HOST DEFENSE PEPTIDES OF THE COAGULATION SYSTEM AND THEIR THERAPEUTIC POTENTIAL

Kasetty, Gopinath

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Host defense peptides of the coagulation system and their therapeutic potential

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With the approval of the Faculty of Medicine at Lund University, this thesis will be defended on January 24th 2014 at 9.00 in the Segerfalk lecture hall, Wallenberg Neuroscience Center, Lund, Sweden

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Prevention and control of infectious diseases is seriously hampered by an increasing prevalence of bacteria that are resistant towards conventional antibiotics. Resistant bacteria are often the root cause of infections that trigger the complex clinical syndrome called sepsis, which is a concern for high morbidity and mortality. Despite extensive basic research and clinical studies, treatment of sepsis remains challenging due to an uncontrolled immune response mediated by various phagocytic cells, and the coagulation and complement cascades. Emerging evidence suggests that host defense peptides may be potential lead structures in sepsis treatment due to their ability to modulate innate immune responses as well as being directly antimicrobial. From that perspective, this thesis aimed to identify and characterize the structure-activity relationships of short antimicrobial peptides (AMPs) derived from serine proteases. In paper I and II, structural features, governing the antimicrobial activity of peptides derived from the C-terminal region of human serine proteases were identified. Moreover the potential of these peptides to modulate inflammatory responses caused by bacterial lipopolysaccharide (LPS) was investigated. Truncated forms of thrombin-derived C-terminal peptides exhibited length- and sequence-dependent antimicrobial and immunomodulating effects in vitro and in vivo as illustrated by increasing survival rates in mouse models of LPS-induced septic shock (paper I). Quantitative structure-activity relationship (QSAR) analysis utilizing biophysical, antimicrobial and immunomodulatory activities of peptides derived from evolutionary conserved C-terminal protease domains of serine proteases, disclosed a set of active peptides with potent selective membrane disrupting effects and promising therapeutic potential in animal models of LPS-induced septic shock (paper II). The data in Paper III demonstrate generation of antimicrobial peptides from the C-terminal region of the human coagulation factor X by proteolytic cleavage with human leukocyte elastase as well as P. aeruginosa elastase. The prototypic peptide RKG25 derived from the core region of the FX protease domain, exhibited multiple biological functions including, antimicrobial, anti-inflammatory and anticoagulant effects. In summary, the findings of multiple immunomodulatory functions of these novel HDPs provides a possible new approach for the development of treatments for bacterial infections.

Key words: Host defense peptides, Sepsis, Serine proteases, Factor X, Immunomodulation

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Doctoral Thesis

by

Gopinath Kasetty

Lund University
Faculty of Medicine

Lund, 2014
I dedicate this thesis to
my family, for their constant support and unconditional love.
I love you all dearly.
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List of papers

This thesis is based on the following papers:


   Identification of a structural core region within coagulation factor X exerting host defense activities. *Manuscript*
List of publications not included in this thesis


Abbreviations

APC Human activated protein C
AMPs Antimicrobial peptides
ATIII Antithrombin III
BK Bradykinin
C. albicans Candida albicans
cfu Colony-forming unit
CL Cardiolipin
C-terminal Carboxy-terminal (COOH-terminal)
D3, 5 Domain 3, 5
DIC Disseminated intravascular coagulation
DNA Deoxyribonucleic acid
E. coli Escherichia coli
ELISA Enzyme-linked immunosorbent assay
F(I-XIII) Coagulation factors I-XIII
FITC Fluorescein isothiocyanate
GAGs Glycosaminoglycans
hBD Human b defensin
hCAP18 Human cationic antimicrobial protein 18kDa
HCII Heparin cofactor II
HDPs Host defense peptides
HLE Human leukocyte (neutrophil) elastase
HK High molecular weight kininogen
HMGB1 High-mobility group protein B1
HRG Histidine-rich glycoprotein
Ig Immunoglobulin
IL Interleukin
INF Interferon
IRAK IL-1 receptor-associated kinase
LBP LPS binding protein
LPS Lipopolysaccharide
LTA Lipoteichoic acid
MBL Mannose binding lectin
MIC Minimal inhibitory concentration
MKK Mitogen-activated protein kinase
MyD88 Myeloid differentiation factor protein 88
NETs Neutrophil extracellular traps
NK-cell Natural killer cell
NO Nitric oxide
N-terminal Amino-terminal (NH2-terminal)
P. aeruginosa Pseudomonas aeruginosa
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PAE</td>
<td><em>Pseudomonas aeruginosa</em> elastase</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activated inhibitor-1</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PARs</td>
<td>Protease activated receptors</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycans</td>
</tr>
<tr>
<td>PK</td>
<td>Plasma kallikrein</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>RDA</td>
<td>Radial diffusion assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin-activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>THP1</td>
<td>Human monocytic cell line</td>
</tr>
<tr>
<td>THRB</td>
<td>Thrombin</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor inducing interferon-β</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>VCA</td>
<td>Viable count assay</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
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Abstract

Prevention and control of infectious diseases is seriously hampered by an increasing prevalence of bacteria that are resistant towards conventional antibiotics. Resistant bacteria are often the root cause of infections that trigger the complex clinical syndrome called sepsis, which is a concern for high morbidity and mortality. Despite extensive basic research and clinical studies, treatment of sepsis remains challenging due to an uncontrolled immune response mediated by various phagocytic cells, and the coagulation and complement cascades.

Emerging evidence suggests that host defense peptides (HDPs) may be potential lead structures in sepsis treatment due to their ability to modulate innate immune responses as well as being directly antimicrobial. From that perspective, this thesis aimed to identify and characterize the structure-activity relationships of small HDPs derived from serine proteases. In paper I and II, structural features, governing the antimicrobial activity of peptides derived from the C-terminal region of human serine proteases were identified. Moreover the potential of these peptides to modulate inflammatory responses caused by bacterial lipopolysaccharide (LPS) was investigated. Truncated forms of thrombin-derived C-terminal peptides exhibited length- and sequence-dependent antimicrobial and immunomodulating effects in vitro and in vivo as illustrated by increasing survival rates in mouse models of LPS-induced septic shock (paper I). Quantitative structure-activity relationship (QSAR) analysis utilizing biophysical, antimicrobial and immunomodulatory activities of peptides derived from evolutionary conserved C-terminal protease domains of serine proteases, disclosed a set of active peptides with potent selective membrane disrupting effects and promising therapeutic potential in animal models of LPS-induced septic shock (paper II). The data in Paper III demonstrate generation of antimicrobial peptides from the C-terminal region of the human coagulation factor X by proteolytic cleavage with human leukocyte elastase as well as P. aeruginosa elastase. The prototypic peptide
RKG25 derived from the core region of the FX protease domain, exhibited multiple biological functions including, antimicrobial, anti-inflammatory and anticoagulant effects. In summary, the findings of multiple immunomodulatory functions of these novel HDPs provides a possible new approach for the development of treatments for bacterial infections.
1 Overview

1.1 The innate immune system

In daily life, a healthy individual is constantly exposed to different microorganisms of which some may cause disease. However, the immune system guards the body effectively against several pathogenic bacteria, preventing the development of invasive microbial diseases (1). The first line of defense mechanisms against an invading pathogen is comprised of physical or anatomical barriers (skin, mucosal lining), mechanical barriers (ciliated cells from the respiratory tracts, tight junctions) and biochemical barriers (antimicrobial peptides, tears or saliva containing antimicrobial lysozyme). In addition, there are two immune defense systems; the innate and the adaptive immune system, which are sequentially activated during infection and work cooperatively to help destroy and clear invading pathogens (2).

The innate immune system recognizes unique microbial features and becomes quickly activated in order to eradicate invading microbes or microbial agents (3). This activation also contributes to the induction of more specific adaptive immune responses that provide a long-standing protection by generating a memory for specific pathogens (2). Unlike the adaptive immune system, the innate system is non-specific and relies on a limited number of receptors, groups of phagocytic cells, antimicrobial peptides as well as the complement and coagulation systems (2). Neutrophils are considered to be the most important cells (4, 5). They are the first cells to be recruited in large numbers to the site of infection, which are later followed by monocytes and immature dendritic cells (4). Together, these phagocytic cells recognize invading microbes, engulf and subsequently destroy them by utilizing antimicrobial enzymes or proteases (6). In addition to AMPs, cytokine and/or chemokine gradients are also established resulting in the recruitment of phagocytes and other immune cells. These substances mediate chemotaxis and influence proliferation and maturation of immune cells. The
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recruited cells are further prompted to produce more cytokines, resulting in an amplification of associated responses. Thus, depending on the activation of specific intracellular signaling cascades, cytokines generate pro-inflammatory or anti-inflammatory responses (7, 8).

Innate immune cells can sense common structural patterns of microbes called pathogen-associated molecular patterns (PAMPs) with the help of pattern recognition receptors (PRRs) expressed at their surface (3). Upon recognition of these conserved microbial structures, intracellular signaling cascades are triggered ultimately culminating in the production of pro-inflammatory cytokines, chemokines and the up-regulation of leukocyte adhesion molecules as an early host response to infection (9). In some cases this activation also induces the production of antimicrobial peptides (10). Another significant part of innate immunity is the complement system, which can be activated on the surface of pathogens and involves various proteins present in blood. These proteins are activated in a cascade fashion and mediate phagocytosis either by opsonization of the microorganisms or by creating pores in the bacterial membrane (11).

The host may combat and limit the invading pathogen by inducing coagulation and inflammation. Recognition of bacterial components by TLRs, induces the expression of tissue factor (TF) on monocytes which triggers the activation of the extrinsic pathway of the coagulation system (12, 13). The inflammatory system sustains TF expression through the action of cytokines and leukocyte elastase, which can down-regulate or degrade natural anticoagulant protein e.g. tissue factor pathway inhibitor (TFPI), ensuring a positive feedback loop to further promote coagulation and inflammation (14). All branches of innate immunity are tightly regulated. An imbalance in the cross talk between the different arms leads to dysregulated inflammatory responses and coagulopathy, which may result in multiple organ failure during severe bacterial invasive disease.
1.2 The coagulation system

Activation of the coagulation cascade and subsequent fibrin deposition are essential parts of the host defense, which not only prevents blood loss but also invasion of microorganisms (15). Therefore, the clotting system has been considered as a crucial part of innate immunity. Coagulation is a complex process comprising the involvement of platelets that serve to form a platelet plug over damaged vessels (primary haemostasis) and actions of multiple proteins called clotting factors that act in concert to produce a fibrin clot (secondary haemostasis).

In its stable state, blood remains liquid despite the presence of a vast excess of coagulation proteins and platelets, due to a number of regulatory measures suppressing the coagulation machinery. Unlike the sub-endothelium, the endothelium which lines blood vessels is devoid of thrombogenic tissue factor and collagen, thereby preventing the activation of platelets and the coagulation cascade (16). The coagulation proteins circulate in the blood in an inactive form (17), and their activation is triggered either by contact activation (intrinsic pathway), or by tissue factor stimulation (extrinsic pathway), which act in cascades to promote the formation of the end product fibrin.

**Intrinsic pathway**

The intrinsic pathway is initiated by components contained within the vascular system. It is comprised of the serine proteinases factor XII (FXII), factor XI (FXI), plasma kallikrein (PK) and the non-enzymatic cofactor, high-molecular weight kininogen (HK) (18). The contact system has been shown to be activated on various cell surfaces and on negatively charged non-physiological surfaces like kaolin, or glass, but also on the outer membrane of bacteria (18, 19), and extracellular DNA from neutrophil extracellular traps (NETs) (20). Pre-kallikrein circulates in complex with HK in plasma. Factor XII is activated via anionic surfaces to its active form, FXIIa. FXIIa then converts pre kallikrein and factor XI to their active forms (21). Once activated, the serine protease activity of FXIa
cleaves its anchoring cofactor HK and diffuses into solution where it activates FIX in the presence of calcium which subsequently activates FX. Among the events associated with the contact system, cleavage of HK by activated plasma kallikrein leads to the release of the potent pro-inflammatory molecule bradykinin (BK). BK mediates an increase of vascular permeability, generation of nitric oxide (NO) and other inflammatory responses, and plays an important role in the pathogenesis of severe infections (18, 22). Furthermore, it has been shown that cleavage of HK also leads to the generation of antimicrobial peptides (AMPs) from domains D3 and D5 of the protein (23, 24).

**Extrinsic pathway**

The extrinsic system is the principal initiating pathway of *in vivo* blood coagulation (25). Unlike the intrinsic system, the extrinsic system requires components extrinsic to the blood to initiate the coagulation cascade. The critical component is tissue factor (TF), a membrane bound glycoprotein constitutively expressed on the surface of fibroblasts and around blood vessels and also in various other tissue cells (26). The only vascular cells expressing TF are monocytes, upon induction by endotoxins like LPS, LTA, PGN or M1 and cytokines like interleukin-6 (12, 13, 27, 28). Rupture of the endothelium exposes the subendothelium, which expresses TF on its cell surface. Once TF is exposed to blood, circulating FVII binds to it and forms a catalytic TF/FVII complex initiating the autoactivation of FVII to FVIIa. In the presence of calcium and membrane phospholipids, the TF/FVIIa complex activates FIX and FX (29). This marks the initiation of the first step of the common pathway.
Common pathway

The activation of FX is the point at which the intrinsic and extrinsic pathways converge to form the common pathway of the coagulation cascade. Factor Xa, regardless of how it is formed, is the active catalytic component of the “prothrombinase” complex, which converts prothrombin to thrombin. Thrombin is known as the master regulator of the coagulation cascade. Thrombin cleaves N-terminal peptides from fibrinogen to form soluble fibrin monomers (30). These subsequently polymerize, trapping platelets, erythrocytes, and leukocytes to form the clot. Thrombin also activates circulating factor XIII, a transglutaminase, which catalyzes the formation of covalent intermolecular crosslinks between fibrin molecules providing the necessary structure for a stable fibrin clot. Thrombin also
activates surrounding platelets that in conjunction with the fibrin clot, seal the breach in the vessel wall. Finally, thrombin also activates FV and FVIII triggering a positive feedback loop, thereby amplifying the activation of the coagulation cascade (31).

Despite these important roles, thrombin is also known to be involved in other host defense functions. The proteolytic cleavage of thrombin by human neutrophil elastase and bacterial proteases like *Pseudomonas aeruginosa* elastase generates HDPs from the C-terminal region of thrombin (32). Coagulation is important in trapping the pathogen and thereby preventing their dissemination. The process results in the generation of several peptides in close proximity to the pathogen, which exhibit antimicrobial activity and are immunomodulatory (23, 32, 33). The work described in the current thesis focuses on the generation of such host defense peptides.

**Regulation of coagulation**

The abnormal activation or excessive activation of the coagulation system results in an acquired coagulation disorder called disseminated intravascular coagulation (DIC). It usually arises as a complication of a variety of life threatening conditions e.g. sepsis, massive tissue injury, and obstetric complications (34). To prevent uncontrolled fibrin formation, natural anticoagulant proteins, present in blood and at the vascular endothelial cell surface, balance this process. The negative regulators of the coagulation cascade include C1-inhibitor (C1 INH), tissue factor pathway inhibitor-I (TFPI-I), heparin cofactor II (HC-II), antithrombin III (ATIII), and protein C (29). In addition to these negative regulators, an efficient fibrinolytic system assists in limiting the amount of cross-linked fibrin formed under normal conditions. Together, coagulation, anticoagulation and fibrinolysis maintain a delicate physiological balance. The fibrinolytic system is activated to degrade fibrin into fibrin degradation products. As a result, plasminogen is catalytically converted into plasmin, which in turn breaks down fibrin clots. The activation of
plasminogen is mediated by the tissue plasminogen activator (tPA), the urokinase plasminogen activator (uPA), kallikrein and FXIIIa. Recently, it was also reported that, derivatives of plasmin cleaved FX bound to phospholipid was shown to bind plasminogen and accelerate plasmin generation by tissue plasminogen activator. These FX derivatives inhibit the clot promoting function of FX and convert the protein into a fibrinolysis cofactor (35). The fibrinolysis mechanism is regulated by the thrombin-activatable fibrinolysis inhibitor (TAFI), plasminogen activated inhibitor-1 (PAI-1), α2-plasmin inhibitor and α-2 macroglobulin (36).

2 Bacteria and infections

Bacterial communities commonly inhabit humans as harmless commensals often benefitting the host. But some bacteria act as pathogens and may cause localised or systemic infections. Bacterial pathogens cause a wide spectrum of diseases in humans. Both Gram positive bacteria like *Staphylococcus aureus* and *Streptococcus pyogenes*, and Gram negative bacteria like *Pseudomonas aeruginosa* reside in the epidermis and nasal cavities of healthy individuals and can give rise to superficial infections (37). But in some extreme cases, they can cause systemic infections that might lead to life threatening conditions like sepsis or toxic shock syndrome.

Emerging evidences suggest that microbial virulence factors including cell surface components and bacterial load contribute to the host inflammatory response and the outcome of sepsis. The initiation of the host response during sepsis involves recognition of highly conserved structures of microbes called PAMPs by large family of pattern recognition receptors (PRRs) and this subsequently trigger intracellular signaling cascades leading to the production of proinflammatory cytokines (3).
2.1 Bacterial membrane components

Bacteria are remarkable organisms that ensure the survival of their species by adapting to extreme environmental conditions. Selective evolutionary changes provided them with the structural components, essential for their survival under severe conditions, one of them being their cell wall (38). The essential functions of bacterial cell walls are to provide mechanical strength to survive in any environment and to cope with even sudden changes in osmolarity and temperature (39). As a result, the cell wall of almost all bacteria is fairly rigid, comprising of an interconnected mesh of cross-linked sugar derivates called the peptidoglycan layer (40). However, the rigidity of peptidoglycan does not affect the permeability for compounds, even for Gram-positive bacteria, which have a thick peptidoglycan layer. The structure and composition of peptidoglycan appears to be relatively consistent among Gram-negative bacteria, but varies greatly among Gram-positive bacteria (41). The thickness of the peptidoglycan layer in most Gram-negative bacteria is around 2-3 nm, whereas for Gram-positives it can extend up to 80 nm (40). In addition to the difference in the thickness of the peptidoglycan layer, Gram-negative bacteria are usually surrounded by an additional outer membrane layer, which separates the cytoplasmic membrane, the periplasmic space and the thin layer of peptidoglycan (42). Outer membrane components often play important roles in the interaction of symbiotic or pathogenic bacteria with their host organisms, but the most important functions of this membrane are to serve as a selective permeation barrier allowing the influx of nutrient molecules and to prevent the entry of toxic compounds (43).

The outer membrane of Gram-negative bacteria is fundamentally built as an asymmetric bilayer of lipids, having lipid A in the outer leaflet and phospholipids as the main structure in the inner membrane. As a result, the unusual slow influx of lipophilic solutes makes the outer membrane a highly efficient selective barrier. The outer membrane also contains a nonspecific channel-forming protein called
‘porin’ suggested to be involved in the uptake of hydrophilic solutes or nutrients and perhaps in the extrusion of waste products (43, 44). The structure of the cytoplasmic membrane, also called the cell membrane or plasma membrane, appears very similar to the outer membrane, both in its thickness and its double-layer appearance. This membrane also influences the permeability of various chemicals needed for metabolism. Chemical analysis further showed that the lipid composition of outer membranes varies substantially between bacterial species and is composed of anionic lipids, typically either phosphatidylglycerol (PG) or cardiolipin (CL) or both, as well as zwitterionic lipids like phosphatidylethanolamine (PE) (45, 46). The most significant variations exist with respect to PE and PG, the two major lipids, which together account for about 95% of the phospholipids. Generally, the outer membrane of Gram-negative bacteria possess more PE than PG (47). The electronegative nature of the bacterial membrane, induced by PG and CL, are thought to be the main contributor of selectivity exerted by AMPs towards bacteria because of their charge-mediated attraction to the positively charged AMPs (46).

Moreover, the outer membrane of Gram-negative bacteria invariably contains unique and abundant glycolipids called lipopolysaccharides (LPS) in addition to proteins and phospholipids. LPS is vital to both the structural and functional integrity of the bacterial outer membrane. The LPS molecule is classified as an endotoxin that elicits a strong immune response during bacterial infections (48). Due to the presence of LPS in the outer leaflet, it is believed that AMPs first interact with negatively charged LPS in the bacterial membrane (49).

2.2 Structure of LPS

The basic structure of LPS in all Gram-negative organisms can be formally classified to contain three separate regions. Lipid A is the most conserved domain of LPS comprising saturated fatty acids, which significantly reduce the membrane fluidity (50). Furthermore, lipid A is the active part of the molecule that accounts
for most of the toxic activity in humans through recognition by the signaling receptor TLR-4. This recognition and subsequent activation of immune responses can potentially lead to sepsis – as discussed in the next section (51). The lipid A is covalently attached to a core oligosaccharide region which itself is divided into an inner and outer core. The inner core is proximal to the lipid A and contains a high proportion of unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep). The outer core extends further from the bacterial surface that serves to link the O-antigen unit and is comprised of more common sugars such as hexoses and hexosamines (48). The O-antigen is a repetitive structure of oligosaccharide subunits typically composed of common hexoses contributing to the overall negative charge of LPS. It is the most heterogeneous part of LPS, which can be truncated or even missing. This variation is the reason for varying molecular weights of LPS (48).

Figure 2: Schematic representation of the general features of LPS structure: a proximal hydrophobic lipid A region, a core oligosaccharide region connecting a distal O-antigen polysaccharide region to lipid A.
2.3 Recognition of bacterial membrane components

Innate immune responses are initiated by pattern recognition receptors (PRRs), which recognize specific microbial components, known as pathogen-associated molecular patterns (PAMPs). PRRs are germline encoded, nonclonal, expressed constitutively on various immune cells, including macrophages, monocyte, neutrophils, dendritic cells, B-cells, specific T-cells, and also on non-immune cells such as fibroblasts and epithelial cells (52). PRRs have been identified to react with specific PAMPs, show distinct expression patterns, trigger specific signaling cascades and lead to distinct anti-pathogen responses (53). Several different structural types of PRRs have been identified. They include the Toll-like receptors (TLRs), the RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs) as well as NOD (nucleotide-binding domain)-like receptors (NLRs) and macrophage scavenger receptors (MSR) (52, 54). Among these different PRRs, TLRs were highlighted as the key recognition structures capable of sensing a wide range of microorganisms like bacteria, fungi, protozoa and viruses (55). TLRs are evolutionally conserved type 1 transmembrane glycoproteins and characterized by multiple extracellular domains containing leucine rich repeats (LRRs) responsible for pattern recognition and contain a cytoplasmic signaling domain similar to the interleukin-1 receptor (IL-1R) called a Toll/IL-1 receptor (TIR) (56). TLRs are expressed at different cell locations (intra- or extracellularly), while certain TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface and TLRs 3, 7, 8 and 9 are found in intracellular compartments and endosomes. TLRs have been shown to be a skillful system of sensing a particular molecular structure associated with a pathogen; TLR4 recognizes LPS from Gram-negative bacteria, TLR3 senses double-stranded viral RNA, TLR5 recognizes bacterial flagellin, TLR7 and TLR8 sense single-stranded viral RNA, and TLR9 recognizes bacterial CpGDNA (57). Whereas TLR2 is able to recognize yeast, spirochete, and fungi, several components of Gram-positive bacteria such as peptidoglycan, LTA, lipoarabinomanan, lipoproteins and LPS from certain Gram-negative bacteria.
Recognition of diverse microbial structures by TLR2 has been attributed to its unique ability to heterodimerize with TLRs 1 and 6. Heterodimerization of TLR2 is required for the discrimination of triacylated lipoproteins from diacylated lipoproteins (57). Furthermore, the expression of these TLRs varies rapidly in response to the variety of pathogens, cytokines, and environmental stress (3). Interaction of microbial components with TLRs triggers different signaling pathways, resulting in the activation of inflammation and different antimicrobial responses including the generation of antimicrobial peptides, reactive oxygen species and cytokines, as well as activation of the coagulation and the complement system (58).

**Recognition of LPS by TLR4**

During a Gram-negative infection, bacteria, owing to their normal growth and cell division, inevitably shed LPS together with other cell components thereby making them available for recognition by macrophages and neutrophils. LPS associates with the LPS binding protein (LBP) in the bloodstream or at cell membranes (59), which subsequently catalyzes the transfer of LPS to CD14, a glycosylphosphatidylinositol (GPI) linked protein expressed on the cell surface of phagocytes (60). Furthermore, LPS is transferred to MD-2, which associates with the extracellular portion of TLR4, followed by formation of a homodimer and initiation of the signaling cascade by transducing the signal to the intracellular TIR domain leading to recruitment of adapter molecules (61). There are four adaptor molecules, MyD-88 (myeloid differentiation factor protein 88), TIRAP (TIR domain-containing adaptor protein) or MAL (MyD88-adaptor-like), TRIF (TIR domain-containing adaptor inducing interferon-β) and TRAM (TRIF-related adaptor molecule) (62). The selective usage of these adaptor molecules by distinct TLR ligands mediates different signaling pathways. TLR4 activation triggers two distinct signaling pathways, the MyD88-dependent, leading to the production of pro-inflammatory cytokines and the MyD88-independent, mainly involved in the
production of type-1 interferons (63). In the MyD88-dependent pathway, MyD88 associates with the cytoplasmic signaling domain of TLR4 and recruits IL-1 receptor-associated kinase 4 (IRAK4) and IRAK1 through the N-terminal death domain of the protein (55). Phosphorylation of IRAK4 initiates the phosphorylation of IRAK1 which subsequently associates with TRAF6 (tumour necrosis factor receptor-associated factor 6) initiating further phosphorylation and ubiquitination of several cytosolic signal proteins, including TAK1 (TGFβ-activated kinase 1) and MKK6 (mitogen-activated protein kinase 6). These then modulate the activation of transcription factors e.g. NF-κB inducing the expression of pro-inflammatory cytokines such as TNF-α or IL-6 (62, 63).

**Figure 3:** Schematic representation of simplified LPS/TLR4 signaling pathway
2.4 Sepsis

When the initial host response to a bacterial infection is amplified, and inappropriately regulated, a serious clinical condition referred to as sepsis can occur. Early clinical manifestations of this complex disease include fever, mental confusion, transient hypotension, diminished urine output or unexplained thrombocytopenia (64). The primary source of these severe infections is the lungs, followed by the abdominal cavity, urinary tract, and infections of the blood stream (64, 65). Sepsis is a potentially life-threatening complication, ranking amongst the top 10 causes of death with a mortality of 50% or greater in patients with the more severe form (66). Studies have shown that early diagnosis and effective treatment within the first hour was associated with increased survival (67, 68).

In the early 1990s a consensus conference between the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) laid out a new definition for sepsis (69, 70):

The *systemic inflammatory response syndrome* (SIRS) is defined by the presence of severe clinical insults, manifested by two or more of the following conditions: alterations in body temperature, changes in white blood cell counts, an increased heart rate or hyperventilation.

*Sepsis* is characterized by a systemic inflammatory response to infection in association with infection and similar manifestations like SIRS.

*Severe sepsis* is defined as a state of sepsis in which at least one organ has become dysfunctional.

*Septic shock* refers to hypotension due to severe sepsis caused by acute circulatory failure characterized by persistently low arterial blood pressure despite adequate fluid resuscitation.
Figure 4: Illustration of sepsis continuum

Pathogenesis and treatment

Various Gram-positive and Gram-negative bacteria, and also fungi and viruses can account for the infections that lead to sepsis. The bacteria *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Escherichia coli* are the main contributors (71). LPS derived from the outer membrane of Gram-negative bacteria has a predominant role in causing sepsis, whereas peptidoglycan and lipoteichoic acid of Gram-positive bacteria are much less active (64). Mononuclear cells play an important role during the infection by releasing pro-inflammatory cytokines e.g. IL-1, IL-6 and TNF-α along with other cytokines like IL-12, IL-15, IL-18 and danger-associated molecules (e.g. HMGB-1). The excessive release of pro-inflammatory mediators, the so called cytokine storm, mediates many
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immunopathological features of septic shock (72). Moreover, LPS and other microbial components trigger activation of coagulation through upregulation of TF expression on the surfaces of the endothelium and monocytes leading to a systemic activation of coagulation and inappropriate intravascular fibrin deposition (73). Furthermore, down regulation of three naturally occurring anticoagulant proteins, antithrombin III (ATIII), tissue factor pathway inhibitor (TFPI) or protein C, is an additional cause for a systemic procoagulant state that contributes to the development of disseminated intravascular coagulation (DIC) (74). The extent of uncontrolled inflammation-induced coagulation significantly contributes to organ dysfunction, failure and death (75). The activated coagulation system further aggravates the excessive inflammatory response and leads to an activation of the classical complement pathway (72, 76). Enhanced activation of endothelial cells results in increased vascular permeability, thereby recruiting immune cells like neutrophils. Neutrophils release proteinases or other enzymes as well as reactive oxygen species (ROS). This not only aids in bacterial killing, but also contributes to tissue damage and organ failure (72).

Despite the remarkable developments in understanding the immunopathology of sepsis, therapeutic advances have been painfully slow (77). In the early stages of sepsis, patients are treated with fluids, blood transfusion and inotropic agents to optimize hemodynamic function and broad-spectrum antibiotics. Previous developments of therapeutic strategies against sepsis had mostly focused on single elements leading to activation of immune cells. Related antagonists against various cytokines or single symptoms related to sepsis have been tested, all with limited success (78). Human activated protein C (APC) which is known to function as an anti-inflammatory, anticoagulant and profibrinolytic protein has been used as adjuvant treatment in severe sepsis to reduce the high rate of motility. Recent data with randomized controlled trials found no effect of the treatment with APC in adults or children with severe sepsis (79). Therefore, understanding of the immunopathology of sepsis and determining the structural components of bacteria
that are responsible for initiation of sepsis facilitated many other approaches in identifying potential therapeutic targets. Recent experimental studies, including research work enclosed in this thesis, suggest HDPs as potential therapeutic drugs for treatment of sepsis. HDPs have many features important for the treatment of sepsis. They target the bacteria, but also neutralize LPS leading to modulation of the immune response, thereby eventually restraining the development of sepsis (80, 81).

3 Antimicrobial peptides as anti-infective drugs

Antimicrobial peptides (AMPs) are essential components of the innate immune system that play a crucial role in preventing bacterial colonization and infection. AMPs are found in virtually all groups of organisms, including mammals, amphibians, insects, and other invertebrates (82). Many natural AMPs are gene-encoded and many are generated through proteolytic cleavage of precursor proteins. The biological effects of AMPs vary greatly and have been shown to possess broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses (32, 81, 83). It has been increasingly evident that many AMPs are also involved in modulating host innate and inflammatory responses such as neutralizing the effect of LPS (anti-endotoxin), affecting chemotaxis, enhancing phagocytosis, promotion of angiogenesis and wound healing, blocking contact activation (anti-coagulant), and activation of the complement system (33, 84-86). Given the plethora of functions, AMPs are also called host defense peptides (HDPs). These important properties of AMPs might be exploited furthermore for developing potent therapeutic candidates for the treatment of sepsis. This thesis mainly focuses on AMPs possessing antibacterial, anti-endotoxin, and anti-coagulant activities.
3.1 Classification and structure

AMPs are classified into three major groups: (i) peptides with an α-helical conformation (eg. human LL-37 and magainin), (ii) cyclic and open-ended cyclic peptides with pairs of cysteine residues (eg. defensins, protegrin), and (iii) peptides with an over-representation of some amino acids (eg. proline rich, histidine rich peptides). There are a number of structural similarities that apply for most AMPs. They are usually relatively short molecules, ranging from 12-100 amino acids, display hydrophobic and cationic properties (+2 to +9), and adopt an amphipathic structure (α-helix or β-hairpin-like β-sheet structures) essential for their antimicrobial action (82). In addition to three major classes of cationic antimicrobial peptides, anionic antimicrobial peptides also exist in nature, possessing a net-charge from -1 to -7 and exhibit broad-spectrum antimicrobial effects against bacteria, fungi and viruses (87).

Many AMPs display a helical structure in proximity to lipid-like environments, and a rather unstructured arrangement in hydrophilic conditions (80). When encountering lipid membranes or LPS, they often seem to adapt distinct amphiphilic characteristics, which in most cases have been identified as a significant prerequisite for antibacterial activity (88). Adapting a conformation-dependent amphipathic structure results in separation of hydrophobic and hydrophilic residues of the peptides into different patches of the secondary structure, allowing interaction with both the polar headgroups of lipids and the hydrophobic lipid tail (89).

In contrast to helical peptides, β-sheet peptides often have several antiparallel β-strands stabilized by intermolecular disulphide bridges. Disulfide-stabilized peptides exhibit a similar three-dimensional structure reflecting a reoccurring amino acid and disulfide bridge pattern, derived from the N- to C-terminal orientation (90). This three-dimensional structural cysteine motif is called the γ-core motif, which is characterized by the presence of an 8 to 16 amino acid residue.
long conserved sequence (CXG or GXC motif) with a net positive charge (+0.5 to +7), and basic residues aligned along the axis of the motif as well as amphipathicity (90).

The cationic AMPs investigated in this thesis are derived from the C-terminal region of serine proteases. These peptides are linear in solution and assume an α-helical confirmation upon interacting with lipid membranes or LPS. The peptides then exert their activity by selectively permeabilizing bacterial cell wall and membranes (80, 81).

3.2 Mechanism of action

In contrast to classical antibiotics, AMPs have a uniquely complex mode of action. Regardless of whether peptides act intracellularly or at the bacterial membrane, they all rely on initial interactions with the bacterial outer membrane (82). It is believed that the selectivity of AMPs interactions rely on electrostatic attraction between the positively charged AMPs and the negatively charged outer membrane structures of bacteria. Gram-negative bacterial membranes are highly negatively charged due the presence of phospholipids and lipopolysaccharides (LPS) whereas Gram-positive bacteria contain teichoic acid motifs in their outer membrane, which give them a partial negative charge. Conversely, an eukaryotic cell membrane consists of cholesterol (up to 25%), and zwitterionic or neutral phospholipids, thereby not mediating considerate electrostatic attraction for AMPs (46, 82).

AMPs can be divided into membrane disintegrating compounds or intracellular targets. Several models have been proposed outlining the membrane permeabilizing effect of AMPs, although they are primarily applied to the membrane interactions of α-helical peptides. The common mechanisms for all the models, is the peptide interaction with negatively charged lipids and membrane disruption, once a threshold concentration of peptides is reached. The three most
well known models to kill microbes are the barrel-stave model, the toroidal pore model and the carpet model (91).

*Barrel-stave model*

This mechanism describes the formation of transmembrane channels/pores by bundles of peptides. AMPs aggregate on the surface of the membrane or within the hydrophobic core of the membrane by undergoing a conformational phase transition. The hydrophobic parts align with the lipid core of the membrane and the hydrophilic regions point inward into the lumen of the pore. This leads to leakage of bacterial intracellular components, thereby killing the microbes (91, 92).

*Toroidal pore model*

Peptides insert perpendicularly into the membrane, where hydrophilic parts of the peptide monomers associate with the polar headgroups of the lipid and simultaneously induce the membrane to bend inwards to form pores. The formation of pores is similar to the barrel-stave model, but differs greatly in the structures of the pores formed (91, 93).

*Carpet model*

This mechanism of membrane disruption is also known as the detergent-like effect. AMPs bind in parallel to the plane of the membrane, such that hydrophilic parts interact with the polar headgroups of the lipids. Upon reaching a threshold concentration, the peptides disrupt parts of the membrane, which break off into micelles causing local membrane disruption (94).

Notably, all of these models rely on the amphipathic peptide structure, adapted in contact with lipids, to allow interaction with both the polar headgroups and hydrophobic acyl chain of lipids. Recent data showed that peptides, in addition to acting on membranes, could have multiple cellular targets or alternative modes of action (91, 92). They are found to interact with intracellular targets such as DNA.
or RNA and inhibit protein synthesis (95). Furthermore, pro inflammatory bacterial cell wall components like LPS are also known to be scavenged and neutralised by AMPs (96). The peptides generated from factorX and thrombin described in this thesis, bind and nullify LPS and LPS induced inflammation, in line with previously characterised LPS neutralising AMPs like LL-37 and human β-defensin.

Figure 5: Schematic drawing of the mechanisms of action of antimicrobial peptides.

3.3 Therapeutic potential

Owing to their many targets and multiple host defense functions, AMPs are promising candidates for the development of novel anti-infective agents. It was shown that some bacteria, either intrinsically or adaptably, make use of various
strategies to prevent the effects of AMPs. It appears that specific changes including modifications of LPS (introduction of NH$_3^+$ group) (97), teichoic acid (D-alanine substitutions) (97) or some lipid entities (addition of Lys) (98), as well as alterations in the composition of membrane lipids (99), reduces the overall negative charge, thereby decreasing the electrostatic attraction of AMPs. In addition, degradation of AMPs by host or bacterial proteases and external trapping and neutralization of AMPs by bacterial proteins or efflux pumps preventing inhibitory concentrations to manifest at the membrane, appear to represent important mechanisms of bacterial resistance against AMPs (97).

The selective pressure towards AMPs resistance amongst bacteria might seem worrisome. However, in view of the high resistance of pathogenic bacteria to antibiotics and, based on current knowledge, proposing biological activities and dynamic modes of action of AMPs, they are promising lead structures for therapeutic compounds against bacterial infections. This is further supported by the fact that most active AMPs are known to display broad-spectrum activity including activity against bacterial strains that are resistant to conventional drugs (100). Moreover, the discovery of AMPs that potently inhibit the formation of bacterial biofilms reveals a specific anti-biofilm defense mechanism that could help to combat even chronic infections (101, 102). Furthermore, recent discovery of pleiotropic immunomodulatory properties of AMPs has created a new wave of interest in both academic as well as in the pharmaceutical industry. However, knowledge of the in vivo interactions and immunomodulatory effects of AMPs must be further elucidated in order to assess safety before being used in large-scale trails. Thus, antimicrobial peptides are interesting, new and potential therapeutic candidates for topical or systemic administration.
4 Present investigation

4.1 Paper I

Structure-activity studies and therapeutic potential of host defense peptides of human thrombin.

Background

Increasing knowledge about the host response in severe infections such as sepsis aids in developing novel therapeutic targets. In this scenario, antimicrobial peptides were recently proposed to be potential candidates for treating multidrug-resistant bacterial infections (84). Thrombin, a key enzyme in the coagulation cascade, was known to elicit numerous cellular responses (103). Recently, thrombin was also shown to be involved in the innate immune response against infections. Thrombin cleavage by neutrophil elastase leads to the generation of host defense peptides (32). These peptides derived from C-terminal region of thrombin were antimicrobial against Gram-positive and Gram-negative bacteria, as well as fungi (32). Furthermore, the prototypic peptide GKY25, containing residues 598–622 of the protease domain of thrombin was anti-inflammatory and reduced bacterial loads in initial in vivo experiments.

Aims

To evaluate the structure-activity relationship of HDPs derived from the C-terminal thrombin peptide GKY25.

To unravel the main structural features and minimal determinants responsible for host defense functions of the peptide GKY25.

To evaluate whether smaller variants of GKY25 retain their therapeutic potential in vivo.
Results and conclusion

The peptides, generated through sequential truncation of GKY25 from the N, C, or N and C terminus, respectively, showed a length- and sequence-dependent broad-spectrum antibacterial activity against Gram-negative bacteria *E. coli* and *P. aeruginosa*, the Gram-positive bacterium *S. aureus*, and the fungus *Candida albicans*. Peptides with a minimum length of 19 to 20 amino acids or higher also retained immunomodulatory properties, as illustrated by blocking of nitric oxide (NO) induction in LPS, LTA and Zymosan-stimulated mouse macrophages. Furthermore, by sequential replacement of R and K residues with S, we determined that the two central K residues, adjacent to the evolutionary conserved W1 sequence, are most important for the antimicrobial activity. Moreover, smaller peptides variants (12-20 amino acids) displayed high cell membrane selective behavior. They efficiently permeabilized bacterial cell membranes, but showed low hemolysis and reduced toxicity against eukaryotic cells. In mouse models of *P. aeruginosa* infection, lipopolysaccharide-induced septic shock treatment with the peptide GKY20 resulted in improved survival rates of animals. Further GKY20 treatment improved the animal status as evidenced by decreased fibrin deposition and leakage in the lungs and reduced pro-inflammatory cytokine levels. The therapeutic potential, *in vivo* supports thrombin C-terminal peptides as promising lead molecules in the development of new anti-infective agents.

4.2 Paper II

The C-terminal sequence of several human serine proteases encodes host defense functions.

Background

Serine proteases of the S1 family are versatile enzymes involved in a variety of biological processes, including blood coagulation and fibrinolysis, the complement
and kallikrein systems, as well as various signaling pathways (104). The conserved C-terminal sequence within the protease domain determines the functional diversity and wide variety of substrate specificities and also modulates enzyme activity (105). In a previous study by Papareddy et al., it was shown that C-terminal peptides of human thrombin constitute a novel class of HDPs with bactericidal and anti-inflammatory properties (32). This study has defined new HDPs and expanded the field of antimicrobial peptides to thrombin and the coagulation system. During the course of these studies, it was also noted that the C-terminal sequence of thrombin, as well as other related coagulation factors, comply with a pattern sequence X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X(5,26) (PROSITE pattern (106)) found not only in these proteases of the coagulation system, but are also present in the vast and diverse family of S1 peptidases (32). This pattern sequence is interspersed with conserved hydrophobic residues, which give an amphipathic α-helical conformation to the C-terminal region of S1 peptidases. These structural properties make S1 peptidases a novel source for the generation of HDPs.

**Aims**

To investigate whether the concept of HDPs of coagulation factors could be extended to the family of S1 peptidases (Are S1 peptidases a novel source of AMPs?)

**Results and conclusion**

The application of the pattern sequence X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X(5,26) captured 68 S1 peptide sequences, which were synthesized and screened for antimicrobial and immunomodulatory effects. Studies employing 20 amino acid long peptides, corresponding to the C-terminal region of S1 peptidases, demonstrated that a significant proportion of the sequences of S1 peptidases displayed potent antimicrobial activities against both Gram-positive and Gram-negative bacteria and also the fungus *C. albicans*. 
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Interestingly, peptides derived particularly from proteases of the coagulation and kallikrein systems, blocked nitric oxide production of LPS- and zymosan-stimulated RAW macrophages. Furthermore, in a mouse model of LPS-induced septic shock, selected peptides from thrombin, factor X, hyaluronic acid binding protein-2 (also called factor VII activating protease) and kallikrein 8, significantly reduced animal mortality. In treated animals, reduced levels of pro-inflammatory cytokines paired with an increase of anti-inflammatory IL-10 were observed. Taken together, the results indicate the possibility that several S1 peptidases may give rise to new host defense peptides upon proteolysis.

4.3 Paper III

Identification of a structural core region within coagulation factor X exerting host defense activities.

Background

Coagulation factor X/Xa has a pivotal role as a regulator of the coagulation cascade by converting prothrombin to thrombin (17). In addition to hemostatic functions, activated factor X (FXa) is known to trigger various inflammatory responses on a wide range of cells by activating protease-activated receptors (107). It has been recently reported that proteolytic cleavage of FX by plasmin generates bioactive peptides that enhance fibrinolysis, revealing a new physiological function of FX as a fibrinolysis cofactor (35). It is also known that FX has potential heparin–binding sites in its C-terminal region (108). This is interesting because many heparin-binding peptides are antimicrobial (109). Furthermore, previous predictions based on biophysical and sequence considerations, in combination with peptide analyses (32, 80) indicated that the C-terminal part of FX indeed encodes for antimicrobial and LPS-inhibitory activity.
**Aims**

To further explore the activities of the antimicrobial domain in the C-terminal region of FX.

To investigate whether proteolytic cleavage of FX generates HDPs and whether these are present in vivo

**Results and conclusion**

Analysis of peptides overlapping the sequence of the serine protease domain of FX, identified a C-terminal core region from amino acid G\textsuperscript{402} to K\textsuperscript{427} exhibiting antimicrobial and anti-endotoxin effects. These activities were dependent on cationic residues around a central and evolutionary conserved W-I sequence. In addition, a prototypic peptide RKG25 derived from this core region, displayed potent antimicrobial activity against the Gram-negative *E. coli* and *P. aeruginosa*, and the Gram-positive *S. aureus* in physiological buffer conditions. It also exhibited selective permeabilization of bacterial membranes. Additionally, RKG25 displayed potent immunomodulatory activities, including inhibition of LPS-induced pro-inflammatory cytokine production in human blood and inhibition of activation of the intrinsic pathway of coagulation at negatively charged surfaces such as bacterial membranes or kaolin. Finally, we found that C-terminal fragments from FX are generated by proteolytic cleavage with human leukocyte elastase and also *P. aeruginosa* elastase. Those fragments are also found in patient-derived wound fluid and on bacteria. Taken together, a structural core region within coagulation factor X exerts multiple host defense functions, which could be utilized in the development of novel anti-infective and anti-inflammatory therapies.
5 Summary

An essential new approach for the treatment of infectious diseases is the modulation of host immune responses in order to enhance clearance of infectious agents and reduce tissue damage by uncontrolled inflammation. Apart from comprising diverse antimicrobial and immunomodulatory functions, HDPs also exhibit other important functions of the host innate immune response by being chemotactic, mitogenic, and promoting wound healing (7, 110, 111). Our recent advances in understanding the generation and function of AMPs have highlighted the possibility of their use as potential alternatives to conventional antibiotics by serving as adjuncts and/or replacements to traditional antibiotics, although more research is clearly needed to develop these promising tools (32, 80, 81, 85).

In this present work using computational models and functional analysis, we show the structure-activity relationships of short AMPs derived from the C-terminal region of several serine proteases. We also ascertain that these peptides share characteristics common with classical AMPs of innate immunity and comprise host defense functions. The contemporary approach in the present study by which we identify the structural determinants required for optimal activity, and testing of the efficacy of HDPs in in vitro and in vivo models would contribute to the high throughput discovery of novel endogenous or synthetic HDPs. Furthermore, to validate our hypothesis concerning the generation and existence of these antimicrobial peptides during wounding, we demonstrated that proteolytic cleavage of coagulation factor X with elastase from neutrophils and Pseudomonas generates host defense peptides. In conclusion, host defense peptides derived from human thrombin and factor X are promising candidates for the development of new therapies targeting severe bacterial infections. Hence our findings support the fact that, apart from possessing direct antimicrobial effects, HDPs play a crucial role in modulating both coagulation and inflammation.
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