg/ml) resulting in a 99% reduction compared with water (**Fig. 10**). Further, CEN1 HC reduced viable counts of both *P. aeruginosa* and MRSA *in vivo*, where the highest concentration (2 mg/ml) resulted in a 99% and 78% reduction, respectively, compared with water (**Fig. 11**).

4.3. Paper III

In paper III, we evaluated the potential of PXL150 for safe and effective topical treatment of SSTIs. For this purpose, *in vitro* assays for microbicidal activity, anti-inflammatory effect, cytotoxicity, hemolytic effect, and resistance development were

used, as well as an *ex vivo* wound infection model on pig skin and an *in vivo* excision wound infection model in rats. A preliminary assessment of acute systemic toxicity using a single SC injection of PXL150 was also performed. PXL150 was formulated in water.

The results showed that PXL150, under standard assay conditions, killed a broad spectrum of Gram-positive and Gram-negative bacteria, including multidrug-resistant strains, as well as several *Candida* strains, with MMC values similar or better than mupirocin and fusidic acid (**Table 4**). The effect against *S. aureus* and MRSA was negatively influenced by the salt and/or serum in SWF_{dil}, which was not the case for the antibiotics. PXL150 demonstrated no significant hemolytic effect against human erythrocytes, suggesting a selectivity of PXL150 for bacteria *versus* human cells. Under selection pressure *in vitro* for 21 days, no resistance development to PXL150 was observed for *S. aureus* and MRSA, whereas resistance to gentamicin was developed. PXL150 caused a rapid depolarization of the cytoplasmic membrane of *S. aureus*, where both time and concentration correlated with the microbicidal activity. PXL150 showed anti-inflammatory activity by inhibiting secretion of TNF-α from LPS-stimulated macrophages and secretion of PAI-1 from IL-1β-stimulated mesothelial cells, with no cytotoxicity observed. In the *ex vivo* model, PXL150 significantly reduced viable counts of *S. aureus* compared with water in a dose–response fashion, with the highest concentration (2 mg/ml) resulting in a > 99% reduction of bacteria compared with water (**Fig. 10**). Further, PXL150 reduced viable counts of MRSA in the *in vivo* excision wound infection model, where the highest concentration (2 mg/ml) resulted in a 88% reduction compared with water (**Fig. 11**). A kinetic study using the same *in vivo* model demonstrated that the antimicrobial effect of PXL150 was rapid (at 30 minutes after treatment, 5 mg/ml caused a > 99.9% reduction compared with water) and lasted for at least 4 hours after treatment. Finally, PXL150 showed no signs of systemic toxicity after SC administration.

In summary, the *in vitro* experiments demonstrated that PXL150 exhibited a broad microbicidal effect, at least in part caused by depolarization of the bacterial cytoplasmic membrane, no hemolytic effect, and no propensity to select for resistance, in combination with an anti-inflammatory action. In *ex viv*o and *in vivo* studies, the peptide demonstrated pronounced anti-infective effect and no signs of local adverse effects or systemic toxicity.

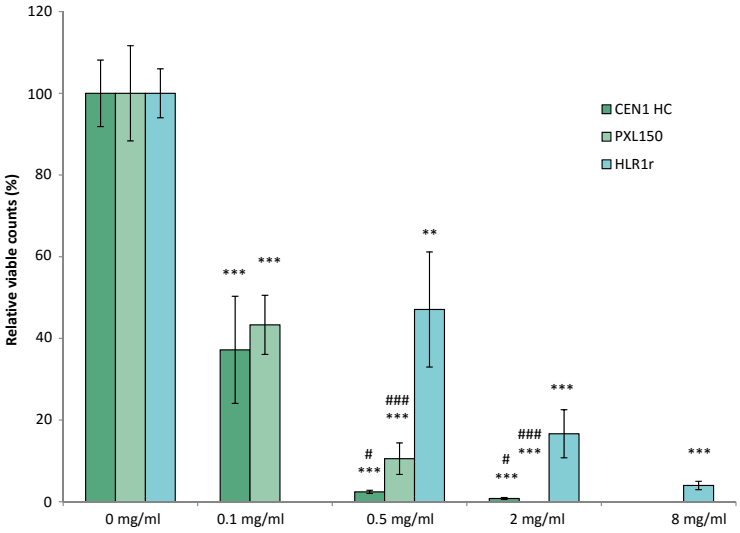

Figure 10. *Compilation of the anti-infectious effect of three of the AMPs against S. aureus using the ex vivo pig skin wound infection model (paper II, III, VI). The concentration of 0.1 mg/ml was only tested for CEN1 HC and PXL150. The concentration of 8 mg/ml was only tested for HLR1r. Data are presented as average relative viable counts (%) compared with placebo (water) ± SEM. The statistical methods used were unpaired Student's t-test (CEN1 HC and PXL150) and Mann–Whitney U test (HLR1r). #, p ≤ 0.05; **, p ≤ 0.01; ***, ###, p ≤ 0.001, where the comparison between the placebo group and AMP is indicated by "*" and the comparison between 0.1 mg/ml and the higher concentrations is indicated by "#".*

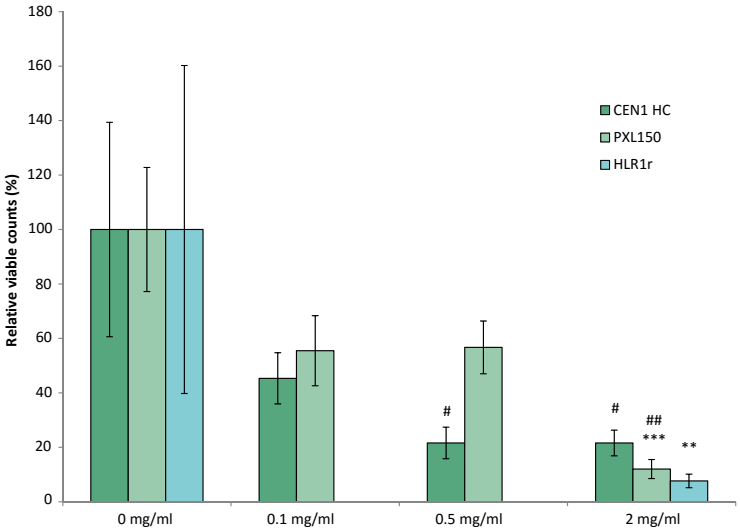

Figure 11. *Compilation of the anti-infectious effect of three of the AMPs against MRSA using the in vivo excision wound infection model in rats (paper II, III, VI). The concentrations of 0.1 and 0.5 mg/ml were only tested for CEN1 HC and PXL150. Data are presented as average relative viable counts (%) compared with placebo (water) ± SEM. The statistical methods used were unpaired Student's t-test (CEN1 HC and PXL150) and Mann–Whitney U test (HLR1r). #, p ≤ 0.05; **, ## p ≤ 0.01; *** p ≤ 0.001, where the comparison between the placebo group and AMP is indicated by "*" and the comparison between 0.1 mg/ml and the higher concentrations is indicated by "#".*

4.4. Paper IV

In paper IV, we continued to evaluate the potential of PXL150 for safe and effective treatment of topical infections, now with focus on infected surgical wounds. For this purpose, a surgical site infection model in mice was used and the peptide was formulated in either water or in HPC.

The results showed that PXL150 in water caused a significant dose–response reduction of *S. aureus* compared with water. Further, PXL150 in HPC caused a significant dose–response reduction of *S. aureus* compared with both water and HPC, with the highest concentration tested (10 mg/g) resulting in > 95% reduction compared with water. HPC showed no significant effect *per se* and no synergistic effect together with the peptide. Notably, the peptide was more efficient in water than in HPC.

4.5. Paper V

In paper V, we continued to evaluate the potential of PXL150 for safe and effective treatment of topical infections, now with focus on infected burn wounds. To study the efficacy, we used an *in vitro* microbicidal assay and an *in vivo* mouse model of infected burn wounds. To study the *in vivo* safety, we performed a nonclinical safety program of repeated dose toxicity in rats and rabbits. PXL150 was formulated in either aqueous (or saline) solution or in HPC.

The results confirmed the previous results of paper III that PXL150 in BHI_{dil} efficiently killed *P. aeruginosa in vitro*. Upon formulation in HPC, the effect was enhanced with increasing concentrations of HPC. The highest concentration of HPC (1.5%) had a clear microbicidal effect *per se in vitro*, although the lower concentrations did not show any detectable killing activities *per se* (< 98% killing). In the infected burn wound model, PXL150 in HPC (in concentrations of 2.5 mg/g or higher) caused a pronounced anti-infective effect after one day of treatment. PXL150 in HPC administered twice daily for four consecutive days resulted in significantly reduced *P. aeruginosa* compared with no treatment and HPC, with 5 mg/g resulting in a > 99.9% reduction compared with no treatment. No further increase in efficacy was observed for the higher concentrations. Unlike the *in vitro* experiment, HPC had no significant antibacterial effect *per se in vivo*. Notably, PXL150 in water was not tested in this model and thus any possible synergistic effect of HPC and PXL150 could not be determined.

High doses of PXL150 in saline induced some local reactions in the rats, but there were no findings (except for a change in blood cell counts in some rats with unknown cause) indicating any treatment-related systemic toxicity after 5 and 14 days of daily SC administrations in the feasibility and pivotal toxicity studies in rats, re-

spectively (**Table 5**). No toxicokinetic analysis was possible since the plasma concentrations of PXL150 were below limit of detection. In the rabbits, there were no toxicologically relevant local reactions and no signs of any treatment-related systemic toxicity after 28 days of daily topical administrations of clinically relevant doses of PXL150 in HPC (1 ml of 2 mg/g and 10 mg/g PXL150 in HPC). Further, no treatment-related abnormalities were found in rabbits receiving a single ocular dosage of PXL150.

4.6. Paper VI

In paper VI, we evaluated the potential of HLR1r for local treatment of SSTIs. For this purpose, *in vitro* assays for microbicidal activity, anti-inflammatory effect, and cytotoxicity were used, as well as a mouse model of cutaneous candidiasis, an *ex vivo* wound infection model on pig skin, and an *in vivo* excision wound infection model in rats.

The results showed that HLR1r displayed a broad spectrum of microbicidal activity against both Gram-positive and Gram-negative bacteria and *C. albicans* under standard assay conditions, with low MMC_{99} values for all microbes except the anaerobic *P. acnes* (**Table 4**). In the presence of salt and/or serum, the bactericidal activity was markedly reduced or abolished against all tested strains ($MMC_{99} > 200\mu g/ml$). HLR1r demonstrated anti-inflammatory efficacy by reducing LPS-induced release of TNF-α from macrophages and by repressing IL-1β-induced secretion of IL-6 and PAI-1 from mesothelial cells, without showing any cytotoxicity. In the mouse model of cutaneous candidiasis, two treatments of HLR1r caused a significant antifungal effect compared with water, with the highest concentration tested (20 mg/ml) resulting in a 75% reduction of viable cells. In the *ex vivo* model, HLR1r significantly reduced the viable counts of *S. aureus* in a dose–response fashion compared with water, with the highest concentration tested (8 mg/ml) resulting in a $\geq 95\%$ reduction compared with water **(Fig. 10)**. In the excision wound infection model, HLR1r (2 mg/ml) caused a significant reduction of viable counts of MRSA compared with water (92%) **(Fig. 11)** and compared with 20 mg/g mupirocin.

5. Discussion

5.1. Methodological considerations

Preclinical research involves evaluation of potential therapeutic interventions using *in vitro, ex vivo*, and *in vivo* assays. To draw accurate conclusions about the safety and clinical benefit of new drug candidates, clinical studies on humans ultimately need to be performed. However, clinical studies cannot be performed without a solid set of data supporting the safety and efficacy from preclinical models mimicking the clinical situation. It must be emphasized that such models are simplified versions of the reality. The living body is an extremely complex functional system, thus it is difficult to study specific mechanisms and interactions in such a system. Using *in vitro*/*ex vivo* assays, a specific property can be studied without the interference of systemic factors, such as other cell types or soluble mediators. *In vivo* studies, on the other hand, offer a whole-body approach; however, due to species variability precautions should always be taken when extrapolating the results from preclinical models to the clinical situation. Importantly, no single preclinical method/model can provide all the desired information and therefore several complementary methods must be used (as has been done in this thesis), together creating a more accurate prediction of the efficacy and safety in humans.

5.2. *In vitro* antimicrobial effect and mechanism

5.2.1. *In vitro* microbicidal effect

Under standard low-salt conditions (BHI_{dil}) in the MMC assay, the tested peptides displayed similar microbicidal potency, although HLR1r was less efficient against *P. acnes* compared with other peptides (paper I, II, III, VI) (**Table 4**). Of the five variants of CEN1 HC-Br studied in paper II, no differences in bactericidal activity were observed. In comparison to the antibiotic controls, the AMPs tested displayed a wider activity spectrum, covering both Gram-positive (aerobic and anaerobic) and Gram-negative bacteria, as well as *Candida* species (only tested for CEN1 HC, PXL150, and HLR1r).

Dissolving an AMP in a formulation for clinical use might affect the microbicidal properties of the product; the formulation can have effect *per se* and the effect of the AMP can be altered. In paper V, 1.5% HPC alone displayed a bactericidal effect against *P. aeruginosa in vitro*. In addition, HPC showed synergistic effects together with PXL150, substantially increasing the antibacterial activity of the peptide.

5.2.2. *In vitro* salt and serum sensitivity

The microbicidal activities of all AMPs tested were lower in SWF_{dil} as compared to in BHI_{dil} (paper I, II, III, VI). Although negatively affected by salt and/or serum in SWF_{dil}, CEN1 HC and PXL150 still exerted a bactericidal effect, while the activity of HLR1r was abolished ($MMC_{99} > 200$ µg/ml) (PXL01 was not tested) (**Table 4**). Neither the effect of mupirocin nor fusidic acid was sensitive to the salt or serum, as reflected by a preserved effect in SWF_{dil}. The salt insensitivity of these antibiotics is confirmed in the literature [229, 272], while the lower effect of the antibiotics in serum described in the literature [229, 231, 232] was not observed in our experiments.

The relative salt tolerability of CEN1 HC, may reflect the assumption that AMPs from marine organisms are more tolerant to high-ionic strength conditions since they are functioning *in vivo* in salt-rich environments. Notably, CEN1 HC (Ser) had lower microbicidal activity under salt/serum conditions than its unsubstituted variant with a free cysteine, hence the free cysteine seemed to be important for the activity under such circumstances (paper II). The observation that the C-terminally truncated peptides, also lacking the cysteine, were more susceptible to salt/serum in the medium (paper II), further support this claim. In the literature, the importance of free cysteines in AMPs seems to vary between peptides [273, 274].

In general, the negative impact of salt on bactericidal effect of the AMPs appeared to be more pronounced against the Gram-positive *S. aureus* than against the Gram-negative *E. coli* and *P. aeruginosa*. This could possibly be due to differences in cell envelope properties of Gram-positive and Gram-negative bacteria. However, this difference in salt sensitivity could not be unambiguously confirmed in the literature [108, 275, 276].

Consistent with the findings in this thesis, several other research groups have previously reported that many AMPs display strong antimicrobial activity at low salt and serum concentrations *in vitro* but at physiological concentrations *in vitro* the effect is often considerably weaker. However, as mentioned in the introduction, this does not necessarily translate to the *in vivo* situation since many studies demonstrate that salt-sensitive AMPs have ability to reduce bacterial loads in infection models *in vivo* [125-127].

5.2.3. Mechanism of action (MOA)

To study the MOA of PXL150 to kill bacteria, the $DiSC_3(5)$ assay in combination with viable cell counts was used (paper III). PXL150 caused a dose-dependent increase in fluorescence, with maximum depolarization occurring within 2–5 minutes after addition of the AMP. The depolarization time reported in the literature for different AMPs ranges from a few minutes up to 60 minutes [277, 278], thus PXL150 belongs to the group of AMPs causing a very rapid membrane permeabilization.

In general, AMPs show substantial heterogeneity in this assay when it comes to correlation between depolarization and killing, thus supporting the concept of AMPs having multiple targets for bactericidal activity; membrane disruption and/or internal targets [63]. For example, it has been reported that polymyxin rapidly kills bacterial cells without causing any pronounced depolarization, whereas gramicidin S causes rapid depolarization but slow killing [64]. The latter further supports the theory that membrane permeabilization is not always *per se* a lethal event, but may be required in order to reach an intracellular target [63, 64, 241]. For PXL150, we found a clear correlation between membrane permeabilization and bacterial killing with respect to both time and concentration, indicating that membrane depolarization is at least one part of the mechanism of PXL150 to kill target bacteria. Notably, a much higher concentration of PXL150 was needed to kill *S. aureus* in this assay compared to in the MMC assay using BHI_{dil}. This finding has been reported by others and could possibly be due to the presence of high concentrations of KCl (100 mM) in the depolarization medium [241, 277].

Besides being used for evaluation of AMPs on cytoplasmic membranes of Gram-positive bacteria [241, 279], the model, with some modifications, has also been shown useful on the Gram-negative species *E. coli* and *P. aeruginosa* [63, 64, 275, 277, 280]. In this thesis; however, we only evaluated PXL150 on the Gram-positive *S. aureus*.

5.3. *In vitro* resistance development

Bacteria are suggested to be less efficient in developing resistance towards AMPs than towards conventional antibiotics, possibly due to the multiple mechanisms and targets of AMPs [28, 34, 65]. To study resistance development, bacteria were cultured under selection pressure of peptides in a multistep resistance assay (paper II and III). For PXL150 and CEN1 HC, no resistance development was observed in *S. aureus* and MRSA during serial passage in sub-MIC concentrations of the peptides during 12-21 passages. This low ability to select for resistance has been observed for other AMPs in similar assays [103, 134], although the opposite has also

been shown [136, 138]. In contrast, within the same number of passages, we found that pronounced resistance development towards the two antibiotic controls, gentamicin and mupirocin, occurred in the two bacterial strains. This discrepancy between AMPs and conventional antibiotics in terms of propensity to select for resistance, has previously been reported by other groups [103, 134].

5.4. *In vitro* selectivity and toxicity

To determine the selectivity of AMPs for microbial cells *versus* human cells, hemolytic activity to human erythrocytes and cytotoxicity to human cell lines were assessed. A large difference between hemolytic (or cytotoxic) concentration *versus* antimicrobial concentration is desirable for a safe AMP (i.e. high therapeutic index) [275, 281]. Of the peptides studied, only PXL150 was assayed for its ability to cause hemolysis (paper III). PXL150 did not display any significant hemolytic activity using the same concentrations as tested in the MMC assay indicating a high selectivity, although it should be noted that the two assays use different assay media. In addition, no peptide, except CEN1 HC-Br, displayed significant cytotoxicity against human cell lines using the same concentrations as were effective in anti-inflammatory/fibrinolytic assays using these cells, thus also indicating a high selectivity (paper I, II, III, VI).

5.5. *In vitro* immunomodulatory effect

In this thesis, we studied the immunomodulatory properties of the AMPs with focus on anti-inflammatory and fibrinolytic activities. For this purpose we used macrophages (differentiated THP-1 cells) and mesothelial cells (MeT-5A), both cell types playing an important part of the innate immunity by e.g. producing mediators involved in the inflammatory and fibrinolytic response [13, 20, 179, 246]. Using these cells, the AMPs tested had ability, although to varying extent, to suppress the secretion of proinflammatory cytokines and the chemokine IL-8, in response to LPS and/or IL-1β stimulation, indicating anti-inflammatory properties (paper I, II, III, and VI). Although some AMPs are known to bind and sequester LPS from interacting with the TLR4/CD14 receptor complex, the suppression of cytokines observed could not be explained by such a LPS-scavenging effect only. This is because the peptides were added to the cells 30 minutes after addition of LPS, thereby giving the LPS sufficient time to bind LBP and TLR4/CD14 receptor complex [75, 159].

Besides the classical role of PAI-1 as a fibrinolysis inhibitor with increased levels of PAI-1 associated with scarring/adhesion formation [182], PAI-1 is also a mediator

of inflammatory response [20-22]. In this thesis, PXL01, PXL150, and HLR1r suppressed the secretion of PAI-1 by IL-1β-stimulated MeT-5A cells, thereby exerting both anti-inflammatory as well as anti-scarring/adhesion properties (paper I, III, and VI). Notably, these immunomodulatory activities were present in assay medium containing both salt and serum, suggesting a salt/serum insensitive effect.

Further, no peptide, except CEN1 HC-Br, displayed cytotoxicity against the human cell lines tested and thus the possibility that the suppression of the production of inflammatory/fibrinolytic markers by the peptides could be due to peptide-induced cytotoxicity, could be ruled out.

5.6. *In vivo* efficacy and safety as anti-adhesion agent

5.6.1. Postsurgical adhesion model in rats

In this model, PXL01 in SH showed marked ability to prevent adhesion formation and the effect was more pronounced compared to PXL01 in water and SH only (paper I). The limited effect of PXL01 in water could be due to rapid elimination of the water solution from the peritoneal surface, since three administrations of PXL01 in water were somewhat more effective than one administration, thus suggesting a beneficial effect of slow release. The positive effect of combining PXL01 with SH could be explained by SH acting as a physical barrier (since SH had effect *per se*), as well as by SH providing a slow release of the peptide. The *in vitro* release experiment indicated a relatively brief period of PXL01 release from SH, suggesting that the duration of the drug release required for adhesion prevention *in vivo* may be rather short. Notably, since the first 36 hours after surgery are suggested to be the most critical for adhesion formation, maintaining sufficient peptide concentration and barrier effect at the site of injury for a rather short time after injury should be sufficient to reduce adhesion formation [248].

5.6.2. Intestinal anastomosis healing model in rats

Since inflammation is a crucial part of the wound healing process, there is a risk that anti-inflammatory substances would negatively affect the healing. For example, non-steroidal anti-inflammatory drugs have shown a suppressive effect on wound healing [282]. In the intestinal anastomosis healing model in rats, PXL01 in SH did not show

any negative impact on the healing of the anastomosis, although exhibiting anti-inflammatory properties *in vitro* (paper I).

5.7. *In vivo* efficacy and safety as anti-infectious agents

5.7.1. *Ex vivo* pig skin wound infection model

In this assay, the AMPs tested significantly reduced the viable counts of *S. aureus* compared with water (paper II, III, VI). Notably, the effective concentrations are much higher in this model (as well as in the other infection models) compared to the bactericidal concentrations *in vitro*, a finding that we have observed in publications of other AMPs as well [147, 150]. Generally, *in vitro* results are not directly translatable to the *ex vivo* and *in vivo* situation. This may be due to environmental effects of the wound milieu on the activity of AMPs, such as salt, serum, proteases, pH and/or accessibility of AMPs to the bacteria.

5.7.2. Excision wound infection model in rats

In this model, all peptides tested, except CEN1 HC (Ser), significantly reduced the viable counts of MRSA compared with water (paper II, III, VI) and they were also more efficient than mupirocin (paper VI). CEN1 HC was also effective against *P. aeruginosa* infection (the only peptide tested). Notably, a rapid reduction (within 30 minutes) of viable counts was achieved by PXL150, which is in line with the previous reports on killing activities of AMPs *in vitro* [101-103]. Ultrapure water also resulted in significant decrease of viable counts using this model; however, after 1 hour the bacteria started to grow in contrast to PXL150 where the effect lasted for at least 4 hours. This phenomenon could possibly be explained by the ultrapure water disrupting the bacteria by means of osmotic pressure [283].

5.7.3. Surgical site infection model in mice

In this model, stationary phase bacteria were used to inoculate the wounds, in contrast to the other infected wound models using actively replicating bacteria. It could therefore be argued that the two-hour interval was too short to allow log-phase

growth and to create an established infection. However, the viable counts of the bacteria in the wounds increased exponentially during the first 4 hours, reaching $> 10^5$ CFU already within 2 hours (paper IV), which is the generally accepted bacterial load indicating an established wound infection in clinic [190].

PXL150 caused significant reduction of viable counts compared with water and HPC, with a more pronounced effect when dissolved in water compared with when formulated in HPC (paper IV), possibly due to a slow release of the peptide from HPC resulting in a lower actual concentration of the peptide in the wound. The level of reduction caused by PXL150 was comparable to that of repeated administration of retapamulin and mupirocin ointments reported for this model in the literature [264]. The formulation used in this study, HPC, showed no effect on bacterial counts *per se*, compared with administration of water only. This is in contrast to several other formulations used in this model, which have been reported to cause an increase in bacterial load, possibly by increasing the moisture content of the wound by preventing water loss or by supporting the growth of pathogens *per se* [267].

5.7.4. Infected burn wound model in mice

In this assay, twice-daily application of PXL150 in HPC showed a rapid (during the first day) and pronounced anti-infective effect (paper V). This very rapid effect is consistent with our finding that PXL150 quickly depolarizes the bacterial membrane *in vitro* and reduces viable counts within 30 minutes (paper III).

Burns are commonly associated with biofilm formation; however, a two-hour time interval between infection and treatment initiation used in this model is too short to allow formation of mature *P. aeruginosa* biofilms (10–72 hours are required [284, 285]). Nevertheless, *P. aeruginosa* isolated from burn wounds produces biofilm exopolysaccharide matrix already 5 hours after inoculation *in vitro*, suggesting that wound bacteria possess ability to build a biofilm early in the infection process [284] and thus efforts to prevent or inhibit bacterial growth and biofilm formation should preferably be made as soon as possible after wounding/infection.

5.7.5. Cutaneous candidiasis model in mice

In this model, HLR1r demonstrated significant ability to reduce viable counts compared with water (paper VI). The AMP was more effective in comparison to previously related lactoferrin-derived peptides [169]. Compared to the other *in vivo/ex vivo* infection models, concentrations needed to efficiently reduce viable counts were

approximately 10 × higher for the candidiasis model; however, 10–100 × more cells were added per wound compared to the bacterial loads used in the other assays.

5.7.6. Nonclinical safety studies

Due to their complex MOA, there is a general concern regarding the safety of AMPs as pharmaceuticals (in particular for systemic administration), although this issue has not been well studied and/or documented [26, 52]. In this thesis, all peptides studied in the *in vivo* efficacy models were well tolerated without causing any adverse events. Notably, PXL150 was the only peptide assessed in nonclinical safety studies to support clinical trials (paper V). In clinic, PXL150 in HPC is planned to be dermally applied in a maximal concentration of 10 mg/ml, 0.3 ml per application (twice per day during 5 days), resulting in clinical doses of 0.005–0.05 mg/kg for a patient of 60 kg and 0.03–0.3 mg/kg for a child of 10 kg. The dose levels in the nonclinical safety studies were selected to demonstrate sufficient safety margins compared to the targeted dose range in clinical application (**Table 5**), which according to ICH guidelines should be up to a 50 × margin (usually based on plasma levels) for acute and repeated dose toxicity [271].

In the preliminary study of single dose toxicity, PXL150 did not cause any clinical or behavioral signs of systemic toxicity in rats after a SC injection with doses 50 and 250 × higher than the highest planned clinical dose in adults (**Table 5**). In the comprehensive nonclinical safety program, two repeated dose toxicity studies were performed in rats; a 5-day feasibility study and a 14-day pivotal study, both using SC administrations of PXL150. Although there were findings of local reactions at some of the treatment sites in both studies, there were no findings of concern indicating any treatment-related systemic toxicity. Further, although very high doses were administered in the feasibility study, blood plasma contained undetectable levels of PXL150, suggesting that the systemic exposure of the peptide was very low. The highest dose used in rats (5 mg/kg) was 100–1000 × higher than the estimated adult dermal dose in clinic and 16.7–167 × higher than the targeted dose in children, hence the peptide demonstrated sufficient safety margins for systemic toxicity, at least for adults (**Table 5**).

In the local tolerance study in rabbits, clinically relevant doses of PXL150 in HPC (i.e. 2 mg/g and 10 mg/g PXL150 in HPC), dermally administered on both healthy and abraded skin for 28 days, were shown to be safe and well tolerated. Further, since it is warranted that local tolerance at sites that accidentally could be exposed to the product should be determined, ocular tolerance towards the product was evaluated, revealing no treatment-related abnormalities in rabbits receiving a single ocular dosage of PXL150.

Table 5. *Dose levels of PXL150 administered in rats in nonclinical safety studies and the corresponding multiples of estimated clinical doses.*

Study	Dose level (mg/kg)	Multiples of low adult dose 0.3 mg, 0.005 mg/kg	Multiples of high adult dose 3 mg, 0.05 mg/kg	Multiples of low child dose 0.3 mg, 0.03 mg/kg	Multiples of high child dose 3 mg, 0.3 mg/kg
Preliminary single dose study	2.5	500	50	83	8.3
	12.5	2500	250	417	41.7
5-day pilot study	10	2000	200	333	33
	50	10000	1000	1667	167
14-day pivotal study	1	200	20	33	3.3
	5	1000	100	167	16.7

5.8. Potential and challenges

As described in the Introduction, AMPs have several advantages in comparison to conventional antibiotics. One of these advantages is bactericidal effect, which was confirmed for the AMPs in this study using the *in vitro* MMC assay. Another advantage is the rapid effect of the AMPs, and using the membrane permeabilization assay, a very rapid effect was indeed observed for PXL150. Furthermore, AMPs are generally described to have a broad spectrum of activity, and this was demonstrated for the peptides investigated in this thesis as well, since they were able to kill both Gram-positive and Gram-negative species, including multidrug-resistant strains, as well as yeast, both *in vitro* and *ex vivo*/*in vivo*. The activities were, to a varying extent, suppressed by addition of salt and/or serum in the assay medium *in vitro*, which is commonly reported for AMPs. However, since the peptides exerted *in vivo* activities together with immunomodulatory effects *in vitro* (in the presence of salt and serum in cell assay medium), the salt/serum sensitivity might not give any reason for concern. Although bacteria are described to have less probability to develop resistance to AMPs compared to towards conventional antibiotics, there are recent studies showing that bacteria quite rapidly can acquire resistance to certain AMPs under selection pressure *in vitro* [132, 138]. Notably, none of the two peptides evaluated in the resistance development assay in this thesis selected for resistance development in bacteria.

As described in details in the Introduction, the challenges generally associated with the development of AMPs as therapeutic agents, include high manufacturing

costs, sensitivity toward proteolytic degradation, and potential risk for toxicity. The peptides tested in this thesis were produced using SPPS, which is an expensive method, and since some of the peptides tested are of considerable length, production costs will be relatively high at least compared to conventional antibiotics. To reduce costs, shortening of the peptides could be considered, as well as change of production method. However, compared to the economic burden in terms of increased healthcare costs and loss of productivity associated with antibiotic-resistant infections [286], a higher price compared to conventional antibiotics might be justified for effective and safe lifesaving products.

Since AMPs in this thesis were evaluated for indications requiring local application, there is no great concern for systemic toxicity. Notably, in the nonclinical safety studies performed on PXL150, the peptide appeared to be well tolerated and safe. In addition, the plasma levels of PXL150 were low, suggesting a low systemic exposure. Moreover, based on the rapid local action of the AMPs (at least PXL150), the possible protection offered by the formulations used (SH and HPC), and, most importantly, the proven effect of the peptides *in vivo*, there is no great concern regarding the *in vivo* stability of the peptides.

6. Conclusion

In summary, the results showed that all AMPs tested (PXL03, PXL150, HLR1r, and five variants of CEN1 HC-Br) had broad antimicrobial spectra *in vitro* with varying sensitivity to salt and serum. Furthermore, PXL150 caused a rapid permeabilization of bacterial membrane *in vitro*, indicating that this is at least one part of the MOA of this peptide. Under selection pressure *in vitro*, bacteria did not develop resistance to the peptides tested, i.e. PXL150 and CEN1 HC. Interestingly, all peptides tested showed anti-inflammatory activities by inhibiting the secretion of proinflammatory cytokines and chemokines from stimulated human cell lines. In addition, PXL01, PXL150, and HLR1r demonstrated fibrinolytic ability *in vitro* by suppressing the release of PAI-1. In *ex vivo* and *in vivo* skin/wound infection models, the peptides reduced the number of viable bacterial and yeast cells. Further, PXL01 decreased postsurgical adhesion formation *in vivo* without negatively affecting the wound healing. Notably, nonclinical studies showed that PXL150 was safe and well tolerated for topical application in the intended clinical concentration.

In conclusion, several of the peptides evaluated in this thesis demonstrated a promising preclinical efficacy and safety profile motivating further development as drug candidates for local treatment of infectious and inflammatory conditions.

7. Future perspective

Several follow-up experiments would be of interest to perform. A biofilm study has already been initiated to test the ability of the AMPs in this thesis to prevent formation and/or cause eradication of bacterial biofilms. As a first step, we have recently implemented an *in vitro* biofilm model according to previously published methods [287-289], in which bacteria are grown in microplate wells, followed by addition of AMPs at different time points and staining of the biofilm with crystal violet. Starting from this simple model, the plan is then to further modify and refine the model, to be able to test different substrate materials for biofilm growth, different staining techniques, and different visualization methods.

A protease sensitivity experiment would also be of interest to perform, particularly if the planned route of administration is changed from topical to systemic. This could be done by incubating the peptide with proteases followed by separation of the components in the incubation mixture by dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent staining with Coomassie blue [147, 150]. Using this method, proteolytic degradation is observed as reduced/absent staining compared to untreated control.

The main focus of this thesis was preclinical efficacy and safety characterization of the AMPs, with only limited focus on the MOA. A deeper characterization of the structure activity relationship of the AMPs to gain understanding of the different structural parameters important for antimicrobial and anti-inflammatory action would be of interest; however, these studies were beyond the scope of this thesis.

Notably, based on the findings of this thesis, PXL01 has been further studied in efficacy studies *in vivo* with regards to its ability to reduce adhesion formation after tendon surgery in rabbits [290, 291]. Recently, the efficacy and safety of PXL01 as an anti-adhesion agent for prevention of adhesion formation after hand surgery, has been assessed in a phase II randomized controlled clinical trial. The study showed that a single treatment with PXL01 in SH reduced adhesion formation after flexor tendon repair surgery [292]. The peptide is currently further evaluated in a phase III trial.

I hope that my research will contribute to the development of new, effective, and safe drugs that will improve the health of people.

Acknowledgements

I would like to thank everyone who in one way or another has supported me during my PhD studies and contributed to the work of this thesis. Especially I would like to thank:

My main supervisor Margit Mahlapuu. I have learned so much from you. Your drive and energy is impressive and inspiring. Thank you for all the time and effort you have invested in me and in the work of this thesis.

My co-supervisor and colleague Joakim Håkansson. Thanks to your recommendation, I ended up at SP. Thank you for all your support.

My co-supervisor Inger Mattsby-Baltzer. Your expertise in this research field has been highly valuable to me.

My boss Benny Lyvén. Thank you for employing me and for giving me this once in a lifetime opportunity to pursue PhD studies.

My colleagues at SP. It is such a joy working with you. A special thanks to you Patrik, for guiding me through all practicalities associated with PhD studies and for the photo on the cover of this thesis. Thank you, Jukka and Johan, for proofreading this thesis.

My former colleagues at PharmaSurgics and Pergamum. Thank you for those great years. Especially I would like to thank you Mikael, for helping me to get my first job in the Biotech industry, and you Kerstin for everything you taught me.

All co-authors of the papers in this thesis. Thanks for all your hard work and great teamwork.

My fellow (and former) members of Margit's research group, especially Manoj and Emmelie, for all information provided and all questions answered.

Slutligen vill jag rikta ett tack till:

Mina vänner utanför jobbet. Tack för all glädje ni ger mig. Tack för att ni ger mig perspektiv på tillvaron och för att ni inte vet någonting om antimikrobiella peptider.

Mina svärföräldrar Gunvor och Arne. Man kan inte önska sig bättre svärföräldrar än er.

Mina föräldrar Bodil och P-A. Tack för all omsorg, all tid, allt stöd och all kärlek.

Mina systrar Jennie och Natalie. Vi är olika men ända så lika. Tack för att ni alltid finns där för mig.

Mina barn Linnea, Maja och Stina. Jag är så oerhört glad och tacksam att jag har er. Glöm aldrig att ni är viktiga, värdefulla och innerligt älskade.

Min Ove. Tack för att du älskar mig. Trots att jag är den jag är. För att jag är den jag är. Utan dig hade denna avhandling inte blivit skriven.

This work was financially supported by SP Technical Research Institute of Sweden, PharmaSurgics AB, Pergamum AB, Västra Götalandsregionen, Vinnova, Mabit, and University of Tromsø. This work was made in collaboration between SP Technical Research Institute of Sweden and the Department of Molecular and Clinical Medicine, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg.

References

[1] Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell. New York: Garland Science; 2002.

[2] Si-Tahar M, Touqui L, Chignard M. Innate immunity and inflammation--two facets of the same anti-infectious reaction. Clin Exp Immunol 2009;156:194-8.

[3] Reddy KV, Yedery RD, Aranha C. Antimicrobial peptides: premises and promises. Int J Antimicrob Agents 2004;24:536-47.

[4] Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 2000;8:402-10.

[5] Wang G, Li X, Wang Z. APD3: the antimicrobial peptide database as a tool for research and education. Nucleic Acids Res 2016;44:D1087-93.

[6] Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nature reviews Microbiology 2005;3:238-50.

[7] Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 1981;292:246-8.

[8] Zasloff M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A 1987;84:5449-53.

[9] Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI. Primary structures of three human neutrophil defensins. J Clin Invest 1985;76:1436-9.

[10] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;415:389-95.

[11] Yount NY, Yeaman MR. Emerging themes and therapeutic prospects for anti-infective peptides. Annu Rev Pharmacol Toxicol 2012;52:337-60.

[12] Turvey SE, Broide DH. Innate immunity. J Allergy Clin Immunol 2010;125:S24-32.

[13] Striz I, Brabcova E, Kolesar L, Sekerkova A. Cytokine networking of innate immunity cells: a potential target of therapy. Clin Sci (Lond) 2014;126:593-612.

[14] Kabelitz D, Medzhitov R. Innate immunity--cross-talk with adaptive immunity through pattern recognition receptors and cytokines. Curr Opin Immunol 2007;19:1-3.

[15] Hancock RE, Nijnik A, Philpott DJ. Modulating immunity as a therapy for bacterial infections. Nature reviews Microbiology 2012;10:243-54.

[16] Beutler B. Innate immunity: an overview. Mol Immunol 2004;40:845-59.

[17] Fournier B, Philpott DJ. Recognition of *Staphylococcus aureus* by the innate immune system. Clin Microbiol Rev 2005;18:521-40.

[18] Sun H. The interaction between pathogens and the host coagulation system. Physiology (Bethesda, Md) 2006;21:281-8.

[19] Hellebrekers BW, Kooistra T. Pathogenesis of postoperative adhesion formation. Br J Surg 2011;98:1503-16.

[20] Wang ZH, Ren WY, Zhu L, Hu LJ. Plasminogen activator inhibitor-1 regulates LPS induced inflammation in rat macrophages through autophagy activation. TheScientificWorldJournal 2014;2014:189168.

[21] Ren W, Wang Z, Hua F, Zhu L. Plasminogen activator inhibitor-1 regulates LPS-induced TLR4/MD-2 pathway activation and inflammation in alveolar macrophages. Inflammation 2015;38:384-93.

[22] Xu X, Wang H, Wang Z, Xiao W. Plasminogen activator inhibitor-1 promotes inflammatory process induced by cigarette smoke extraction or lipopolysaccharides in alveolar epithelial cells. Exp Lung Res 2009;35:795-805.
[23] Hassan M, Kjos M, Nes IF, Diep DB, Lotfipour F. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol 2012;113:723-36.
[24] Marr AK, Gooderham WJ, Hancock RE. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. Curr Opin Pharmacol 2006;6:468-72.
[25] Yeung AT, Gellatly SL, Hancock RE. Multifunctional cationic host defence peptides and their clinical applications. Cell Mol Life Sci 2011;68:2161-76.
[26] Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 2006;24:1551-7.
[27] Jenssen H, Hancock RE. Therapeutic potential of HDPs as immunomodulatory agents. Methods Mol Biol 2010;618:329-47.
[28] Fjell CD, Hiss JA, Hancock RE, Schneider G. Designing antimicrobial peptides: form follows function. Nat Rev Drug Discov 2012;11:37-51.
[29] Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 2001;414:454-7.
[30] Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med 2002;347:1151-60.
[31] Hancock RE, Chapple DS. Peptide antibiotics. Antimicrob Agents Chemother 1999;43:1317-23.
[32] Hancock RE. Cationic antimicrobial peptides: towards clinical applications. Expert Opin Investig Drugs 2000;9:1723-9.
[33] Boman HG. Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 1995;13:61-92.
[34] Lai Y, Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. Trends Immunol 2009;30:131-41.
[35] Bals R. Epithelial antimicrobial peptides in host defense against infection. Respir Res 2000;1:141-50.
[36] Wang G. Improved methods for classification, prediction, and design of antimicrobial peptides. Methods Mol Biol 2015;1268:43-66.
[37] Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. Crit Rev Biotechnol 2012;32:143-71.
[38] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 2003;55:27-55.
[39] Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol 2011;29:464-72.
[40] Takahashi D, Shukla SK, Prakash O, Zhang G. Structural determinants of host defense peptides for antimicrobial activity and target cell selectivity. Biochimie 2010;92:1236-41.
[41] Protein Data Bank in Europe. http://pdbe.org
[42] Epand RM, Vogel HJ. Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta 1999;1462:11-28.
[43] Hunter HN, Demcoe AR, Jenssen H, Gutteberg TJ, Vogel HJ. Human lactoferricin is partially folded in aqueous solution and is better stabilized in a membrane mimetic solvent. Antimicrob Agents Chemother 2005;49:3387-95.
[44] Yount NY, Bayer AS, Xiong YQ, Yeaman MR. Advances in antimicrobial peptide immunobiology. Biopolymers 2006;84:435-58.
[45] Powers JP, Hancock RE. The relationship between peptide structure and antibacterial activity. Peptides 2003;24:1681-91.
[46] Rozek A, Friedrich CL, Hancock RE. Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. Biochemistry 2000;39:15765-74.

[47] Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clin Microbiol Rev 2006;19:491-511.
[48] Bahar AA, Ren D. Antimicrobial peptides. Pharmaceuticals (Basel) 2013;6:1543-75.
[49] Giuliani A, Pirri G, Nicoletto S. Antimicrobial peptides: an overview of a promising class of therapeutics. Cent Eur J Biol 2007;2:1-33.
[50] Ebenhan T, Gheysens O, Kruger HG, Zeevaart JR, Sathekge MM. Antimicrobial peptides: their role as infection-selective tracers for molecular imaging. BioMed research international 2014;2014:867381.
[51] Lin TY, Weibel DB. Organization and function of anionic phospholipids in bacteria. Appl Microbiol Biotechnol 2016;100:4255-67.
[52] Matsuzaki K. Control of cell selectivity of antimicrobial peptides. Biochim Biophys Acta 2009;1788:1687-92.
[53] Jiang Z, Kullberg BJ, van der Lee H, Vasil AI, Hale JD, Mant CT, et al. Effects of hydrophobicity on the antifungal activity of α-helical antimicrobial peptides. Chem Biol Drug Des 2008;72:483-95.
[54] Bowman SM, Free SJ. The structure and synthesis of the fungal cell wall. Bioessays 2006;28:799-808.
[55] Clifton LA, Skoda MW, Le Brun AP, Ciesielski F, Kuzmenko I, Holt SA, et al. Effect of divalent cation removal on the structure of gram-negative bacterial outer membrane models. Langmuir 2015;31:404-12.
[56] Hancock RE. Peptide antibiotics. Lancet 1997;349:418-22.
[57] Dathe M, Nikolenko H, Meyer J, Beyermann M, Bienert M. Optimization of the antimicrobial activity of magainin peptides by modification of charge. FEBS Lett 2001;501:146-50.
[58] Khandelia H, Ipsen JH, Mouritsen OG. The impact of peptides on lipid membranes. Biochim Biophys Acta 2008;1778:1528-36.
[59] Dathe M, Wieprecht T. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. Biochim Biophys Acta 1999;1462:71-87.
[60] Toke O. Antimicrobial peptides: new candidates in the fight against bacterial infections. Biopolymers 2005;80:717-35.
[61] Brandenburg L-O, Merres J, Albrecht L-J, Varoga D, Pufe T. Antimicrobial Peptides: Multifunctional Drugs for Different Applications. Polymers 2012;4:539-60.
[62] Eckert R. Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development. Future Microbiol 2011;6:635-51.
[63] Hancock RE, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. FEMS Microbiol Lett 2002;206:143-9.
[64] Zhang L, Dhillon P, Yan H, Farmer S, Hancock RE. Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2000;44:3317-21.
[65] Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nature reviews Microbiology 2006;4:529-36.
[66] Harris M, Mora-Montes HM, Gow NA, Coote PJ. Loss of mannosylphosphate from *Candida albicans* cell wall proteins results in enhanced resistance to the inhibitory effect of a cationic antimicrobial peptide via reduced peptide binding to the cell surface. Microbiology 2009;155:1058-70.
[67] van der Weerden NL, Bleackley MR, Anderson MA. Properties and mechanisms of action of naturally occurring antifungal peptides. Cell Mol Life Sci 2013;70:3545-70.
[68] Vylkova S, Li XS, Berner JC, Edgerton M. Distinct antifungal mechanisms: β-defensins require *Candida albicans* Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. Antimicrob Agents Chemother 2006;50:324-31.

[69] Helmerhorst EJ, Breeuwer P, van't Hof W, Walgreen-Weterings E, Oomen LC, Veerman EC, et al. The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. J Biol Chem 1999;274:7286-91.

[70] Robinson WE, Jr., McDougall B, Tran D, Selsted ME. Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils. J Leukoc Biol 1998;63:94-100.

[71] Yasin B, Wang W, Pang M, Cheshenko N, Hong T, Waring AJ, et al. θ defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. J Virol 2004;78:5147-56.

[72] Sinha S, Cheshenko N, Lehrer RI, Herold BC. NP-1, a rabbit α-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2. Antimicrob Agents Chemother 2003;47:494-500.

[73] Torrent M, Pulido D, Rivas L, Andreu D. Antimicrobial peptide action on parasites. Curr Drug Targets 2012;13:1138-47.

[74] Mookherjee N, Brown KL, Bowdish DM, Doria S, Falsafi R, Hokamp K, et al. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. J Immunol 2006;176:2455-64.

[75] Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA, Mattsby-Baltzer I. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-κB. Cell Immunol 2002;220:83-95.

[76] Davidson DJ, Currie AJ, Reid GS, Bowdish DM, MacDonald KL, Ma RC, et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. J Immunol 2004;172:1146-56.

[77] van der Does AM, Bogaards SJ, Ravensbergen B, Beekhuizen H, van Dissel JT, Nibbering PH. Antimicrobial peptide hLF1-11 directs granulocyte-macrophage colony-stimulating factor-driven monocyte differentiation toward macrophages with enhanced recognition and clearance of pathogens. Antimicrob Agents Chemother 2010;54:811-6.

[78] Afacan NJ, Yeung AT, Pena OM, Hancock RE. Therapeutic potential of host defense peptides in antibiotic-resistant infections. Curr Pharm Des 2012;18:807-19.

[79] De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, et al. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 2000;192:1069-74.

[80] Niyonsaba F, Ushio H, Nakano N, Ng W, Sayama K, Hashimoto K, et al. Antimicrobial peptides human β-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. J Invest Dermatol 2007;127:594-604.

[81] Khine AA, Del Sorbo L, Vaschetto R, Voglis S, Tullis E, Slutsky AS, et al. Human neutrophil peptides induce interleukin-8 production through the P2Y6 signaling pathway. Blood 2006;107:2936-42.

[82] Mattsby-Baltzer I, Roseanu A, Motas C, Elverfors J, Engberg I, Hanson LA. Lactoferrin or a fragment thereof inhibits the endotoxin-induced interleukin-6 response in human monocytic cells. Pediatr Res 1996;40:257-62.

[83] Sun Y, Shang D. Inhibitory Effects of Antimicrobial Peptides on Lipopolysaccharide-Induced Inflammation. Mediators Inflamm 2015;2015:167572.

[84] Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, et al. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. J Clin Invest 2003;111:1665-72.

[85] Park HJ, Cho DH, Kim HJ, Lee JY, Cho BK, Bang SI, et al. Collagen synthesis is suppressed in dermal fibroblasts by the human antimicrobial peptide LL-37. J Invest Dermatol 2009;129:843-50.

[86] Hilchie AL, Wuerth K, Hancock RE. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. Nat Chem Biol 2013;9:761-8.

[87] Simsek O, Con AH, Akkoc N, Saris PE, Akcelik M. Influence of growth conditions on the nisin production of bioengineered *Lactococcus lactis* strains. J Ind Microbiol Biotechnol 2009;36:481-90.
[88] Shin JM, Gwak JW, Kamarajan P, Fenno JC, Rickard AH, Kapila YL. Biomedical applications of nisin. J Appl Microbiol 2016;120:1449-65.
[89] Lipsky BA, Holroyd KJ, Zasloff M. Topical versus systemic antimicrobial therapy for treating mildly infected diabetic foot ulcers: a randomized, controlled, double-blinded, multicenter trial of pexiganan cream. Clin Infect Dis 2008;47:1537-45.
[90] Trotti A, Garden A, Warde P, Symonds P, Langer C, Redman R, et al. A multinational, randomized phase III trial of iseganan HCl oral solution for reducing the severity of oral mucositis in patients receiving radiotherapy for head-and-neck malignancy. Int J Radiat Oncol Biol Phys 2004;58:674-81.
[91] Fox JL. Antimicrobial peptides stage a comeback. Nat Biotechnol 2013;31:379-82.
[92] Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol 2004;173:2909-12.
[93] Weber G, Heilborn JD, Chamorro Jimenez CI, Hammarsjo A, Torma H, Stahle M. Vitamin D induces the antimicrobial protein hCAP18 in human skin. J Invest Dermatol 2005;124:1080-2.
[94] Yamshchikov AV, Desai NS, Blumberg HM, Ziegler TR, Tangpricha V. Vitamin D for treatment and prevention of infectious diseases: a systematic review of randomized controlled trials. Endocr Pract 2009;15:438-49.
[95] Hancock RE. Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infect Dis 2005;5:209-18.
[96] Walsh C. Molecular mechanisms that confer antibacterial drug resistance. Nature 2000;406:775-81.
[97] Finberg RW, Moellering RC, Tally FP, Craig WA, Pankey GA, Dellinger EP, et al. The importance of bactericidal drugs: future directions in infectious disease. Clin Infect Dis 2004;39:1314-20.
[98] Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008;3:163-75.
[99] French GL. Bactericidal agents in the treatment of MRSA infections--the potential role of daptomycin. J Antimicrob Chemother 2006;58:1107-17.
[100] Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis 2001;1:156-64.
[101] Friedrich C, Scott MG, Karunaratne N, Yan H, Hancock RE. Salt-resistant α-helical cationic antimicrobial peptides. Antimicrob Agents Chemother 1999;43:1542-8.
[102] Powers JP, Martin MM, Goosney DL, Hancock RE. The antimicrobial peptide polyphemusin localizes to the cytoplasm of *Escherichia coli* following treatment. Antimicrob Agents Chemother 2006;50:1522-4.
[103] Steinberg DA, Hurst MA, Fujii CA, Kung AH, Ho JF, Cheng FC, et al. Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with *in vivo* activity. Antimicrob Agents Chemother 1997;41:1738-42.
[104] Kollef MH. Broad-spectrum antimicrobials and the treatment of serious bacterial infections: getting it right up front. Clin Infect Dis 2008;47 Suppl 1:S3-13.
[105] Menousek J, Mishra B, Hanke ML, Heim CE, Kielian T, Wang G. Database screening and *in vivo* efficacy of antimicrobial peptides against methicillin-resistant *Staphylococcus aureus* USA300. Int J Antimicrob Agents 2012;39:402-6.
[106] Boucher HW, Talbot GH, Benjamin DK, Jr., Bradley J, Guidos RJ, Jones RN, et al. 10 x '20 Progress--development of new drugs active against gram-negative bacilli: an update from the Infectious Diseases Society of America. Clin Infect Dis 2013;56:1685-94.

[107] Page MG, Bush K. Discovery and development of new antibacterial agents targeting Gram-negative bacteria in the era of pandrug resistance: is the future promising? Curr Opin Pharmacol 2014;18:91-7.
[108] Maisetta G, Di Luca M, Esin S, Florio W, Brancatisano FL, Bottai D, et al. Evaluation of the inhibitory effects of human serum components on bactericidal activity of human β defensin 3. Peptides 2008;29:1-6.
[109] Bowdish DM, Davidson DJ, Lau YE, Lee K, Scott MG, Hancock RE. Impact of LL-37 on anti-infective immunity. J Leukoc Biol 2005;77:451-9.
[110] Dorschner RA, Lopez-Garcia B, Peschel A, Kraus D, Morikawa K, Nizet V, et al. The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides. FASEB J 2006;20:35-42.
[111] Tanaka D, Miyasaki KT, Lehrer RI. Sensitivity of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. to the bactericidal action of LL-37: a cathelicidin found in human leukocytes and epithelium. Oral Microbiol Immunol 2000;15:226-31.
[112] Sanchez-Gomez S, Lamata M, Leiva J, Blondelle SE, Jerala R, Andra J, et al. Comparative analysis of selected methods for the assessment of antimicrobial and membrane-permeabilizing activity: a case study for lactoferricin derived peptides. BMC Microbiol 2008;8:196.
[113] Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM. Human β-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell 1997;88:553-60.
[114] Park IY, Cho JH, Kim KS, Kim YB, Kim MS, Kim SC. Helix stability confers salt resistance upon helical antimicrobial peptides. J Biol Chem 2004;279:13896-901.
[115] Tam JP, Lu YA, Yang JL. Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized β-strand antimicrobial peptides. J Biol Chem 2002;277:50450-6.
[116] Yu HY, Tu CH, Yip BS, Chen HL, Cheng HT, Huang KC, et al. Easy strategy to increase salt resistance of antimicrobial peptides. Antimicrob Agents Chemother 2011;55:4918-21.
[117] Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sonksen CP, Ludvigsen S, et al. Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature 2005;437:975-80.
[118] Harwig SS, Waring A, Yang HJ, Cho Y, Tan L, Lehrer RI. Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations. Eur J Biochem 1996;240:352-7.
[119] Lee IH, Cho Y, Lehrer RI. Effects of pH and salinity on the antimicrobial properties of clavanins. Infect Immun 1997;65:2898-903.
[120] Ciornei CD, Sigurdardottir T, Schmidtchen A, Bodelsson M. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. Antimicrob Agents Chemother 2005;49:2845-50.
[121] Svenson J, Brandsdal BO, Stensen W, Svendsen JS. Albumin binding of short cationic antimicrobial micropeptides and its influence on the *in vitro* bactericidal effect. J Med Chem 2007;50:3334-9.
[122] Sorensen O, Bratt T, Johnsen AH, Madsen MT, Borregaard N. The human antibacterial cathelicidin, hCAP-18, is bound to lipoproteins in plasma. J Biol Chem 1999;274:22445-51.
[123] Nguyen LT, Chau JK, Perry NA, de Boer L, Zaat SA, Vogel HJ. Serum stabilities of short tryptophan- and arginine-rich antimicrobial peptide analogs. PLoS One 2010;5.
[124] Wang Y, Agerberth B, Lothgren A, Almstedt A, Johansson J. Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37. J Biol Chem 1998;273:33115-8.
[125] Chennupati SK, Chiu AG, Tamashiro E, Banks CA, Cohen MB, Bleier BS, et al. Effects of an LL-37-derived antimicrobial peptide in an animal model of biofilm *Pseudomonas* sinusitis. American journal of rhinology & allergy 2009;23:46-51.

[126] Rivas-Santiago B, Rivas Santiago CE, Castaneda-Delgado JE, Leon-Contreras JC, Hancock RE, Hernandez-Pando R. Activity of LL-37, CRAMP and antimicrobial peptide-derived compounds E2, E6 and CP26 against *Mycobacterium tuberculosis*. Int J Antimicrob Agents 2013;41:143-8.

[127] Maiti S, Patro S, Purohit S, Jain S, Senapati S, Dey N. Effective control of *Salmonella* infections by employing combinations of recombinant antimicrobial human β-defensins hBD-1 and hBD-2. Antimicrob Agents Chemother 2014;58:6896-903.

[128] Bals R, Wang X, Wu Z, Freeman T, Bafna V, Zasloff M, et al. Human β-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. J Clin Invest 1998;102:874-80.

[129] Nizet V. Antimicrobial peptide resistance mechanisms of human bacterial pathogens. Curr Issues Mol Biol 2006;8:11-26.

[130] Baltzer SA, Brown MH. Antimicrobial peptides: promising alternatives to conventional antibiotics. Journal of molecular microbiology and biotechnology 2011;20:228-35.

[131] Peschel A. How do bacteria resist human antimicrobial peptides? Trends Microbiol 2002;10:179-86.

[132] Andersson DI, Hughes D, Kubicek-Sutherland JZ. Mechanisms and consequences of bacterial resistance to antimicrobial peptides. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 2016;26:43-57.

[133] Cole JN, Nizet V. Bacterial Evasion of Host Antimicrobial Peptide Defenses. Microbiology spectrum 2016;4.

[134] Zhang L, Parente J, Harris SM, Woods DE, Hancock RE, Falla TJ. Antimicrobial peptide therapeutics for cystic fibrosis. Antimicrob Agents Chemother 2005;49:2921-7.

[135] Perron GG, Zasloff M, Bell G. Experimental evolution of resistance to an antimicrobial peptide. Proc Biol Sci 2006;273:251-6.

[136] Samuelsen O, Haukland HH, Jenssen H, Kramer M, Sandvik K, Ulvatne H, et al. Induced resistance to the antimicrobial peptide lactoferricin B in *Staphylococcus aureus*. FEBS Lett 2005;579:3421-6.

[137] Pranting M, Negrea A, Rhen M, Andersson DI. Mechanism and fitness costs of PR-39 resistance in *Salmonella enterica* serovar Typhimurium LT2. Antimicrob Agents Chemother 2008;52:2734-41.

[138] Habets MG, Brockhurst MA. Therapeutic antimicrobial peptides may compromise natural immunity. Biol Lett 2012;8:416-8.

[139] Pranting M, Andersson DI. Mechanisms and physiological effects of protamine resistance in *Salmonella enterica* serovar Typhimurium LT2. J Antimicrob Chemother 2010;65:876-87.

[140] Lofton H, Pranting M, Thulin E, Andersson DI. Mechanisms and fitness costs of resistance to antimicrobial peptides LL-37, CNY100HL and wheat germ histones. PLoS One 2013;8:e68875.

[141] Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? Nature reviews Microbiology 2010;8:260-71.

[142] Bell G, Gouyon PH. Arming the enemy: the evolution of resistance to self-proteins. Microbiology 2003;149:1367-75.

[143] Fleitas O, Franco OL. Induced Bacterial Cross-Resistance toward Host Antimicrobial Peptides: A Worrying Phenomenon. Frontiers in microbiology 2016;7:381.

[144] Antoniadou A, Kontopidou F, Poulakou G, Koratzanis E, Galani I, Papadomichelakis E, et al. Colistin-resistant isolates of *Klebsiella pneumoniae* emerging in intensive care unit patients: first report of a multiclonal cluster. J Antimicrob Chemother 2007;59:786-90.

[145] Napier BA, Burd EM, Satola SW, Cagle SM, Ray SM, McGann P, et al. Clinical use of colistin induces cross-resistance to host antimicrobials in *Acinetobacter baumannii*. mBio 2013;4:e00021-13.

[146] Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug discovery today 2010;15:40-56.

[147] Malmsten M, Kasetty G, Pasupuleti M, Alenfall J, Schmidtchen A. Highly selective end-tagged antimicrobial peptides derived from PRELP. PLoS One 2011;6:e16400.
[148] Brinckerhoff LH, Kalashnikov VV, Thompson LW, Yamshchikov GV, Pierce RA, Galavotti HS, et al. Terminal modifications inhibit proteolytic degradation of an immunogenic MART-1(27-35) peptide: implications for peptide vaccines. Int J Cancer 1999;83:326-34.
[149] Rink R, Arkema-Meter A, Baudoin I, Post E, Kuipers A, Nelemans SA, et al. To protect peptide pharmaceuticals against peptidases. J Pharmacol Toxicol Methods 2010;61:210-8.
[150] Schmidtchen A, Pasupuleti M, Morgelin M, Davoudi M, Alenfall J, Chalupka A, et al. Boosting antimicrobial peptides by hydrophobic oligopeptide end tags. J Biol Chem 2009;284:17584-94.
[151] Lio PA, Kaye ET. Topical antibacterial agents. Infect Dis Clin North Am 2009;23:945-63, ix.
[152] Amblard M, Fehrentz JA, Martinez J, Subra G. Methods and protocols of modern solid phase Peptide synthesis. Mol Biotechnol 2006;33:239-54.
[153] Legrand D, Pierce A, Elass E, Carpentier M, Mariller C, Mazurier J. Lactoferrin structure and functions. Adv Exp Med Biol 2008;606:163-94.
[154] Puddu P, Valenti P, Gessani S. Immunomodulatory effects of lactoferrin on antigen presenting cells. Biochimie 2009;91:11-8.
[155] Legrand D. Lactoferrin, a key molecule in immune and inflammatory processes. Biochem Cell Biol 2012;90:252-68.
[156] Ward PP, Conneely OM. Lactoferrin: role in iron homeostasis and host defense against microbial infection. Biometals 2004;17:203-8.
[157] Legrand D, Elass E, Carpentier M, Mazurier J. Lactoferrin: a modulator of immune and inflammatory responses. Cell Mol Life Sci 2005;62:2549-59.
[158] Suzuki YA, Lopez V, Lonnerdal B. Mammalian lactoferrin receptors: structure and function. Cell Mol Life Sci 2005;62:2560-75.
[159] Elass-Rochard E, Legrand D, Salmon V, Roseanu A, Trif M, Tobias PS, et al. Lactoferrin inhibits the endotoxin interaction with CD14 by competition with the lipopolysaccharide-binding protein. Infect Immun 1998;66:486-91.
[160] Mann DM, Romm E, Migliorini M. Delineation of the glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin. J Biol Chem 1994;269:23661-7.
[161] Elass-Rochard E, Roseanu A, Legrand D, Trif M, Salmon V, Motas C, et al. Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* 055B5 lipopolysaccharide. Biochem J 1995;312 (Pt 3):839-45.
[162] Nibbering PH, Ravensbergen E, Welling MM, van Berkel LA, van Berkel PH, Pauwels EK, et al. Human lactoferrin and peptides derived from its N terminus are highly effective against infections with antibiotic-resistant bacteria. Infect Immun 2001;69:1469-76.
[163] Chapple DS, Mason DJ, Joannou CL, Odell EW, Gant V, Evans RW. Structure-function relationship of antibacterial synthetic peptides homologous to a helical surface region on human lactoferrin against *Escherichia coli* serotype O111. Infect Immun 1998;66:2434-40.
[164] Gifford JL, Hunter HN, Vogel HJ. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. Cell Mol Life Sci 2005;62:2588-98.
[165] Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K, Tomita M. Identification of the bactericidal domain of lactoferrin. Biochim Biophys Acta 1992;1121:130-6.
[166] Haversen LA, Baltzer L, Dolphin G, Hanson LA, Mattsby-Baltzer I. Anti-inflammatory activities of human lactoferrin in acute dextran sulphate-induced colitis in mice. Scand J Immunol 2003;57:2-10.
[167] Haversen LA, Engberg I, Baltzer L, Dolphin G, Hanson LA, Mattsby-Baltzer I. Human lactoferrin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary tract infection in mice. Infect Immun 2000;68:5816-23.

[168] Haversen L, Kondori N, Baltzer L, Hanson LA, Dolphin GT, Duner K, et al. Structure-microbicidal activity relationship of synthetic fragments derived from the antibacterial α-helix of human lactoferrin. Antimicrob Agents Chemother 2010;54:418-25.
[169] Kondori N, Baltzer L, Dolphin GT, Mattsby-Baltzer I. Fungicidal activity of human lactoferrin-derived peptides based on the antimicrobial αβ region. Int J Antimicrob Agents 2011;37:51-7.
[170] Sperstad SV, Haug T, Blencke HM, Styrvold OB, Li C, Stensvag K. Antimicrobial peptides from marine invertebrates: challenges and perspectives in marine antimicrobial peptide discovery. Biotechnol Adv 2011;29:519-30.
[171] Li C, Haug T, Moe MK, Styrvold OB, Stensvag K. Centrocins: isolation and characterization of novel dimeric antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis*. Dev Comp Immunol 2010;34:959-68.
[172] Otero-Gonzalez AJ, Magalhaes BS, Garcia-Villarino M, Lopez-Abarrategui C, Sousa DA, Dias SC, et al. Antimicrobial peptides from marine invertebrates as a new frontier for microbial infection control. FASEB J 2010;24:1320-34.
[173] Haug T, Kjuul AK, Styrvold OB, Sandsdalen E, Olsen OM, Stensvag K. Antibacterial activity in *Strongylocentrotus droebachiensis* (Echinoidea), *Cucumaria frondosa* (Holothuroidea), and *Asterias rubens* (Asteroidea). J Invertebr Pathol 2002;81:94-102.
[174] Sharma M, DahimaR, Gupta AK. A review on adhesions and their prevention. Journal of Drug Discovery and Therapeutics 2013.
[175] Menzies D, Ellis H. Intestinal obstruction from adhesions--how big is the problem? Ann R Coll Surg Engl 1990;72:60-3.
[176] Liakakos T, Thomakos N, Fine PM, Dervenis C, Young RL. Peritoneal adhesions: etiology, pathophysiology, and clinical significance. Recent advances in prevention and management. Dig Surg 2001;18:260-73.
[177] Monk BJ, Berman ML, Montz FJ. Adhesions after extensive gynecologic surgery: clinical significance, etiology, and prevention. Am J Obstet Gynecol 1994;170:1396-403.
[178] ten Broek RP, Issa Y, van Santbrink EJ, Bouvy ND, Kruitwagen RF, Jeekel J, et al. Burden of adhesions in abdominal and pelvic surgery: systematic review and met-analysis. BMJ 2013;347:f5588.
[179] Holmdahl L, Ivarsson ML. The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. Eur J Surg 1999;165:1012-9.
[180] Muzii L. Survey among members of the roman group of gynecologic endoscopy on the use of agents for postoperative adhesion prevention. J Am Assoc Gynecol Laparosc 2004;11:248-51.
[181] Holmdahl L, Eriksson E, al-Jabreen M, Risberg B. Fibrinolysis in human peritoneum during operation. Surgery 1996;119:701-5.
[182] Falk K, Bjorquist P, Stromqvist M, Holmdahl L. Reduction of experimental adhesion formation by inhibition of plasminogen activator inhibitor type 1. Br J Surg 2001;88:286-9.
[183] Mutsaers SE. The mesothelial cell. Int J Biochem Cell Biol 2004;36:9-16.
[184] Whawell SA, Thompson JN. Cytokine-induced release of plasminogen activator inhibitor-1 by human mesothelial cells. Eur J Surg 1995;161:315-8.
[185] van Hinsbergh VW, Kooistra T, van den Berg EA, Princen HM, Fiers W, Emeis JJ. Tumor necrosis factor increases the production of plasminogen activator inhibitor in human endothelial cells *in vitro* and in rats *in vivo*. Blood 1988;72:1467-73.
[186] Bothin C, Midtvedt T, Perbeck L. Orally delivered antibiotics which lower bacterial numbers decrease experimental intra-abdominal adhesions. Langenbecks Arch Surg 2003;388:112-5.
[187] Tulandi T, Al-Shahrani A. Adhesion prevention in gynecologic surgery. Curr Opin Obstet Gynecol 2005;17:395-8.
[188] Arung W, Meurisse M, Detry O. Pathophysiology and prevention of postoperative peritoneal adhesions. World J Gastroenterol 2011;17:4545-53.

[189] Dryden MS. Complicated skin and soft tissue infection. J Antimicrob Chemother 2010;65 Suppl 3:iii35-44.
[190] White J, R. A charcoal dressing with silver in wound infection: Clinical evidence. Br J Community Nurs 2001;6:S4-11.
[191] Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev 2001;14:244-69.
[192] Sartelli M, Malangoni MA, May AK, Viale P, Kao LS, Catena F, et al. World Society of Emergency Surgery (WSES) guidelines for management of skin and soft tissue infections. World J Emerg Surg 2014;9:57.
[193] Moet GJ, Jones RN, Biedenbach DJ, Stilwell MG, Fritsche TR. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY Antimicrobial Surveillance Program (1998-2004). Diagn Microbiol Infect Dis 2007;57:7-13.
[194] Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. J Clin Invest 2003;111:1265-73.
[195] Miller LG, Daum RS, Creech CB, Young D, Downing MD, Eells SJ, et al. Clindamycin versus trimethoprim-sulfamethoxazole for uncomplicated skin infections. N Engl J Med 2015;372:1093-103.
[196] Kumar N, David MZ, Boyle-Vavra S, Sieth J, Daum RS. High *Staphylococcus aureus* colonization prevalence among patients with skin and soft tissue infections and controls in an urban emergency department. J Clin Microbiol 2015;53:810-5.
[197] Mangram AJ, Horan TC, Pearson ML, Silver LC, Jarvis WR. Guideline for prevention of surgical site infection, 1999. Hospital Infection Control Practices Advisory Committee. Infect Control Hosp Epidemiol 1999;20:250-78; quiz 79-80.
[198] Leaper DJ, van Goor H, Reilly J, Petrosillo N, Geiss HK, Torres AJ, et al. Surgical site infection - a European perspective of incidence and economic burden. Int Wound J 2004;1:247-73.
[199] Kirby JP, Mazuski JE. Prevention of surgical site infection. Surg Clin North Am 2009;89:365-89, viii.
[200] Giacometti A, Cirioni O, Schimizzi AM, Del Prete MS, Barchiesi F, D'Errico MM, et al. Epidemiology and microbiology of surgical wound infections. J Clin Microbiol 2000;38:918-22.
[201] Anderson DJ, Sexton DJ, Kanafani ZA, Auten G, Kaye KS. Severe surgical site infection in community hospitals: epidemiology, key procedures, and the changing prevalence of methicillin-resistant *Staphylococcus aureus*. Infect Control Hosp Epidemiol 2007;28:1047-53.
[202] Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. Clin Microbiol Rev 2006;19:403-34.
[203] Santucci SG, Gobara S, Santos CR, Fontana C, Levin AS. Infections in a burn intensive care unit: experience of seven years. J Hosp Infect 2003;53:6-13.
[204] Ekrami A, Kalantar E. Bacterial infections in burn patients at a burn hospital in Iran. Indian J Med Res 2007;126:541-4.
[205] Appelgren P, Bjornhagen V, Bragderyd K, Jonsson CE, Ransjo U. A prospective study of infections in burn patients. Burns 2002;28:39-46.
[206] Moore EC, Padiglione AA, Wasiak J, Paul E, Cleland H. *Candida* in burns: risk factors and outcomes. Journal of burn care & research : official publication of the American Burn Association 2010;31:257-63.
[207] Altoparlak U, Erol S, Akcay MN, Celebi F, Kadanali A. The time-related changes of antimicrobial resistance patterns and predominant bacterial profiles of burn wounds and body flora of burned patients. Burns 2004;30:660-4.
[208] Greenhalgh DG, Saffle JR, Holmes JHt, Gamelli RL, Palmieri TL, Horton JW, et al. American Burn Association consensus conference to define sepsis and infection in burns. Journal of burn care & research : official publication of the American Burn Association 2007;28:776-90.

[209] Dai T, Huang YY, Sharma SK, Hashmi JT, Kurup DB, Hamblin MR. Topical antimicrobials for burn wound infections. Recent patents on anti-infective drug discovery 2010;5:124-51.
[210] Kennedy P, Brammah S, Wills E. Burns, biofilm and a new appraisal of burn wound sepsis. Burns 2010;36:49-56.
[211] Donlan RM, Costerton JW. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clin Microbiol Rev 2002;15:167-93.
[212] Shiau AL, Wu CL. The inhibitory effect of *Staphylococcus epidermidis* slime on the phagocytosis of murine peritoneal macrophages is interferon-independent. Microbiol Immunol 1998;42:33-40.
[213] de la Fuente-Nunez C, Reffuveille F, Haney EF, Straus SK, Hancock RE. Broad-spectrum anti-biofilm peptide that targets a cellular stress response. PLoS Pathog 2014;10:e1004152.
[214] Bjarnsholt T. The role of bacterial biofilms in chronic infections. APMIS Suppl 2013:1-51.
[215] Reffuveille F, de la Fuente-Nunez C, Mansour S, Hancock RE. A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms. Antimicrob Agents Chemother 2014;58:5363-71.
[216] Otto M. Bacterial evasion of antimicrobial peptides by biofilm formation. Curr Top Microbiol Immunol 2006;306:251-8.
[217] de la Fuente-Nunez C, Korolik V, Bains M, Nguyen U, Breidenstein EB, Horsman S, et al. Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide. Antimicrob Agents Chemother 2012;56:2696-704.
[218] Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock RE. Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun 2008;76:4176-82.
[219] Lee JK, Chang SW, Perinpanayagam H, Lim SM, Park YJ, Han SH, et al. Antibacterial efficacy of a human β-defensin-3 peptide on multispecies biofilms. J Endod 2013;39:1625-9.
[220] Helmerhorst EJ, Hodgson R, van 't Hof W, Veerman EC, Allison C, Nieuw Amerongen AV. The effects of histatin-derived basic antimicrobial peptides on oral biofilms. J Dent Res 1999;78:1245-50.
[221] Minardi D, Ghiselli R, Cirioni O, Giacometti A, Kamysz W, Orlando F, et al. The antimicrobial peptide tachyplesin III coated alone and in combination with intraperitoneal piperacillin-tazobactam prevents ureteral stent *Pseudomonas* infection in a rat subcutaneous pouch model. Peptides 2007;28:2293-8.
[222] Achkar JM, Fries BC. *Candida* infections of the genitourinary tract. Clin Microbiol Rev 2010;23:253-73.
[223] Dabas PS. An approach to etiology, diagnosis and management of different types of candidiasis.
[224] Kashem SW, Kaplan DH. Skin Immunity to *Candida albicans*. Trends Immunol 2016.
[225] Perlroth J, Choi B, Spellberg B. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med Mycol 2007;45:321-46.
[226] Cheng SC, Joosten LA, Kullberg BJ, Netea MG. Interplay between *Candida albicans* and the mammalian innate host defense. Infect Immun 2012;80:1304-13.
[227] Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nature reviews Microbiology 2012;10:112-22.
[228] Chakrabarti A, Chatterjee SS, Rao KL, Zameer MM, Shivaprakash MR, Singhi S, et al. Recent experience with fungaemia: change in species distribution and azole resistance. Scand J Infect Dis 2009;41:275-84.
[229] Sutherland R, Boon RJ, Griffin KE, Masters PJ, Slocombe B, White AR. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. Antimicrob Agents Chemother 1985;27:495-8.
[230] Capobianco JO, Doran CC, Goldman RC. Mechanism of mupirocin transport into sensitive and resistant bacteria. Antimicrob Agents Chemother 1989;33:156-63.

[231] Collignon P, Turnidge J. Fusidic acid *in vitro* activity. Int J Antimicrob Agents 1999;12 Suppl 2:S45-58.
[232] Biedenbach DJ, Rhomberg PR, Mendes RE, Jones RN. Spectrum of activity, mutation rates, synergistic interactions, and the effects of pH and serum proteins for fusidic acid (CEM-102). Diagn Microbiol Infect Dis 2010;66:301-7.
[233] Chaudhary M, Kesava Naidu G, Kumar S, Payasi A. Comparative antibacterial activity of a novel semisynthetic antibiotic: etimicin sulphate and other aminoglycosides. World J Microbiol Biotechnol 2012;28:3365-71.
[234] Fraser JR, Laurent TC, Laurent UB. Hyaluronan: its nature, distribution, functions and turnover. J Intern Med 1997;242:27-33.
[235] Ramachandran S, Chen S, Etzler F. Rheological characterization of hydroxypropylcellulose gels. Drug Dev Ind Pharm 1999;25:153-61.
[236] Eng RH, Padberg FT, Smith SM, Tan EN, Cherubin CE. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob Agents Chemother 1991;35:1824-8.
[237] Lupetti A, Paulusma-Annema A, Welling MM, Senesi S, van Dissel JT, Nibbering PH. Candidacidal activities of human lactoferrin peptides derived from the N terminus. Antimicrob Agents Chemother 2000;44:3257-63.
[238] Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, et al. *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β-defensins and CAP18, expressed by human keratinocytes. Infect Immun 2003;71:3730-9.
[239] Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human β -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 2001;276:5707-13.
[240] Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, et al. Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, β-defensins and LL37, produced by human epithelial cells. J Antimicrob Chemother 2005;55:888-96.
[241] Friedrich CL, Moyles D, Beveridge TJ, Hancock RE. Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria. Antimicrob Agents Chemother 2000;44:2086-92.
[242] Kosowska-Shick K, Clark C, Credito K, McGhee P, Dewasse B, Bogdanovich T, et al. Single- and multistep resistance selection studies on the activity of retapamulin compared to other agents against *Staphylococcus aureus* and *Streptococcus pyogenes*. Antimicrob Agents Chemother 2006;50:765-9.
[243] Yamakawa T, Mitsuyama J, Hayashi K. *In vitro* and *in vivo* antibacterial activity of T-3912, a novel non-fluorinated topical quinolone. J Antimicrob Chemother 2002;49:455-65.
[244] Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: an *in vitro* cell model for immune modulation approach. Int Immunopharmacol 2014;23:37-45.
[245] Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia 1991;47:22-31.
[246] Batra H, Antony VB. Pleural mesothelial cells in pleural and lung diseases. J Thorac Dis 2015;7:964-80.
[247] Haug T, Kjuul AK, Stensvag K, Sandsdalen E, Styrvold OB. Antibacterial activity in four marine crustacean decapods. Fish Shellfish Immunol 2002;12:371-85.
[248] Harris ES, Morgan RF, Rodeheaver GT. Analysis of the kinetics of peritoneal adhesion formation in the rat and evaluation of potential antiadhesive agents. Surgery 1995;117:663-9.
[249] Arnold PB, Green CW, Foresman PA, Rodeheaver GT. Evaluation of resorbable barriers for preventing surgical adhesions. Fertil Steril 2000;73:157-61.
[250] Diamond MP, Wexner SD, diZereg GS, Korell M, Zmora O, Van Goor H, et al. Adhesion prevention and reduction: current status and future recommendations of a multinational interdisciplinary consensus conference. Surg Innov 2010;17:183-8.
[251] Ryan GB, Grobety J, Majno G. Postoperative peritoneal adhesions. A study of the mechanisms. Am J Pathol 1971;65:117-48.

[252] Diamond MP, Daniell JF, Feste J, Surrey MW, McLaughlin DS, Friedman S, et al. Adhesion reformation and de novo adhesion formation after reproductive pelvic surgery. Fertil Steril 1987;47:864-6.
[253] Caglayan K, Erdogan N, Avci B, Gungor B, Cinar H, Arslan N. Effect of Beta-glucan on intestinal anastomoses in a rat model. Advances in clinical and experimental medicine : official organ Wroclaw Medical University 2013;22:157-63.
[254] Holmer C, Praechter C, Mecklenburg L, Heimesaat M, Rieger H, Pohlen U. Anastomotic stability and wound healing of colorectal anastomoses sealed and sutured with a collagen fleece in a rat peritonitis model. Asian J Surg 2014;37:35-45.
[255] Mansson P, Zhang XW, Jeppsson B, Thorlacius H. Anastomotic healing in the rat colon: comparison between a radiological method, breaking strength and bursting pressure. Int J Colorectal Dis 2002;17:420-5.
[256] Habibi M, Oner OZ, Oruc MT, Bulbuller N, Ozdem S, Ozdemir S, et al. Effects of a Glutamine Enema on Anastomotic Healing in an Animal Colon Anastomosis Model. Annals of coloproctology 2015;31:213-21.
[257] Tingstedt B, Nehez L, Axelsson J, Lindman B, Andersson R. Increasing anastomosis safety and preventing abdominal adhesion formation by the use of polypeptides in the rat. Int J Colorectal Dis 2006;21:566-72.
[258] McDonnell G, Haines K, Klein D, Rippon M, Walmsley R, Pretzer D. Clinical correlation of a skin antisepsis model. J Microbiol Methods 1999;35:31-5.
[259] Adhirajan N, Shanmugasundaram N, Shanmuganathan S, Babu M. Collagen-based wound dressing for doxycycline delivery: *in-vivo* evaluation in an infected excisional wound model in rats. J Pharm Pharmacol 2009;61:1617-23.
[260] Chalekson CP, Neumeister MW, Jaynes J. Treatment of infected wounds with the antimicrobial peptide D2A21. J Trauma 2003;54:770-4.
[261] Lin YH, Hsu WS, Chung WY, Ko TH, Lin JH. Evaluation of various silver-containing dressing on infected excision wound healing study. J Mater Sci Mater Med 2014;25:1375-86.
[262] McRipley RJ, Whitney RR. Responsiveness of experimental surgical-wound infections to topical chemotherapy. Antimicrob Agents Chemother 1976;10:45-51.
[263] Gisby J, Bryant J. Efficacy of a new cream formulation of mupirocin: comparison with oral and topical agents in experimental skin infections. Antimicrob Agents Chemother 2000;44:255-60.
[264] Rittenhouse S, Singley C, Hoover J, Page R, Payne D. Use of the surgical wound infection model to determine the efficacious dosing regimen of retapamulin, a novel topical antibiotic. Antimicrob Agents Chemother 2006;50:3886-8.
[265] Huang L, Dai T, Xuan Y, Tegos GP, Hamblin MR. Synergistic combination of chitosan acetate with nanoparticle silver as a topical antimicrobial: efficacy against bacterial burn infections. Antimicrob Agents Chemother 2011;55:3432-8.
[266] Saymen DG, Nathan P, Holder IA, Hill EO, Macmillan BG. Infected surface wound: an experimental model and a method for the quantitation of bacteria in infected tissues. Appl Microbiol 1972;23:509-14.
[267] McRipley RJ, Whitney RR. Characterization and quantitation of experimental surgical-wound infections used to evaluate topical antibacterial agents. Antimicrob Agents Chemother 1976;10:38-44.
[268] Dale RM, Schnell G, Wong JP. Therapeutic efficacy of "nubiotics" against burn wound infection by *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2004;48:2918-23.
[269] Riedel CU, Casey PG, Mulcahy H, O'Gara F, Gahan CG, Hill C. Construction of p16Slux, a novel vector for improved bioluminescent labeling of gram-negative bacteria. Appl Environ Microbiol 2007;73:7092-5.
[270] Sohnle PG, Hahn BL. Epidermal proliferation and the neutrophilic infiltrates of experimental cutaneous candidiasis in mice. Arch Dermatol Res 1989;281:279-83.

[271] The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3(R2). ICH Harmonised Tripartite Guideline 2009.

[272] Saginur R, Stdenis M, Ferris W, Aaron SD, Chan F, Lee C, et al. Multiple combination bactericidal testing of staphylococcal biofilms from implant-associated infections. Antimicrob Agents Chemother 2006;50:55-61.

[273] Lee J, Lee D, Choi H, Kim HH, Kim H, Hwang JS, et al. Synthesis and antimicrobial activity of cysteine-free coprisin nonapeptides. Biochem Biophys Res Commun 2014;443:483-8.

[274] Vignal E, Chavanieu A, Roch P, Chiche L, Grassy G, Calas B, et al. Solution structure of the antimicrobial peptide ranalexin and a study of its interaction with perdeuterated dodecylphosphocholine micelles. Eur J Biochem 1998;253:221-8.

[275] Wang J, Chou S, Xu L, Zhu X, Dong N, Shan A, et al. High specific selectivity and Membrane-Active Mechanism of the synthetic centrosymmetric α-helical peptides with Gly-Gly pairs. Sci Rep 2015;5:15963.

[276] Herbel V, Wink M. Mode of action and membrane specificity of the antimicrobial peptide snakin-2. PeerJ 2016;4:e1987.

[277] Wu M, Hancock RE. Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane. J Biol Chem 1999;274:29-35.

[278] Nakajima Y, Ishibashi J, Yukuhiro F, Asaoka A, Taylor D, Yamakawa M. Antibacterial activity and mechanism of action of tick defensin against Gram-positive bacteria. Biochim Biophys Acta 2003;1624:125-30.

[279] Friedrich CL, Rozek A, Patrzykat A, Hancock RE. Structure and mechanism of action of an indolicidin peptide derivative with improved activity against gram-positive bacteria. J Biol Chem 2001;276:24015-22.

[280] Zhu X, Dong N, Wang Z, Ma Z, Zhang L, Ma Q, et al. Design of imperfectly amphipathic α-helical antimicrobial peptides with enhanced cell selectivity. Acta biomaterialia 2014;10:244-57.

[281] Chen Y, Mant CT, Farmer SW, Hancock RE, Vasil ML, Hodges RS. Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. J Biol Chem 2005;280:12316-29.

[282] Anderson K, Hamm RL. Factors That Impair Wound Healing. The journal of the American College of Clinical Wound Specialists 2012;4:84-91.

[283] Chalabaev S, Anderson CA, Onderdonk AB, Kasper DL. Sensitivity of *Francisella tularensis* to ultrapure water and deoxycholate: implications for bacterial intracellular growth assay in macrophages. J Microbiol Methods 2011;85:230-2.

[284] Harrison-Balestra C, Cazzaniga AL, Davis SC, Mertz PM. A wound-isolated *Pseudomonas aeruginosa* grows a biofilm *in vitro* within 10 hours and is visualized by light microscopy. Dermatol Surg 2003;29:631-5.

[285] Mertz PM, Alvarez OM, Smerbeck RV, Eaglstein WH. A new *in vivo* model for the evaluation of topical antiseptics on superficial wounds. The effect of 70% alcohol and povidone-iodine solution. Arch Dermatol 1984;120:58-62.

[286] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P & T : a peer-reviewed journal for formulary management 2015;40:277-83.

[287] O'Toole GA. Microtiter dish biofilm formation assay. Journal of visualized experiments : JoVE 2011.

[288] Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985;22:996-1006.

[289] Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 2000;40:175-9.

[290] Wiig M, Olmarker K, Hakansson J, Ekstrom L, Nilsson E, Mahlapuu M. A lactoferrin-derived peptide (PXL01) for the reduction of adhesion formation in flexor tendon surgery: an experimental study in rabbits. J Hand Surg Eur Vol 2011;36:656-62.
[291] Hakansson J, Mahlapuu M, Ekstrom L, Olmarker K, Wiig M. Effect of lactoferrin peptide (PXL01) on rabbit digit mobility after flexor tendon repair. J Hand Surg Am 2012;37:2519-25.
[292] Wiig ME, Dahlin LB, Friden J, Hagberg L, Larsen SE, Wiklund K, et al. PXL01 in Sodium Hyaluronate for Improvement of Hand Recovery after Flexor Tendon Repair Surgery: Randomized Controlled Trial. PLoS One 2014;9:e110735.