Characterization of antimicrobial and immunomodulatory activities displayed by short proline-rich antimicrobial peptides *in vitro* and *in vivo*

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**„Ausdauer wird früher oder später belohnt –
meistens aber später.“**

Wilhelm Busch
(15. April 1832 – 9. Januar 1908)

Für meine Familie

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Characterization of antimicrobial and immunomodulatory activities displayed by short proline-rich antimicrobial peptides *in vitro* and *in vivo*

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Dissertation

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Antimikrobielle Peptide (AMPs) sind evolutionär konservierte Bestandteile des angeborenen Immunsystems von Vertebraten und Invertebraten. Ihre antimikrobielle Wirkung spielt eine wichtige Rolle bei der unmittelbaren, direkten Abwehr von Pathogenen. Darüber hinaus wirken viele AMPs regulierend auf Zellen des Immunsystems und üben somit einen zusätzlichen, indirekten Einfluss auf die Pathogenabwehr aus. Im Gegensatz zu lytischen AMPs inhibieren Prolin-reiche AMPs wichtige intrazelluläre bakterielle Moleküle, wie das Hitzeschockprotein DnaK. Die Prolin-reichen Insekten-AMP-Derivate Oncocin, Onc72, Apidaecin 1b sowie Api88 sind insbesondere gegen Gram-negative Bakterien wirksam. In dieser Arbeit konnte gezeigt werden, dass, nach Ausschluss einer zytotoxischen Wirkung auf murine Immunzellen *in vitro* und *in vivo*, das optimierte Peptid Onc72 Mäuse vor den Folgen einer Infektion mit einer letalen Dosis von *Escherichia coli* schützen konnte. Im Folgenden sollte daher untersucht werden, ob stimulierende oder modulierende Wirkungen der Peptide auf wichtige Zellen des angeborenen Immunsystems festzustellen sind. Es wurde zunächst gezeigt, dass keines der Peptide chemotaktisch auf Dendritische Zellen (DC) wirkt. Darüber hinaus wurden jeweils Kulturen von DC oder Makrophagen bzw. Splenozyten und Peritoneal Exsudat Zellen mit den Peptiden in An- und Abwesenheit von Lipopolysaccharid (LPS) inkubiert. Das als immunmodulierend bekannte AMP „cathelicidin-related antimicrobial peptide“ (CRAMP) diente hierbei als Kontrollpeptid. Im Gegensatz zu CRAMP modulierten die vier getesteten Insekten-AMP-Derivate die LPS-induzierte Immunantwort nicht. Weiterhin wirkte keines der Prolin-reichen Peptide immunstimulierend. Diese Daten deuten darauf hin, dass die getesteten Peptide auch *in vivo* ausschließlich direkt antibakteriell wirksam sind, ohne DC oder Makrophagen zu modulieren. Somit werden weitergehende pharmazeutische Untersuchungen für die Entwicklung von Insekten-Peptiden als Therapeutika vereinfacht.

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ABBREVIATIONS

°C	degree (in Celsius)
µg	microgram
µl	microliter
µM	micromolar
AMP	antimicrobial peptide
AMP-P5	antimicrobial peptide-P5
APC	antigen presenting cell
APO	all peptide optimized
A3-APO	pyrrhocoricin-derivative with the synthesis number A3 (all concerning amino acid positions are optimized)
Api 1b	apidaecin 1b
Api88	optimized apidaecin No. 88 (optimized sequence derived from apidaecin 1b)
Bac	bactenecin
BMDC	bone marrow-derived dendritic cells
BMDM	bone marrow-derived macrophages
CD	cluster of differentiation
cDC	conventional dendritic cells
cDNA	complementary desoxyribonucleic acid
cfu	colony forming unit
CRAMP	(murine) cathelicidin-related antimicrobial peptide
CTL	cytotoxic T cell
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell sorter / flow cytometry
FBS	fetal bovine serum
FcR	Fc receptor
FITC	fluoresceinisothiocyanate
Flt3-ligand	fms-like tyrosine kinase-ligand

hBD-1/2	human beta-defensin 1/2
HEPES	4-(2-Hydroxymethyl)-1-piperazine ethanesulfonic acid
HPF	high power field
GM-CSF	granulocyte macrophage colony-stimulating factor
h	hour
H & E	hematoxylin and eosin
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
i.p./ip	intraperitoneal
KLH	keyhole limpet hemocyanin
K. pneumoniae	*Klebsiella pneumoniae*
LPS	lipopolysaccharide
LBP	lipopolysaccharide-binding protein
M	molar
mBD-2	murine beta-defensin 2
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
min	minute
MIP-1α	macrophage inflammatory protein-1 alpha
ml	milliliter
mM	millimolar
nm	nanometer
nM	nanomolar
Onc	oncocin (optimized sequence derived from *Oncopeltus* antibact. peptide 4)
Onc72	optimized oncocin No. 72 (optimized sequence derived from oncocin)
P. aeruginosa	*Pseudomonas aeruginosa*
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cells
PE	phycoerythrin
PEC	peritoneal exudate cells

PerCp	peridinin-chlorophyll-protein complex
pfu	plaque forming units
PHA	phythemagglutinin
PrAMP	proline-rich antimicrobial peptide
PRR	pattern recognition receptor
ROI	reactive oxygen intermediates
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium
RSV	respiratory syncytial virus
S. aureus	*Staphylococcus aureus*
TFA	trifluoroacetic acid
Th1/2/17	T helper cell 1/2/17
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
x g	times gravity

1 INTRODUCTION

1.1 OVERVIEW: INNATE AND ADAPTIVE IMMUNE SYSTEM OF VERTEBRATES

The ability of eukaryotic organisms to distinguish between "self" and "non-self" is a necessity for their survival. Eukaryotic hosts co-exist with a multiplicity of different kinds of potential pathogens such as bacteria, viruses or fungi. Therefore, it is of significant importance that a host identifies and combats possible threats which are derived from such "non-self" sources. Moreover, eukaryotic organisms have to distinguish, whether or not single cells of its "self" are degenerated and have to be eliminated. As a result, higher eukaryotic organisms have evolved an immune system that contains various mechanisms for host defense, homeostasis, tissue and wound repair, differentiation, memory, tolerance and a lot of more tasks.

The immune system of higher eukaryotic organisms consists of two axes. On the one hand, the innate immune system reacts immediately as a first and effective barrier for intruding pathogens. One part of this barrier is a physical barricade, built by e.g. epithelia, mucosal surfaces, and intestinal peristalsis. Commensal bacteria compete with potential pathogenic intruders for nutrients and space. Innate immunity contains a number of components (e.g. phagocytes, receptors, complement, and soluble mediators) which recognize and counter conserved structures of pathogens. Moreover, innate immunity comprises a chemical barricade, built by e.g. fatty acids on the skin, mucus secretion, antimicrobial peptides (AMPs) and enzymes like pepsin (in the stomach) or lysozyme (in tears, sweat or saliva), and others [1]. In many cases, various mechanisms of innate immunity are sufficient to successfully combat pathogens, e.g. especially newborns rely on innate immune responses due to their insufficiently developed adaptive immunity [2].

On the other hand, higher eukaryotes possess an adaptive immune system that is able to adjust to specific antigenic characteristics by rearrangement of T and B lymphocyte receptors. B cells express membrane-bound B cell receptors and are able to generate antibodies, which play an important role for the control of extracellular pathogens [3]. The other lymphocyte population are T cells, which comprise two major subgroups: CD8-positive cytotoxic T cells (CTLs) kill virus-infected cells, while CD4-positive T helper cells (Th) support activation of other immune cells by cell-cell-interaction followed by cytokine secretion [3]. Moreover, the

adaptive immune system is characterized by the existence of organized lymphoid tissues, a primary immune response and an immunological memory [4].

Recognizing an intrusion and developing an antigen-specific and therefore highly effective adaptive immune response takes some time in advance. Hence, an immediately working innate immunity is indispensable and forms an effective first line of defense against pathogenic intruders. If a pathogen has overcome the physical and chemical barriers described above, it will be antagonized by innate immune mechanisms such as natural killer cells (for intracellular antigens), or phagocytes and complement (for extracellular antigens). Natural killer cells induce apoptosis in virus-infected cells, whereas dendritic cells (DC), macrophages and neutrophilic granulocytes act as phagocytes. Together with B lymphocytes, DC and macrophages are defined as professional antigen presenting cells (APC). APCs permanently collect and process samples of their environment and present fragments of those possible antigens to T cells in lymphatic tissue. The occurrence of "danger signals" in the context of antigen presentation affects the resulting immune response [5]. DC are the only APC type that is able to activate naïve T cells in lymphoid tissues [6]. Thus, DC take a special part in the regulation of immune responses regarding the interplay between the innate and adaptive immune system [7]. In contrast to DC, macrophages present antigens to pre-activated T cells in lymphatic tissue [6] and they exert regulatory and effector immune functions [8,9]. Macrophages and T cells mutually promote their effector functions. Altogether, DC and macrophages are important parts of innate immunity, which are indispensable to support and regulate adaptive immune responses.

1.2 CYTOKINES AND CHEMOKINES

Cytokines are important regulatory elements of communication between immune (and non-immune) cells. They are small proteins (up to 25 kDa), which can be secreted by different immune and non-immune cell types [10]. Cytokines are involved in all aspects of orchestration of immune responses and can have different effects on adjacent cells in the tissue, depending on the cells' cytokine receptor expression. Therefore, cytokines regulate adjacent cells (defined as paracrine action) as well as the cytokine-releasing cell itself (defined as autocrine action). Even distant cells can be affected by cytokines acting in an endocrine manner [10]. The resulting immune response is a consequence of different factors involved, e.g. the stimulus inducing cytokine release, the site and time point of release, and

the combination of cytokines present [11]. According to structural and / or functional properties, cytokines are grouped into several subfamilies (e.g. chemokines, interleukins, and growth factors) [10]. Chemokines are an exceptional cytokine subfamily. Its members are able to attract especially immune cells. Mammalian cell migration plays a significant role in diverse situations, e.g. for organization of tissues and organs during development, wound healing, recruitment of immune cells to the site of infection, location of lymphocytes inside lymphoid organs, or guidance of sperm to the oocyte or homeostasis [12-14]. Cancer cells also use chemotaxis for metastasizing, which is the leading cause for cancer death [12,13,15,16]. Chemokines can be produced by various cell types in response to infection, damage, or other processes. Even some bacterial peptides act as chemoattractors for eukaryotic immune cells [10]. Interestingly, Eisenbach *et al.* and others identified at least five chemoattractant proteins for human sperm cells [15,17,18]. The authors raise the question of the purpose, for which this high amount of perhaps redundant chemoattractors is needed. Probably, the chemotaxis process may be more complex as previously assumed and a combination of different chemoattractors is needed to navigate cells correctly [15].

According to structural differences, chemokines are divided into four subgroups (Table 1). These chemokine groups are designated according to the number of non-cysteine amino acids ("X") separating two adjacent cysteines ("C"). As shown in table 1, CC and CXC chemokines each comprise most of the known chemokines. The two other groups, C chemokines and CX3C chemokines, include only one (CX3C chemokines) or two (C chemokine) members, respectively [10,19]. Only cells expressing an adequate receptor can be attracted by chemokines. All chemokine receptors are integral membrane proteins with seven membrane-spanning helices [10]. The expression of chemokine receptors depends on the identity as well as the activation status of the cell. After maturation most DC lose their sensitivity for several chemokines which attracted them in an immature status [20]. For instance, immature DC, but not mature DC, express the chemokine receptors CCR1, CCR5, CCR2A/B, and others. Thus, only immature DC respond to the chemokines MIP-1α (via CCR1 and CCR5) and MCP-1 (via CCR2A/B) [20].

Table 1. Chemokine groups, a selection of chemokines and their target cells (modified according to Murphy *et al.* [3], with additional information from Yoshida *et al.* [21] and Dieu-Nosjean *et al.* [20])

chemokine group	members	target cells
C-chemokines	lymphotactin α (XCL1)	T cells, NK cells
	lymphotactin β (XCL2)	lymphocytes [21]
CC-chemokines	MIP-1α (CCL3)	monocytes, macrophages, T cells, NK cells, basophilic granulocytes, immature DC, bone marrow cells
	MCP-1 (CCL2)	T cells, monocytes, basophilic granulocytes, immature dendritic cells [20]
	RANTES (CCL5)	monocytes, macrophages, T cells, NK cells, basophilic granulocytes, eosinophilic granulocytes, DC
	MIP-3α (CCL20)	T cells, mononuclear cells of the peripheral blood, bone marrow cells, DC
	MIP-3β (CCL19)	naïve T cells, mature DC, B cells
	eotaxin	eosinophilic granulocytes
CXC-chemokines	IL-8 (CXCL8)*	neutrophilic granulocytes
	GCP-2 (CXCL6)*	neutrophilic granulocytes
	IP-10 (CXCL10)	activated T cells
	SDF-1α/β (CXCL12)	$CD34^+$ bone marrow cells, T cells, activated $CD4^+$ T cells, DC, B cells, naïve B cells
CX3C-chemokines	fractalkine	T cells, monocytes, neutrophilic granulocytes (?)

* contains the sequence motif ELR immediately before the first cysteine residue; ELR^+ chemokines chemoattract neutrophilic granulocytes [3].

1.3 EVOLUTION OF THE INNATE IMMUNE SYSTEM

Several genes participating in innate host defense of vertebrates have homologous genes in invertebrates and plants. These homologous gene products are often involved in host defense, too. For instance, genome analyses of plants and animals indicates that a common ancestor of both groups already possessed elements of the Toll signal pathway of NFκB activation [22]. In vertebrates, Toll-like receptors act as pattern-recognition receptors (PRRs), which sense pathogen-associated molecular patterns (PAMPs) derived from different pathogens, and promote innate immunity [23]. In 1985, *toll* was identified in *Drosophila melanogaster* by Nobel Prize laureate Christiane Nüsslein-Volhard [24,25] and colleagues. They showed that the *toll* gene product is involved in embryogenesis and establishment of the dorso-ventral

body axis. Later it was demonstrated that Toll triggers immune responses in *D. melanogaster* [26,27]. For example, in 1996, Lemaitre *et al.* showed that Toll is involved in defense of *D. melanogaster* against fungal infections [27]. Structurally similar Toll-like receptors were identified by several laboratories in vertebrates. Their importance in vertebrate innate immunity as well as in promotion of adaptive immune system activation was shown in numerous reports [28-30].

1.4 OVERVIEW: INNATE IMMUNE SYSTEM OF INSECTS

There are various elements of the insect immune system that can be compared to elements of innate and even adaptive immunity of vertebrates. For instance, alternative splicing of PRRs in insect immunity allows adaptation to a variety of pathogens [31]. Insect as well as vertebrate immunity comprise humoral and cellular components. Insects possess free blood cells which are termed “hemocytes” [32]. Some authors discriminate between hemocytes and immunocytes, while others rank immunocytes among hemocytes [31,32]. Plasmatocytes are a prominent hemocyte subpopulation, which phagocytoses pathogens and apoptotic cells and is able to secrete antimicrobial peptides (AMPs) and extracellular matrix proteins. Additionally, they can be attracted by chemokines [31]. Besides plasmatocytes, various other hemocyte cell types were described in different insect families e.g. granulocytes, crystal cells, thrombocytoids, or lamellocytes [31]. Insects as well as vertebrates use cytokines for communication between cells. Importantly, cytokines from mammals and insects are not homologous. However, despite obvious differences between insect and mammal cytokines, they seem to use the same crucial downstream signaling processes, e.g. JAK/STAT pathway [31]. Additionally, as mentioned above, invertebrates and vertebrates use homologous PRRs, e.g. Toll-like receptor and they work with similar mechanisms such as opsonization, melanization, phagocytosis, or secretion of complement and AMPs [31,33]. Several insects secrete AMPs on their epithelial surface to protect themselves from colonization and penetration by pathogens [33]. Besides the fat body, which represents the main source of AMP expression in insects, AMPs are also produced by hemocytes and several epithelia [31,32]. According to Hetru *et al.*, the maximal AMP concentration of all AMPs combined in hemolymph in response to infection is 300 µM. This concentration has no cytotoxic effect on eukaryotic host cells [31,34].

1.5 PARTICULAR ASPECTS OF HONEY BEE IMMUNITY

Honey bees (*Apis mellifera*) are social animals. High population densities may implicate an increased risk of infections. Therefore, honey bees possess group-related and individual defense mechanisms for protection against pathogens. Group immunity includes a variety of precautionary mechanisms e.g. separating and removing of infected larvae, building the beehive with antimicrobial material and feeding larvae as well as adult bees with strongly antimicrobial royal jelly [33,35]. Moreover, honey bees protect themselves from pathogens by using inhibitory effects of microbial non-pathogenic bacterial: Evans and Armstrong reported that symbiotic bacteria which were isolated from honey bee larvae strongly inhibited growth of *Paenibacillus larvae*, which represents one of the main bacterial pathogens of honey bees [36]. Genomic analyses revealed that the honey bee *Apis mellifera* possesses only one third of the amount of genes involved in immunity than typically assumed for insects. This suggests that the group immunity may either be strong enough to protect individual bees from most pathogens or that the variety of co-evolved pathogens may be limited [32,33].

1.6 ANTIMICROBIAL PEPTIDES

1.6.1 OVERVIEW: ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs) are important conserved components of the innate immune system. Those structurally diverse peptides can be found in a variety of organisms ranging from plants and arthropods to higher animals [37] and they are defined as AMPs based on their common direct antimicrobial activity especially against bacteria. Another very important effect of their antimicrobial activity is suppression of biofilm formation [38]. Some AMPs were described to exert antifungal (e.g. against *Candida albicans* or *Cryptococcus neoformans*) [39,40] or antiviral effects (e.g. against adenoviruses, influenza virus, vaccinia virus, SARS), [41-45] as well as strong protective effects against parasites (e.g. against *Plasmodium falciparum*) [46]. Several AMPs are additionally able to exert an indirect antimicrobial activity by activation and modulation of the host's immune system (see chapter 1.6.7).

Additionally to anti-infective effects, some AMPs were reported to kill cancer cells or at least to stop tumor growth [47] by cell membrane perturbation or induction of apoptosis, or inhibitory effects on angiogenesis [47]. Many AMPs are also involved in various other tasks, e.g. wound healing [38] or cell proliferation [48].

By combining of a few distinct AMPs, the host is able to control almost all potential threatening pathogens. For instance, insects express only a limited number of AMPs and each of it has its own spectrum of potential target pathogens [49-51]. Important AMP subgroups are defensins, cathelicidins, short proline-rich peptides, histatins, neuropeptides, or peptide hormones [52]. In 1973, Fernandez de Caleya *et al.* were the first to describe antimicrobial acting peptides in plants. These basic polypeptides were named "purothionins α and β" and were found in endosperm of wheat [53,54]. The first AMPs in animals were originally isolated from hemolymph of pupae of the giant silk moth *Hyalophora cecropia* by Boman *et al.* in 1980/1981 [55,56]. Besides the two cecropin peptides P9A and P9B, Boman *et al.* additionally purified lysozyme [55-57]. Lysozyme is bactericidal against Gram-positive bacteria, as previously shown by Fleming [58], whereas the cecropins P9A and P9B exert antimicrobial activity against Gram-negative bacteria [56]. In contrast to the structurally related bee venom peptide melittin, the two small basic cecropins are only lytic

for bacteria, but not for eukaryotic cells [56,57]. After Boman's first description of AMPs, several other AMPs were isolated from invertebrate and vertebrate tissues, e.g. neutrophilic granules, insect hemolymph, frog skin, saliva, or intestine of mammals. In 2010, isolation and identification of more than 1500 different AMPs from animal tissues was estimated [59]. In humans, phagocytes and epithelial cells are the main sources of AMP expression [52]. AMPs are constitutively expressed or their expression can be induced by endogenous or exogenous signals. For instance, human neutrophilic granulocytes constitutively express the cathelicidin LL-37 or the α-defensins HNP 1-3. Upon infection, pro-inflammatory cytokines and endotoxins induce LL-37 expression in monocytes, mast cells, T lymphocytes, keratinocytes, and barrier epithelial cells [60,61]. It was reported that T helper cell 2 (Th2) cytokines suppress expression of defensins and cathelicidins, whereas bacterial components as well as T helper cell 1 (Th1) and especially T helper cell 17 (Th17) cytokines support AMP secretion [62,63]. Interestingly, several AMPs such as cathelicidins and defensins were described to be present in amniotic fluid, emphasizing their important role as early soluble antimicrobial mediators in innate immunity [2].

1.6.2 STRUCTURAL ASPECTS OF AMPS

Despite structural differences, diverse subgroups of AMPs share several features. Most AMPs are small (12 - 50 amino acids) and possess a net charge of +2 to +7 and up to 50 % hydrophobic amino acids [37,52]. In addition to the majority of cationic AMPs, a few anionic AMPs are known [53]. Examples for this small group of AMPs are propieces of ovine trypsinogen or PYL^{a} (which is the synthetic derivative of PGL^{a}) peptide from *Xenopus laevis* skin secretion [53,64,65]. Most AMPs are amphiphatic, which means that they consist of hydrophilic (charged) as well as hydrophobic (uncharged) regions [37]. Amphiphatic AMPs are able to penetrate and disrupt bacterial membranes [52]. Besides lytic AMPs, some AMPs bind intracellular bacterial targets and thus inhibit bacterial growth or kill bacteria (see chapter 1.6.3).

According to their structural features and their content of particular amino acids, AMPs are divided into several sub-groups. Depending on different authors, there are several classifications of AMPs. Brogden divided AMPs into five subgroups (Table 2): Besides the above mentioned group of anionic AMPs, there are linear cationic α-helical peptides (e.g. LL-37), cationic peptides enriched for one specific amino acid (e.g. proline-rich

apidaecins and oncocin), anionic and cationic peptides with disulfide bonds (e.g. defensins), and anionic and cationic fragments of larger proteins (e.g. lactoferricin) [66].

Table 2. Structurally diverse groups of AMPs and selection of important group members according to Brogden (extended and modified according to Brogden [66])

AMP group	members
anionic peptides	- ovine **trypsinogen** - **PYL[a] / PGL[a]** from *Xenopus laevis* - **dermcidin** from humans - **maximin H5** from amphibians
linear cationic α-helical peptides	- **LL-37** from humans, **CRAMP** from mice - **CAP18** from rabbits - **magainin 2** from amphibians - **BMAP, SMAP** from cattle, sheep, pigs - **cecropin**, **melittin** from insects
cationic peptides, enriched for one specific amino acid	- proline-and arginine-rich insect peptides from the honey bee *Apis mellifera* (e.g. **apidaecin 1b**), ***Oncopeltus* antibacterial peptide 4** from the large milkweed bug *Oncopeltus fasciatus*, **pyrrhocoricin** from the European sap-sucking bug, **bactenecins** from cattle (**Bac7, Bac5**), sheep and goats, **PR-39** from pig, drosocin from *Drosophila spp.*, - optimized proline-rich peptides (**oncocin, Onc72, Api88, A3-APO**) - tryptophan-containing peptides such as **indolicidin** from cattle - small histidine-rich salivary polypeptides such as **histatins** from humans
anionic and cationic peptides that contain cysteine and form disulfide bonds	- **α-defensins** from humans (e.g. **HNP1-3**), rabbits and rats, **β-defensins** from humans (e.g. **hBD1-4**), cattle, mice (e.g. **mBD-2**), rats, pigs - insect defensins (**defensin A**)
anionic and cationic fragments of larger proteins	- **lactoferricin** from lactoferrin - antimicrobial domains from bovine α-lactalbumin, human hemoglobin, lysozyme and ovalbumin

Several AMPs show posttranslational modifications which may profoundly affect their activity [53]. Formation of disulfide bonds may have an enormous impact on receptor-mediated activities which require a specific three-dimensional structure of the ligand. Other possible posttranslational modifications are e.g. glycosylations, amidations, phosphorylations, or usage of *D*-amino acids. These modifications can have protective effects against proteinases. They may augment the specific binding of the peptide to the pathogen, change the mechanism of antibacterial action, and thereby modulate the peptide's antimicrobial activity [53,67-72].

1.6.3 MODES OF ANTIMICROBIAL ACTION

While most AMPs have Gram-negative and Gram-positive bacterial targets, there are only a very few AMPs known which exclusively kill Gram-positive bacteria or Gram-negative bacteria [47]. Some AMPs disturb or disrupt several bacterial key cell processes and components, e.g. cell integrity, bacterial metabolism, protein synthesis, DNA transcription, or replication. A number of AMPs are even able to use several modes of antibacterial action [73]. Most AMPs exert their antibacterial activity by permeabilization of the bacterial membrane [53]. This killing mechanism is used by e.g. cathelicidins, defensins, cecropins, or magainins. [53,74-76]. The first step in this mode of action is electrostatic attraction between oppositely charged components of the bacterial membrane and the AMP. Afterwards, the peptide traverses a layer of polysaccharides (Gram-negative bacteria) or polysaccharides, teichoic acids, and lipoteichoic acids (Gram-positive bacteria) before interacting with the bacterial cytoplasmic membrane [66]. The AMP integrates into the membrane which is followed by pore forming, destruction of the transmembrane potential and of the pH gradient, inhibition of respiration, and ultimately resulting in leakage of the cell [66]. There are several models to describe membrane perturbation by AMPs, e.g. toroidal model, barrel-stave model, or carpet model [73]. Correlation between increased positive charge of the peptide and permeabilization activity was shown for several AMPs [53]. In contrast, too many positive charges in an AMP can have the opposite effect: Matsuzaki *et al.* showed that a strongly positively charged magainin analogue has a reduced antimicrobial activity. This effect may be due to strong attraction of membrane and peptide leading to very fast internalization of the peptide. Thus, the remaining time for the peptide to form pores on the membrane is too short. Another explanation may be that the strongly positively charged peptides may repulse each other, so that they destabilize membrane pores or they can not cooperate to form membrane pores [53,72].

The *D*-enantiomer of the lytically acting AMP magainin 2 is antibacterially active, too [77]. This demonstrates clearly that the lytic AMP magainin 2 does not require a specific receptor peptide but rather acts via charge-specific interactions with the bacterial membrane. Antibacterial activities of AMPs are influenced by several bacterial membrane features such as membrane potential, phospholipid composition, or sterol content [53]. This raises the question, if AMPs are toxic for their host cells. AMP-mediated killing of eukaryotic cells seems to occur only at high AMP concentrations. Many authors suggest that eukaryotic host

cells protect themselves with high cholesterol content from AMP attack [78]. Bacterial membranes do not contain cholesterol, which is known to condensate phospholipid bilayers and therefore hampers AMP penetration [78]. Moreover, in eukaryotic cell membranes, phospholipids are arranged asymmetrically: While the inner layer of the eukaryotic phospholipid bilayer contains lipids with negatively headgroups, the outer layer consists of neutral phospholipids, which further prevent the eukaryotic cell from AMP attraction [78-80]. Another important feature of most AMPs is their sensitivity towards salt and serum components. Even physiological salt concentrations may attenuate many AMPs. However, due to modifications of bacterial membrane composition and gene expression *in vivo*, many bacteria are thus vulnerable for AMPs [78,81]. For instance, the presence of CO_3^{2-} at physiological salt concentrations leads to alterations in the bacterial membrane which makes them more susceptible to AMPs [81].

Besides membrane perturbation, other killing mechanisms are known. These mechanisms affect targets on the bacterial surface as well as within bacteria [66,82]. Intracellular killing mechanisms interfere with crucial processes in cell homeostasis, cell metabolism, or reproduction. Examples for intracellular killing of bacteria by AMPs are activation of bacterial amidases and subsequent degradation of bacterial peptidoglycan by nisin and pep5, inhibition of cell wall synthesis by mersacidin, or inhibition of protein synthesis by indolicidin. [53,66,83-85]. AMPs such as oncocin, apidaecins, histatins, pyrrhocoricin or drosocin enter the bacterium and inhibit bacterial enzymes [53,64,86,87]. This is facilitated either by direct inhibition of the enzyme's activity or by blockade of its substrate binding site [53,86]. For instance, the proline-rich AMPs oncocin as well as apidaecin 1b were described to specifically bind the bacterial heat-shock protein DnaK as well as other target molecules [86,87]. Otvos *et al.* demonstrated that a related proline-rich AMP, pyrrhocoricin, indeed binds DnaK, but does not interact with Hsp70, the human equivalent of DnaK [86]. It is assumed that intracellular bacterial killing by proline-rich AMPs consists of three consecutive steps: First, the AMP passively traverses the outer bacterial membrane which is followed by active transport of the peptide from the periplasmic space to the cytoplasm. It was suggested that the peptide may be transferred to the cytoplasm by a permease-type transporter [51], where it binds DnaK and possibly additional other targets [87,88]. As a result, bacterial protein synthesis and chaperone-assisted protein-folding is inhibited and the bacterium is subsequently killed by these AMPs [51,86,87,89,90]. Interestingly, it was reported that insertion of prolines into α-helical peptides leads to decrease of the lytic activity of these

peptides [51,91]. The apidaecin *D*-enantiomer is not antimicrobially active suggesting specific binding of a bacterial target rather than charge-specific bacterial killing [92]. A similar mode of bacterial killing was proposed for the eukaryotic proline-rich cathelicidin PR-39, which is known as mammalian relative of the insect-derived short-proline-rich AMPs (PrAMPs) [51,83,93].

To date, a few AMPs were tested in various *in vivo* bacteraemia models [51,94-96]. The PrAMP pyrrhocoricin protected mice at doses of 10 or 25 mg/kg (administered intravenously) in a septicaemia model using *Escherichia coli* (*E. coli*) [51]. In another study, the pyrrhocoricin-analog A3-APO was shown to be antibacterially active in different septicaemia mouse models [96]. Furthermore, a backbone-protected pyrrhocoricin-derivative was also effective in a local *Haemophilus influenzae* infection [51].

Some α-helical cathelicidins, β-defensins or histatins were described to be also active against several fungal strains such as *Candida albicans* or *Cryptococcus neoformans* [97-99]. The MIC values for the antifungal activity performed by α-helical cathelicidins were reported to be similar or only slightly higher than the MIC values in antibacterial assays [98]. Interestingly, indolicidin, which kills bacteria via inhibition of bacterial DNA, RNA and protein synthesis, was shown to disrupt fungal cell membranes [82,100,101]. It is assumed that antifungal activity performed by α-helical cathelicidins is facilitated by membrane permeabilization [98]. Also, promotion of reactive oxygen intermediates (ROI) production or depletion of fungal mitochondria by distinct AMPs is discussed [82,102].

1.6.4 MICROBIAL RESISTANCE AGAINST AMPS

In contrast to conventional antibiotics, which mainly target a small number of essential bacterial proteins, many AMPs permeabilize bacterial membranes. While in recent years, increasing numbers of bacterial species with resistances for conventional antibiotics were reported, the development of AMP resistances was slower than expected [38,103,104]. Thus, extensive numbers of bacterial strains resistant against AMPs have not been reported until yet. This may be due to the fact that bacteria might overcome the antibacterial action of conventional antibiotics by alteration of a single element [78]. In contrast to that, different kinds of AMPs may be secreted by surrounding cells at an infection site. Therefore, it is likely that several antibacterial modes of action are involved, which makes it more difficult for

bacteria to escape AMP activity. However, as it is known for antibiotics, microorganisms try to develop resistance strategies to evade AMP killing. There are only a few bacterial species that have evolved resistance mechanisms against AMPs [38]. These resistance mechanisms include all aspects of antimicrobial action, e.g. expression of AMP, attachment, insertion, and membrane perturbation, peptide transport, as well as peptide cleavage [66]. Various bacteria such as *Shigella spp.* down-regulate secretion of AMPs in monocytes and epithelial cells [78,105]. Moreover, Gram-negative bacteria as well as the Gram-positive bacterial strain *Staphylococcus aureus* (*S. aureus)* try to reduce their net negative surface charge in order to minimize attachment of positively-charged AMPs. This includes reduction of membrane anionic phospholipid content as well as chemical modification of surface molecules, e.g. with positively charged amino acids [53,66]. It was observed that *S. aureus* integrates the basic amino acid *L*-lysine into its anionic membrane [66,106]. Besides, Gram-negative bacteria change lipid A or the fluidity of their outer membrane to prevent AMP attachment. Another mechanism is the modification of expression of outer membrane proteins associated with AMP resistance performed by *Yersinia enterocolitica* [66,107].

There are also several mechanisms to prevent bacteria from killing by intracellularly acting AMPs. Bacteria use efflux pumps to export AMPs taken up intracellularly [66,108]. Additionally, several resistant bacteria utilize influx pumps for subsequent proteolytic AMP degradation [66,78]. However, considering these various strategies of bacterial resistance, high expression of a variety of AMPs using different modes of bacterial killing mechanisms at the infection site increases the chance to combat the pathogen [78,80]. Bacteria have to balance between possible risks of evasion mechanisms and the requirements of metabolism and structural integrity for cell homeostasis [78].

1.6.5 APIDAECINS AND ONCOCINS

Apidaecins, *Oncopeltus* antibacterial peptide 4, drosocin or pyrrhocoricin consist of 20-35 amino acids and belong to the group of insect-derived short proline rich AMPs (PrAMPs) [74,109-111]. The apidaecin family consists of 20 members, which were originally isolated from hemolymph of bees and wasps [109]. Honey bees (*Apis mellifera*) express three different apidaecins (apidaecin 1a and b as well as apidaecin 2), which are functionally identical [109]. According to Casteels-Josson *et al.*, in honey bees apidaecin-coding genes are arranged to gene clusters [112]. Following transcription and expression, the translated

precursor proteins are further cleaved into up to 12 apidaecin peptides, which can be different apidaecin isoforms. This enhancement of peptide production is unique among insects and may compensate the fact that transcriptional activation in honey bees is relatively slow [112]. It is assumed that constant domains in the amino acid sequence of the peptide are associated with their general antibacterial activity, whereas variable sequences are responsible for their specific activity against particular bacterial strains. This may represent an adaptation to species-specific pathogens [51].

Adult honey bees express mature and active apidaecins, whereas bee larvae possess less and non-mature apidaecins [109]. Due to their high proline content, apidaecin peptides do not form helices. Moreover, they are stable at high temperatures as well as at a low pH [109]. Apidaecins are known to be antibacterially active especially against Gram-negative pathogens [109]. The antibacterial activity of apidaecins decreases under nutrient-free assay conditions, supporting the finding that short PrAMPs inhibit bacterial metabolism [51,109].

Oncopeltus antibacterial peptide 4 was initially isolated by Schneider *et al.* from the large milkweed bug *Oncopeltus fasciatus* [111]. The amino acid sequence of the 2 kDa AMP was determined using Edman degradation, resulting in a lack of 1 - 3 unidentified amino acids (sequence VDKPPYLPRP(X/P)PPRRIYN(NR)). The so far identified *Oncopeltus* antibacterial peptide 4 consists of 30 % proline residues as well as 25 % cationic amino acids and thus belongs to the family of proline-rich AMPs [113]. According to Knappe *et al.*, *Oncopeltus* antibacterial peptide 4 is not active against *Micrococcus luteus* and *E. coli* in an *in vitro* antibacterial assay. Therefore, the questionable amino acids were substituted, the peptide was shortened from 20 to 19 amino acids and a C-terminal amide was added. The resulting antibacterially active designer peptide was named oncocin (with the amino acid sequence VDKPPYLPRPRPPRRIYNR-NH_2) [113]. Oncocin was described to be non-hemolytic and relatively stable in serum. Furthermore, the peptide passes through the bacterial cell membrane without being lytic for the bacterium. This observation supports the assumption that, similar to apidaecins, oncocin acts antibacterially via binding and inhibition of an intracellular target [113].

Chemically synthesized apidaecins encompass the same bacteriostatic features as naturally occurring apidaecins [109]. This is also true for other AMPs, but especially for those which do not require complex folding via disulfide bonds to exert their activities, such as short

proline-rich AMPs. Insect-derived short PrAMPs can be easily synthesized on solid phase in relatively large amounts. Moreover, PrAMPs are primarily active against Gram-negative bacteria [90], although most of them show less antibacterial activities to be used therapeutically [38]. Therefore, some laboratories optimize naturally occurring PrAMPs concerning their antibacterial activity against various bacterial strains [90,113,114]. Api88 and Onc72 are derived from wild-type apidaecin 1b or the designer peptide oncocin, respectively. The optimized PrAMPs Onc72 and Api88 exert enhanced antibacterial activities against several bacterial strains such as *E. coli*, *Klebsiella pneumoniae (K. pneumoniae)*, and *Pseudomonas aeruginosa (P. aeruginosa)* [115,116], while e.g. wild-type apidaecin 1b was previously shown to be highly active against *E. coli*, less active against *K. pneumoniae*, but nearly inactive against *P. aeruginosa* [90]. This illustrates the clear effect of single substitutions on the peptide's activity spectrum. In addition, optimization of oncocin to Onc72 led to increased stability against serum proteases [113,117]. The *in vitro* and *in vivo* antibacterial capacities of native and optimized PrAMPs such as Api88, oncocin, A3-APO (an optimized pyrrhocoricin-derivative) or Bac7 (1-35) (a bactenecin fragment) have been examined in several studies [113,114,118].

1.6.6 CATHELICIDINS

The first cathelicidin was identified in the early 1990's by Zanetti *et al.* [119] who cloned the cDNA from Bac5, a bovine cathelicidin stored in neutrophilic granules [98,120]. Cathelicidins are prevalent in vertebrates as well as invertebrates, which suggests an important role in host defense [78]. They have a broad-spectrum antimicrobial activity and they are known to neutralize endotoxin [98]. Immature cathelicidin peptides contain a relatively large highly conserved N-terminal pro-region which is known as "cathelin domain" [78,98]. Thus, even structurally diverse peptides are grouped according to the presence of the cathelin domain in their pro-peptides. Besides the cathelin-domain, cathelicidins from the same as well as from different species show only little similarity to each other. Altogether, there are α-helical cathelicidins such as murine CRAMP as well as cathelicidins with a high content of specific amino acids such as PR-39 or indolicidin, or even more complex cathelicidins with disulfide bonds such as bactenecin [98].

Structurally diverse cathelicidin peptides use different modes of action to perform their antibacterial activity. Several authors consider the proline- and arginine-rich pig cathelicidin PR-39 as relative of apidaecin. PR-39 has a similar amino acid composition and is considered to use the same antimicrobial mechanism as PrAMPs such as apidaecin and oncocin [98]. Other cathelicidins are known to permeabilize bacterial membranes. Some cathelicidins such as the bovine peptides BMAP-27 or BMAP-28 are toxic for eukaryotic cells, whereas others, such as CRAMP, were reported to be less or not at all cytotoxic for eukaryotic cells [98,121]. Interestingly, certain cell types, such as erythrocytes or strongly proliferating cells (e.g. tumor cells) are more susceptible to cytotoxic actions than e.g. non-proliferating cells [98,121].

Neutrophilic granulocytes are the most prominent constitutive cathelicidin source. Besides DC and macrophages, neutrophilic granulocytes represent another important cell type of the innate immune system. Inducible secretion of cathelicidins was also reported for other immune cells than neutrophilic granulocytes, such as macrophages, NK cells, mast cells, as well as various kinds of epithelial cells [78]. This suggests an important role of cathelicidin as part of the first defense line against pathogens. Humans express only one cathelicidin peptide, LL-37. The immature pro-peptide of LL-37, hCAP18, is stored in neutrophilic granules prior to activation of the cell and further processing of the cathelicidin by elastase [98]. LL-37 consists of a sequence containing 37 amino acids, beginning with two leucins. It has been reported that the pro-peptide can also be found associated with lipoproteins in human plasma, which possibly indicates an additional biological function [98,122]. LL-37 expression in airway epithelia suggests a protective role against pulmonary infections [98,123]. On human skin, the mature cathelicidin is further cleaved into smaller peptide fractions with diverse antimicrobial activities [78,124]. After processing, mature LL-37 exhibits a broad-spectrum antimicrobial activity. CRAMP and LL-37 show antifungal activity against *Candida albicans* at low salt concentrations [125]. Moreover, antiviral activity of LL-37 against herpes simplex virus, vaccinia virus, influenza virus, and adenovirus was reported [41-43,126]

Besides direct antimicrobial and antiviral activities, LL-37 is known to modulate the host's immune responses in order to control pathogens (see chapter 1.6.7). Although LL-37 causes lytic effects to bacteria, the peptide is able to translocate eukaryotic membranes in order to induce chemokine release [38,127,128]. Moreover, LL-37 is known to neutralize endotoxin [98,129,130]. CRAMP ("cathelicidin-related antimicrobial peptide") is the murine ortholog of the human cathelicidin LL-37 and was identified by Gallo *et al* in 1997 [131]. The expression

of CRAMP is developmentally regulated [98,131,132] and CRAMP is antibacterially active against Gram-positive as well as Gram-negative bacterial strains by disruption of the inner bacterial membrane [133].

The effective antimicrobial activity of CRAMP was confirmed *in vivo* in several infection studies. In mice lacking CRAMP expression or correct CRAMP processing, several defects in host defense were described. For instance, NE2/2 mice, which are deficient in neutrophilic elastase, cannot process CRAMP propeptide maturation. Therefore, these mice are particularly susceptible to gram-negative bacteria [98,134]. Another study demonstrated that the intracellular macrophage pathogen *Salmonella typhimurium* shows increased survival in CRAMP knockout mice. In the same manner as stated above, this activity is influenced by intracellular elastase-like serine protease. Moreover, it was revealed that CRAMP and host proteases cooperate to finally eliminate the intracellular pathogen [135].

1.6.7 IMMUNOSTIMULATION AND IMMUNOMODULATION BY AMPS

It was reported before that several AMPs stimulate or modulate immune responses and therefore have an additional indirect effect on host defense. This assumption is supported by the fact that the physiological concentration of many AMPs is not sufficient to kill microbes directly. Moreover, salt as well as serum are known to inhibit AMP activity [52]. Besides co-operation of different AMPs, regulation of a variety of effects related to inflammation, innate and adaptive immune responses is suggested to contribute to antimicrobial action. Modulation of immune responses by AMPs may also promote elimination of the pathogen and may inhibit immunopathological effects (e.g. by induction of regulatory T cells) [136]. Immuno-modulation includes all facets of immune responses ranging from direct immune cell activation and inhibition to indirect effects, e.g. support of antigen uptake by dendritic cells [137], binding of extracellular self-DNA, followed by entering of pDC and triggering of an IFN-γ response [138,139], augmentation of systemic IgG and IgM production by B cells [52], or modulation of cytokine or chemokine secretion enabling activation and recruitment of immune cells [140-142]. Numerous AMPs were reported to chemoattract immune cells such as neutrophilic granulocytes, monocytes or T cells to sites of infection [143,144]. Involvement of AMPs was also reported in mechanisms like wound healing [145], angiogenesis [146], cell proliferation [48], promotion of phagocytosis [52], or binding and neutralization of endotoxin [147].

AMPs may stimulate cells of innate and adaptive immune system, e.g. DC, monocytes, macrophages, mast cells, or T cells [148-152]. These stimulatory effects comprise up-regulation of activation and co-stimulatory markers or an enhanced expression of inflammatory cytokines. B-defensins, which are known as BMDC-stimulators [148], as well as the human cathelicidin LL-37, were also shown to induce histamine, IL-2, IL-4, IL-6, IL-31, GM-CSF, leukotriene C_4 etc. in human mast cells [141]. This strong effect may be involved in the pathogenesis of several mast cell-induced skin diseases [141]. Furthermore, it was shown that LL-37 traverses eukaryotic membranes, which is important to stimulate chemokine secretion [38,127,128]. LL-37 was recently reported to up-regulate IL-8 expression in human gingival fibroblasts [153] and it stimulates IL-1β expression in human monocytes, both via $P2X_7$ receptor [62,154]. LL-37 also induces expression of IL-8 in monocytes using an unknown mechanism that does not involve a G-protein-coupled receptor [62,155]. Moreover, LL-37 co-operates with IL-1β in promotion of cytokine and chemokine production (e.g. IL-6, IL-10, MCP-1 etc.) in human PBMC [78,156].

Induction of chemotaxis is another mode of direct immune cell activation exhibited by various AMPs. The cathelicidins LL-37 and CRAMP were both described to chemoattract human monocytes and macrophages, neutrophilic granulocytes, and murine peripheral blood leukocytes using the human formyl peptide receptor-like-1 (FPRL-1) and murine FPRL-2 [157]. Biragyn *et al.* reported that murine β-defensins 2 and 3 chemoattract murine immature but not mature BMDC via CC-chemokine receptor CCR6 [143]. Chemotactic attraction of immature DC supports cell influx, phagocytosis of pathogens, and subsequent antigen presentation in order to enable adaptive immune responses. In addition to direct chemoattraction, LL-37 as well as α-defensins also contribute indirectly to immune cell chemotaxis by induction of chemokines like IL-8 [52].

Several authors demonstrated that AMPs may also have inhibitory effects on immune responses. This enables processes such as tissue repair or tolerance. The proline and arginine-rich pig AMP PR-39 is known to induce syndecan-1 and syndecan-4 in fibroblasts and endothelia [131,158]. Syndecan-1 and -4 are involved in several processes such as wound repair, where they facilitate cell proliferation and cell migration to the appropriate area [98]. Additionally, it was shown that PR-39 participates in modulation of HIF-1α-regulated VEGF-induction and therefore plays a role in angiogenesis and tissue vascularization [98,159]. Moreover, a functionally active fragment of PR-39 translocates the eukaryotic membrane and selectively binds cytoplasmic signaling molecules containing SH3 domains such as

$p130^{Cas}$ [98,160]. Interaction with signaling molecules such as $p130^{Cas}$ may lead to induction of various signaling pathways in order to induce diverse effects, e.g. induction of neutrophil chemotaxis or inhibition of phagocyte NADPH oxidase activity [98,161]. Phagocyte NADPH oxidase is involved in generation of ROI, which play a significant role in the control of pathogens but may also cause tissue damage.

Additionally to direct stimulatory or inhibitory effects of the peptide *per se*, several AMPs indirectly affect immune cells, e.g. by modulation of immune responses to PAMPs. Some AMPs bind and neutralize TLR ligands such as lipopolysaccharide (LPS; TLR4) or lipoteichoic acid (TLR2) from Gram-negative and Gram-positive bacteria, respectively. As a result, activation of (especially innate) immune cells and the resulting adaptive immune response is abrogated or at least reduced [52]. Kändler *et al.* demonstrated that co-stimulation of LPS, flagellin or lipoteichoic acid with LL-37 leads to decreased activation of human DC. As a result, DC show reduced secretion of cytokines such as IL-6, IL-12, TNF-α, decreased expression of stimulatory and co-stimulatory markers such as MHC-II, CD80, or CD86 as well as diminished expression of chemokine receptor CCR7 [162]. Upon these initial inhibitory effects on innate immune cells, adaptive immune responses, which are facilitated by interaction of activated innate professional antigen presenting cells (APCs), are also suppressed. Simultaneous stimulation of DC with LPS and LL-37 followed by washing of the cells and co-cultivation of DC with $CD4^+$ T cells results in reduced IL-2 and IFN-γ release as well as diminished $CD4^+$ T cell proliferation. Kändler *et al.* also demonstrated that after co-cultivation with LPS/LL-37-pre-treated DC, memory T cells show diminished IL-2 and IFN-γ secretion in presence of the recall antigen [162]. Importantly, Rosenfeld *et al.* reported that LL-37 is able to dissociate LPS aggregates, which are assumed to be necessary for binding of LPS-binding protein (LBP) followed by activation of the immune cell via CD14 and TLR4 [163]. Besides interaction with soluble as well as membrane-bound LPS, LL-37 also binds CD14 and competes with (LBP-bound) LPS [62,163-165]. Some AMPs also interfere with LPS-induced signal transduction. As an example, Pinheiro da Silva *et al.* reported that even after removal of CRAMP and washing of murine BMDM, pre-incubation of the cells with the cathelicidin significantly inhibited the immune response (e.g. pERK activation) to a later LPS-stimulation [166]. Interestingly, simultaneous stimulation of macrophages with LPS and LL-37 leads to suppression of genes involved in sepsis. Thus, mechanisms of immune suppression include cytokine secretion and NO production, whereas

ROI production is further enhanced, and anti-inflammatory effectors are not affected [62,167,168].

LPS binding and neutralization by AMPs plays an important role in several *in vivo* models. For instance, Kirikae *et al.* as well as Sawa *et al.* reported that the human cathelicidin LL-37/hCAP18 protects *P. aeruginosa*-infected mice from antibiotic-induced endotoxin shock rather than being bactericidal itself [98,169,170]. Injection of an LL-37-fragment can also protect mice in an *in vivo* sepsis model [62,147,169].

LL-37 was furthermore shown to induce pro- and anti-apoptotic effects in different cell types. For instance, it was reported that LL-37 promotes caspase-dependent apoptosis in lung epithelial cells *in vitro* and *in vivo*, whereas it protects neutrophilic granulocytes from caspase 3-induced apoptosis (via FPRL-1 and $P2X_7$) [62,171,172]. Antitumoral activity against the adenocarcinoma cell line SAS-H1 was described for an LL-37 fragment, which promotes caspase-independent apoptosis [62,173].

Taken together, some AMPs may have strong effects on pathogen-induced immune responses. Therefore, knowledge of accurate regulation of inflammation by AMPs is of particular importance. Concerning a possible application of AMPs as therapeutic agents and since some AMPs (such as LL-37) were reported to be involved in the development of diseases such as psoriasis [138,139], there is special interest to clarify the function and mechanisms of the peptides as signaling molecules between immune cells or between infected tissue and arriving immune cells.

1.7 AIMS OF THE STUDY

In recent years, the increasing number of bacteria multi-resistant for conventional antibiotics led to extensive investigations in the field of antimicrobial peptides. AMPs which specifically bind crucial bacterial but not eukaryotic targets represent the most promising candidates for pharmaceutical investigation.

In this study, the potential of Onc72 in an *in vivo* septicaemia model was evaluated. Furthermore, this study investigates the abilities of oncocin, Onc72, apidaecin 1b and Api88 to stimulate or modulate immune responses of murine innate immune cells which may impact possible antimicrobial activities *in vivo*. Together, this study addresses the following questions:

1. ***Do the PrAMPs oncocin, Onc72, apidaecin 1b and Api88 exert cytotoxic effects on murine immune cells, when tested in* in vivo *and* in vitro *studies?***
   Possible cytotoxic effects on immune cells displayed by the oncocin and apidaecin derivatives were determined *in vitro* and further analyses regarding toxicity were done for Onc72 also *in vivo*. For *in vivo* testing, mice were treated with high doses of the peptide and after five days, several organs were removed and analyzed histopathologically. Furthermore, cytotoxic effects on murine bone marrow-derived dendritic cells (BMDC) were assessed *in vitro* using flow cytometric analyses.

2. ***Can Onc72 protect mice from septicaemia after infection with a lethal dose of Escherichia coli ATCC 25922?***
   The optimized peptide Onc72 was tested in an acute septicaemia model in order to evaluate its protective effect against a lethal infection with *E. coli*. Mice were clinically monitored and analysis was done five days post infection. In this respect, several organs were analyzed histopathologically and bacterial counts in organs and body fluids as well as body weight changes were evaluated in further detail.

3. ***Are oncocin, Onc72, apidaecin 1b and Api88 immunostimulatory or immunomodulatory for murine bone marrow-derived dendritic cells (BMDC) or macrophages (BMDM)? Do the peptides need additional cell-cell-interactions between distinct immune cell types to exert immunostimulatory or immunomodulatory effects?***

   AMPs may modulate innate immune responses. Thus, immunostimulatory and immunomodulatory effects of the four insect PrAMP-derivatives on isolated murine BMDC and BMDM were studied and compared to the well-known immunomodulatory peptide CRAMP [62,157,162,166,174]. Possible immunostimulatory or immunomodulatory activities of the peptides on DC and/or macrophages may depend on their interaction with other immune cell types. Thus, splenocytes and peritoneal exudate cells (PEC) were used for further *in vitro* peptide stimulation studies.

2 PUBLICATION I: ONCOCIN DERIVATIVE ONC72 IS HIGHLY ACTIVE AGAINST *ESCHERICHIA COLI* IN A SYSTEMIC SEPTICAEMIA INFECTION MOUSE MODEL

To date, the antibacterial efficacy of only a few AMPs was tested in acute infection models *in vivo* [51,94-96,170,175]. Although the relatively low risk of induction of the host's immunity is advantageous, degradation in serum and renal clearance of small peptide antibiotics may impede the use of AMPs as medication for bacterial infections [96]. To test the antibacterial efficacy of the optimized PrAMP derivative Onc72 *in vivo*, the designer peptide was evaluated in acute septicaemia models also used by the pharmaceutical industry, where the peptide antibiotic is administered within one hour after infection [159,161].

To test the peptide's antibacterial efficacy in an acute septicaemia model, female mice of the outbred strain NMRI were infected i.p. with a lethal dose of *E. coli* strain ATCC 25922 containing 2.5 % mucin. Mice of an outbred strain are genetically diverse and therefore suitable to represent a population of different individuals. Thus, the utilization of outbred mouse strains is important for testing of therapeutic compounds [176]. Following infection with *E. coli*, mice were medicated three times with Onc72. Ciprofloxacin hydrochloride injection served as positive control, whereas aquaeous glucose solution served as negative control for antibacterial treatment. Due to the fact that most common human pathogen bacterial strains are not naturally virulent for mice, including staphylococci, enterococci or Enterobacteriaceae, infection models using those pathogens require additives to locally and temporally suppress immediate immune responses [177]. The commonly used glycoprotein mucin promotes establishment of bacterial infection by inhibition of the local macrophage activity for two to three hours [177,178] and consequently decreases the lethal bacteria dose to approximately $1x10^6$ colony forming units (cfu) per mouse [116]. After infection and subsequent medication with Onc72, ciprofloxacin hydrochloride (positive control) or glucose solution (negative control), the mice were clinically monitored, weighed and scored for five days. Subsequently, bacterial counts of body fluids and organs were assessed.

All *in vivo* studies were performed in close collaboration with Dr. Uwe Müller and Dr. Daniel Knappe. Parts of these data were presented in several publications, amongst others also in Dr. Knappe's doctoral thesis [116].

2.1 MAJOR RESULTS

Injection of the proline-rich antimicrobial peptide Onc72:

- was not toxic for female NMRI mice if administered at concentrations of 20 mg/kg or 40 mg/kg for four times.
- protected mice in an acute septicaemia model using *E. coli* strain ATCC 25922 in a dose-dependent manner. The effective dose (ED_{50}) of Onc72, which assures a survival rate of 50 %, can be estimated at approx. 2 mg/kg (three injections).
- reduced body weight loss caused by infection as well as the bacterial burden in body fluids and in the analyzed organs in a dose-dependent manner. Furthermore, each consecutive injection led to significantly decreased bacterial blood and organ burden within 24 h. Finally, organs of all Onc72-medicated mice, which survived infection, were sterile five days post infection.
- Furthermore, *in vivo* imaging studies using fluorophore-labeled Onc72 showed that the fluorescence signal could be detected in mice for a few hours post injection.

2.2 CONCLUSIONS

Onc72 efficiently protected mice from septicaemia resulting from infection with a lethal dose of *E. coli* strain ATCC 25922. This dose-dependent protective effect of Onc72 included reduction of bacterial burden in blood, peritoneal lavage and all tested organs. Due to the fact that all mice of the negative control group succumbed the bacterial infection, it can be assumed that most bacteria were killed by Onc72 alone or by the synergistic antibacterial action of the peptide together with the host's immune system. Whether Onc72 additionally affects the host's immune system by stimulation or modulation, or whether the direct antibacterial effect of Onc72 buys time for additional action by the host's immunity, needed to be further clarified (see publication II). Taken together, Onc72 is an effective and promising lead compound, which should be considered for further investigations, e.g. in infection models using other pathogens.

2.3 PUBLICATION I

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Oncocin derivative Onc72 is highly active against *Escherichia coli* in a systemic septicaemia infection mouse model

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Objectives: The antimicrobial oncocin derivative Onc72 is highly active against a number of Gram-negative bacteria, including resistant strains. Here we study its toxicity and efficacy in a lethal mouse infection model.

Methods: In an acute toxicity study, purified Onc72 was administered to NMRI mice in four consecutive injections within a period of 24 h as an intraperitoneal bolus. The animals' behaviour was monitored for 5 days, before several organs were examined by histopathology. A lethal *Escherichia coli* infection model was established and the efficacy of Onc72 was evaluated for different peptide doses considering the survival rates of each dose group and the bacterial counts in blood, lavage and organs.

Results: Intraperitoneal bolus injections with single doses of 20 or 40 mg of Onc72 per kg of body weight did not result in any abnormal animal behaviour. No mouse became moribund or died within the studied period. Histopathological examinations revealed no toxic effects. When infected with *E. coli* at a lethal dose, none of the untreated animals survived the next 24 h, whereas all animals treated three times with Onc72 at doses of $\geq$5 mg/kg survived the observation period of 5 days. No bacteria were detected in the blood of treated animals after day 5 post-infection. The effective dose (ED_{50}) was ~2 mg/kg.

Conclusions: No toxic effects were observed for Onc72 within the studied dose range up to 40 mg/kg, indicating a safety margin of >20.

Keywords: antimicrobial peptides, AMPs, *Escherichia coli* ATCC 25922, histopathology, proline rich peptides, repeated toxicity, therapeutic window

Introduction

The broad and often inappropriate usage of antibiotics in recent decades has selected bacteria that have acquired one or several resistance mechanisms, which is especially threatening for hospital-acquired (nosocomial) infections. The prevalence of nosocomial infections in intensive care units is ~50%. In recent years, the most notable increase in resistance has been observed for Gram-negative bacteria. A very recent example highlighting the problem is the occurrence of deaths in Europe caused by bacteria carrying New Delhi metallo-β-lactamase (NDM-1).[1] Despite all efforts, including prophylactic measures, the number of nosocomial infections is still increasing worldwide. This illustrates an urgent medical need to develop new antibiotics that kill the bacteria by novel mechanisms to overcome acquired resistance mechanisms.

One promising strategy relies on antimicrobial peptides (AMPs) to gain a new option in the 'war against germs'. AMPs of different lengths and sequences are found in all higher organisms.[2,3] They can act by different mechanisms, such as lysis of the bacterial membrane or inhibiting certain targets on the surface or within the bacteria.[4,5] This huge pool of active substrates provides an ideal starting point for developing novel antibiotics to treat humans. Typical limitations of peptide drugs are, however, their fast enzymatic degradation and low absorption rates, which often prevent their oral administration. With respect to nosocomial infections this might be a benefit, as peptide antibiotics would be given intravenously by skilled personnel, i.e. clinicians, under well-controlled conditions (dose, treatment intervals and period as well as therapy success), preventing, or at least reducing, the risk of resistance development.

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The family of proline-rich AMPs present in insects and mammals has recently attracted several research groups to investigate their therapeutic potential and to optimize their structures for *in vivo* applications.[6,7] The early promise of *in vitro* tests has been fully confirmed in mouse models. Moreover, an artificial peptide sequence called A3-APO showed an even broader activity spectrum in mice than the *in vitro* screens suggested.[8,9] Recently, we introduced another promising AMP, called oncocin, which was optimized for its activity *in vitro* against *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, starting from a native AMP originally isolated from *Oncopeltus fasciatus* (large milkweed bug).[10,11] Oncocin was highly active *in vitro* and non-toxic to mammalian cells. In an ongoing drug development process we were able to increase its stability against serum and bacterial proteases by modifying the cleavage sites, which were deduced from the metabolites identified after incubating the peptides with mouse serum and *E. coli* lysates.[12,13] Lead compound Onc72, for example, inhibited the growth of *E. coli* strain ATCC 25922 (also designated as DSM 1103) in a microdilution assay at a concentration of 4 mg/L (MIC) in 33% TSB medium.

Here we report the first animal study to evaluate systemic toxicity after repeated injections of Onc72 and its efficacy in a lethal systemic infection model with NMRI mice being infected intraperitoneally (ip) with *E. coli* strain ATCC 25922.

Materials and methods

Peptide synthesis

Onc72 (VDKPPYLPRPRPPROIYNO-NH_2, where O represents L-ornithine) was synthesized on a microwave-assisted peptide synthesizer (Liberty; CEM, Matthews, NC, USA) at a 250 μmol scale using Rink amide resin (MultiSynTech GmbH, Witten, Germany), as described recently.[14] The side chains of trifunctional amino acids were protected with 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pbf) for Arg, *tert*-butyl (^{t}Bu) for Asp and Tyr, *tert*-butyloxycarbonyl (Boc) for Lys and L-ornithine (Orn) and trityl (Trt) for Asn (MultiSynTech GmbH, Witten, Germany and Orpegen Pharma GmbH, Heidelberg, Germany). Amino acids were activated with HBTU/DIPEA and coupled in a 4-fold excess using the standard protocols recommended for microwave-assisted solid phase peptide synthesis. The final peptide resin was washed with dichloromethane, dried and cleaved with trifluoroacetic acid (TFA) containing 12.5% (v/v) of a scavenger mixture (ethandithiole, m-cresole, thioanisole and water, 1:2:2:2 by volume) and precipitated with cold diethyl ether after 2 h. The precipitated peptide was washed twice with cold diethyl ether, dried and purified on a C_{18} phase using a linear aqueous acetonitrile gradient in the presence of 0.1% (v/v) TFA as ion pair reagent (Jupiter C_{18} column, 21.2 mm internal diameter, 250 mm length, 15 μm particle size, 30 nm pore size; Phenomenex Inc., Torrance, USA). The purity of the peptide was judged by reversed-phase (RP)-HPLC on a Jupiter C_{18} column (4.6 mm internal diameter, 150 mm length, 5 μm particle size, 30 nm pore size; Phenomenex) by recording the absorbance at 214 nm. The molecular weight was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS; 4700 Proteomic Analyzer; AB Sciex, Darmstadt, Germany). The lyophilized peptide was dissolved in 0.1% (v/v) aqueous acetic acid and lyophilized again. This step was repeated once to remove residual TFA.

Animals

For *in vivo* experiments, female NMRI outbred mice aged 6–8 weeks and weighing 25–32 g were used. Food and water were given *ad libitum*. The mice were housed in an IVC system (Ebeco, Castrop-Rauxel, Germany) under specific pathogen-free conditions. The experiments were in accordance with the guidelines approved by the Animal Care and Usage Committee of the state agency Leipzig (Landesdirektion Leipzig, file number: 24-9168.11/12/22).

E. coli and infection

For infection of mice the *E. coli* strain ATCC 25922 was used. In a first step, host-adapted bacteria were generated by a two-step passage through mice, i.e. 10^6–10^7 bacteria were given ip and isolated 17 h later from blood cultures. From this isolate aliquotted frozen stocks were generated and used for infection. The aliquots had a concentration of $5.5 \pm 1.0 \times 10^8$ cfu/mL. As the virulence of these host-adapted bacteria remained low, each mouse was infected ip with $1.1 \pm 0.2 \times 10^6$ cfu in the presence of 2.5% (w/v) mucin (300 μL in total) in accordance with established protocols.[15] Mice were checked three times per day for their health status [mobility, breathing, feeding, fur appearance (plain or scruffy) etc.] for a total period of 5 days and weighed 1 day before and 1 and 5 days after infection. Moribund mice, in accordance with the guidelines of the Animal Care and Usage Committee of the Landesdirektion Leipzig, were euthanized.

Toxicity analyses and antimicrobial therapy

To check the toxicity of the optimized oncocin, two high doses were used with Onc72 at concentrations of 20 and 40 mg/kg body weight. The AMP was given four times (0, 3, 7 and 24 h). Mice were checked for behavioural abnormalities and were analysed histopathologically after a period of 5 days. The antibacterial therapy relied on ciprofloxacin as the positive control using the recommended dose of 40 mg/kg injected three times ip after infection (1, 4 and 8 h). The negative control was the solution in which the antimicrobial substances were diluted, i.e. 5% glucose in water, as recommended for ciprofloxacin. In addition, Onc72 was administered in five different doses from 20 to 1.25 mg/kg, which were also administered three times in the manner described above for ciprofloxacin.

Ex vivo analyses

Blood samples were taken 1, 4, 8, 24 h and 5 days post-infection and checked for bacterial growth on nutrient agar plates (Carl Roth GmbH, Karlsruhe, Germany). Blood was taken by puncture of the submandibular plexus or at the end by puncture of the heart. After sacrificing the mice, five organs (heart, liver, spleen, kidney and brain) and peritoneal lavage were collected from all animals under sterile conditions. Parts of the organs were minced (Ultraturrax, IKA-Werke, Staufen, Germany) and an aliquot plated to check for the bacterial burden.

Pathology

Spleens were weighed and thereafter all removed organs (e.g. liver, kidneys, pancreas, ovary, urinary bladder, lung, brain and spleen) were fixed in 4% buffered formalin and embedded in paraffin. The extent and morphology of a potential inflammatory response were studied by haematoxylin and eosin (H&E) and Giemsa staining of organ sections.

Statistical analyses

The statistical significance of differences between experimental groups of animals was determined using the log rank test for survival analysis and the one-tailed Mann–Whitney test for bacterial burden, organ weight and body weight changes.

Results

Synthesis of Onc72

The antimicrobial peptide Onc72 was obtained in high yields and purities (≥95% as indicated by RP-HPLC), with only minor peptidic by-product impurities detected by MALDI-MS. The dried peptide was lyophilized twice in one batch using 0.1% aqueous acetic acid as solvent to reduce the content of acetonitrile and especially TFA. The final peptide batch was quantified from the RP chromatogram (absorbance at 214 nm) relative to an oncocin standard injected on the same column in similar quantities, which was quantified absolutely by amino acid analysis.[16] Thus, the peptide amounts used in this animal study should be relatively accurate, with an expected error of ~10%.

Toxicity and pathological effects of Onc72

Two groups of NMRI mice with seven animals each were treated four times (0, 3, 7 and 24 h) ip with doses of 20 or 40 mg/kg Onc72. The mice showed no signs of abnormality and showed their normal behaviour, and no mouse became moribund or died during the treatment. After 5 days of examination the mice were sacrificed. Spleens were weighed (Figure 1a) and fixed for further histopathological analysis. Moreover, liver, kidneys, pancreas, ovary, urinary bladder, lung and brain were examined histopathologically. The analysed tissues showed the normal structure, as shown for spleen (Figure 1). Detailed histopathological analysis following H&E (Figure 1b–d) and Giemsa staining (data not shown) revealed the regular anatomical structure of the organs with no signs of any toxic side effects or internal bleeding, including the kidney as the likely clearance pathway. There were no signs of inflammation in any of the organs studied.

Systemic septicaemia infection model

Mice were infected ip with 1×10^6 cfu of *E. coli* strain ATCC 25922 in 2.5% mucin to restrict acute macrophage activation and promote establishment of infection. In a first step, the distribution rate and change in bacterial burden after inoculation were studied. The bacterial burden increased over time in all tested body fluids and organs (Figure S1, available as Supplementary data at *JAC* Online). The highest levels of infection were found in the peritoneal cavity, where the inoculum was administered. During the first 8 h the bacterial burden in blood, liver, lung, spleen and kidneys was 100–1000 times lower in comparison with the peritoneal cavity. Importantly, the bacteria were systemically distributed in all animals and reached all organs except the brain within the first hour. This confirms that the blood–brain barrier was still intact and reduced the dissemination of bacteria to the CNS. Moreover, all infected animals showed signs of severe symptoms of illness within 1 h post-infection, providing a good measure for the following therapeutic intervention. Based on these data, we started to medicate the mice 1 h post-infection. Two more therapeutic injections with the same dose were scheduled 3 and 7 h later, which resembled the time intervals of the toxicity study.

Onc72 treatment in infection

Mice were infected as described above and were then medicated three times ip with Onc72, ciprofloxacin (positive control) or received no antibiotic (negative control). Within 1 h of infection, all mice showed severe signs of infection, including reduced mobility, scruffy fur and apathy. Based on the toxicity study, we started the medication with doses of 20, 10 or 5 mg of Onc72 per kg per injection (Figure 2a). No mice of the ciprofloxacin or any of three AMP groups succumbed to the infection, whereas all of the untreated mice (negative control) had to be euthanized

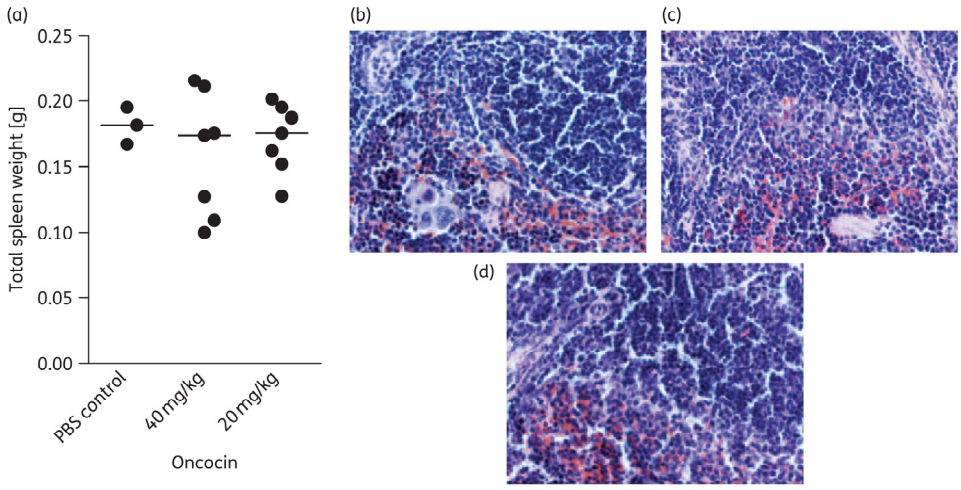

Figure 1. In a toxicity test with 20 and 40 mg/kg oncocin given four times over a period of 24 h, spleen weights, as a measure of systemic immune response, were examined after 5 days (a). Representative splenic micrographs are depicted for the PBS control (b) and Onc72 doses of 20 mg/kg (c) and 40 mg/kg (d). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

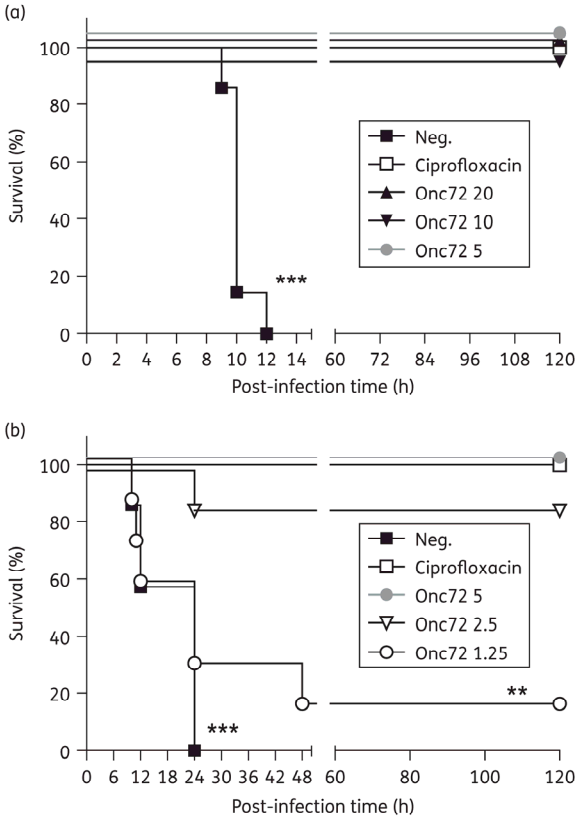

Figure 2. Survival rates of NMRI mice infected with *E. coli* ip and medicated with high doses of Onc72 (i.e. 20, 10 and 5 mg/kg) (a) or low doses of Onc72 (i.e. 5, 2.5 and 1.25 mg/kg) (b). Seven mice per group were used. Survival data were compared with the ciprofloxacin group: $^{**}P<0.01$; $^{***}P<0.001$.

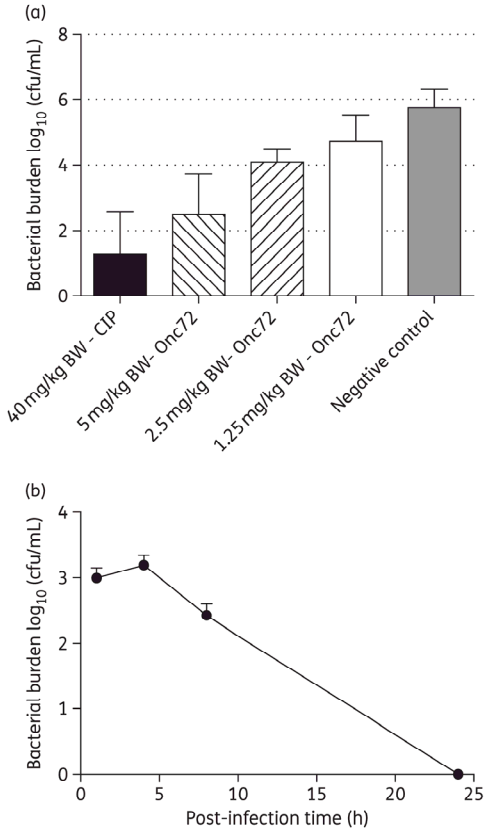

Figure 3. Bacterial blood burden at 8 h post-infection in NMRI mice medicated twice with ciprofloxacin or Onc72 1 and 4 h post-infection (four animals per group) (a) or with Onc72 at a dose of 5 mg/kg 1, 4 and 8 h post-infection (seven animals per group) (b). BW, body weight; CIP, ciprofloxacin.

within the first 12 h of infection. All treated mice recovered completely within the 5 day observation period, i.e. all animals showed normal behaviour. Thus, we reduced the doses of Onc72 further (Figure 2b). In the second experiment all mice of the control group had to be sacrificed within 24 h, whereas all mice treated with a dose of 5 mg/kg Onc72 survived. At a dose of 2.5 mg/kg, six out of the seven animals survived, but they took longer to return to their normal behaviour pattern. At the lowest dose of 1.25 mg/kg only one out of seven mice survived. We estimate the effective dose (ED_{50}), i.e. the Onc72 dose that guaranteed a 50% survival rate, at 2 mg/kg if injected 1, 4 and 8 h post-infection.

Furthermore, the bacterial counts were determined in different body fluids and organs in response to the different doses of Onc72. Untreated mice showed the highest bacterial burden in blood, followed by the group treated with the lowest dose of Onc72 (1.25 mg/kg) (Figure 3a); this is in agreement with the survival rates obtained for the two groups (Figure 2b). All Onc72 doses showed a correlation between increasing peptide concentrations and decreasing bacterial counts. Importantly, the bacterial burden was significantly reduced in blood by each injection of Onc72 at a dose of 5 mg/kg, providing almost sterile blood after 24 h (Figure 3b). In addition, the weight loss of the animals 24 h after infection was a good marker for the antimicrobial effect of the therapeutic agent that was given (Figure 4). The smallest weight losses were measured for ciprofloxacin treatment ($3\pm1\%$) and the high doses of Onc72 ($\geq$10 mg/kg). For lower doses of 5 and 2.5 mg/kg, the weight decreased on day 1 by $8\pm1\%$ and $12\pm3\%$, respectively, before the animals returned almost to their pre-infection weight at day 5 ($-1\pm1\%$ and $-3\pm1\%$). This confirms again that Onc72 is more effective given at 5 mg/kg than at 2.5 mg/kg.

For blood taken from the facial vein 8 h post-infection, bacterial burdens were 4×10^4–2×10^6 cfu/mL (negative control), 2×10^3–1×10^6 cfu/mL (Onc72 doses of 1.25 and 2.5 mg/kg) and $<7\times10^3$ cfu/mL (Onc72 doses of 5 mg/kg and ciprofloxacin). Next, we studied the bacterial counts in peritoneal lavage, heart

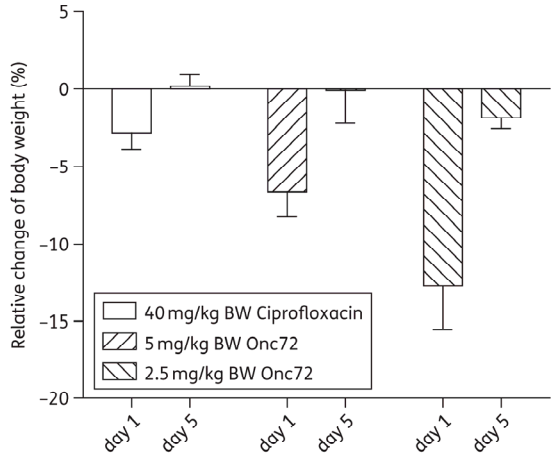

Figure 4. Body weight changes for infected mice (1 million *E. coli* ip in 2.5% mucin) treated with ciprofloxacin and Onc72 at two different doses. The bars indicate body weight 1 and 5 days post-infection (in relation to the weight at timepoint 0). BW, body weight.

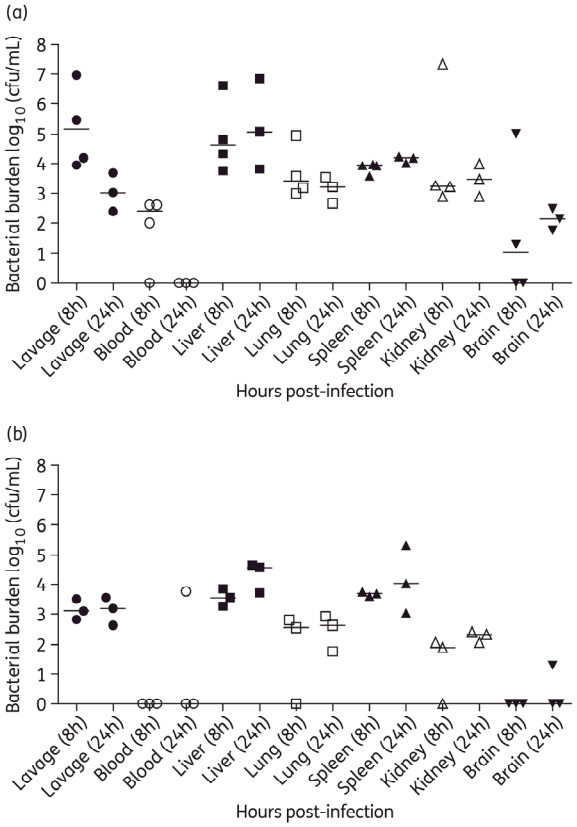

Figure 5. Bacterial burden in infected animals treated with Onc72 at a dose of 5 mg/kg (a) or 20 mg/kg (b) 8 and 24 h post-infection. Three to four mice were analysed at each timepoint and concentration.

blood, liver, lung, spleen, kidney and brain for animals treated with 20 and 5 mg/kg Onc72, i.e. the highest and lowest doses that provided a 100% survival rate (Figure 5). The bacterial burden detected 8 h post-infection was lower in the high-dose group (20 mg/kg) than in the low dose group (5 mg/kg). After 24 h the bacterial burden was at a similar level in both groups, except for the liver sample, where the cfu count was ~100-fold higher in the low-dose group. This finding correlates well with the survival curves for high (Figure 2a) and low (Figure 2b) doses of Onc72. Most importantly, after 5 days of infection no bacteria were isolated in any organ examined from any of the animal groups treated with ciprofloxacin or Onc72, even for the lowest doses of Onc72 (1.25 and 2.5 mg/kg). After these 5 days of infection no differences or signs of toxicity could be found in the histology of the kidneys of mice treated with ciprofloxacin (Figure S2a, H&E staining, available as Supplementary data at *JAC* Online) or Onc72 (10 mg/kg) (Figure S2b, H&E staining, available as Supplementary data at *JAC* Online).

Discussion

The increasing number of resistance mechanisms in Gram-negative pathogens and especially the occurrence of single strains that show multiple resistances against a broad range of antibiotics demands new treatment options for both nosocomial and community-acquired infections. A promising treatment option is represented by AMPs from organisms evolutionarily distant from humans, which will prevent bacteria from developing resistance mechanisms against antimicrobial mechanisms of the innate immune system (e.g. mammalian AMPs). For the same reason it appears favourable to modify the structure of natural AMPs chemically to prevent immunomodulatory effects on the defence mechanisms of the corresponding hosts. Following this hypothesis, we have modified the sequence of the *Oncopeltus* antimicrobial peptide 4 at several positions and also shortened the C-terminal sequence.[14] The resulting oncocin lead compound Onc72 thus contains two L-ornithine residues, additionally reducing its degradation by serum proteases, as indicated by its half-life in serum.[11] Recent studies have shown its superior antibacterial activity spectrum against several threatening Gram-negative pathogens *in vitro*, i.e. *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Acinetobacter baumannii*, and have also revealed how Onc72 inhibits the substrate-binding domain of chaperone DnaK.[13]

To evaluate the therapeutic potential of Onc72 for systemic *E. coli* infections, we first established a fast lethal sepsis model in NMRI outbred mice. The bacteria injected into the peritoneum disseminated to blood, liver, lungs, spleen and kidneys within 1 h, reaching a bacterial burden of 10000–100000 cfu/mL homogenate. This bacterial burden increased a further ~10-fold over the next 3 h and again over the following 4 h period, if not medicated. *E. coli* reached the brain with some delay, but increased relatively fast to around 10000 cfu/mL homogenate after 8 h. Thus, the treatment started at a point when the systemic infection was already established in the animals and its further aetiopathology no longer depended on the bacterial load of the

peritoneum. In this respect the established model provides a good measure to evaluate the efficacy of antibiotics. This was also confirmed by the dose–response curve obtained for lower Onc72 doses, ranging from 1.25 to 5 mg/kg.

Onc72 was highly efficient, providing a 100% survival rate already at a dose of 5 mg/kg and an ED_{50} of ~2 mg/kg. These values are similar to those for other antibiotics, including ciprofloxacin, used in this study, although it is difficult to compare such values among different mouse models. Nevertheless, we showed that Onc72 possesses a strong antibacterial activity *in vivo*. Interestingly, the lowest dose rescuing all animals (i.e. 5 mg/kg) basically reduced bacterial growth from the first injection and then reduced the number of living bacteria after the second and third treatments, providing sterile blood after 24 h. A similar effect was obtained for the studied organs, although they were not sterile within the 24 h period, but were at the end of the observation period of 5 days. Most likely the bacteria present after 24 h in the organs were later cleared by the host's immune system rather than by Onc72 as the treatment was not further continued. This raises the question of how long the peptide is active. *In vivo* imaging using DY675–Onc72, i.e. Onc72 carrying the near-infrared fluorophore DY675 (Dyomics, Jena, Germany) at the N-terminus, injected ip indicated that the dye-labelled peptide distributes systemically within 30 min. The fluorescence intensity increased continuously in the mice for the 2 h observation period, the peptide being slowly released from the peritoneum, which provides a depot (data not shown). After 2 h fluorescence was detected in the heart, bladder, ovaries, duodenum, kidneys and liver, but not in the brain. Thus we assume that the peptide was present at significant blood concentrations ~30 min after its first injection (i.e. 1.5 h post-infection) and remained there for at least the next 2 h. Considering the time–kill kinetics reported earlier for Onc72,[12] which indicated a reduction of the cfu by a factor of 1000 within 1 h *in vitro*, most bacteria should be killed by Onc72 directly. Based on the treatment intervals of 3 and 4 h, it appears likely that Onc72 was still present at low levels when the next dose was administered, providing continuous protection of the animals for at least 10 h. The highest fluorescence intensities were obtained in the kidneys and liver, which indicates that the dye was cleared through both organs at similar rates. The *in vivo* imaging data, however, have to be taken with caution as the peptide was chemically modified, with the dye influencing its properties significantly. More importantly, it is not possible to distinguish the fluorescence of the full peptide from degraded peptide fragments. Therefore, we will study this in more detail by using a radio-labelled peptide.

Proline-rich AMPs expressed in mammals are part of the innate immune system and thus can also modulate cells of the immune system during infection. These immunomodulatory effects contribute significantly to the effective elimination of pathogens, besides their immediate antimicrobial effect. Despite the evolutionary gap between insects and mammals, such immunomodulatory effects have recently been suggested to explain the high efficacy of A3-APO in a murine infection model with *Staphylococcus aureus* and confirmed for apidaecin.[8,17] Such effects can be excluded for Onc72, at least for key innate immune cells such as dendritic cells and macrophages (unpublished results) as also shown for Api88 recently.[18] The high efficacy of Onc72 might also be explained by synergisms with other substances present in blood, such as endogenous antimicrobial peptides or components of the complement system. Thus further studies for such synergistic interactions are necessary. Currently, we are studying the activities of Onc72 and some other AMPs in murine serum and blood samples.

Besides its high efficacy, Onc72 was not toxic within the studied concentration range up to 40 mg/kg when administered four times within 1 day, which indicates a therapeutic window of at least 10. Further studies will be necessary to address adverse effects in more detail using a panel of biomarkers to identify toxic effects on the kidneys and liver with a higher sensitivity and for a longer period of time (e.g. daily injections of Onc72 for 10 days).

Conclusions

The presented data indicate that Onc72 is highly efficient in female NMRI mice and clears the bacterial burden within 24 h in blood and reduces it during this period significantly in all studied organs. Considering the 'no observed adverse effect limit' (NOAEL) at repeated ip injections of 40 mg/kg from the toxicity study and an ED_{100} between 5 and 2.5 mg/kg, the therapeutic window can be estimated as $\geq$10. Thus, Onc72 is a promising lead compound with favourable characteristics that should be evaluated further concerning possible toxic side effects and its efficacy in other animal infection models.

Acknowledgements

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Funding

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Transparency declarations

R. H. is a cofounder of AMP-Therapeutics GmbH (Leipzig, Germany) and owns 12.5% of the stocks. All other authors: none to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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2.4 SUPPLEMENTARY DATA TO PUBLICATION I

Supplementary data

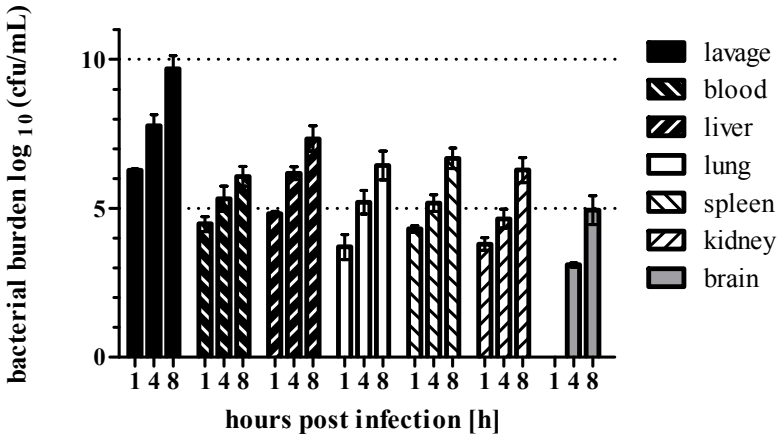

Figure S1. Bacterial burden in the designated organs of *E. coli*-infected NMRI outbred mice without therapeutic invention. Mice were sacrificed 1, 4 or 8 h after infection, as stated and the bacterial burdens of the depicted organs were monitored. Per time point four individual mice were analyzed.

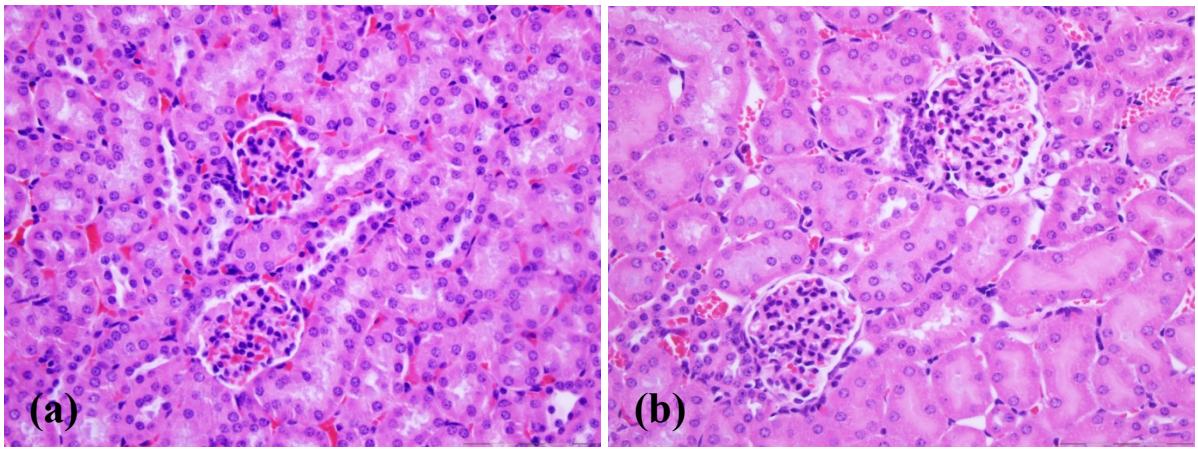

Figure S2. Histological analyses of the kidneys of ciprofloxacin- and Onc72-treated NMRI mice after clearance of the bacterial infection 5 days post-infection with *E. coli* strain ATCC 25922. Shown are representative H&E-stained micrographs for dose groups of 40 mg/kg [ciprofloxacin (a)] and 10 mg/kg [Onc72 (b)] at a magnification of 400.

3 PUBLICATION II: ABSENCE OF *IN VITRO* INNATE IMMUNOMODULATION BY INSECT-DERIVED SHORT PROLINE-RICH ANTIMICROBIAL PEPTIDES POINTS TO DIRECT ANTIBACTERIAL ACTION *IN VIVO*

Besides direct antimicrobial activities, several AMPs were described to exert stimulatory or regulatory effects on several immune cell types [148-152]. Immunomodulation of DC and macrophages by AMPs is of particular interest, due to the fact that they are both central cells of the immediately acting innate immunity as well as professional antigen-presenting cells.

Apidaecin 1b was recently reported to modulate immune responses by human monocytes and macrophages, but has no influence in human DC [179]. Furthermore, immunomodulation by another PrAMP, A3-APO, was proposed to be responsible for its strong protective effect in several murine *in vivo* infection models using *S. aureus*, mixed *K. pneumoniae* - *Acinetobacter baumannii* - *Proteus mirabilis* wound infection, or *K. pneumoniae* lung infection [96,180].

Stimulation or modulation of immune responses may support the potent antibacterial activity of the optimized PrAMPs Onc72 (see publication I) and Api88 [90] in the *in vivo* septicaemia models. Therefore, immunostimulatory and immunomodulatory capacities of the PrAMPs Onc72 and Api88, as well as of oncocin and apidaecin 1b on murine BMDC and BMDM were analyzed. Additionally, the peptides were also tested using PEC and splenocytes to evaluate their effects in the context of possible interactions between different immune cell types.

3.1 MAJOR RESULTS

The proline-rich antimicrobial peptides oncocin, Onc72, apidaecin 1b and Api88:

- were not cytotoxic for BMDC at a concentration up to 400 µg/ml (approximately 170-190 µM).
- were not immunostimulatory for BMDC and BMDM, splenocytes and peritoneal exudate cells as assessed by flow cytometric analysis of activation markers and/or detection of inflammatory cytokines in the cell supernatants.
- did not induce chemotaxis of BMDC at a concentration range from 1 ng/ml to 1 µg/ml.
- did not modulate the LPS-induced immune response by BMDC and BMDM, peritoneal exudate cells and splenocytes (in contrast to CRAMP) as assessed by flow cytometric analysis of activation markers and/or detection of inflammatory cytokines in the cell supernatants.

3.2 CONCLUSIONS

This study shows that oncocin, Onc72, apidaecin 1b and Api88 neither stimulated BMDC, BMDM, PEC and splenocytes, nor modulated their LPS-induced immune response. This outcome indicates that the PrAMPs and especially Onc72 in the septicaemia model (see publication I), exerted a strong antibacterial activity *in vivo*, most likely without influencing dendritic cells or macrophages. Based on these results, further pharmaceutical analyses regarding insect PrAMPs as antibacterial treatment will be simplified.

3.3 PUBLICATION II

Research Article

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Absence of *in vitro* innate immunomodulation by insect-derived short proline-rich antimicrobial peptides points to direct antibacterial action *in vivo*

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Some antimicrobial peptides (AMPs) have been described to exert immunomodulatory effects, which may contribute to their *in vivo* antibacterial activity. Very recently, we could show that novel oncocin and apidaecin derivatives are potently antibacterially active *in vivo*. Therefore, we studied oncocin and apidaecin derivatives for their effects on murine dendritic cells (DC) and macrophages and compared them with well-known immunomodulatory activities of murine cathelicidin-related antimicrobial peptide (CRAMP). To characterize the immunomodulatory activity of the peptides on key cells of the innate immune system, we stimulated murine DC and macrophages with the oncocin and apidaecin derivatives alone, or in combination with lipopolysaccharide (LPS). We analyzed the secretion of pro-inflammatory cytokines, the expression of surface activation markers, and the chemotactic activity of the AMPs. In contrast to LPS, none of the oncocin and apidaecin derivatives alone has an influence on cytokine or surface marker expression by DC and macrophages. Furthermore, the tested oncocin and apidaecin derivatives do not modulate the immune response after LPS stimulation, whereas CRAMP shows a reduction of the LPS-mediated immune response as expected. All peptides tested are not chemotactic for DC. Together, lack of *in vitro* immunomodulatory effects by oncocin and apidaecin derivatives on key cells of the innate murine immune system suggests that their potent *in vivo* antibacterial activity relies on a direct antibacterial effect. This will simplify further pharmaceutical investigation and development of insect peptides as therapeutic compounds against bacterial infections. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information can be found in the online version of this article.

Keywords: proline-rich antimicrobial peptides; oncocin; apidaecin; immunomodulation

Introduction

Eukaryotic hosts are confronted with different kinds of pathogens such as bacteria, viruses or fungi, which led to the evolution of various host defense mechanisms. AMPs are gene-encoded and highly conserved innate immune effector molecules, which contribute to the first line of defense against microbial infection. With only a few exceptions known, extensive resistance against AMPs has not been described yet [1,2]. Thus, AMPs appear to be promising novel candidates for therapeutic control of bacterial infections. AMPs can be classified by their mode of action in peptides that disturb or lyse the bacterial membrane and peptides that block specific bacterial targets. The latter class appears especially promising for therapeutic application because of the usually relatively low toxic side effects on mammalian cells. Among these peptides, proline-rich AMPs (PrAMPs), which are produced in mammals and insects, have gained much interest in recent years [3]. Insect-derived PrAMPs, e.g. apidaecin, drosocin, pyrrhocoricin and *Oncopeltus* antibacterial peptide 4 [4–7], are basic, around 20–35 residues long peptides with a high proportion of proline residues. Recently, several laboratories have tested native PrAMPs or related designer peptides that were optimized for clinical applications, such as A3-APO [8], Bac7(1–35) [9], oncocin [10] and Api88 [11]. Api88 and oncocin are structurally modified versions of apidaecin 1b (Api 1b) and *Oncopeltus* antibacterial

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Abbreviations: *AMP, antimicrobial peptide; Api 1b, apidaecin 1b; Api88, optimized apidaecin-derivative (sequence derived from apidaecin 1b); BMDC, bone marrow-derived dendritic cells; BMDM, bone marrow-derived macrophages; CD, cluster of differentiation; cDC, conventional dendritic cells; CRAMP, cathelicidin-related antimicrobial peptide; DC, dendritic cells; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorter/flow cytometry; FBS, fetal bovine serum; Flt3-ligand, fms-like tyrosine kinase-ligand; HEPES, 4-(2-Hydroxymethyl)-1-piperazine ethanesulfonic acid; HPF, high power field; Ig, immunoglobulin; Il., interleukin; LPS, lipopolysaccharide; mBD-2, murine β-defensin 2; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; MIC, minimal inhibitory concentration; MHC-II, major histocompatibility complex II; MIP-1α, macrophage inflammatory protein-1 alpha; Onc, oncocin (optimized sequence derived from Oncopeltus antimicrobial peptide 4); Onc72, optimized oncocin-derivative (sequence derived from oncocin); pDC, plasmacytoid dendritic cells; PEC, peritoneal exudate cells; PrAMP, proline-rich antimicrobial peptide; TFA, trifluoroacetic acid; TLR, Toll-like receptor; TNF, tumor necrosis factor*

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peptide 4 to improve the activities against *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with MIC ranging from around 0.5 to 8 μg/ml in low salt conditions [10–12]. Api88 contains ornithine and arginine in positions one and ten instead of glycine and glutamine, respectively, in native Api 1b. Furthermore, Api88 contains a N-terminal N,N,N′,N′-tetramethylguanidino group and an amidated C-terminus, which are both essential for its broad spectrum activity [11]. Onc72 was optimized for reduced degradation in serum by substituting arginine residues 15 and 19 with ornithine [10,13]. Onc72 and Api88 as well as the other designer peptides appear to kill the bacteria by the same mechanism as the native PrAMPs by entering the bacteria and then inhibiting the 70 kDa bacterial chaperone DnaK [11,14–16]. Most importantly, A3-APO, Bac7(1–35), Onc72 and Api88 were highly efficient in different rodent infection models indicating that they are promising lead compounds with a high clinical potential [11,12,17,18].

Onc72 and Api88 have very recently been shown to be potently been antibacterial active in an acute septicaemia model using *Escherichia coli* ATCC 25922 *in vivo* [11,12]. This strong effect may result from both, direct antimicrobial activity as well as activation of innate immunity. In addition to their direct antimicrobial properties, several AMPs have been found to be immunomodulatory, which can contribute on the one hand to a rapid and effective elimination of the pathogen and on the other hand to suppression of immunopathological effects, e.g. by induction of regulatory T cells [19]. The described immunomodulatory effects include direct immune cell activation and amplification of immune responses, e.g. by chemotactic attraction of immune cells to sites of infection [20,21]. Moreover, AMPs are involved in various other processes, for instance wound healing [22], angiogenesis [23], induction of cytokines [24,25] or antigen-uptake by DC [26].

In this study, we compare the immunomodulatory activity of oncocin (Onc), Api 1b, as well as of the optimized PrAMP derivatives Onc72 and Api88 and compare them to murine CRAMP, which has been shown to be immunomodulatory *in vitro* and *in vivo* [27–31]. AMPs may modulate innate immunity in a species-specific manner. A recent study by Tavano *et al.* showed that, albeit being evolutionary distant, the honey bee Api 1b exhibits *in vitro* immunomodulatory activity on human monocytes and macrophages, but not on human DC [32]. Thus, we studied the immunomodulatory effect of the optimized PrAMPs Onc72 and Api88 on isolated murine bone marrow-derived dendritic cells (BMDC) generated in the presence of fms-like tyrosine kinase-3-ligand (Flt3-ligand) as well as isolated murine bone marrow-derived macrophages (BMDM) differentiated in the presence of M-CSF. The BMDC and BMDM we use in our studies closely resemble *in vivo* occurring murine DC and macrophages [33–37]. Moreover, we wished to detect immunomodulatory activities of AMPs on DC and/or macrophages, which may depend on the interaction with other immune cell types. Therefore, we *ex vivo* stimulated splenocytes and peritoneal exudate cells (PEC), which are mixed cultures of different immune cell types of the innate and adaptive immune system (e.g. DC, macrophages, natural killer cells, B and T cells etc.).

Dendritic cells and macrophages are important cells in the dialog between the innate and the adaptive immune system. DC are sentinel cells, which ingest samples of their environment, process them and subsequently present antigens to T cells. The resulting immune response depends on the presence of additional 'danger signals'. Therefore, DC play a very important role in the orchestration of immune responses [38]. Macrophages are phagocytes with many regulatory and effector immune functions [39,40].

In this study, we show that oncocin and Api 1b as well as their optimized derivatives Api88 and Onc72 neither induce expression of proinflammatory cytokines or activation markers nor modulate the immune response to LPS by murine DC or macrophages. The PrAMPs studied were found to be not chemotactic for BMDC. This points to a direct antibacterial activity without immunomodulation involving DC or macrophages.

Materials and Methods

Peptide synthesis

Oncocin (VDKPPYLPRPRPPRRIYNR-NH_2), Onc72 (VDKPPYLPRPRP-PROIYNO-NH_2), Api 1b (GNNRPVYIPQPRPPHPRL-OH), Api88 (Gu-ONNRPVYIPRPRPPHPRL-NH_2, with Gu = tetramethylguanidino), and CRAMP (GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ-NH_2) were synthesized automatically on solid phase by using the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/^{t}Bu)-strategy. Oncocin, Api 1b and CRAMP were synthesized by activating the Fmoc-amino acid derivatives with diisopropylcarbodiimide in the presence of 1-hydroxy-benzotriazole [10], whereas the syntheses of Api88 and Onc72 relied on 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate (HBTU) activation in the presence of *N*,*N*-diisopropylethylamine (DIPEA) as base [11,12]. The N,N,N′,N′-tetramethylguanidino group at the N-terminus of Api88 was incorporated with ten equivalents of HBTU in the presence of DIPEA or *N*-methylmorpholine in *N*,*N*-dimethylformamide as reported recently [12,41]. All peptides were cleaved with trifluoroacetic acid (TFA) containing 12.5% (v/v) of a scavenger mixture (ethanedithiol, m-cresol, thioanisol and water, 1:2:2:2 by volume) for 2 h. The peptides were precipitated with diethyl ether and purified on a C_{18}-phase using an aqueous acetonitrile gradient in the presence of 0.1% (v/v) TFA as ion pair reagent (Jupiter C_{18}-column, 21.2 mm internal diameter, 250 mm length, 15 μm particle size, 30 nm pore size; Phenomenex Inc., Torrance, USA). The peptide purities were determined by analytical RP-HPLC (Jupiter C_{18}-column, 2 mm internal diameter, 150 mm length, 5 μm particle size, 30 nm pore size; Phenomenex Inc., Torrance, USA) to be higher than 95% based on the peak areas detected by absorbance at 214 nm. Furthermore, the masses were confirmed by MALDI-TOF/TOF-MS (4700 Proteomic Analyzer; AB Sciex, Darmstadt, Germany). The mass spectra did not display any intense signals indicating impurities, except for Api88 that contained a small content of the non-guanidated sequence (Supplement, Figure S5). Api88 and Onc72 used in this study were synthesized in the same batch as the compounds used for the NMRI infection model. The purification was performed at different time points, but the RP chromatograms and MALDI-MS indicated very similar purities (>95%).

The control peptide CRAMP showed a potent antimicrobial activity against both, Gram-negative (e.g. *E. coli* MIC: 16–32 μg/ml) and Gram-positive bacteria strains (e.g. *Staphylococcus aureus* DSM 6247 MIC: 4 μg/ml) (data not shown).

Cell culture generation of BMDC and BMDM

All named cell culture reagents were purchased from PAA Laboratories GmbH (Coelbe, Germany), if not stated otherwise. Femora and tibiae from female 8–10-week old C57BL/6J mice were removed, and the bone marrow was flushed with ice-cold PBS containing 5% FBS (purchased from Gibco, Life technologies GmbH, Darmstadt, Germany). For generation of BMDC, bone marrow was centrifuged at 300 × g and 4°C for 8 min, and

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erythrocytes were lysed using Gey's solution. The lysis was stopped by addition of ice-cold PBS. Cells were counted and seeded at a concentration of 1.75×10^6 cells/ml in BMDC differentiation medium and incubated for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. The BMDC differentiation medium contained RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol (Sigma, Taufkirchen, Germany) and 200 ng/ml human fms-like tyrosine kinase-3-ligand (Flt3-ligand) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After incubation of bone marrow cells with Flt3-ligand-containing differentiation medium, the resulting BMDC culture consisted of a mixture of two DC subsets, 65.6 ± 6.2% cDC, positive for CD11c, highly positive for CD11b and negative for B220, and 17.7 ± 2.9% pDC, positive for CD11c and B220 as well as intermediately positive for CD11b.

For generation of BMDM, flushed bone marrow was centrifuged, counted and seeded at a concentration of 1×10^5 cells/ml in BMDM differentiation medium. The cell suspension was filled in teflon bags and incubated for 10 days at 37°C in humidified atmosphere containing 5% CO_2. BMDM differentiation medium contained Dulbecco's Modified Eagle's medium supplemented with 15% sterile-filtered M-CSF containing fibroblast L929 supernatant, 10% heat-inactivated FBS, 5% heat-inactivated horse serum (PAA, Pasching, Austria), 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol (Sigma, Taufkirchen, Germany). Following *in vitro* production, we characterized the resulting differentiated BMDM by markers specific for macrophages (i.e. F4/80) confirming their identity.

Preparation of splenocytes and PEC for *in vitro* stimulation

Peritoneal exudate cells (PEC) were isolated from 8-week old female C57BL/6J mice by lavage using 5 ml ice-cold PBS containing 50 mM EDTA. Furthermore, spleens were removed, and single cell suspension was prepared using a 100 μm Nylon cell strainer (BD Falcon, Franklin Lakes, NJ), followed by erythrocyte lysis using Gey's solution. The cells were resuspended in Iscove's Modified Dulbecco's medium containing 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The number of viable PEC and splenocytes was assessed by trypan blue staining. In the subsequent flow cytometry staining, the different immune cell subpopulations were identified by expression of the following markers: macrophages ($F4/80^+$, $CD11c^-$, $B220^-$), total DC ($CD11c^+$), cDC (cDC; $CD11c^+$, $B220^-$) and B cells ($B220^+$, $CD11c^-$).

In vitro stimulation of BMDC, BMDM, PEC and splenocytes

For *in vitro* stimulation, cells were washed twice, counted and seeded into 96-well plates in their appropriate culture medium at a concentration of 5×10^5 cells/ml (for BMDC and BMDM), 5×10^6 cells/ml (for splenocytes) or 2.5×10^6 cells/ml (for PEC), respectively. For immunostimulation assays, cells were stimulated for 24 h with 100 μg/ml PrAMPs, which corresponds to 41.9 μM Onc, 43.3 μM Onc72, 47.4 μM Api 1b and 43.7 μM Api88, respectively. Immunomodulation was analyzed by stimulation of the cells with the PrAMPs in the aforementioned concentrations in combination with 0.5 μg/ml (for BMDC and BMDM) or 10 ng/ml (for splenocytes and PEC) highly purified LPS (TLRgrade LPS from *Salmonella abortus equi*; Alexis, Bergruen Germany). The LPS concentrations were chosen on the basis of induction of maximal cytokine responses requiring only 10 ng/ml for stimulation of PEC and splenocytes (data not shown). In all stimulation assays, LPS and 20 μg/ml CRAMP (5.2 μM) without LPS served as controls. For analysis of possible cytotoxic effects, we stimulated DC for 24 h with up to 167.4 μM Onc, 173.6 μM Onc72, 189.8 μM Api 1b and 174.7 μM Api88, respectively, equalling 400 μg/ml of the peptides. The cathelicidin CRAMP was tested at a concentration of up to 97 μg/ml (25 μM). After stimulation, the cell suspensions were centrifuged for 8 min at 300 × g and 4°C. The cell supernatants were harvested for further cytokine measurement by sandwich ELISA. Stimulated cells were collected for further analyses of activation marker expression by flow cytometry.

Quantification of cytokines in the supernatant of stimulated cells by ELISA

The concentration of TNF-α as well as the interleukins (IL) IL-12p40 and IL-6 in the supernatants of stimulated BMDC, BMDM, splenocytes and PEC was assessed by sandwich ELISA performed in 96-well roundbottom ELISA plates (Thermo Fisher Scientific/nunc, Roskilde, Denmark). The cytokines were measured using ELISA kits (BD Pharmingen, Heidelberg, Germany), which provided capture and detection antibody pairs. The analysis was performed according to the manufacturer's description. Recombinant murine IL-12p70 for ELISA standard was provided by Dr M. Gately (Hoffmann-LaRoche, Nutley, NJ). Recombinant IL-6 and TNF-α were purchased at Peprotech (Hamburg, Germany). Samples and standards were diluted using Serum Diluent (PBS containing 0.5% (w/v) BSA, 0.1% (v/v) gelatine, 0.05% (v/v) Tween 20). Peroxidase-linked streptavidin (Streptavidin-HRP, 1:3000; Southern Biotechnology Associates, Birmingham, AL) was used for detection of the biotinylated antibodies. 3,3′,5,5′-Tetramethylbenzidine (TMB) Microwell Peroxidase System (KPL, Gaithersburg, MD) served as substrate for the colorimetric reaction, which was stopped by addition of 50 μl 1M H_3PO_4 and measured at 450 and 630 nm with a Spectra-max 340 ELISA reader (Molecular Devices, Biberach a.d. Riss, Germany). The ELISA plate measures were analyzed using the software SoftMax Pro (Molecular Devices, Biberach a.d. Riss, Germany).

Flow cytometry

If not stated otherwise, antibodies and viability dye were purchased from eBioscience (Frankfurt, Germany). All staining steps were performed at 4°C. *In vitro* stimulated cells were harvested, washed with PBS and stained with the fixable viability dye eFluor780 according to the manufacturer's description. To verify the use of eFluor780, we analyzed mixed samples of viable and heat-treated cells. After viability staining, cells were washed first with PBS and afterwards twice with FACS buffer (3% FBS, 0.1% NaN_3 in PBS). Following pre-incubation with anti-CD16/CD32 FcR block (BD Pharmingen, Heidelberg, Germany), cells were stained using mouse IgG2a-isotype control-fluorescein isothiocyanate (FITC), rat IgG2a-isotype control-FITC/PE/PerCpCy5.5/PeCy7, rat IgG2b-isotype control-FITC/PeCy7 (BD Pharmingen, Heidelberg, Germany), hamster IgG1-isotype control -allophycocyanin (BD Pharmingen, Heidelberg, Germany), anti-mouse $I\text{-}A^b$-FITC (clone AF6-120.1; BD Pharmingen, Heidelberg, Germany), anti-mouse I-A/I-E-FITC (clone M5/114.15.2; BioLegend, San Diego, CA), anti-mouse CD86-PE (clone GL1, BD Pharmingen, Heidelberg, Germany), anti-mouse CD11b-PECy7 (clone M1/70), anti-mouse F4/80-PECy7 (clone BM8), anti-mouse B220-PerCp Cy5.5 (clone RA3-6B2) or anti-mouse CD11c-allophycocyanin (clone N418; BioLegend, San Diego, CA). After 20 min incubation at 4°C, cells were washed

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twice with FACS buffer followed by one wash step with PBS. Subsequently, cells were fixed in 2% (v/v) paraformaldehyde in PBS, washed once with PBS and afterwards with FACS buffer. The samples were analyzed with a BD FACS Canto II (Becton Dickinson, Heidelberg, Germany) flow cytometer using BD FACS Diva (Becton Dickinson, Heidelberg, Germany) and FlowJo (Tree Star, Inc., Ashland, OR) software.

Chemotaxis assay

For migration assays, BMDC were collected and suspended in chemotaxis medium (RPMI 1640 medium containing 1% bovine serum albumin, 20 mM HEPES, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin) at 2×10^6 cells/ml. The migration of murine BMDC in response to the oncocin and apidaecin derivatives and CRAMP, as well as the positive controls MCP-1 and MIP-1α, was determined using a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD). Chemotaxis medium served as negative control. Chemotattractants were diluted in chemotaxis medium and filled into the lower well of the chemotaxis chamber. For chemotaxis assays, dilution series with a range of 0.42 to 420 nM Onc, 0.43 to 430 nM Onc72, 0.47 to 470 nM Api 1b, 0.44 to 440 nM Api88 or 0.26 to 260 nM CRAMP, respectively, equalling a range from 1 ng/ml to 1 μg/ml, were tested. The cell suspension was filled into the wells of the upper compartment. Upper and lower compartment were separated by a 5 μm polycarbonate filter membrane (NeuroProbe, Cabin John, MD). The migration chamber was incubated for 1.5 h at 37°C and 5% CO_2 in humidified air, and afterwards, the filter was removed, scraped and stained with the DiffQuik staining kit (Dade Behring, Newark, DE). Migrated cells were counted under a light microscope by using the analySIS software (Soft Imaging System, Münster, Germany), and the results were displayed as cells per high power field (HPF) with an area of 0.0192 mm^2 at a magnification of 400.

Statistical analysis

Indicated significance (***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$) of differences between groups were calculated by unpaired Mann–Whitney test.

Results

The oncocin and apidaecin derivatives are not immunomodulatory for DC.

Dendritic cells are the primary antigen presenting cells in the immune system and therefore key cells in the dialog between the innate and the adaptive immune system [38]. Several AMPs such as β-defensins have been described to be immunostimulatory for cells of the innate immune system, e.g. DC, monocytes and macrophages [42–46]. To study immunomodulatory effects of insect-derived PrAMPs on DC, we generated BMDC in the presence of Flt3-ligand, which consist of two major subsets, conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDC) [33–35,37]. Following *in vitro* generation, we characterized the resulting differentiated BMDC by markers specific for DC (i.e. CD11c, CD11b, B220) confirming their identity (supplement figure data S1). We stimulated DC for 24 h with up to 400 μg/ml (170–190 μM) of the PrAMP derivatives, to test whether the peptides have a cytotoxic effect on BMDC. The cathelicidin CRAMP was tested at a concentration of up to 97 μg/ml (25 μM). To investigate potential cytotoxic activity of the PrAMP derivatives, dead BMDC were detected by flow cytometry using the fixable viability dye eFluor780. No difference in the viability between BMDC incubated with the various peptides, and medium-incubated or LPS-incubated cells was detectable (data not shown). To clarify whether oncocin (Onc), Onc72, Api 1b, Api88 and CRAMP show an immunostimulatory effect on DC, murine BMDC were stimulated for 24 h with 100 μg/ml of these peptides. None of the tested peptides induced the secretion of IL-12p40, IL-6 or TNF-α, whereas LPS significantly increased secretion of these cytokines compared with medium incubation (Figure 1). In agreement with the cytokine data, after PrAMP stimulation, we could not detect an increased number of cells positive for the activation markers CD86 and MHC-II in the total $CD11c^+$ DC population comprising cDC and pDC (Figure 2). Independently from the constitutive expression level (Figure 2D *versus* Figure 2F), expression of MHC-II was strongly up-regulated by LPS stimulation. When cDC and pDC were analyzed separately for expression of CD86 and MHC-II, neither subpopulation of the PrAMP-stimulated BMDC showed an increase in the percentage of activation marker-positive cells (supplement figure S2 and data not shown). Even when BMDC were stimulated with a maximal PrAMP concentration as high as 400 μg/ml, expression of CD86 and MHC-II remained unchanged, and induction of IL-12p40, IL-6 or TNF-α was not detectable (data not shown). In contrast to the data obtained from ELISA and analysis of the MHC-II expression, in some experiments, the cDC subpopulation of BMDC showed a minor but significant increase of CD86-positive cells after stimulation with CRAMP (Figure 2E and supplement figure S2B).

Another well-known immunostimulatory activity of several AMPs is the induction of chemotaxis of different immune cell types [21,30,42,47–49]. Therefore, we investigated if oncocin, Onc72, Api 1b, Api88 and CRAMP are chemotactic for immature BMDC. MCP-1 and MIP-1α served as positive controls, which are known to chemoattract immature DC via CCR2A/B and CCR5, respectively [50]. As published, MCP-1 and MIP-1α proved to be chemotacticly active in our studies (Figure 3) in a dose-dependent manner (data not shown). We could not detect chemotaxis induction of BMDC for any of the four studied PrAMPs in a concentration range from 1 ng/ml to 1 μg/ml (Figure 3). CRAMP showed no chemotactic activity on DC either as described earlier [30].

Besides a direct immunostimulatory effect, some AMPs were shown to have an immunomodulatory effect on immune responses caused by TLR activation [27,28,31,51]. Therefore, we incubated murine BMDC simultaneously with the TLR4 agonist LPS and the four studied PrAMPs. Because stimulation of human DC with LPS was previously shown to be inhibited by LL-37, the human orthologue of CRAMP [28], we used the immunomodulatory effect of CRAMP as control. Although CRAMP markedly down-regulated the LPS-induced cytokine secretion (Figure 1) and the percentage of surface activation marker-positive cells as published for murine macrophage cell lines before [29], none of the tested oncocin or apidaecin derivatives showed an immunomodulatory effect (Figure 2). Even if LPS was combined with a maximal insect AMP concentration as high as 400 μg/ml, no modulation of the LPS-induced immune response could be observed (data not shown).

Similar to DC, the optimized PrAMPs Onc72 and Api88 as well as oncocin and Api 1b are not immunomodulatory for macrophages.

In addition to DC, macrophages are central players of the innate immunity. Macrophages are phagocytes with many regulatory and

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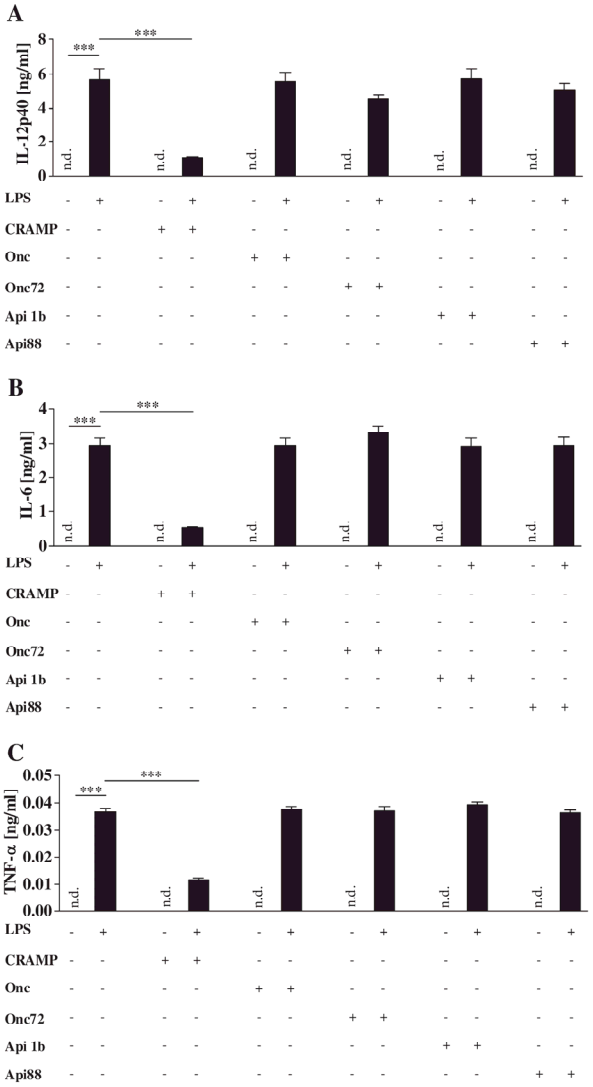

Figure 1. Oncocin, Onc72, as well as Api 1b and Api88 do not activate or modulate the secretion of inflammatory cytokines by DC. *In vitro* generated BMDC were stimulated for 24 h with 100 μg/ml (corresponding to 41.9 μM Onc, 43.3 μM Onc72, 47.4 μM Api 1b and 43.7 μM Api88) of the PrAMPs alone or in combination with 0.5 μg/ml LPS. Cell supernatants were collected and the concentration of IL-12p40 (**A**), IL-6 (**B**) and TNF-α (**C**) was assessed by sandwich ELISA. LPS alone or 20 μg/ml CRAMP (5.2 μM) w/o LPS served as controls. Pooled data from three individual experiments are shown (mean ± SEM, each experiment was done with triplicate samples). For statistical analysis, unpaired Mann–Whitney test was used. n.d. (not detectable) indicates that cytokine levels were below detection level.

effector immune functions [39,40]. As described previously, some AMPs have been described to be immunostimulatory or immunomodulatory for macrophages, monocytes and DC [32,42–46]. The identity of the BMDM was confirmed using flow cytometrical detection of the F4/80 antigen as a typical macrophage marker (data not shown). To elucidate the immunostimulatory properties of the insect AMP derivatives, murine BMDM were stimulated for 24 h with the peptides. Similar to the data for BMDC, none of the peptides studied induced macrophage secretion of pro-inflammatory cytokines such as IL-12p40 (Figure 4), TNF-α or IL-6 (data not shown).

Besides, we analyzed, if the peptides were able to modulate the LPS-induced immune response of BMDM. None of the tested PrAMP derivatives modulated the LPS-induced IL-12p40 secretion (Figure 4), while in both experiments, CRAMP showed a tendency to inhibit LPS-induced IL-12p40 expression without reaching statistical significance (Figure 4). Down-regulation of the LPS-induced cytokine production by CRAMP could be further enhanced by increasing the ratio of CRAMP *versus* LPS (data not shown).

Novel oncocin and apidaecin derivatives display no immunomodulatory activities on PEC and splenocytes.

Because the experiments described previously relied only on isolated DC or macrophages, we further studied innate responses of immune cells derived from the peritoneal cavity (i.e. the infection and treatment site used in [11,12]), or from the spleen. This approach should allow cellular interactions potentially necessary to enable immunostimulation by AMPs. To this end, we stimulated splenocytes as well as PEC with the four PrAMPs alone or in presence of LPS. Upon incubation with one of the four studied PrAMPs or CRAMP, we did not find induction of the inflammatory cytokines IL-6 (Figure 5), IL-12p40 and TNF-α as well as the activation marker CD86 on the different immune cell types (data not shown). Moreover, we could not observe a modulatory effect of any PrAMP on the LPS-induced immune response either on the splenocyte or on the PEC subpopulations as assessed by ELISA (Figure 5) and flow cytometry (data not shown). CRAMP, however, modulated the LPS-induced IL-6 expression of the splenocytes (Figure 5A) but not of PEC (Figure 5B), suggesting tissue-specific immunomodulation by CRAMP that may depend on cellular interactions. Together, these data strengthen the conclusions drawn before for isolated BMDC and BMDM (Figures 1, 2, and 4). Even in a mixed culture system of spleen or peritoneal cells, the insect peptides studied did not induce or modulate dendritic cell and macrophage activation.

Discussion

Because of the increasing number of single or multiple resistances in bacteria and fungi, new approaches for the control of microbial infections are required. Considering the different modes of action used, the diverse group of AMPs shows a broad antibacterial spectrum. Therefore, several members of AMPs turn out to be promising candidates for the treatment of microbial infections. Insect immunity shows significant similarities to vertebrate innate immunity. Comparable to vertebrates, (innate) immunity of arthropods contains humoral as well as cellular elements [52]. Another intriguing similarity between insects and mammals is the utilization of mechanisms such as phagocytosis, melanization, opsonization or secretion of complement and AMPs [53,54]. In contrast to membrane-perturbating AMPs, PrAMPs, such as oncocin and apidaecin, inhibit the intracellular bacterial target chaperone DnaK [14,16,55]. Otvos *et al.* showed that pyrrhocoricin, another member of the short proline-rich peptide family, is able to inhibit DnaK, but cannot bind Hsp70, the human homologue of DnaK [16]. Moreover, the amino acid sequence homology between the bacterial chaperone DnaK and the eukaryotic heat-shock protein Hsp70 is only 48% [56]. Thus, the risk of pathological effects against eukaryotic cells seems to be

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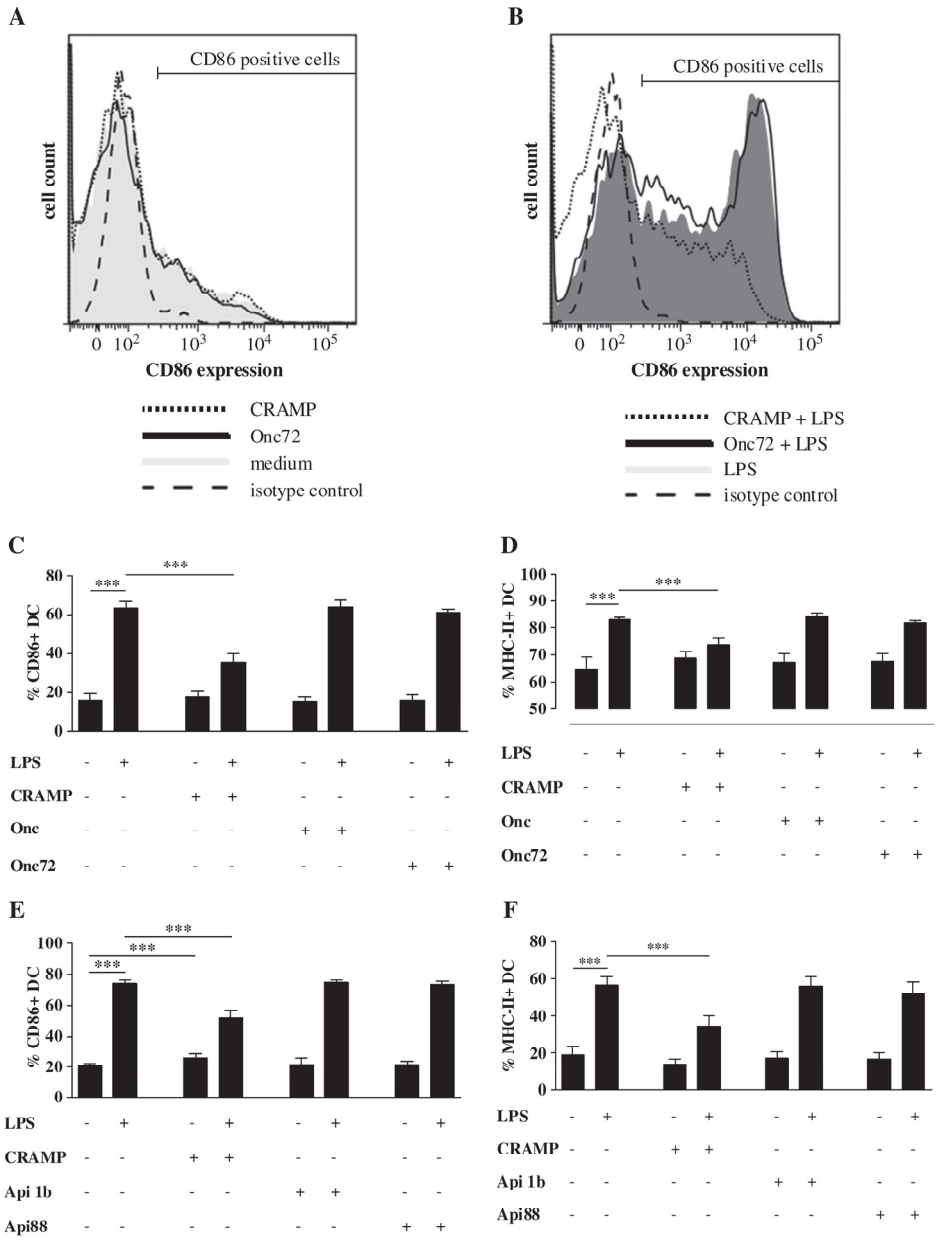

Figure 2. In contrast to CRAMP, the insect PrAMPs are not immunostimulatory or immunomodulatory for DC. After 24 h stimulation of BMDC with 100 μg/ml of the four tested PrAMPs alone (equalling 41.9 μM Onc, 43.3 μM Onc72, 47.4 μM Api 1b and 43.7 μM Api88) or in combination with 0.5 μg/ml LPS, cells were harvested and stained for dendritic cell markers (e.g. CD11c, CD11b, B220) and expression of activation markers CD86 and MHC-II. LPS alone or 20 μg/ml CRAMP (5.2 μM) w/o LPS served as controls. **A** shows a representative overlay of the CD86 expression by BMDC incubated with medium (light gray curve), Onc72 (solid line) or CRAMP (dotted line). In **B** a representative overlay of the CD86 expression by LPS (dark gray filled curve), Onc72 with LPS (solid line) or CRAMP with LPS (dotted line) stimulated BMDC is presented. Marker position was set according to the isotype control (dashed line) to determine the proportion of CD86 positive cells (figure **A** and **B**). Percentages of CD86 and MHC-II positive DC are depticted for oncocin w/o LPS stimulated cells (**C**, **D**) as well as apidaecin w/o LPS stimulated BMDC (**E, F**). Pooled data from $n = 2$ individual experiments for Onc72, $n = 4$ individual experiments for Onc wt and $n = 3$ individual experiments for Api88 and Api 1b are shown (mean ± SD, triplicate samples for apidaecins, quadruplicate samples for oncocins). For statistical analysis, unpaired Mann–Whitney test was used.

unlikely [16], especially as PrAMPs only enter some cell lines of the immune system without being toxic to them, but are excluded from other mammalian cells [57–59]. All these aspects make them interesting as therapeutic agents against bacteria and other pathogens.

We have shown that the lead compounds Onc72 and Api88 are potent antibiotics in an *in vivo* septicaemia model [11,12]. Besides, a direct antibacterial activity activation or modulation of the innate immunity may be involved in this strong antimicrobial effect. Therefore, first of all, we clarified that the four studied

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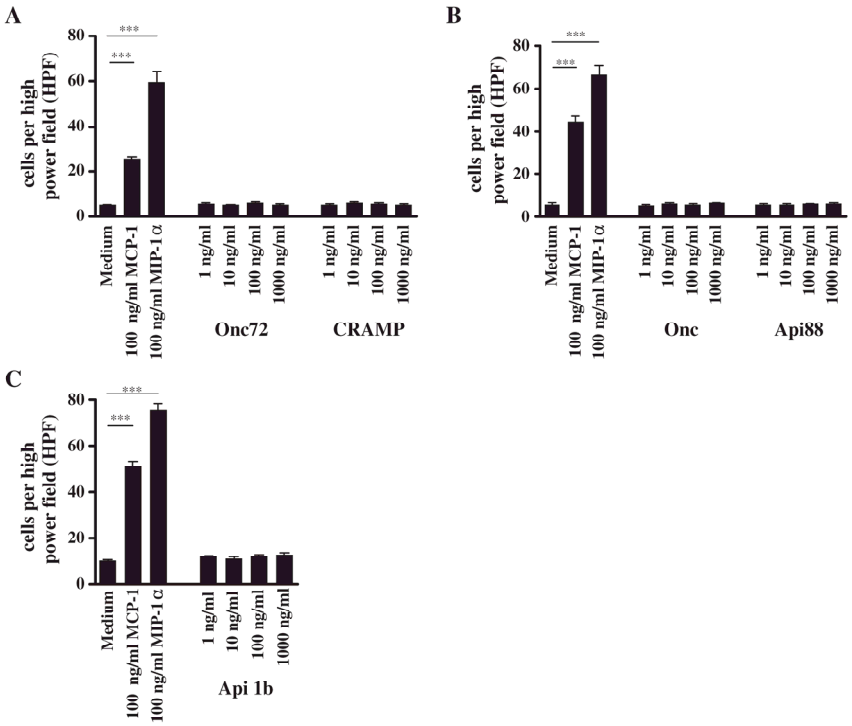

Figure 3. Oncocin, Onc72, Api 1b and Api88 are not chemotactic for DC. Migration of murine BMDC in response to Onc72 (**A**), oncocin (**B**), Api88 (**B**), Api 1b (**C**) and CRAMP (**A**) was determined. MCP-1 and MIP-1α were used as positive controls. For chemotaxis assays, 0.42 to 420 nM Onc, 0.43 to 430 nM Onc72, 0.47 to 470 nM Api 1b, 0.44 to 440 nM Api88 or 0.26 to 260 nM CRAMP, respectively, equalling a range from 1 ng/ml to 1 μg/ml peptide, were tested. One representative experiment out of at least two is shown. Data are represented as number of cells per HPF, with an area of 0.0192 mm^2 at a magnification of 400 (mean ± SEM). For statistical analysis, unpaired Mann–Whitney test was done.

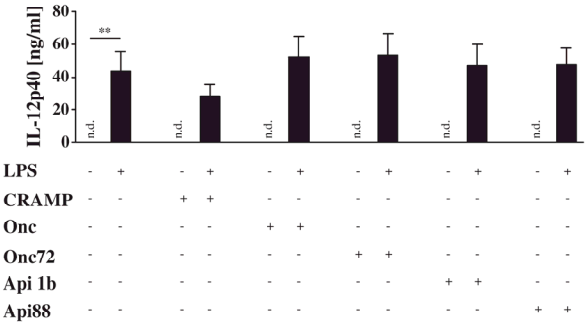

Figure 4. Similar to DC, the oncocin and apidaecin derivatives show no activation or modulation of cytokine secretion by macrophages. BMDM were stimulated for 24 h with 100 μg/ml of the PrAMPs alone (corresponding to 41.9 μM Onc, 43.3 μM Onc72, 47.4 μM Api 1b and 43.7 μM Api88) or in combination with 0.5 μg/ml LPS. Concentration of IL-12p40 in cell supernatants was assessed by sandwich ELISA. LPS alone or 20 μg/ml CRAMP (5.2 μM) w/o LPS served as controls. Pooled data from $n-2$ experiments are shown (mean ± SEM, triplicate samples). n.d. (not detectable) indicates that cytokine levels were below detection level. Unpaired Mann–Whitney test was used for statistical analysis.

PrAMPs and CRAMP do not have an elevated cytotoxic effect on BMDC (data not shown). In contrast to apidaecins and oncocins, the cathelicidin CRAMP is a membrane-active AMP [60], and therefore, it is more likely that CRAMP may have toxic effects on eukaryotic cells [29]. For instance, LL-37, the human orthologue of CRAMP, is toxic for eukaryotic cells because of its N-terminal hydrophobic region. In contrast to that, the murine cathelicidin CRAMP does not have such a strong cytotoxic effect [61], which is in accordance with our data.

As mentioned previously, several mammalian AMPs such as β-defensins, have been described to be immunostimulatory for cells of the innate immune system, e.g. DC, monocytes or macrophages [42–46]. For instance, Biragyn *et al.* demonstrated that after 18 h of incubation with murine β-defensin 2 (mBD-2), murine BMDC showed a markedly increased expression of the activation markers CD86 and CD40 as well as the cytokines IL-12p40, IL-1α and IL-1β. Moreover, amongst others, elevated mRNA expression of TNF-α after 6 h of incubation with mBD-2 could be assessed [42]. Moreover, the human orthologue of CRAMP, LL-37, was recently shown to induce mRNA transcription and protein expression of the pro-inflammatory cytokine IL-8 in human gingival fibroblasts via the $P2X_7$ receptor [62]. In contrast to LL-37, we could not find an immunostimulatory activity of the murine cathelicidin CRAMP, which may be due to the different species as well as different cell types observed. Additionally, in accordance to Tavano *et al.* [32], we could not detect an immunostimulation of murine BMDC by Api 1b, as assessed by analysis of the expression of surface activation markers and/or inflammatory cytokines (Figures 1 and 2). This was also true for oncocin, Onc72 and Api88. However, Tavano *et al.* showed that stimulation with Api 1b up-regulated the expression of inflammatory cytokines and surface activation markers by human macrophages, which we could not confirm for murine BMDM stimulated with Api 1b or with any or the other

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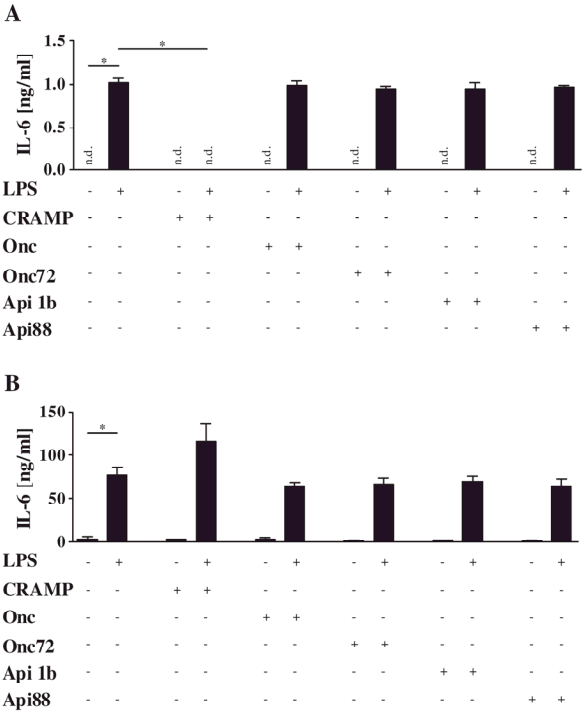

Figure 5. The PrAMP derivatives do not induce or modulate the IL-6 secretion by splenocytes and PEC. Freshly isolated splenocytes (**A**) and PEC (**B**) were stimulated with 100 μg/ml oncocin, Onc72, Api 1b and Api88 alone (equalling 41.9 μM Onc, 43.3 μM Onc72, 47.4 μM Api 1b and 43.7 μM Api88) or in presence of 10 ng/ml LPS for 24 h. LPS alone or 20 μg/ml CRAMP (5.2 μM) w/o LPS served as controls. After stimulation, concentration of IL-6 was determined in the cell supernatants. Pooled data from $n = 2$ individual experiments are shown (± SEM, duplicate samples). For statistical analysis, unpaired Mann–Whitney test was used. n.d. (not detectable) indicates that cytokine levels were below detection level.

three PrAMPs (Figure 4). Moreover, we could exclude that innate immune cells (e.g. macrophages and DC) could be activated by the four PrAMPs tested, in the context of possible cellular interactions between different immune cell types derived from spleen or peritoneal cavity (Figure 5 and data not shown).

As another immunostimulatory feature, it was shown that several AMPs can be chemotactic for some immune cell types. For instance, CRAMP has been described to recruit human monocytes, neutrophils, macrophages and mouse peripheral blood leukocytes via the murine formyl peptide receptor-like 2 (FPRL-2) or its human homologue FPRL-1 [30]. Moreover, LL-37, the human orthologue of CRAMP, was shown to use at least one other receptor involving the G_i-protein-phospholipase C-signaling pathway to act chemotactic for rat mast cells [47]. Moreover, it was reported that the proline-rich cathelicidin PR-39 is chemotactic for neutrophils, too [63]. In our chemotaxis experiments, none of the four proline-rich oncocin and apidaecin peptides exhibited any chemotactic activity for BMDC (Figure 3). Furthermore, CRAMP did not chemoattract murine BMDC, either. This result is in accordance with the finding that albeit being chemotactic for human monocyte-derived macrophages, CRAMP has no chemotactic effect on human monocyte-derived immature DC even in concentrations up to 10 μM [30].

Besides an immunostimulatory activity, some AMPs have been described to act as immunomodulators [28,32,64]. An immunomodulatory effect may be an advantage in the case of an infection, because additional immune cells can be recruited or their immune responses can be enhanced to better control the pathogens. However, in some cases, immunomodulation may be disadvantageous. This is true for the case that the AMP may cause immunopathological effects, e.g. over-production of pro-inflammatory cytokines, which can result in excessive inflammation. We investigated whether oncocins and apidaecins have a modulatory effect on the innate immune response against a TLR agonist. In accordance to the literature, co-incubation of murine macrophages (as well as BMDC and splenocytes) with CRAMP and LPS resulted in a reduced expression of inflammatory cytokines (Figures 1, 2, 4 and 5A for splenocytes) [29]. On the one hand, this is in agreement with the finding that the Pro/Arg-rich ovine cathelicidin OaBac5mini shows a significant reduction of *S. aureus* strain Cowan-induced IL-12 response by murine J774A cells [64]. On the other hand, our results for CRAMP are contrary to the fact that there is no modulatory effect of OaBac5mini on the *S. aureus* strain Cowan-induced expression of the cytokines IL-6, IL-10 and TNF-α [64], which may be due to the different pathogen-associated molecular patterns used to induce an immune response (LPS from gram-negative bacteria *versus* gram-positive bacteria). However, in contrast to CRAMP, we could not detect a modulatory effect of the tested PrAMPs on the LPS-induced expression of activation marker and/or inflammatory cytokines by isolated BMDC and BMDM cultures, as well as mixed immune cell cultures of splenocytes and PEC (Figures 1, 2, 4, 5). The molar mass of CRAMP is around 1.6–1.8× higher than that of the insect AMP derivatives. Although we used a 9× higher amount of insect AMP molecules in comparison to CRAMP for co-stimulation with LPS, we could not detect a modulatory effect as observed with the well-known immunomodulator CRAMP. Again, our data confirm the results of the study by Tavano *et al.*, who showed that Api 1b has no modulatory effect on the LPS-induced immune response of human DC [32]. In contrast to Tavano *et al.*, we could not find immunomodulation of BMDM by the peptides tested (Figures 1, 2, 4 and 5). Explanations for these differences may be that we used cells from another species as well as LPS from another bacterium.

It is known that CRAMP can bind and inhibit LPS, flagellin and lipoteichonic acid, and therefore reduces the immune response to these TLR ligands [29,31]. However, it has been shown that similar to co-stimulation of CRAMP with LPS, pre-incubation of murine BMDM with CRAMP, followed by removal of CRAMP and stimulation of the cells with LPS resulted in a strong inhibition of pERK activation [31]. This indicates that additional to LPS binding, CRAMP interferes directly with the LPS-induced immune response. It has been controversially discussed if apidaecin is also able to bind LPS. Whereas Otvos *et al.* reported that apidaecin binds LPS [16], Dutta *et al.* asserted that apidaecin shows no or only a very weak binding to LPS [65]. Moreover, they interpret the modest binding of fluorescein-labeled apidaecin to LPS seen by Otvos *et al.* as non-active ionic interaction [65]. It is assumed that non-lytic AMPs first have to interact with a bacterial outer membrane molecule, e.g. LPS, to facilitate cell entry [16]. It remains unclear, if even a weak binding would lead to inhibition of LPS and therefore diminish the immune response to LPS stimulation. However, we could not see a modulatory effect of oncocin, Onc72, Api 1b and Api88 on the LPS-induced immune response.

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Together, in this study, we show that the strong antibacterial effects obtained for Onc72 and Api88 *in vitro* and *in vivo* cannot be explained by immunostimulatory or immunomodulatory effects on central cells of the innate immune system. Thus, it seems likely that their efficacy in murine infection models relies exclusively on a direct effect against the pathogen. This will simplify further pharmaceutical investigation and development of insect peptides as therapeutic compounds against bacterial infections.

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3.4 SUPPLEMENTARY DATA TO PUBLICATION II

Absence of *in vitro* innate immunomodulation by insect-derived short proline-rich antimicrobial peptides points to direct antibacterial action *in vivo*

Stefanie Fritsche, Daniel Knappe, Nicole Berthold, Heiner von Buttlar, Ralf Hoffmann, and Gottfried Alber

Supporting Information

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Flow cytometry

If not stated otherwise, antibodies and viability dye were purchased from eBioscience (Frankfurt, Germany). All staining steps were performed at 4°C. *In vitro* stimulated BMDC were harvested, washed with PBS and stained with the fixable viability dye eFluor780 according to the manufacturer's description. To verify the use of eFluor780 we analyzed mixed samples of viable and heat-treated cells. After viability staining, cells were washed first with PBS and afterwards twice with FACS buffer (3 % FBS, 0.1 % NaN_3 in PBS). Following pre-incubation with anti-CD16/CD32 FcR block (BD Pharmingen, Heidelberg, Germany), cells were stained using rat IgG2a-isotype control- PE / PerCp Cy5.5, rat IgG2b-isotype control-FITC / PeCy7 (BD Pharmingen, Heidelberg, Germany), hamster IgG1-isotype control APC (BD Pharmingen, Heidelberg, Germany), anti-mouse I-A/I-E-FITC (clone M5/114.15.2; BioLegend, San Diego, CA), anti-mouse CD86-PE (clone GL1, BD Pharmingen, Heidelberg, Germany), anti-mouse CD11b-PECy7 (clone M1/70), anti-mouse B220-PerCp Cy5.5 (clone RA3-6B2) or anti-mouse CD11c-APC (clone N418; BioLegend, San Diego, CA). After 20 min incubation at 4°C, cells were washed twice with FACS buffer followed by one wash step with PBS. Subsequently, cells were fixed in 2 % (v/v) paraformaldehyde in PBS, washed once with PBS and afterwards with FACS buffer. The samples were analyzed with a BD FACS Canto II (Becton Dickinson, Heidelberg, Germany) flow cytometer using BD FACS Diva (Becton Dickinson, Heidelberg, Germany) and FlowJo (Tree Star, Inc., Ashland, OR) software.

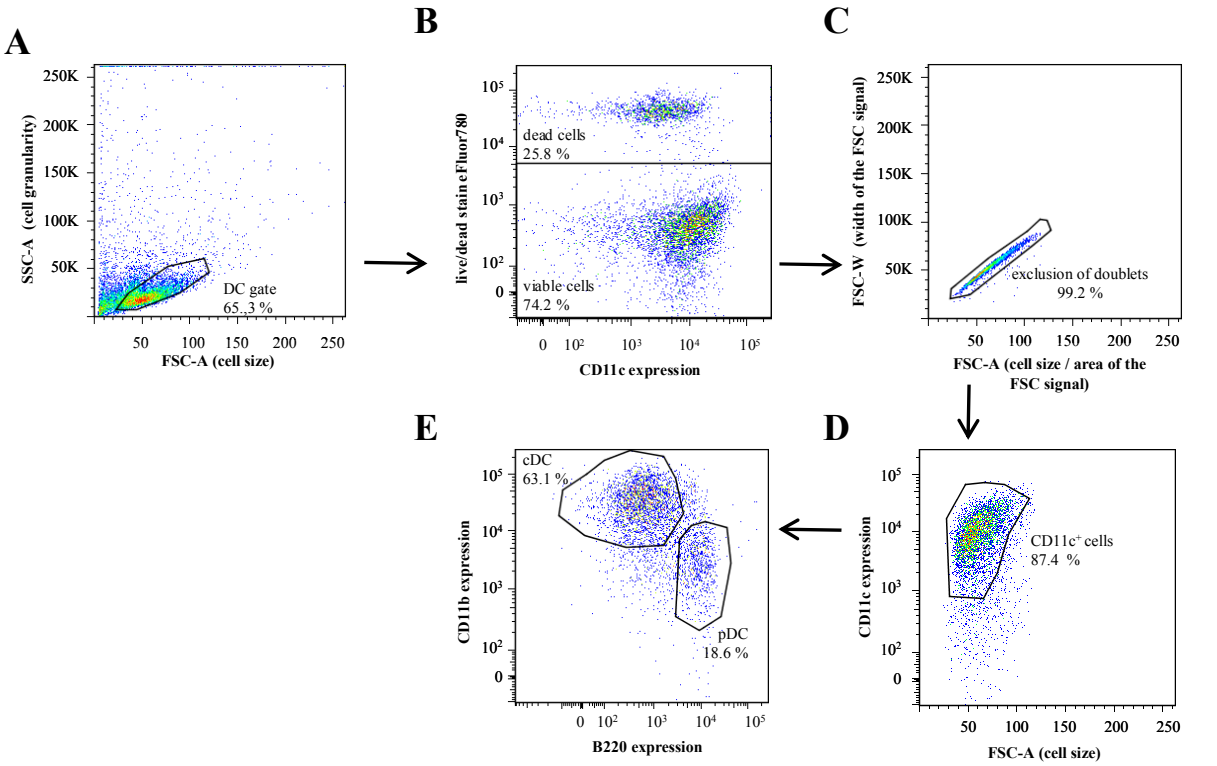

Figure S1. Gating strategy for bone marrow-derived dendritic cells (BMDC)
After incubation of bone marrow cells with Flt3-ligand-containing differentiation medium for seven days, the resulting BMDC culture was stained using antibodies against murine CD11c, CD11b, B220, CD86 and MHC-II. Cells were gated with respect to their size and granularity (**A**). Living cells (**B**) were further gated in order to exclude cellular doublets via FCS-A vs. FCS-W comparison (**C**). Based on this gating strategy, BMDC are all $CD11c^{+}$ cells (**D**) which are further classified in 65.6 ± 6.2 % cDC ($CD11c^{+}$, $CD11b^{++}$, $B220^{-}$) and 17.7 ± 2.9 % pDC ($CD11c^{+}$, $CD11b^{med}$, $B220^{+}$) (**E**). One representative experiment out of n = 353 samples tested is presented.

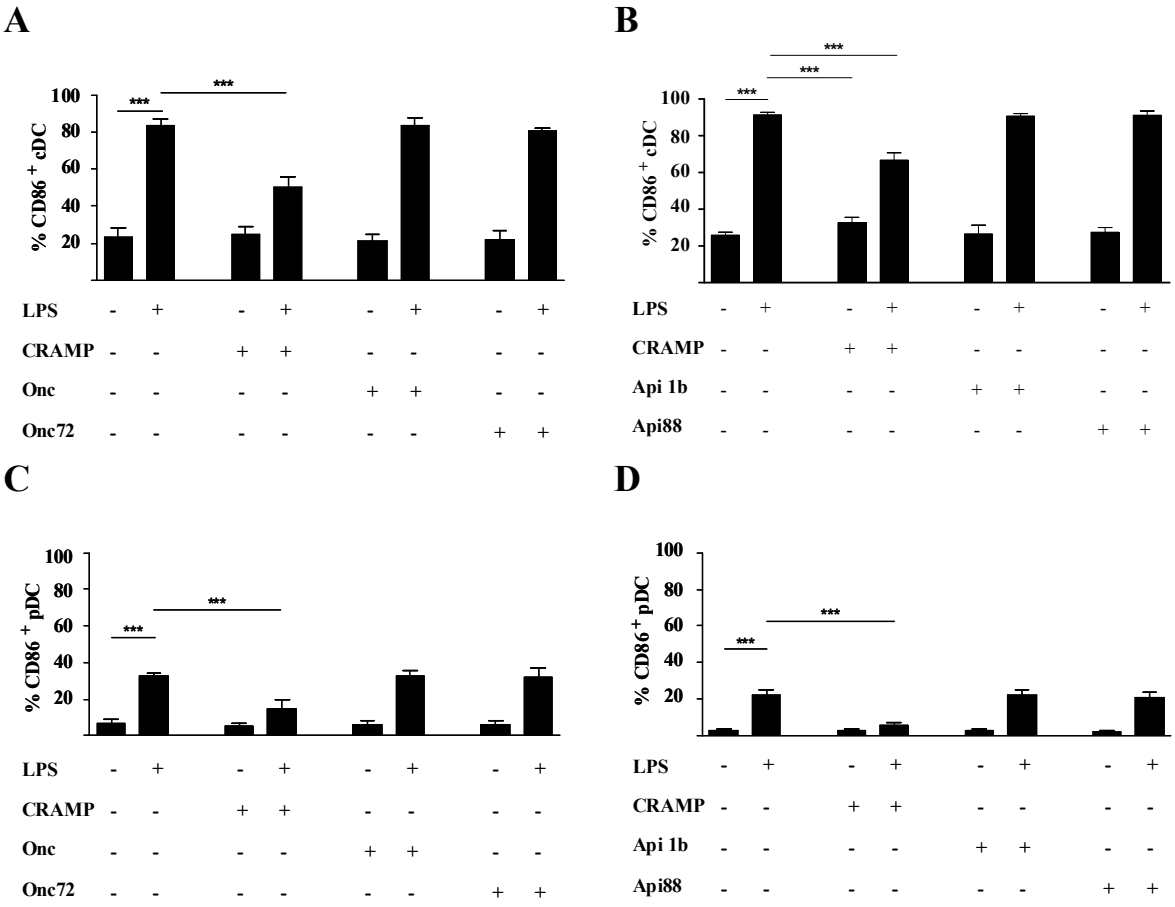

Figure S2. The oncocin and apidaecin derivatives do not stimulate or modulate the activation status of conventional (cDC) and plasmacytoid dendritic cells (pDC).
Following 24 h stimulation of BMDC with 100 µg/ml of the four oncocin and apidaecin derivatives in presence or absence of 0.5 µg/ml LPS, cells were stained for dendritic cell markers (e.g. CD11c, CD11b, B220) and expression of the activation marker. LPS alone or 20 µg/ml CRAMP w/o LPS served as controls. Proportions of CD86 positive cDC (**A**, **B**) and pDC (**C**, **D**) following PrAMP stimulation in presence or absence of LPS stimulation are depicted. Pooled data from n = 2 individual experiments for Onc72, n = 4 individual experiments for oncocin and n = 3 individual experiments for Api88 and apidaecin 1b are shown (mean ± SD, triplicate samples for apidaecins, quadruplicate samples for oncocins). For statistical analysis, unpaired Mann-Whitney-test was used**.**

Table S1: Sequences and analytical data of the peptides used in this study. Calculated (calc) and experimental (exp) monoisotopic masses of the quasimolecular ion $[M+H]^+$ recorded by MALDI-MS.

Peptide	Sequence[1]	Monoisotopic mass	
		exp	calc
Api 1b wt	GNNRPVYIPQPRPPHPRL-OH	2108.12	2108.16
Api88	Gu-ONNRPVYIPRPRPPHPRL-NH_2	2290.40	2289.35
Onc	VDKPPYLPRPRPPRRIYNR-NH_2	2389.36	2389.38
Onc72	VDKPPYLPRPRPPROIYNO-NH_2	2305.45	2305.33
CRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ-NH_2	3877.24	3877.30

[1]O and Gu denotes ornithine and N,N,N',N'-tetramethylguanidino

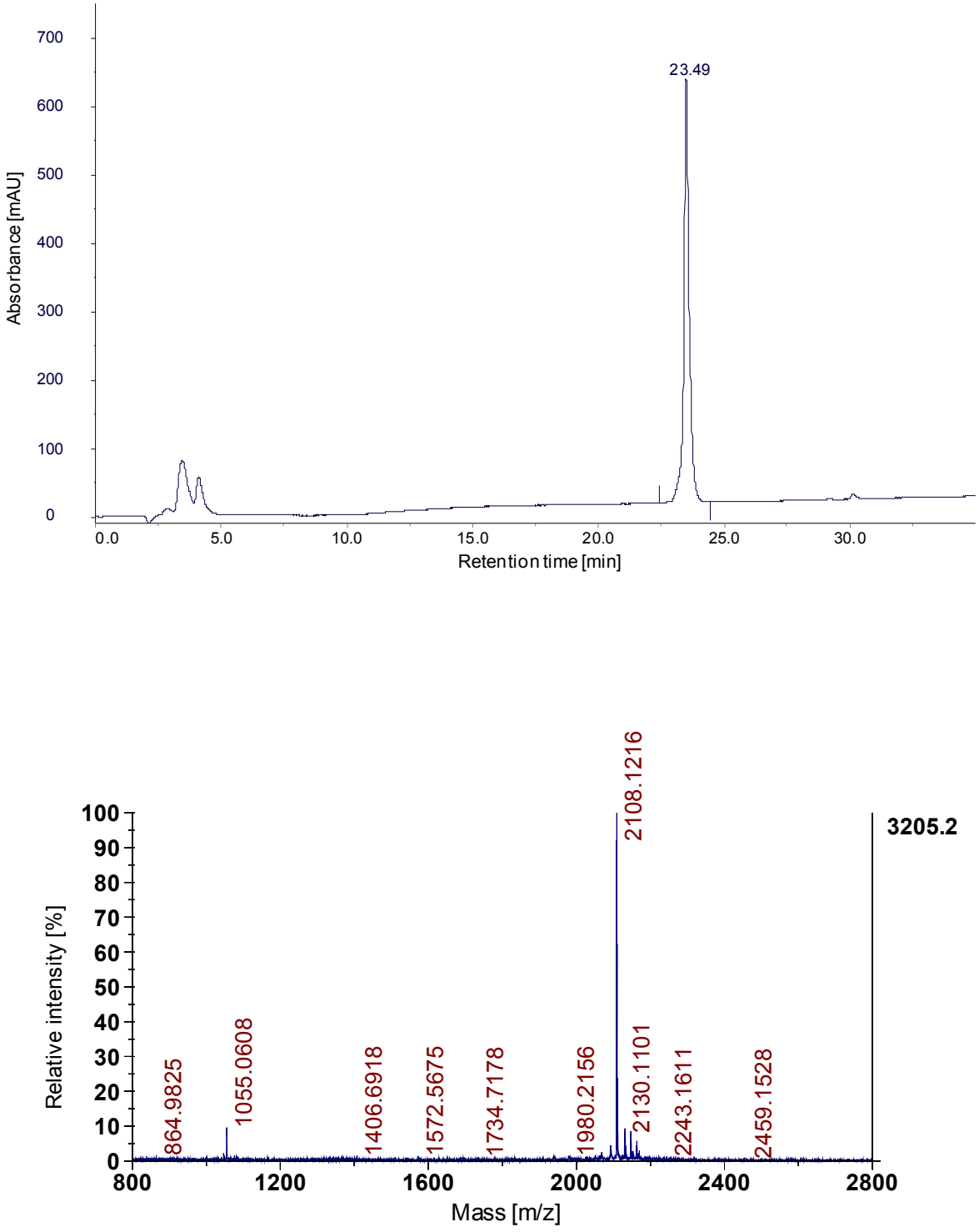

Figure S3: RP-HPLC (top) and MALDI-MS (bottom) of purified apidaecin 1b.

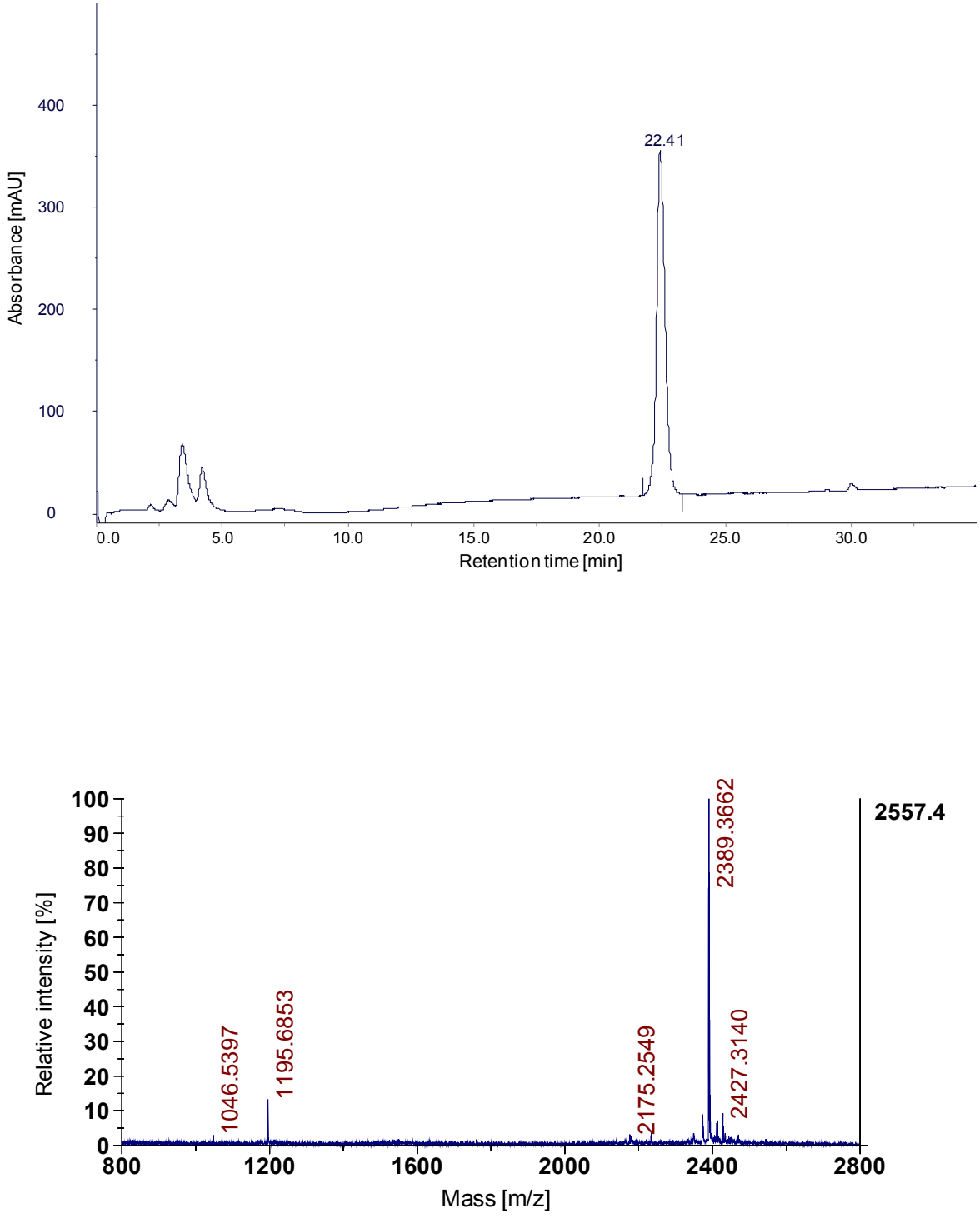

Figure S4: RP-HPLC (top) and MALDI-MS (bottom) of purified oncocin.

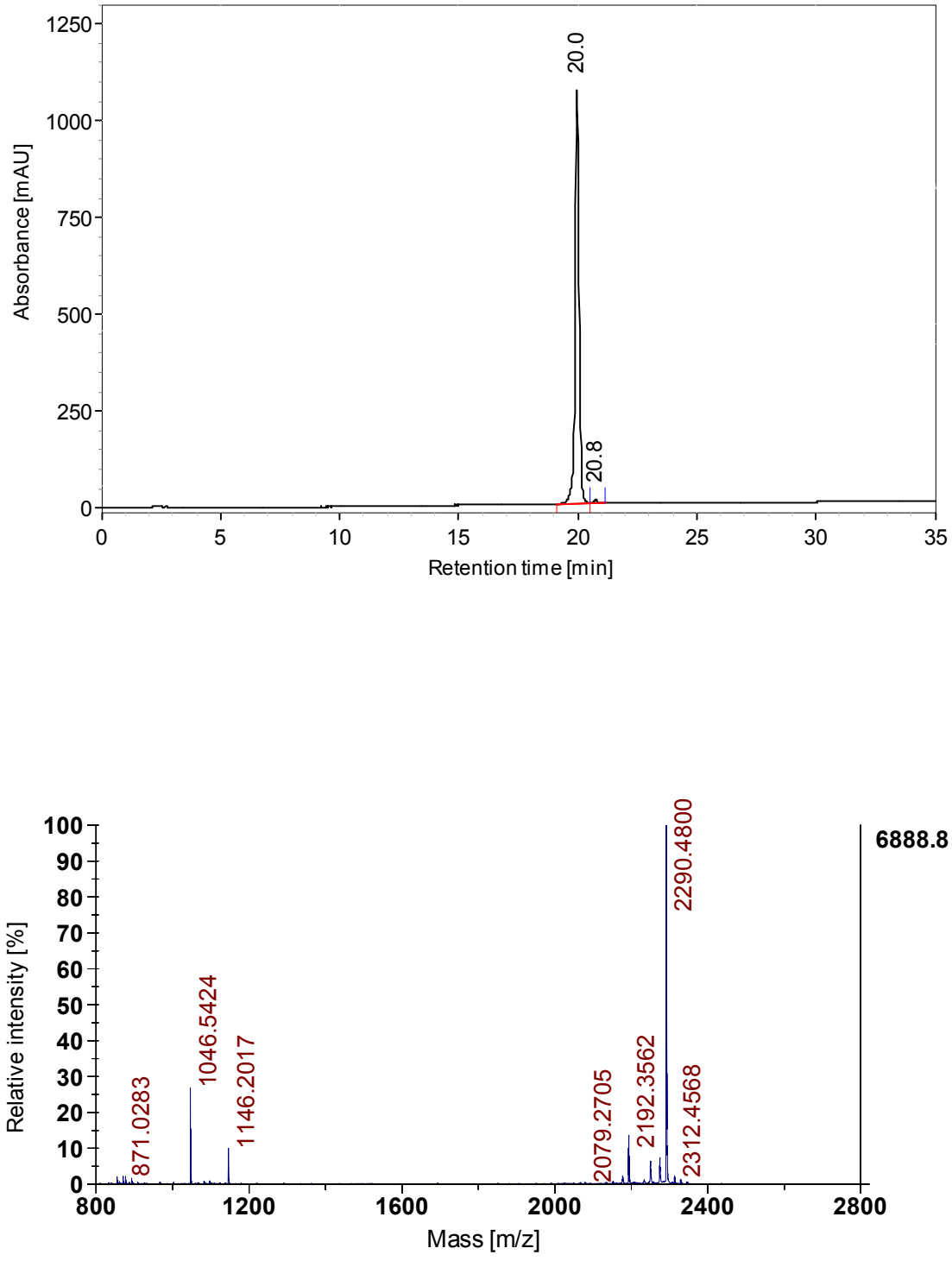

Figure S5: RP-HPLC (top) and MALDI-MS (bottom) of purified Api88.

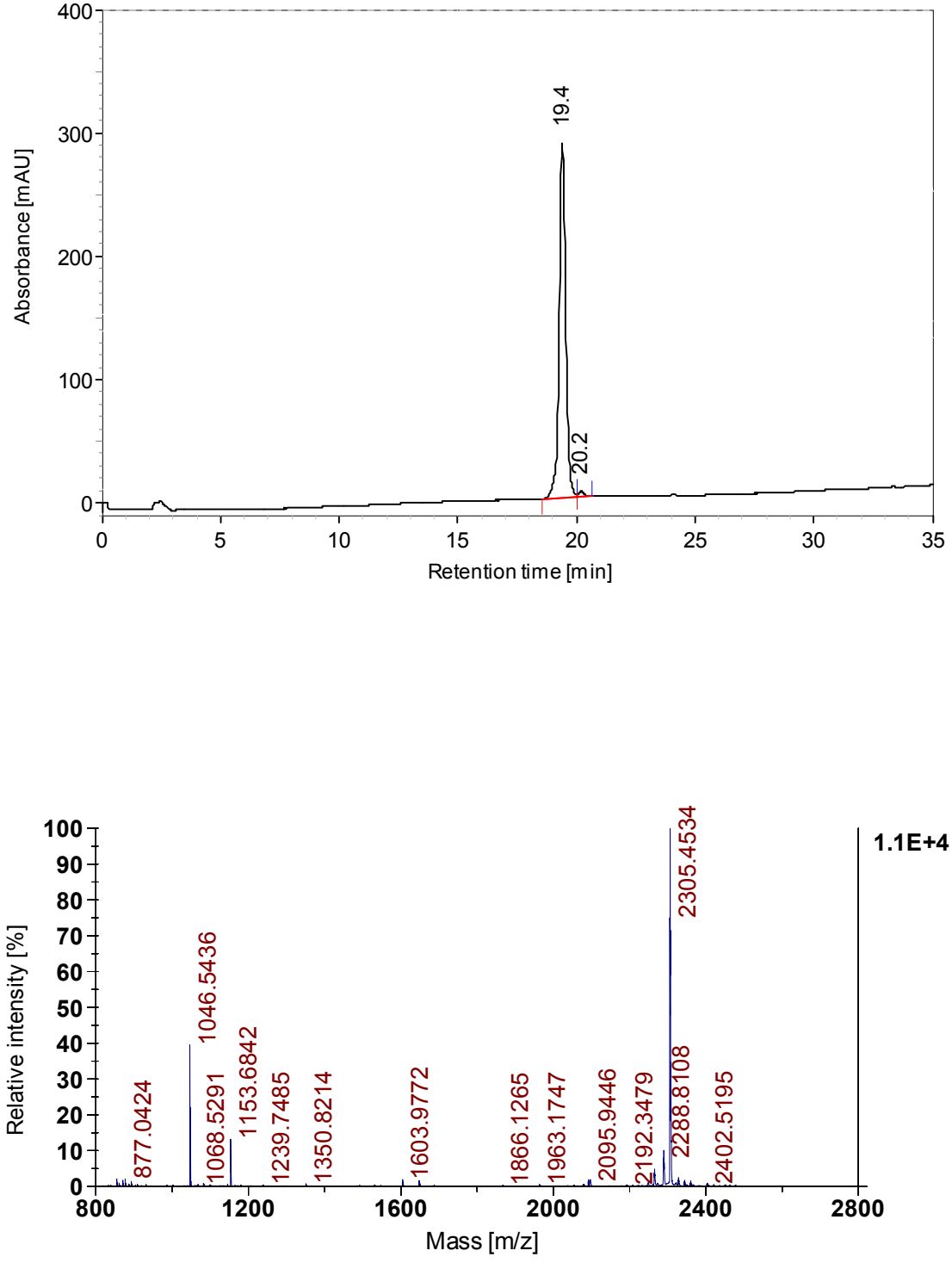

Figure S6: RP-HPLC (top) and MALDI-MS (bottom) of purified Onc72.

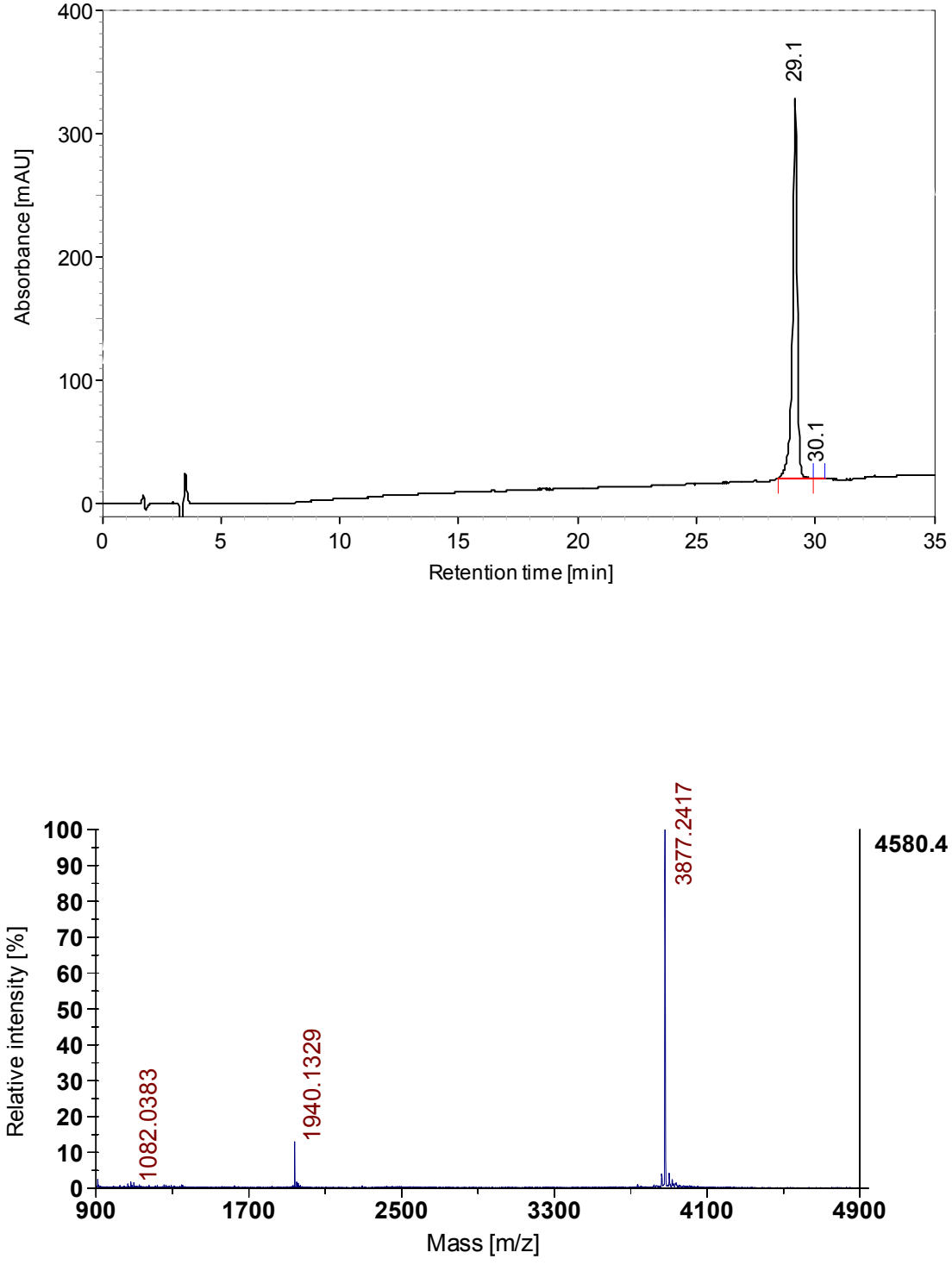

Figure S7: RP-HPLC (top) and MALDI-MS (bottom) of purified CRAMP.

4 FINAL COMMENTS FOR DISCUSSION

In recent years, immensely increasing numbers of single or multiple antibiotic-resistant bacteria (which have more than three resistances against antibiotics [181]) occurred. This development impedes the treatment of microbial infections and is the reason for serious complications up to increased mortality rates, especially for infected elder or severely ill patients, and patients with immune deficiencies [181,182]. Especially nosocomial infections, which are acquired in hospitals or care units, are progressively becoming a problem. For instance, in France, 3,500 death cases per year were estimated due to nosocomial infections [183].

There are several groups of conventional antibiotics known, which exhibit different mechanisms of antibacterial action. B-lactams such as penicillins, cephalosporins or carbapenems interfere with the bacterial cell wall synthesis, whereas sulfonamides inhibit metabolic processes of the bacteria [181,184]. Other modes of antibacterial action displayed by certain conventional antibiotics include perturbation of the bacterial membrane (e.g. polymyxins) as well as interference with bacterial protein (e.g. tetracyclines, macrolides) or nucleic acid synthesis (rifampicin, fluoroquinolones) [181,184,185]. In the year 2011, penicillins were reported to be the most prevalent prescribed class of antibiotics in Europe. In Germany, they made up 28.7% of all prescribed conventional antibiotics [186]. Application of conventional antibiotics leads to the development of bacterial exit strategies and selection of antibiotic-resistant bacteria. The main reasons for dramatically increasing numbers of bacterial resistances against conventional antibiotics are short-term medication of bacterial infections or treatment with inadequate antibiotics (e.g. an antibiotic, for which the pathogen is not susceptible) [182]. Interestingly, prolonged application of certain antibacterial drugs was also reported to induce low-level resistances [181,187]. There is a large difference in the use of antibiotics within several European countries: While in 2009 in Latvia per day approximately 1.05 % of the patients were treated ambulatory with antibiotics, in Greece in the same year the number was up to 4 times higher (3.5 – 4 % of the patients) [186]. In Germany, in 2009 approximately 1.5-2.0 % of the patients were treated ambulatory with antibiotics [186].

Bacteria may obtain resistance mechanisms against conventional antibiotics by mutation or by gaining resistance genes from other bacteria, facilitated by plasmids [181]. Known bacterial resistance mechanisms include expression of efflux pumps for the antibacterial drug, expression of enzymes that catabolize the conventional antibiotic, alteration of antibiotic target molecules, or metabolic processes [181,188]. Highly prevalent antibiotic-resistant bacteria are for instance extended-spectrum β-lactamase-(ESBL) producing *E. coli*, which are able to hydrolyze and inactivate β-lactams, or methicillin-resistant *S. aureus* (MRSA), which express a modified penicillin-binding protein (PBP2a) [181,189,190].

Besides taking necessary precautions and hygiene measures, treatment of bacterial infections with suitable and correctly dosed antibiotics for the appropriate duration are of highest importance. Since increasing numbers of bacteria strains emerge which are resistant to nearly all conventional antibiotics (e.g. New Delhi metallo-beta-lactamase 1- (NDM-1) expressing bacteria), new strategies for the control of microbial infections need to be elucidated [47].

Numerous AMPs are known to protect their hosts from a broad spectrum of Gram-negative and Gram-positive bacteria, fungi, viruses, parasites, or even cancer cells [47]. Therefore, the diverse group of AMPs provides several promising candidates for pharmaceutical investigation which exert different modes of action and thus can be developed as a new class of antibiotics to treat microbial infections. According to Li *et al.*, medication of bacterial infections with AMPs is very promising due to the more global antimicrobial mechanism used by AMPs (e.g. membrane perturbation) compared to conventional antibiotics [47]. However, a possible induction of bacterial resistances against AMPs needs to be considered and may be limited, e.g. by appropriate modification of the peptide.

Some AMPs, such as pyrrhocoricin or the optimized pyrrhocoricin-derivative A3-APO, were shown to be very protective in various *in vivo* bacteraemia models [51,94-96]. In addition to their antibacterial activity, the PrAMP derivatives oncocin, Onc72 and Api88, as well as peptides with their corresponding reverse amino acid sequence, exert antifungal effects against the human pathogen *Cryptococcus neoformans* var. *neoformans* (strain 1841, serotype D) in an *in vitro* antifungal study (unpublished data). These peptides possess a net positive charge larger than +6. In contrast to that, apidaecin 1b and apidaecin 1b reverse, both possessing a net positive charge of +4, did not influence fungal growth (unpublished data). This result indicates that the antifungally active peptides act via a structure- and sequence-

independent mechanism, which may depend on peptide characteristics such as their charge. Thus, in further experiments, the antifungal assay was performed using the peptides Onc72 all-*D* and Api88 all-*D* consisting of *D*-amino acids instead of *L*-amino acids. *D*-amino acids possess the same charge as *L*-amino acids, but the isoforms differ in their structure. Therefore, peptides comprising *D*-amino acids show reciprocal chiral structures [191] to peptides containing *L*-amino acids, whereas both do not differ in their net charge. Similarly to the antifungally active *L*-peptides (see above), incubation of the fungus with Onc72 all-*D* or Api88 all-*D* dose-dependently reduced survival of fungi demonstrated by the decreased amount of cfu on agar plates. Therefore, the antifungal effect of Api88 and Onc72 seems to rely rather on charge-specific effects than on structural aspects.

As mentioned above, various AMPs were shown to exert anti-parasitic (e.g. against *Plasmodium falciparum*) [46] or antiviral activities, e.g. against adenoviruses, influenza virus, SARS, vaccinia virus, and others [41,43-45,192]. While the cathelicidins LL-37 and CRAMP showed a strong antiviral effect against the enveloped vaccinia virus, the human β-defensins hBD-1 and hBD-2 did not alter the amount of plaque forming units (pfu) or vaccinia mRNA expression in the *in vitro* assays performed by Howell *et al.* [43]. In contrast to that, the authors demonstrated in another study that human β-defensin hBD-3 exerts an antiviral activity against vaccinia virus [44]. Interestingly, hBD-2 was shown to inhibit another enveloped, respiratory syncytial virus (RSV), by disruption of the viral envelope and inhibition of viral entry [193,194].

In addition to an anti-infective application, some AMPs were also tested by several authors as anti-cancer treatment [47]. Soman *et al.* demonstrated in an *in vivo* study that the membrane-active insect AMP melittin kills tumor cells and reduces tumor growth [195]. To enable specific delivery of melittin to the tumor, the peptide was incorporated into the outer lipid monolayer of a molecularly targeted nanoparticle [195]. With this approach, the authors avoided the negative effects of nonspecific cytotoxicity and degradation.

In this study, Onc72 as well as the optimized apidaecin derivative Api88 were shown to be strongly antibacterially active without inducing any toxic side effects *in vivo* [90]. Even when the peptide was injected at high doses such as 20 mg/kg or 40 mg/kg, tissue sections of all analyzed organs displayed a regular anatomical structure without indicating inflammation, internal bleedings or toxicity at day five post infection. As PrAMPs such as Onc72 specifically bind and inhibit the intracellular bacterial chaperone DnaK [86,87,196], the

occurrence of pathological effects against eukaryotic cells is unlikely [86]. In contrast to the data obtained for Onc72 and Api88 [90], Szabo *et al.* reported that a single intraperitoneal (i.p.) injection of 50 mg/kg of the PrAMP A3-APO was lethal for all mice. Even after reducing the dose to a single injection of 25 mg/kg as well as three injections of 40 mg/kg A3-APO, severe transient side effects such as internal bleedings occurred [96].

Onc72 and Api88, as well as apidaecin 1b and oncocin did not modulate or stimulate immune responses by BMDC, BMDM, splenocytes and PEC *in vitro*. Furthermore, the data in this study and shown by Czihal *et al.* [90] suggest a direct and potent antimicrobial activity by Onc72 and Api88 without any toxic or other negative side effects *in vivo* and *in vitro*. In further studies, influences of consecutive peptide administration for a longer time period and possible toxic effects at the sites of peptide clearance (liver and kidneys) have to be elucidated in more detail using methods with a higher sensitivity. Moreover, detailed analyses regarding pharmacokinetic aspects, such as stability *in vivo*, peptide distribution and residence time either at the injection point or in the body, need to be done.

Some of these questions were addressed in a first *in vivo* imaging-study using near-infrared fluorophore-labeled Onc72. Within two hours after injection, systemic distribution of the fluorophore-labeled peptide could be observed. The peptide was distinguishable in several organs except brain. Thus, it can be assumed that the blood-brain-barrier was still intact at this time point. Czihal *et al.* demonstrated an influx of the optimized PrAMP Api88 from serum into the brain achieving almost as much as double of the brain distribution volume determined for the control peptide BSA [90]. Therefore, another explanation for the lack of Onc72-influx into the brain may be that the fluorophore-labeling hampers the peptide in overcoming the blood-brain-barrier. Since the peptide may be catabolized by serum proteases, the fluorescence-signal may be detected also from not peptide-bound fluorophore-dye. Non-labeled peptide may also have reached the brain in the *in vivo* imaging study without being detected. The fluorophore-label represents a chemical modification of the peptide and may therefore alter the peptide's characteristics. Additional studies, e.g. with a radio-labeled peptide, should provide further and more sensitive data in order to elucidate the peptide's pharmacokinetic properties *in vivo*. Nevertheless, this first *in vivo* study suggests i) how the peptide distributes, ii) that the fluorescence signal is detectable for several hours and iii) that the peptide is cleared by liver and kidney at the same rate (data not shown).

For a therapeutic use of the peptides, numerous additional important pharmacokinetic indices such as the fielding concentration and dosage, the maximum plasma concentration, the bioavailability, the elimination half-life, the dosaging interval, and the type of application (e.g. intravenous application or intramuscular application) need to be elucidated. Several other important points such as induction of bacterial resistances or allergic immune responses, the distribution to the targeted organ(s) [90], or possible toxic effects on distinct cell types should also be considered in further analyses.

Importantly, treatment of bacterial infections with insect AMPs may avoid bacterial resistances against mammalian AMPs. Insect AMPs should also not induce the host's immune mechanisms (e.g. by activation of mast cells), which may be facilitated by the usage of antibacterially active, non-immunogenic AMPs (haptens) or modification of potentially immunogenic peptides. The question, if Onc72 or Api88 induce mammalian immunity, is currently addressed by Dr. Daniel Knappe and colleagues. In this *in vivo* study, Onc72, Api88, or keyhole limpet hemocyanin-(KLH-) conjugated peptides (as positive control for the induction of an immune response) are consecutively injected and their immunogenic capacities are further analyzed. KLH was originally isolated from the marine mollusc *Megathura crenulata* (giant keyhole limpet) and is used for 30 years as a potent stimulatory protein, which activates both, cellular as well as humoral immune mechanisms [197]. It is used as hapten-carrier for small molecules which cannot induce immune responses by themselves and is therefore used e.g. for the treatment of several carcinomas [197].

Besides a direct therapeutic use of AMPs against infections or cancer, they may also be used for other aspects of protection against pathogens or biofilms, e.g. for coating of medical devices [47]. As stated above, some β-defensins were reported to be stimulatory for cells of the innate immune system [148-152]. Mei *et al.* used the murine β-defensin mBD-2 as adjuvant in an immunotherapy against melanoma, which resulted in potent NK cell and tumor-specific cytotoxic T cell (CTL) activation as well as secretion of IL-12 and IFN-γ. The specific anti-tumor response led to survival of mBD-2-treated mice due to inhibition of tumor development and progression [47,198].

In addition to their use in the health sector, cultivation of AMP-expressing transgenic plants [199] as well as application of AMPs in food additives e.g. for preservation or as unspecific immunostimulant is also considered [47]. Lactoferrin-supplementation of weanling pig's diet was used as immunostimulant in a growth study. The stimulatory effect was indicated by e.g. up-regulated phythemagglutinin- (PHA-) induced peripheral and spleen lymphocyte proliferation, increased serum IgG, IgA, IgM, IL-2 and complement 4 levels, and led to a reduced risk of diarrhea in comparison to animals of the control group [200]. Application of the iron-binding AMP lactoferrin or of bacteriocins is also used for food preservation [199,201]. Since bacteriocins, such as the lantibiotic nisin, are highly active against several pathogens which are responsible for most of the food borne illnesses, this group has gained much interest for a use as food preservatives [199].

Due to the wide range of possible indications, AMPs are of special interest for therapeutic applications. Particularly those peptides which exert multiple functions such as antimicrobial activities against several kinds of pathogens, anti-cancerous action, limitation of inflammatory diseases and / or precise tuned modulation of the host's immunity, are promising new candidates for pharmaceutical research and development. Optimization of the peptides will enhance their activities, while potential risks such as induction of microbial resistances or allergic immune responses need to be avoided. Further research on AMPs will provide a basis for their future therapeutic application in various kinds of diseases and probably, more activities exerted by the peptides will be discovered.

5 SUMMARY

Antimicrobial peptides (AMP) are a structurally diverse group of conserved components of the innate immune system, which can be found in a variety of organisms ranging from plants to amphibians, arthropods, and higher animals [37]. Besides their antibacterial activity, some AMPs were reported to have antifungal, antiviral, or antiparasitic effects [39-47].

In recent years, the number of single or multiple resistances in bacteria increased dramatically. Thus, new therapeutic compounds for antibacterial treatment are required. Considering the different modes of action used, the diverse group of AMPs shows a broad antibacterial spectrum against Gram-negative and Gram-positive bacteria strains. Short proline-rich AMPs (PrAMPs) do not require complex folding via disulfide bonds to exert their activities and can be easily synthesized on solid phase. Thus, insect PrAMPs are of special interest for further pharmaceutical investigations.

Since many wild-type AMPs exert only a weak antibacterial activity [38], a few of them were optimized concerning their stability against serum proteases as well as their antimicrobial activity [90,113,114,117]. Api88 and Onc72 are optimized derivatives from wild-type apidaecin 1b or the designer peptide oncocin, respectively. Both optimized peptides show improved antibacterial activities against several bacterial strains such as *E. coli* or *P. aeruginosa* [115,116]. Onc72 was also reported to be more stable in serum than oncocin [113,117]. Thus, optimized insect PrAMPs, such as Onc72, turn out to be promising candidates for the medication of bacterial infections.

In the present study, possible cytotoxic effects of the insect PrAMP derivatives oncocin, Onc72, apidaecin 1b, and Api88 on bone marrow-derived dendritic cells (BMDC) were evaluated *in vitro*. To elucidate the antimicrobial potential of the optimized PrAMP Onc72 under *in vivo* conditions, it had to be clarified that the peptide has no toxic effects on mice when injected intraperitoneally at high doses. Following the toxicity analysis, Onc72 was tested in an acute septicaemia model using a lethal dose of *E. coli* strain ATCC 25922 to confirm its antimicrobial activity *in vivo*. Moreover, possible immunostimulatory and immunomodulatory effects of the four insect PrAMP-derivatives oncocin, Onc72, apidaecin 1b and Api88 on murine bone marrow-derived dendritic cells (BMDC) and macrophages (BMDM) were studied and compared to the well-known immunomodulatory

peptide CRAMP. Possible immunostimulatory or immunomodulatory activities of the peptides on DC and/or macrophages may depend on the cells' interaction with other immune cell types. To elucidate, whether the peptides may stimulate or modulate immune responses by a mixture of different immune cell types, splenocytes and peritoneal exudate cells (PEC) were used for further *in vitro* studies.

The main results of the present study are:

1. **The insect PrAMP derivatives oncocin, Onc72, apidaecin 1b and Api88 are not cytotoxic for bone marrow-derived dendritic cells (BMDC).**
   Even after incubation with up to 400 µg/ml (approximately 170-190 µM) oncocin, Onc72, apidaecin 1b, or Api88, murine BMDC showed no signs of reduced viability, as assessed by flow cytometric detection of dead cells using the fixable viability dye eFluor780.

2. **Consecutive intraperitoneal injections of 20 mg/kg or 40 mg/kg of the optimized insect PrAMP Onc72 are not toxic for mice.**
   Five days after four injections of Onc72 at high doses such as 20 mg/kg or even 40 mg/kg, mice showed no clinical symptoms. Furthermore, tissue sections of all analyzed organs (spleen, liver, kidney, lung, heart, pancreas, ovary, urinary bladder and brain) displayed regular anatomical structures without any signs of inflammation, internal bleedings, or toxicity. In addition, spleens of Onc72-treated mice and control mice neither differed in weight nor showed eosinophilic or basophilic granulocyte infiltration.

3. **The optimized PrAMP Onc72 efficiently protects mice in an acute septicaemia model using *Escherichia coli* (strain ATCC 25922) in a dose-dependent manner.**
   After intraperitoneal infection with a lethal dose of *E. coli* (strain ATCC 25922), mice of the outbred strain NMRI were medicated three times i.p. with 1.25 to 20 mg/kg Onc72. While all mice of the negative control group died or had to be sacrificed within the first 24 h post infection, dose-dependent survival of the Onc72-medicated mice could be observed. The effective dose (ED_{50}), which assures a survival rate of 50 %, can be estimated at approximately 2 mg/kg.

4. **Treatment of *E. coli* (strain ATCC 25922)-infected mice with Onc72 decreases bacterial burden in body fluids and all analyzed organs.**

   Repeated application of Onc72 significantly reduced bacterial counts in blood and peritoneal lavage as well as in spleen, liver, kidney, lung and brain following each consecutive injection. In addition, Onc72 decreased organ and body fluid bacterial burden in a dose-dependent manner. Importantly, five days post infection, organs of all surviving medicated animals were sterile and exhibited regular anatomical structures, without any signs of inflammation or toxicity.

5. ***In vivo* imaging studies using fluorophore-labeled Onc72 showed that the fluorescence signal can be detected in mice for a few hours post injection.**

   Fluorescence data indicated that considerable levels of Onc72 are distributed systemically (excluding brain). Furthermore, the *in vivo* imaging experiments confirmed that kidney and liver are responsible for peptide clearance at the same rate (data not shown).

6. **Oncocin, Onc72, apidaecin 1b and Api88 do not activate BMDC, BMDM, splenocytes, and PEC.**

   Stimulation with up to 400 µg/ml of the PrAMP derivatives had no effect on the expression of inflammatory cytokines (IL-12p40, IL-6, TNF-α) and / or the proportion of activation-marker positive cells among BMDC, BMDM, PEC, or splenocytes compared to medium-incubated cells.

7. **The four insect PrAMP derivatives as well as CRAMP are not chemotactic for immature BMDC.**

   While the positive controls MCP-1 and MIP-1α proved to be chemotacticly active as published, the four PrAMPs and CRAMP were not chemotactic for immature BMDC in a concentration range from 1 ng/ml to 1 µg/ml.

8. **None of the PrAMPs oncocin, Onc72, apidaecin 1b and Api88 modulates the LPS-induced immune responses by BMDC, BMDM, PEC, and splenocytes.**
   In contrast to CRAMP, none of the PrAMP derivatives modulated the LPS-induced immune response (inflammatory cytokine secretion and / or surface activation marker expression) of BMDC, BMDM, or splenocytes. Furthermore, the LPS-induced IL-6 response of PEC was not influenced by the four PrAMPs oncocin, Onc72, apidaecin 1b, and Api88.

Together, the present study shows that the optimized proline-rich AMP Onc72 exerted a strong direct antibacterial activity in an acute septicaemia model without any toxic side effects. The effective elimination of the pathogen seems not to depend on additional immunomodulatory or immunostimulatory effects of the peptide, since the PrAMP derivatives oncocin, Onc72, apidaecin 1b and Api88 did not influence central cells of the innate immune system. These results may promote further analyses and development of proline-rich insect AMPs as treatment against microbial infections.

6 ZUSAMMENFASSUNG

Antimikrobielle Peptide (AMPs) sind wichtige Komponenten des angeborenen Immunsystems. Die strukturell sehr unterschiedlichen Peptide sind in einer Vielzahl verschiedener Organismen, wie z.B. Pflanzen aber auch in Amphibien, Arthropoden und in evolutionär hoch entwickelten Tieren, zu finden [37]. Primär erfolgt die Zuordnung von Peptiden zur Gruppe der AMPs anhand ihrer gemeinsamen antimikrobiellen Aktivität insbesondere gegen Bakterien. Daneben wurden für einige AMPs auch antifungale, antivirale oder antiparasitäre Wirkungen beschrieben [39-47].

Durch die steigende Anzahl einzel- oder multiresistenter Bakterien sind neue Ansätze für die Behandlung mikrobieller Infektionen notwendig. Aufgrund der verschiedenen Wirkweisen einzelner Peptide zeigt die vielseitige Gruppe der AMPs ein breites antibakterielles Spektrum gegen Gram-negative wie auch Gram-positive Bakterienstämme. Kleine Prolin-reiche AMPs (PrAMPs) weisen keine komplexen, durch Disulfidbrücken gebildeten Tertiärstrukturen auf und können somit relativ einfach in großen Mengen mittels Festphasensynthese hergestellt werden. Aus diesen Gründen sind PrAMPs für eine weitergehende pharmazeutische Entwicklung von besonderem Interesse.

Da viele Wildtyp-AMPs keine sehr stark ausgeprägte antibakterielle Wirkung aufweisen [38], wurden einige natürlich vorkommende Peptide hinsichtlich ihrer Serumstabilität und antibakteriellen Aktivität gegen verschiedene Bakterienstämme optimiert [90,113,114,117]. Beispielsweise zeigen die PrAMPs Api88 und Onc72, welche optimierte Derivate des Wildtyp-Apidaecin 1b und des Oncocins darstellen, eine erhöhte antibakterielle Aktivität gegen z.B. *Escherichia coli* oder *Pseudomonas aeruginosa* [115,116]. Darüber hinaus ist das optimierte Peptid Onc72 stabiler gegenüber Serumproteasen als Oncocin [113,117]. Zusammenfassend betrachtet, sind optimierte Insekten-PrAMPs, wie Onc72, vielversprechende Kandidaten für die Entwicklung neuer Behandlungsmöglichkeiten mikrobieller Infektionen.

In der vorliegenden Arbeit wurden *in vitro* zunächst mögliche zytotoxische Effekte der Insekten-PrAMP-Derivate Oncocin, Onc72, Apidaecin 1b und Api88 auf murine, aus Knochenmarkszellen generierte, Dendritische Zellen (BMDC) untersucht. Um später die antimikrobielle Wirksamkeit des optimierten Peptids Onc72 *in vivo* analysieren zu können,

sollte zunächst bestätigt werden, dass die intraperitoneale Applikation einer hohen Dosis des Peptids keine toxischen Effekte auf die Mäuse hat. Im Anschluss an die Toxizitäts-Untersuchungen wurde Onc72 in einem akuten Septikämiemodell eingesetzt, bei dem den Mäusen zuvor eine letale Dosis *Escherichia coli* ATCC 25922 intraperitoneal appliziert wurde. Des Weiteren wurden mögliche immunstimulierende und immunmodulierende Effekte der PrAMP-Derivate Oncocin, Onc72, Apidaecin 1b und Api88 auf aus Knochenmark generierten Dendritischen Zellen (BMDC) und Makrophagen (BMDM) analysiert und mit der Wirkung des bekannten immunmodulierenden Peptids CRAMP verglichen. Um zu klären, ob die Peptide möglicherweise einen stimulierenden oder modulierenden Einfluss auf aus verschiedenen Immunzelltypen bestehende Zellverbände haben, wurden Milzzellen und Peritoneal Exsudat Zellen (PEC) für weitere Untersuchungen genutzt.

Zusammenfassung der Ergebnisse:

1. **Die Insekten-PrAMP-Derivate Oncocin, Onc72, Apidaecin 1b und Api88 sind nicht zytotoxisch für BMDC.**
   Nach Inkubation mit bis zu 400 µg/ml (ca. 170-190 µM) der vier PrAMP-Derivate war keine zytotoxische Wirkung der Peptide auf BMDC festzustellen. Der Anteil toter Zellen wurde mittels durchflusszytometrischer Analyse unter Nutzung des fixierbaren Lebend-Tot-Farbstoffes eFluor780 bestimmt.

2. **Mehrfache intraperitoneale Injektionen von 20 mg/kg oder 40 mg/kg des optimierten Peptids Onc72 sind nicht toxisch für Mäuse.**
   Fünf Tage nachdem den Tieren in vier aufeinanderfolgenden Injektionen jeweils 20 mg/kg oder 40 mg/kg des Peptids appliziert wurden, zeigten die Mäuse keine klinischen Auffälligkeiten. Ebenso wiesen die Organschnitte von Milz, Leber, Niere, Lunge, Herz, Bauchspeicheldrüse, Ovarien, Blase und Gehirn einen regulären anatomischen Aufbau, ohne Anzeichen für Inflammation, innere Blutungen oder toxische Schäden, auf. Darüber hinaus war keine signifikante Differenz im Milzgewicht von Onc72-behandelten Tieren und Kontrolltieren feststellbar. Auch war keine Infiltration von basophilen oder eosinophilen Granulozyten in die Milz von Peptid-behandelten Tieren und Kontrolltieren zu erkennen.

3. **Das optimierte PrAMP Onc72 schützt Mäuse dosisabhängig in einem akuten *Escherichia coli* - Septikämie Modell.**

   Mäuse des Auszuchtstammes NMRI wurden intraperitoneal mit einer letalen Dosis *E. coli* (Stamm ATCC 25922) infiziert und anschließend mittels drei ebenfalls intraperitonealer Injektionen von 1,25 bis 20 mg/kg Onc72 behandelt. Während alle Tiere der Negativ-Kontrollgruppe innerhalb von 24 Stunden starben oder euthanasiert werden mussten, überlebten die Tiere der Onc72-Gruppen abhängig von der Dosis des eingesetzten Peptids. Somit kann die effektive Dosis (ED_{50}), welche eine Überlebensrate von 50 % der Tiere garantiert, auf ca. 2 mg/kg eingeschätzt werden.

4. **Die Behandlung von mit *E. coli* (Stamm ATCC 25922) infizierten Mäusen mit Onc72 sorgt für eine Erregereliminierung im Blut, in der Peritoneal-Lavage sowie in allen analysierten Organen.**

   Nach jeder Applikation von Onc72 reduzierte sich deutlich die Anzahl nachzuweisender Bakterien im Blut und in der Peritoneal-Lavage sowie in Milz, Leber, Niere, Lunge und Gehirn. Diese Abnahme war ebenfalls Peptid-dosisabhängig. Fünf Tage nach der Infektion und Medikation waren die analysierten Organe aller überlebenden behandelten Tiere steril und wiesen einen regulären anatomischen Aufbau ohne Anzeichen für eine Entzündung oder toxische Schäden auf.

5. **Bei *in vivo*-Imaging-Analysen von Mäusen, die mit Fluorophor-markiertem Onc72 behandelt wurden, konnte das Fluoreszenzsignal für mehrere Stunden nach Injektion im Organismus detektiert werden.**

   Aufgrund der Fluoreszenzdaten kann angenommen werden, dass signifikante systemische Peptid-Konzentrationen (außer im Gehirn) von Onc72 erreicht werden. Weiterhin konnten mittels *in vivo*-Imaging gleichermaßen Niere und Leber als Orte des Peptidabbaus und der Peptidausscheidung nachgewiesen werden (Daten nicht gezeigt).

6. **Oncocin, Onc72, Apidaecin 1b und Api88 aktivieren BMDC, BMDM, Milzzellen und PEC nicht.**
   Im Vergleich zu Medium-inkubierten Zellen zeigten BMDC, BMDM, PEC und Milzzellen nach Stimulation mit bis zu 400 µg/ml der PrAMP-Derivate keine erhöhte Expression pro-inflammatorischer Zytokine (IL-12p40, IL-6, TNF-α) und / oder keine erhöhte Anzahl von Aktivierungsmarker-positiven Zellen.

7. **Die vier Insekten-PrAMP-Derivate und CRAMP sind nicht chemotaktisch für unreife BMDC.**
   Während die Positivkontrollen MCP-1 und MIP-1α wie erwartet chemotaktisch auf BMDC wirkten, übten die vier PrAMPs und CRAMP in einem Konzentrationsbereich von 1 ng/ml bis 1 µg/ml keinerlei chemotaktische Wirkung auf BMDC aus.

8. **Oncocin, Onc72, Apidaecin 1b und Api88 modulieren die durch LPS-Stimulation induzierte Immunreaktion von BMDC, BMDM, PEC und Milzzellen nicht.**
   Im Gegensatz zu CRAMP modulierte keines der vier PrAMP-Derivate die durch LPS-Stimulation induzierte Immunantwort (Zytokinsekretion und / oder Expression von Aktivierungsmarkern) von BMDC, BMDM oder Milzzellen. Daneben wurde auch die durch LPS-Stimulation induzierte IL-6 Sekretion durch die vier PrAMPs Oncocin, Onc72, Apidaecin 1b und Api88 nicht beeinflusst.

Zusammengefasst zeigt die vorliegende Arbeit, dass das optimierte PrAMP Onc72 eine starke protektive Wirkung in einem akuten Septikämie-Modell ausübte, ohne dass toxische Nebenwirkungen feststellbar waren. Das Ausbleiben immunstimulierender oder immunmodulierender Wirkungen der PrAMP-Derivate Oncocin, Onc72, Apidaecin 1b und Api88 auf wichtige Zellen des angeborenen Immunsystems deutet darauf hin, dass die Effektivität des Peptids im murinen Infektionsmodell ausschließlich auf einer direkten antibakteriellen Wirkung beruht. Diese Ergebnisse vereinfachen weitergehende pharmazeutische Untersuchungen und die Entwicklung von Insekten-Peptiden als Therapeutika gegen bakterielle Infektionen.

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NACHWEIS ÜBER ANTEILE DER CO-AUTOREN

Characterization of antimicrobial and immunomodulatory activities displayed by short proline-rich antimicrobial peptides *in vitro* and *in vivo*

Nachweis über Anteile der Co-Autoren:

Titel:	**Oncocin derivative Onc72 is highly active against *Escherichia coli* in a systemic septicaemia infection mouse model**
Journal:	Journal of Antimicrobial Chemotherapy
Autoren:	Daniel Knappe*, Stefanie Fritsche*, Gottfried Alber, Gabriele Köhler, Ralf Hoffmann, Uwe Müller

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Anteil Dr. Daniel Knappe (gemeinsame Erstautorenschaft mit Stefanie Fritsche):

- Optimierung und Peptidsynthese von Onc72
- Etablierung des Septikämie-Modells gemeinsam mit Dr. U. Müller
- Analysen im Rahmen des Septikämie-Modells (z.B. Keimzahlbestimmungen, Organpräparation etc.)
- Analyse und Verarbeitung von Daten
- Schreiben der Publikation

Anteil Stefanie Fritsche (gemeinsame Erstautorenschaft mit Dr. Daniel Knappe):

- Durchführung der Toxizitätsstudie sowie Beteiligung am Septikämie-Modell (z.B. Blutentnahme, Organpräparation, Gewinnung der Peritoneal-Exsudat-Zellen mittels Peritoneal-Lavage, durchflusszytometrische Bestimmung immunologisch relevanter Marker etc.)
- Analyse und Verarbeitung von Daten
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Anteil Prof. Dr. Gottfried Alber:

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Anteil Prof. Dr. Gabriele Köhler:

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Nachweis über Anteile der Co-Autoren:

Titel: **Absence of *in vitro* innate immunomodulation by insect-derived short proline-rich antimicrobial peptides points to direct antibacterial action *in vivo***

Journal: Journal of Peptide Science

Autoren: Stefanie Fritsche*, Daniel Knappe, Nicole Berthold, Heiner von Buttlar, Ralf Hoffmann, Gottfried Alber

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Anteil Stefanie Fritsche (Erstautorin):

- Konzeption des Projektes
- Zellkulturen
- Präparation von Milz und Knochenmark sowie Gewinnung der Peritoneal-Exsudat-Zellen (PEC) mittels Peritoneal-Lavage; Herstellen von PEC und Splenozyten-Einzelzellsuspensionen
- Generierung von Dendritischen Zellen und Makrophagen aus Knochenmarks-Stammzellen
- Durchführung aller Zellstimulationen und nachfolgende durchflusszytometrische Messungen sowie Zytokinbestimmungen in den Zellüberständen mittels ELISA
- Etablierung der Bedingungen für Chemotaxis-Analysen sowie Durchführung und Auswertung der Chemotaxis-Experimente
- Analyse und Verarbeitung aller Daten
- Schreiben der Publikation

Anteil Dr. Daniel Knappe:

- Optimierung von Oncocin und Onc72
- Peptidsynthese von Oncocin, Onc72 und CRAMP
- beteiligt am Schreiben der Publikation

Anteil Nicole Berthold:

- Peptidsynthese von Apidaecin 1b und Api88 (optimierte Sequenz von Api88 beruht auf den Vorarbeiten von Dr. Patricia Czihal)
- Bestimmung der minimalen inhibitorischen Konzentration (MIC) verschiedener Peptidderivate

Anteil Dr. Heiner von Buttlar:

- Knochenmarkspräparation
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Anteil Prof. Dr. Ralf Hoffmann:

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Anteil Prof. Dr. Gottfried Alber:

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Hiermit versichere ich, die vorliegende Dissertation, bis auf die genannten Beiträge der Co-Autoren, selbstständig ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt zu haben. Dabei wurden keine unzulässigen oder andere als die angegebenen Hilfsmittel in Anspruch genommen. Direkt oder indirekt aus fremden Quellen übernommene Informationen wurden als solche kenntlich gemacht.

Bei der geistigen Herstellung der Arbeit wurde kein Promotionsberater in Anspruch genommen. Auch wurden vom Antragsteller keine unmittelbar oder mittelbar geldwerten Leistungen an Dritte vergeben, um im Gegenzug Leistungen zu erhalten, welche mit dem Inhalt der vorgelegten Dissertation in Verbindung stehen.

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Leipzig, den 6. September 2012

Stefanie Fritsche

PUBLIKATIONSLISTE

PUBLIKATIONEN

1. Knappe, D.*, S. Fritsche*, G. Alber, G. Koehler, R. Hoffmann, and U. Mueller. 2012. Oncocin derivative Onc72 is highly active against *Escherichia coli* in a systemic septicaemia infection mouse model. *J. Antimicrob. Chemother.* in press DOI: 10.1093/jac/dks241

2. Fritsche, S.*, D. Knappe, N. Berthold, H. von Buttlar, R. Hoffmann, and G. Alber. 2012. Absence of *in vitro* innate immunomodulation by insect-derived short proline-rich antimicrobial peptides points to direct antibacterial action *in vivo*. *J. Pept. Sci.* in press

3. Czihal, P.*, D. Knappe, S. Fritsche, N. Berthold, S. Piantavigna, U. Mueller, S. van Dorpe, N. Herth, A. Binas, G. Koehler, B. de Spiegeleer, L. L. Martin, O. Nolte, N. Strater, G. Alber, and R. Hoffmann. 2012. Api88 is a novel antibacterial designer peptide to treat systemic infections with multidrug-resistant Gram-negative pathogens. *ACS Chem. Biol.* in press
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4. Czihal, P.*, N. Berthold*, S. Fritsche, D. Knappe, U. Sauer, G. Schiffer, G. Alber, and R. Hoffmann. 2012. Second generation apidaecin 1b analogs with superior serum stabilities to treat Gram-negative pathogens. in preparation

POSTER

1. Fritsche, S.*, D. Knappe., H. von Buttlar, N. Berthold, R. Hoffmann, and G. Alber. 2011. Optimized derivatives of invertebrate antimicrobial peptides show broad antimicrobial activity without immunomodulatory side effects on dendritic cells. 2011 Joint annual meeting of the Italian Society of Immunology, Clinical Immunology and Allergology (SIICA) and the German Society for Immunology (DGfI), Riccione, Italy

2. Fritsche, S.*, D. Knappe., H. von Buttlar, N. Berthold, R. Hoffmann, and G. Alber. 2011. Optimized derivatives of invertebrate antimicrobial peptides show broad antimicrobial activity without immunomodulatory side effects on dendritic cells. 10th Research Festival for Life Sciences 2011, Leipzig, Germany

3. Fritsche, S.*, D. Knappe., H. von Buttlar, N. Berthold, R. Hoffmann, and G. Alber. 2012. Proline-rich insect antimicrobial peptides display potent antibacterial activity *in vivo* without being immunomodulatory for innate immune cells *in vitro*. Saxon Biotechnology Symposium 2012, Leipzig, Germany

4. Fritsche, S.*, D. Knappe., H. von Buttlar, N. Berthold, R. Hoffmann, and G. Alber. 2012. Proline-rich insect antimicrobial peptides display potent antibacterial activity *in vivo* without being immunomodulatory for innate immune cells *in vitro*. International Meeting on Antimicrobial Peptides 2012, Leipzig, Germany

5. Knappe, D.*, U. Müller, M. Zahn, S. Fritsche, G. Alber, N. Sträter, and R. Hoffmann. Oncocin is highly active against *E. coli* in a mouse model of peritoneal sepsis and binds to the conventional substrate binding site of chaperone DnaK. Modern solid phase peptide synthesis and its applications symposium 2011, Mackay, Australien

6. Knappe, D.*, U. Müller, M. Zahn, S. Fritsche, G. Alber, N. Sträter, and R. Hoffmann. *In vivo* antibacterial activity of oncocin derivative Onc72 against *Escherichia coli* in a systemic septicaemia infection mouse model. 10th Research Festival for Life Sciences 2011, Leipzig, Germany

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* Erstautoren

VORTRÄGE

1. Gemeinsames Kolloquium des Instituts für Immunologie (Veterinärmedizinische Fakultät, Universität Leipzig) und des Institute of Immunology (Friedrich-Schiller-Universität Jena), 2009, Leipzig
   Beitrag: „Effects of new antimicrobial peptides on dendritic cells (DCs)“

2. Doktorandenkolloquium des BBZ 2011, Leipzig
   Beitrag: „Untersuchung der immunmodulatorischen Aktivität von antimikrobiellen Peptiden“

3. Doktorandenkolloquium des BBZ 2012, Leipzig
   Beitrag: „Untersuchung der immunmodulatorischen Wirkung Prolin-reicher antimikrobieller Peptide *in vitro* und *in vivo*“