Antimicrobial Peptides

Edited by
William M. Shafer

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William M. Shafer (Ed.)

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About the Guest Editor

William M. Shafer received the Ph.D. degree in 1979 from Kansas State University and then performed post-doctoral studies at the University of North Carolina-Chapel Hill. In 1982 he moved to Emory University School of Medicine (Atlanta, Georgia USA) where he is now Professor of Microbiology and Immunology. He is also a Senior Research Career Scientist Award in the Medical Research Service of the Department of Veterans Affairs (VA). His laboratory is engaged in research dealing with the mechanisms used by the sexually transmitted pathogen *Neisseria gonorrhoeae* to develop resistance to antibiotics and host-derived cationic antimicrobial peptides (CAMPs). The gonococcus causes over 100 million cases of gonorrhea worldwide each year and many strains causing disease are now resistant to multiple antibiotics. His group was the first to show that a bacterial drug efflux pump that recognizes antibiotics can also export host-derived CAMPs. Professor Shafer has been funded by the NIH and VA for over 30 years for studies dealing with mechanisms of bacterial pathogenesis and antimicrobial resistance. He has published over 150 manuscripts, book chapters and reviews many of which report his findings on mechanisms of bacterial resistance to CAMPs.

Preface

Antimicrobial peptides (AMPs) are gene-encoded, ancient (and important) mediators of innate host defense that exert direct or indirect antimicrobial action as well as possessing other important biologic activities (e.g., neutralization of endotoxin and anti-biofilm action) that help to protect vertebrates, invertebrates and plants from invading pathogens. While the emergence of multi-antibiotic resistant pathogens (and the desperate need to develop new anti-infectives) has been a recent force driving the field, interest in AMPs has an earlier origin in studies of how phagocytes kill bacteria by oxygen-independent processes. AMPs responsible for such killing of microbes by rabbit and human neutrophils were later purified by Ganz, Selsted and Lehrer, which they termed defensins; at the time of this writing, literally thousands of defensin-based publications can be found in the scientific literature! The initial reports on defensins and the earlier report by Boman's group on the purification and action of an insect AMP represented a historical and defining point for the AMP field as they, in hindsight, demanded the recognition of AMP research as a unique discipline that has important linkages to other important fields of medicine, especially those of microbiology, infectious diseases and immunology. On a personal note, I remember conferences on phagocytes and host defense in the early 1980s where the topic of AMPs was relegated to one short session in a five day period! Now, we have hundreds of international "AMPologists" with expertise in chemistry, biochemistry, molecular and structural biology, cell biology, microbiology, pharmacology, or medicine who have built their research careers around AMPs and can now attend international conferences dedicated to advances in AMP research.

In the summer of 2014 I was invited by the Editorial Board of *Antibiotics* to organize a special edition for the Journal dedicated to AMPs. I was excited by this invitation because it re-affirmed to me that others recognize that studies on AMPs are in many respects similar to those classical antibiotics. I was allowed considerable freedom in selecting topics and soliciting authors. I was impressed by the favorable response by those contacted; their prompt submission of manuscripts was greatly appreciated! Some papers represent a review of the literature for a given topic while others report results from unpublished studies. The reader can be assured that all papers appearing in this volume were subjected to rigorous peer-review and were accepted only after corresponding authors responded to critiques provided by two-three reviewers and when they modified their manuscripts accordingly.

From the onset, it was not my intent to have manuscripts that cover the entire AMP field. Rather, I selected topics that I envisioned would provide a welcomed learning experience for those new to the AMP field as well as providing a stimulus for established "AMPologists" to consider new areas for their research program; I apologize in advance to my colleagues if your domain of AMP research is not covered herein. Thus, this volume offers to the reader fifteen manuscripts dealing with topical issues of AMP research: identification of novel AMPs, host defense, diverse biologic activities,

mechanisms of killing target bacteria, bacterial resistance (after all, AMPs are by definition antibiotics!), AMP structure-function relationships and modulation of their production by clinically used antibiotics and other compounds (e.g., HDAC inhibitors).

In conclusion, I thank all of the authors for their contribution and the help of the *Antibiotics* production staff for their unwavering help and support in bringing this volume to fruition.

William M. Shafer
Guest Editor

Host Antimicrobial Peptides in Bacterial Homeostasis and Pathogenesis of Disease

Derek R. Heimlich, Alistair Harrison and Kevin M. Mason

Abstract: Innate immune responses function as a first line of host defense against the development of bacterial infection, and in some cases to preserve the sterility of privileged sites in the human host. Bacteria that enter these sites must counter host responses for colonization. From the host's perspective, the innate immune system works expeditiously to minimize the bacterial threat before colonization and subsequent dysbiosis. The multifactorial nature of disease further challenges predictions of how each independent variable influences bacterial pathogenesis. From bacterial colonization to infection and through disease, the microenvironments of the host are in constant flux as bacterial and host factors contribute to changes at the host-pathogen interface, with the host attempting to eradicate bacteria and the bacteria fighting to maintain residency. A key component of this innate host response towards bacterial infection is the production of antimicrobial peptides (AMPs). As an early component of the host response, AMPs modulate bacterial load and prevent establishment of infection. Under quiescent conditions, some AMPs are constitutively expressed by the epithelium. Bacterial infection can subsequently induce production of other AMPs in an effort to maintain sterility, or to restrict colonization. As demonstrated in various studies, the absence of a single AMP can influence pathogenesis, highlighting the importance of AMP concentration in maintaining homeostasis. Yet, AMPs can increase bacterial virulence through the co-opting of the peptides or alteration of bacterial virulence gene expression. Further, bacterial factors used to subvert AMPs can modify host microenvironments and alter colonization of the residential flora that principally maintain homeostasis. Thus, the dynamic interplay between host defense peptides and bacterial factors produced to quell peptide activity play a critical role in the progression and outcome of disease.

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1. Introduction

Antimicrobial peptides (AMPs) are widely distributed in animals and plants and are innate host defense peptides that display a broad spectrum of antimicrobial activity against bacteria, viruses, fungi and protozoa [1]. Lysozyme, lactoferrin, and collectins act primarily by targeting the bacterial outer membrane [2]. Other AMPs are cationic, amphipathic molecules of 12 to 50 amino acids in length that are capable of interacting with a bacterial cytoplasmic membrane, comprised primarily of negatively charged phospholipids. One of the ways in which these peptides can cause cell death is by inserting themselves into the cytoplasmic membrane to form channels that result in leakage of cytoplasmic contents [3–5]. In addition, multiple systems have been described that function to counter the initial lethality of rapid ion efflux (e.g., potassium) from the bacterial cell associated

with AMP exposure [6,7]. Humans produce various types of AMPs, such as cathelicidins, thrombocidins and defensins [8–12].

In this review, we examine the impact of AMP production and dysregulation in select bacterial-mediated diseases and the consequences of AMP function in pathogenesis. Due to our research focus, we primarily focus the scope of this review to the discussion of the interplay of AMPs with bacterial pathogens. For a comprehensive look at bacterial strategies used to subvert AMP killing, Guilhelmelli and colleagues describe the current state of understanding pertaining to bactericidal mechanisms and bacterial responses to AMP exposure [13]. In addition, a number of reviews comprehensively characterize AMP expression within various anatomical locations of the human body, knowledge of which is important to consider to more fully understand the multifactorial nature and evolution of disease progression [14–19].

2. Modulation of AMPs during Viral Infection Facilitates Bacterial Disease

Many bacteria reside in the host as benign commensals, yet are able to relocate and colonize privileged, normally sterile sites of the body, particularly as a consequence of environmental stress. Translocation of commensal bacteria to a privileged niche occurs with the suppression of antimicrobial peptides (AMPs) that serve as one of the host's first lines of defense. In the animal model for ascending otitis media (OM), the initial infection with respiratory syncytial virus suppresses expression of chinchilla β-defensin 1 (cBD-1), an ortholog of human β-defensin-3 (hBD-3), in the upper respiratory tract [20]. This suppression coincides with an increase in colonization of nontypeable *Haemophilus influenzae* (NTHI) in the chinchilla nasopharynx [21]. These data suggest that AMP production restricts levels of bacterial colonization within the normal flora population. Although a correlation between increased bacterial colonization with subsequent infection of the middle ear was not determined, the authors suggest this may explain the higher incidence of OM following viral infection and the polymicrobial nature of disease. In other work, rhinovirus increases the expression of elastase from neutrophils, and decreases expression of the secretory leukocyte peptidase inhibitor (SLPI) and elafin AMPs, enabling secondary *Staphylococcus aureus* colonization in humans with chronic obstructive pulmonary disease (COPD) [22]. Further, influenza type A suppresses TH17 effector cell pathway associated AMP production in mice exposed to *S. aureus*. The suppression of the TH17 pathway impairs bacterial clearance which leads to *S. aureus* colonization in the lung and subsequent development of pneumonia [23]. Viral infection is not, however, the only mediator of AMP dysregulation contributing to bacterial infection.

3. Bacterial Mechanisms of AMP Resistance

In a non-diseased state, pathogenic bacteria rely on AMP resistance mechanisms to survive the innate immune response and as such, initiate disease. Bacteria therefore have evolved a number of different strategies to sense and respond to various AMPs that they encounter at various sites in the body in order to survive.

3.1. Surface Charge Alterations

The most well characterized form of AMP resistance in Gram-negative bacteria is the modification of lipopolysaccharides (LPS) to decrease bacterial surface electronegativity. These changes reduce electrostatic attraction of positively charged AMPs, minimize bacterial association with AMPs, and decrease AMP-mediated killing. For example, the addition of phosphorylcholine (ChoP) to the oligosaccharide portion of NTHI lipooligosaccharide (LOS) results in increased resistance to the cathelicidin LL-37 [24]. Furthermore, the *dra* locus incorporates D-alanine to the outer membrane in *Bordetella pertussis* to similarly alter the surface charge and increase resistance to AMPs [25]. Highlighting the differential targets and killing ability of AMPs in NTHI, an *htrB* mutant, which switches from production of a hexaacylated lipid A to a tetraacyl lipid A, increases susceptibility of NTHI to hBD-2, but not to hBD-3 [26]. Finally, the deletion of *msbB*, which encodes a lipid A secondary acyltransferase in *Vibrio cholerae* El Tor strain C6706, increases susceptibility to polymyxin B, but interestingly does not affect *V. cholerae* classic strain O395 [27]. The difference in susceptibility to AMPs by closely related strains emphasizes the evolutionary differences that may enable bacteria to adapt to different environmental pressures.

Gram-positive bacterial cell walls lack LPS, but surface charge alterations still prove to be an important strategy for AMP resistance through modification of the lipoteichoic acids and cell wall teichoic acids that characterize their surface. Methicillin resistant *S. aureus* clinical isolates demonstrate an increase in resistance to LL-37 [28]. Additionally, the *dlt* operon, which is responsible for the D-alanylation of teichoic acids, increases resistance to a variety of AMPs. A *dlt* knockout mutant in *Enterococcus faecalis* is significantly more susceptible to the AMPs: nisin, polymyxin B, and colistin when compared to the wild type strain [29]. Furthermore, *dlt* knockout mutants in Group A *Streptococcus* (GAS) [30] and *S. aureus* [31,32] are more sensitive to AMP killing.

The importance of lipid decoration in the pathogenesis of Gram-negative bacterial-mediated disease is evident by studies in animal models of infection. A series of NTHI mutant strains that produce varying truncations of the sugar moiety of LOS are more susceptible to AMP killing and are attenuated when mice are infected via an intranasal route [33]. *Francisella* species with mutations in the genes that encode the deacetylase NaxD are unable to add galactosamine to lipid A and so are more susceptible to polymyxin B killing and are attenuated in a subcutaneous mouse model of infection [34]. A *Neisseria gonorrhoeae* mutant deficient in phosphoethanolamine decoration of lipid A is attenuated in the murine and human urogenital tract, likely due to an increase in susceptibility to AMPs [35,36]. Additionally, a *Salmonella* Typhimurium strain deficient in the incorporation of 4-aminoarabinose into lipid A, was less virulent when orally inoculated into mice as compared to mice infected with the wild type strain [37]. Further, *V. cholerae msbB* mutants showed a reduction in colonization rates compared to the wild type strain when inoculated intragastrically into infant mice [27]. Similar effects were observed in Gram-positive model systems. Survival of *Drosophila melanogaster* improves when infected with a *dltA* deficient strain of *S. aureus,* additionally, the *dltA* deletion mutant is less virulent in mice compared to the parental strain [38–40]. An increase in surface charge to reduce electrostatic AMP interactions is an essential and common mechanism for

bacteria to limit AMP interactions, but this is only one of a variety of ways in which bacteria resist AMP killing.

3.2. Efflux Pumps

The active expulsion of AMPs from the bacterial cell via efflux pumps is utilized by many bacteria to avert lethality of AMP molecules and thus promote colonization of the host. An analysis of the *Vibrio parahaemolyticus* proteome indicated that TolC, a multiple drug resistance efflux pump, is significantly upregulated in strains resistant to AMPs, suggesting that this porin plays an important role in AMP resistance [41]. *Neisseria* species deficient in MtrCDE efflux pump proteins are more susceptible to killing by structurally diverse AMPs [42,43]. In addition, absence of the MefE/MefI efflux pump in *Streptococcus pneumoniae* increases susceptibility to LL-37 [44]. Likewise, in *Haemophilus ducreyi*, an *mtrC* mutant is more sensitive to both cathelicidin and β-defensin peptides [45]. Emphasizing the diversity of AMP resistance mechanisms between organisms, the *Escherichia coli* AcrAB and *Pseudomonas aeruginosa* MexAB efflux pumps, despite being similar to the *N. gonorrheoeae* MtrCDE efflux pump in substrates transported and amino acid identity, fail to play a significant role in AMP resistance [46]. Collectively, these data highlight the importance of efflux of AMPs in bacterial resistance to AMP lethality.

3.3. Import of AMPs into the Cytoplasm for Degradation

Recent investigations indicate that Haemophilus possesses a mechanism to actively import AMPs into the cytoplasm followed by intracellular degradation, thus serving to neutralize AMP-mediated lethality coupled with a nutritional benefit of amino acid recycling. The Sap (sensitivity to antimicrobial peptide) ABC transporter was identified through a transposon mutagenesis screen to identify *Salmonella typhimurium* strains that are more susceptible to the melittin and protamine AMPs [47]. In NTHI, *sapA* gene expression is up-regulated in the middle ear during NTHI-induced experimental OM [48]. A mutation in *sapA* significantly attenuates NTHI survival in the nasopharynx and the middle ear [49]. SapA binds the AMP cBD-1 [6] and mutants deficient in the Sap transporter permease proteins, SapB and SapC, lack the ability to transport AMPs to the bacterial cytoplasm, resulting in the accumulation of hBD-3 and LL-37 in the periplasm [50]. Thus, the Sap transporter binds and transports AMPs into the bacterial cytoplasm, for degradation, using proteases that have not yet been clearly elucidated. Importantly, neutralization of cBD-1 in the chinchilla middle ear restored virulence of the *sapA* mutant, indicating a critical role of this AMP resistance mechanism in *Haemophilus* pathogenesis [50]. Additional studies extended the importance of Sap-dependent import of AMP to *Haemophilus ducreyi*, the causative agent of canchroid in immunocompromised individuals. A mutant strain lacking the SapB and SapC permease proteins is more sensitive to LL-37 and is highly attenuated for pustule development in a human challenge model [51]. Interestingly, although SapA expression confers resistance to β-defensin and cathelicidin molecules in NTHI strains, *H. ducreyi* SapA does not appear to mediate defensin resistance. The observation of import of AMP molecules is now being investigated in other microorganisms. For example, in *E. coli*, the SbmA inner membrane transporter binds and imports the AMP Bac7 using an electrochemical

transmembrane gradient [52]. It is intriguing to speculate that degradation of AMPs in the bacterial cytoplasm, subsequent to active transport, may provide a mechanism by which bacteria co-opt AMPs as a nutritional source to enhance their survival.

3.4. Secreted Proteins that Reduce AMP Activity

The mechanisms of bacterial resistance to AMP-mediated killing extend beyond surface modification and transport mechanisms, and include the active secretion of proteins that bind or degrade AMPs in an effort to abrogate their antimicrobial activity. Numerous studies indicate a role for exogenous proteases to neutralize the threat of host AMPs. Elastase production by *P. aeruginosa* was shown to be essential for evasion of LL-37 in chronic leg ulcer fluid [53]. Loss of metalloprotease SepA production in *Staphylococcus epidermidis* significantly impaired resistance to the dermcidin AMP [54]. In support of the importance of bacterial proteases in the development of disease, Puklo and colleagues observed degraded LL-37 in the gingival crevicular fluid that correlated with the presence of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, all of which express large amounts of proteases [55]. Furthermore, degradation of exogenous LL-37 also occurs in *P. gingivalis* infected gingival crevices [56]. Staphylococcal staphylokinase (SAK) and Streptoccocal streptokinase (SKA) are two exogenous proteins that activate the zymogen plasminogen into plasmin, which is essential for fibrinolysis. The use of these bacterial enzymes to dissolve blood clots has been proposed as a novel virulence mechanism for bacteria to evade confinement and to disseminate throughout the host. However, GAS can also use the plasmin to degrade LL-37 [57]. Similarly, in Staphylococcal induced pneumonia, cathelicidins are upregulated and the binding of SAK to host cathelicidins augmented fibrinolysis [58]. However, the role of AMPs in the augmentation of staphylokinase fibrinolysis is not an absolute. For example, α-defensin expression results in a significant decrease of fibrinolysis [59]. Collectively these data indicate that secretion of bacterial factors to neutralize host AMPs may provide an advantage for bacterial colonization at distinct environments in the host. It is also interesting to speculate that exogenous degradation of AMPs may provide a nutritional benefit to bacteria, as a source of amino acids for growth. Collectively, exogenous degradation of AMPs serves to limit bacterial surface interactions with AMPs, and so increase their survival.

3.5. Decoys That Sequester AMPs

The presence of bacterial factors such as LPS and DNA in the extracellular environment can serve as decoys to bind and neutralize AMPs and thereby reduce AMP mediated killing. Gram-negative bacteria produce a large amount of LPS and capsular polysaccharides, thus interaction of AMPs with these surface structures likely serve to delay bactericidal mechanisms. A study of major lung pathogens found that the polysaccharide components released by bacteria inhibited the activity of LL-37 and the cathelicidin-like protein SMAP-39; interestingly, the affinity of polysaccharides and AMPs is not based solely on electrostatic interaction as lower negative net charged particles do not correlate with better binding of LL-37 [60]. Furthermore, the presence of exogenous capsular polysaccharide from *Klebsiella pneumoniae*, *S. pnemuoniae*, and *P. aeruginosa*

have demonstrated the capacity to serve as decoys by binding to AMPs and increasing bacterial resistance to polymyxin B and hNP [61]. Finally, plasmid DNA from *Shigella dysenteriae* reduces the expression of LL-37 and hBD-1 by epithelial cells, suggesting that early invading bacteria that are lysed, release factors that alter host defenses and enable subsequent invading bacteria to survive [62].

The bacterial biofilm matrix contains components that promote survival through associations with AMPs. Curli fibers, which are also known to be associated with *E. coli* biofilms, bind to LL-37, and thus prevent penetration, disruption, and killing of the bacterial biofilm [63]. *S. epidermidis* biofilms produce the extracellular polysaccharide intercellular adhesin, which increases bacterial resistance to a variety of AMPs including LL-37, hBD-3 and dermcidin [64]. The extracellular DNA component of *P. aeruginosa* and *S. Typhimurium* biofilm matrices chelate cations that normally repress the two-component PhoPQ and PmrAB signaling pathways and increases virulence [65,66]. When biofilm matrices are treated with DNase however, hBD-3 is more efficient at diminishing the biofilm formed by NTHI, again underscoring the importance of this matrix component in protection from AMP-mediated killing [67]. During biofilm growth, Gram-negative bacteria produce outer membrane vesicles (OMV) that contain proteins, phospholipids, and other periplasmic factors. The protein composition of OMVs released from *V. cholerae* is altered when strains are grown in the presence of sublethal concentrations of AMPs. One change is an increase in expression of the biofilm-associated extracellular matrix protein Bap1, shown to bind AMPs. A mutant deficient in Bap1 is more susceptible to AMP killing. These data suggest that the presence of Bap1 in OMVs is important for OMV associations with AMP that lead to increased AMP resistance [68]. Through diversionary tactics like LPS and DNA secretion, AMP bacterial interactions are lessened, allowing bacterial biofilms to persist in the host and further disease progression.

4. Colonization and Host Microenvironmental Factors That Influence AMP Activity

Transit of bacteria within the human host exposes the microorganism to various environments that vary in pH, nutrient concentrations, temperature, and host defenses. The environment of the host niche plays a pivotal role in the pathogenesis of disease, as a single new variable can drastically change the progression of disease in favor of the host or of the pathogen. For example, vitamin D has been linked to the host's ability to remediate disease as TLR activation up-regulates expression of vitamin D hydroxylase and the vitamin D receptor, VDR. Activation of VDR through the vitamin D ligand leads to induction of expression of LL-37 [69]. VDR and LL-37 are likewise up-regulated in cirrhotic patients with spontaneous bacterial peritonitis, and cirrhotic patients with ascites exhibit vitamin D deficiency [70]. These data suggest that vitamin D could serve as an important factor in bacterial clearance. Conversely, some bacteria benefit from the effects of vitamin D for survival. For example, when vitamin D induces LL-37 production in human macrophages, GAS responds to subinhibitory concentrations of LL-37 through the CsrRS two-component system to increase expression of virulence factors (e.g., hyaluronic acid capsule, streptolysin O). These virulence factors then protect GAS from the killing effects of host cells [71]. Host body temperature also influences bacterial pathogenesis and AMP interactions. For example, as *Yersinia enterocolitica* moves from a lower temperature (representative of the environmental niche) to an elevated temperature (representative of the host niche), expression of the two-component systems PhoPQ and PmrAB

decrease. This decrease in PhoPQ and PmrAB activity leads to a reduction in lipid A modification which increases bacterial susceptibility to AMPs [72]. Moreover, *Salmonella* species respond to increased pH through PhoPQ and PmrAB mediated expression of virulence genes that increase bacterial resistance to AMPs and facilitate transit through the gastric mucosa [73,74]. Conversely, expression of *phoQ* in *P. aeruginosa* is not activated by AMPs but is weakly activated by acidic environments. Therefore, *Salmonella* and *P. aeruginosa* have evolved different cues for PhoQ to sense the environment. PhoQ from *P. aeruginosa* is optimized to respond to the external microenvironment of the soil, which is both less acidic with less AMP exposure than that encountered by *Salmonella* [74]. Similarly, Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) have evolved differences in both the expression and function of the OmpT protease, shown to mediate AMP degradation in *E. coli*, due to the niche of each strain in the small and large intestines, respectively [75]. Thus, the human host is comprised of diverse microenvironments, each presenting a unique set of opportunities and challenges for pathogens in their continued effort to subvert host AMPs and maintain infection.

4.1. AMP Activity of the Skin

The skin serves as a protective barrier while maintaining a diverse micoflora. However, some bacteria can take advantage of breaches in this barrier to establish infection within the host. To prevent disease, it is important for the skin to maintain a healthy flora, in part, through the action of AMPs. While skin flora maintains homeostasis within the respective niches, foreign invaders can initiate changes that alter the microenvironment in favor of pathogen colonization. For example, *Finegoldia magna*, a skin commensal, maintains colonization through the action of the secreted protease SufA, which reduces the antibacterial activity of the MK and BRAK/CXCL14 AMPs. In contrast, the pathogen *Streptococcus pyogenes* protease SpeB demonstrates more efficient degradation of a wide range of AMPs, and thus favors colonization at this site [76]. The expression of neutrophil derived AMPs can likewise compromise the survival of commensals. LPS types 1A and 1B, from *Propionibacterium acnes*, stimulates hBD-2 expression in sebum producing skin epithelial cells or sebocytes. Whereas hBD-2 expression does not impact viability of *P. acnes*, the immunomodulatory effects of hBD-2 expression results in recruitment of neutrophils and induction of inflammation at the site of infection and can have unintended consequences for commensal survival [77]. It is increasingly clear that changes in the skin microenvironment that potentiate loss of benign commensals may lead to the replacement with pathogenic strains. Therefore, the homeostasis of commensals, governed by AMPs, is critical for the control of pathogenesis.

Lesions compromise the skin's protective barrier and leave the host vulnerable to bacterial infection. When this protective barrier has been compromised, the role of AMPs in disease pathogenesis becomes critical. Mice deficient in an ortholog of LL-37 (CRAMP) infected with β haemolytic GAS display larger skin lesions and prolonged bacterial persistence compared to wild type mice. Furthermore, cathelicidin-resistant GAS is more persistent than cathelicidin-sensitive GAS in a post subcutaneous infection of mice [78]. The interplay between bacteria and AMPs impacts the microenvironment with critical consequences for the pathogenesis of skin disease. In acne vulgaris lesions, *P. acnes* secretes proteases that activate specific receptors that increase

hBD-2 and LL-37 production, as well as a wide array of pro-inflammatory cytokines, contributing to epithelial lesions characteristic of this disease [79]. Likewise, skin lesions from acne inversa contain increased levels of hBD-2 in comparison to uninfected epithelial cells, further suggesting a role for hBD-2 in inflammatory skin disease pathogenesis [80]. Microbe-associated molecular patterns such as flagellin, stimulate host cell receptors that subsequently signal release of AMPs into the environment. Although *P. aeruginosa* flagellin stimulates the release of hBD-2 from keratinocytes, rhamnolipids secreted by *P. aeruginosa* inhibit release, effectively protecting the bacteria from hBD-2 mediated killing [81]. The role of AMPs in protecting the skin from pathogenic bacteria is most critical, as a breach in this line of defense can introduce bacteria into a multitude of new environments within the host.

4.2. Bacterial Homeostasis of the Nasopharynx

The contribution of AMPs in bacterial population management within the nasopharynx is crucial for human health, as carriage of commensal bacteria can influence the incidences of lower and upper respiratory disease. Lysozyme and hBD-1 and 2 contribute to the first line of defense by reducing the viability of major respiratory pathogens [82]. In addition, a reduction in the copy number of the DEFB-CN gene cluster, which encodes multiple beta defensins, leads to an increased risk for concurrent nasopharyngeal colonization with the OM pathogens NTHI, *Moraxella catarrhalis* and *S. pneumoniae* [83]. In support of the idea that AMPs influence residential bacteria critical for disease, the neutralization of cBD-1 increases carriage of NTHI in the nasopharynx [21]. This observation supports the idea that AMPs contribute to maintaining low levels of opportunistic pathogens, to protect from bacterial overgrowth and migration, which can lead to disease [21]. AMP exposure may also drive selection for strains that have a higher propensity to survive in this niche. For example, the presence of either LL-37 or hBD-1 selects for different *S. pneumoniae* isolates *in vitro*, suggesting a role for AMPs in establishment of commensal residents of the nasopharynx [84]. Levels of AMP expression have also been suggested to directly impact disease. For instance, among individuals colonized with *S. aureus*, more LL-37 is recovered from healthy controls than from patients with chronic rhinosinusitis, suggesting that dysregulation of LL-37 expression in the nasopharynx contributes to *S. aureus*-mediated disease [85]. Furthermore, carriers of *S. aureus* demonstrate increased expression of hNP-1, hNP-3, and hBD-2 in their nasal fluids, suggesting that the associations between *S. aureus* and these peptides are important in infection [86]. These data suggest that changes in the level of AMPs associated with inflammation due to invasive bacteria and the differences in AMP expression that exist between individuals impact the survival of normal commensals in the nasopharynx and so, may potentiate disease development.

4.3. AMPs and Lung Disease

The lungs have the second largest epithelial area exposed to the outside environment and the innate immune system is paramount to minimize bacterial invasion and infection. In the normal developing human lung, hBD-2 is the predominant defensin expressed. However, premature infants exhibit less expression of this critical AMP, which may contribute to increased susceptibility to

infection [87]. β-defensins are important factors in host lung defense as demonstrated by the fact that hBD-2 and hBD-3 expression increases in human airway epithelial cells when stimulated by *S. pneumoniae*, an important causative agent of pneumonia [88]. The importance of cathelicidins in pathogenesis is shown by the upregulation of CRAMP in the murine lung following infection with *P. aeruginosa* and *K. pneumoniae*. In addition, CRAMP deficient mice infected with *K. pneumoniae* demonstrate reduced survival as well as an increase in bacterial persistence of *P. aeruginosa* and *K. pneumoniae* in the lungs [89]. Furthermore, bronchial epithelial cells stimulated with bacterial LPS demonstrate an increase in LL-37, which induces cellular apoptosis and IL-8 release, resulting in immune cell recruitment and a concomitant induction of an inflammatory response [90]. This proinflammatory response, mediated in part through AMP induction, likely contributes to chronic lung disease states such as COPD, pneumonia, and cystic fibrosis.

Microenvironmental alterations in AMP activity or concentration may also impact chronic lung diseases and facilitate residence by pathogenic bacteria which would further complicate disease progression. The environment of the cystic fibrosis lung is characterized as hyperinflammatory with a disrupted electrolyte composition due to a mutation in the chloride transporter CFTR. The potential for alterations in salt concentration during cystic fibrosis may impact the antimicrobial activity of AMPs. In fact, high salt concentrations have been shown to decrease the effectiveness of AMP killing [91,92]. High salt concentrations reduce activity of β-defensins [93,94], whereas LL-37 retains activity in high salt concentrations [95]. However, LL-37 activity is suppressed in the lungs of cystic fibrosis patients due to the interaction of LL-37 with bacterial-associated factors such as LPS, DNA as well as host glycosaminoglycans, and the dissociation of these complexes restores LL-37 potency [95,96]. The potential loss of LL-37 activity due to disease manifestation can leave the lung vulnerable to bacterial outgrowth or new bacterial invasion. Conversely, the presence of AMPs can also have unintended consequences for lung disease pathogenesis. For example, LL-37 induces IL-8, which in turn induces Muc5AC mucin production. Overproduction of Muc5AC can further compromise the airways in diseases such as COPD [97]. Exposure to LL-37 also selects for mutations in *P. aeruginosa* that facilitate mucoid conversion, making *P. aeruginosa* more recalcitrant to LL-37 killing [98]. Together these data underscore the importance of environmental factors, including AMPs, in the progression of lung disease.

4.4. AMPs and Periodontal Disease: Oral Cavity

The oral cavity serves as an entry point for pathogenic bacteria to enter the body; therefore, it is important for host defenses to prevent pathogenic bacteria from establishing residence within the oral cavity and causing disease. Periodontal disease is of particular concern, as this bacterial-mediated infection can lead to inflammation and the loss of both gum tissue and teeth. In an effort to eradicate pathogenic bacteria in the oral cavity, α-defensins hNP1-3 are significantly increased [55]. Although neutrophils are the primary source of hNP1-3 in infection, studies suggest that other sources are responsible for hNP1-3 secretion in later disease. For example, chronic periodontitis associates with significantly more hNP1-3 than aggressive periodontitis, yet both disease states shared similar neutrophil responses, which suggests an additional source of AMP secretion in chronic periodontitis [55]. This study highlights the fact that different stages of disease associate with

expression of different AMPs that can have a direct influence on bacterial pathogenesis. The role of neutrophil-derived AMPs in maintaining health of the oral cavity is further suggested by the observation that patients with morbus Kostmann, a severe congenital neutropenia, are prone to chronic periodontal disease. When neutrophil production is stimulated in morbus Kostmann patients, the neutrophils do not express LL-37 and express lower concentrations of α-defensins [99]. In addition, dysregulation of β-defensin production also contributes to oral disease as a single nucleotide polymorphism in DEFB1, the gene that encodes hBD-1, has been linked to cases of periodontitis [100]. Furthermore, expression of hBD-2 mRNA increases in response to live *P. gingivalis* stimulation in a mouse human gingival graft model, which suggests a role for this AMP in prevention of periodontal disease [101]. Interestingly, hBD-3 plays a role in decreasing the deleterious effects of inflammation from disease, as hBD-3 is capable of binding and neutralizing the oral pathogen *P. gingivalis* via interaction with non-fimbral adhesin HagB, thereby interfering with binding of host cell receptors and thus decreasing inflammation [102]. Dysregulation of any of the multitude of AMPs implicated in oral diseases appears to play an important role in the progression of disease.

4.5. AMPs and the Gastric Mucosa

Invading bacteria are normally prevented from colonizing the gastric mucosa through the action of acidic gastric juice, mucus, and AMPs. Despite these harsh conditions, some bacteria are able to circumvent the antimicrobial conditions unique to this microenvironment by responding to environmental cues and changing expression of AMP resistance genes or by disrupting the host environment. *Helicobacter pylori* alters the expression of several key AMPs known to be associated with the gastric mucosa. hBD-2 expression increases in response to bacterial insult, suggesting a role for this peptide in controlling the pathogenesis of *H. pylori* mediated diseases [103–105]. There are conflicting reports in the literature regarding the regulation of transcription of hBD-1, hBD-3 and LL-37 in the gastric mucosa in response to bacterial insult. hBD-1 is constitutively expressed in the normal gut, but may be either upregulated by *H. pylori* infection [106], down regulated by *H. pylori* infection [105] or unchanged between infected and non-infected gastric mucosa [103]. Expression of hBD-3 and LL-37 may be increased [107,108], decreased [104] or undetectable in the gastric mucosa [103] following *H. pylori* infection. The different AMP expression levels observed is likely due to the use of different *H. pylori* strains and the presence of the virulence factor CagA, which can translocate into host cells and inhibit synthesis of hBD-3 via dephosphorylation of epidermal growth factor receptor [109]. Despite these contradictory findings, we can speculate that modulation of the bacterial effects of hBD-3 and LL-37 can regulate infection by *H. pylori* in the gastric mucosa and thus be a critical defense mechanism against *H. pylori* pathogenesis [103].

4.6. Homeostasis and Diseases of the Intestines

The intestines are home to a diverse micro-flora that evolves to withstand AMP killing, since AMPs are routinely expressed in the intestines. Paneth cells continually express AMPs, such as HD-5,

HD-6 and lysozyme that serve as a first line of defense against invading bacteria [110]. Upon infection with *V. cholerae* [110] *Bacteroides fragilis* [111] and various *Pseudomonas* species [112], hBD-2 is upregulated, which suggests that this β-defensin is important in the modulation of disease. CRAMP deficient mice are more susceptible to *Citrobacter rodentium* colonization, epithelial damage and systemic infection, which further implicates a role for AMPs in the small intestine in preventing bacterial pathogens from causing dysbiosis [113]. The influence of AMPs within the intestinal environment has also been demonstrated in mice deficient in α-defensins, as loss of these AMPs leads to significant differences in the composition of intestinal microbiota, although the total bacterial load remains unaffected [114]. Similarly, mice deficient in α-defensins are more attenuated in removal of *Chlamydia trachomatis* from the small intestines [115]. Combined, these studies suggest a role for AMP influence on population shifts, which may impart susceptibility or resistance to diseases of the intestinal tract. In a mouse model of necrotizing enterocolitis, where AMP levels and intestinal inflammation increase, the probiotic *Bifidobacterium bifidum* attenuates disease and decreases AMP levels, by normalizing the microbiota and re-establishing the synergistic relationship between the commensal and the host [116]. However, the influence of AMPs on normal commensal populations may not always be the result of direct AMP and bacterial interactions. For example, LL-37 stimulates mucus production in the human colonic cell line HT-29 and the administration of CRAMP upregulates expression of mucin genes in the colonic tissue of mice with experimentally induced colitis. These data demonstrate that LL-37 can influence the microenvironment of the intestines by stimulating the production of mucus [117,118]. As cathelicidin deficient mice have less thick and non-homogenous mucus layers when compared to wild type animals, *E. coli* are more likely to penetrate the mucus layer and attach to the underlying epithelial cells [119]. The intestinal mucus is therefore an important barrier that prevents bacteria from reaching the intestinal epithelium, so any deficiencies in the production could have a critical impact on bacterial pathogenesis in the intestines. Finally, the scarce availability of oxygen in the intestines is important to consider in AMP-mediated killing. For example, HD-5, is effective at killing facultative anaerobic species, but is ineffective at killing strict anaerobic bacteria, while hBD-3 is only able to kill anaerobic species under aerobic conditions [120]. Therefore, bacterial residents of the intestines display differential susceptibility to AMP killing dependent on their metabolism or O_2 availability, further emphasizing the complex interactions between AMPs and bacteria in pathogenesis. The maintenance of balance between host factors, nutrient status, and commensal populations in the bowel is important to consider particularly with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.

4.7. Urinary Tract Infections

The environment of the urinary tract is generally considered sterile, despite the close proximity to the gastrointestinal tract, and provides unique challenges for invading bacterial attachment due to the shedding of epithelial cells, urine flow, and expression of innate defenses such as AMPs. Cathelicidins are constitutively expressed by both human and mouse urinary epithelia. The importance of cathelicidins in preventing pathogenic bacteria from establishing disease is demonstrated by the observation that cathelicidin resistant *E. coli* are more likely to invade the urinary tract than

cathelicidin susceptible strains, while CRAMP deficient mice are more easily infected by *E. coli* when compared to wild type mice [121]. Additionally, defensins in the urinary tract have diverse functions in the protection of the urothelium from disease. In patients undergoing urinary diversion surgery, there is a significant increase in the expression of HD-5 α-defensin and decrease in expression of hBD-1 in urothelial tissue [122]. As HD-5 shows bactericidal activity towards uropathogenic *E. coli* (UPEC), these data suggest that this peptide mediates a direct role in bacterial clearance in urinary tract infection (UTI) pathogenesis. Becknell and colleagues have observed a reduction in mBD-1 expression in the mouse bladder in response to UPEC infection and furthermore, observed that a mouse deficient in mBD-1 has no significant impairment when challenged with UPEC, suggesting that mBD-1 may not contribute to the control of UPEC mediated infection [123]. In contrast, mBD-3 and mBD-14 display dose-dependent bactericidal activity to UPEC *in vitro*, suggesting a role for AMPs in mucosal immunity in the lower urinary tract [123]. RNase6 and 7 are members of the RNaseA superfamily, and have recently been described as having important roles in urinary tract defense where they exhibit strong antimicrobial activity towards uropathogenic bacteria. RNase7 is important in the urinary tract due to the observation of an increase in levels in urine samples obtained from patients with pyelonephritis as compared to healthy controls [124]. While RNase7 is significantly increased in relation to RNase6 in non-infected human bladder and kidney tissues, RNase6 expression significantly increases during pyelonephritis due to the influx of immune cells [125]. The urinary tract produces multiple classes of AMPs that function in homeostasis of the sterile urinary tract as well as being responsive to disease.

5. Host-Pathogen Tug of War

After a bacterium colonizes a privileged location, the development and subsequent progression of disease is dependent on the successful management of increasingly complex interactions between the host and the bacterium. Convention originally stated that positively charged AMPs primarily exert activity by interaction with negatively charged bacterial surfaces leading to bacterial cell lysis. However, it is becoming increasingly apparent that AMP function is multifactorial. For example, bacteria respond to the presence of AMPs through changes in bacterial gene expression as a result of signaling events. Exposure of *E. coli* to sublethal concentrations of cecropin A changes the transcriptional profile of a subset of genes [126]. Certain AMPs are capable of mediating changes in gene expression profiles of bacterial virulence factors and as such, may influence the dynamics of the pathogen-host interaction. In addition, bacterial responses to environmental pressures may affect host responses and ultimately, may impact disease remediation or continued bacterial infection.

5.1. AMPs Influence Bacterial Gene Expression

Although the host responses and subsequent microenvironmental changes are intended to eradicate infection, pathogenic bacteria sense and respond to environmental cues to regulate transcription of virulence factors. One such environmental cue is the secretion of AMPs by the

host. Importantly for the bacterium, transcription of gene products that contribute to mechanisms of AMP resistance are often upregulated upon bacterial exposure to AMP molecules. A classic example is the PhoPQ regulatory system, a well-studied two-component system that responds to environmental stressors and modulates expression of genes that contribute to modification of LPS, leading to increases in AMP resistance in Gram-negative bacteria [127,128]. Although best described in *S. typhimurium* [129], a role for PhoPQ has been shown for AMP resistance in *P. aeruginosa* [130], *E. coli* [131], *K. pneumoniae* [132], *Shigella flexneri* [133], *Neisseria meningitidis* [134] and *Yersinia pestis* [135]. The conservation of gene function across different species highlights the importance of this mechanism for AMP resistance. Furthermore, PhoPQ regulates expression of another two-component system, PmrAB. PmrAB is involved in the regulation of expression of *pmrE* and *pmrHFIJKLM*, the products of which modify lipid A with 4-aminoarabinose [37,136]. A *S.* Typhimurium *pmrF* insertional mutant, lacking the 4-aminoarabinose modification, displays a decrease in virulence in the mouse when administered orally [37]. While *S.* Typhimurium requires the PmrAB two-component system for regulation of *pmrF* expression, regulation of *pmrF* in *Yersinia pseudotuberculosis* occurs solely through the PhoPQ pathway. Furthermore, *pmrF* expression is not essential for either AMP resistance or survival of *Y. pseudotuberculosis* in the mouse [137]. While the PhoPQ and PmrAB systems are perhaps the most well studied two-component systems involved in AMP resistance in Gram-negative bacteria, other two-component regulatory systems such as ParRS, CprRS, and ColRS increase resistance of *P. aeruginosa* to AMPs through alterations in surface charge [7,138,139].

Gram-positive bacteria also use two-component systems to respond to the presence of AMPs as a way to regulate resistance to bactericidal activity. For example, in *S. aureus* attack by certain AMPs, such as HNP-1 cause a disruption in membrane potential that activates the LytSR two-component system which is protective against AMPs *in vitro* [140]. In addition to the LytSR system, AMPs induce the GraSR regulon (also known as the AMP sensor, or ApsSR) of *S. aureus*, which up-regulates expression of the virulence genes *dlt*, *mprF*, and *vraFG*, and so increases resistance to AMPs [141,142]. Similarly, in GAS, the well-studied two-component system CsrRS (also known as CovRS) can sense LL-37 and alter the expression of virulence genes and thereby increase the resistance to phagocytic killing of this bacterium [143]. Additionally, during progression of disease, GAS transitions to a mucoid phenotype though upregulation of hyaluronic acid capsular polysaccharide biosynthetic enzymes via CsrRS sensing of LL-37 [144]. Finally, growth of *S. pyogenes* in the presence of LL-37 is dependent on the CovRS two-component system *in vitro*. CovS inactivates CovR repression of the *dlt* operon. The *dlt* operon encodes proteins responsible for D-alanylation of the anionic polymers of lipoteichoic and wall lipoteichoic acids. Increased expression of *dlt* may ultimately produce a cell wall with a higher positive charge density and prevent binding of cationic AMPs [145]. The sensing of AMPs and increases in expression of AMP resistance mechanisms via two-component systems allow bacteria to adapt to the innate immune response and persist within the host while establishing infection.

Although regulation of two-component signaling pathways confers AMP resistance to many bacteria, there are additional mechanisms that increase bacterial resistance to AMP molecules. For example, exposure of *P. aeruginosa* to sublethal concentrations of LL-37 increases expression of a number of virulence factors. These virulence factors include genes whose products encode multidrug efflux pumps and those involved in LPS modification as well as in the production of quorum sensing molecules [146]. Host production of the anionic AMP dermcidin increases the expression of the global regulator Agr and decreases the expression of the global regulator SarA in *S. epidermidis*. Regulation of gene expression by Agr and SarA subsequently leads to enhancement of dermcidin proteolysis and thus is protective toward *S. epidermidis* [54]. AMP exposure increases expression of transporters that actively export AMPs to remove them from the cell or, alternatively, import AMPs for degradation and so confer resistance [44,48]. In *P. aeruginosa*, sublethal concentrations of polymyxin B upregulates the expression of *psrA*, which increases AMP resistance through regulation of swarming, motility and biofilm formation [147]. Finally, *in vitro* exposure of *P. aeruginosa* to LL-37 selects for a mutation in the negative regulator of mucoidy, MucA, and so increases alginate synthesis. This increase in alginate production converts bacteria to a mucoid phenotype that provides protection against host AMPs. Moreover, this LL-37 induced mutation in MucA parallels what is observed in the cystic fibrosis lung [98]. Collectively, these examples illustrate how bacterial sensing of AMPs can lead to recalcitrance and so may prove to be the tipping point in the progression of disease.

5.2. AMPs Influence Disease Progression

Whereas regulation of transcription controls microbial virulence mechanisms in response to AMP exposure, the consequence of these responses on pathogenesis continues to be explored in relevant animal models of disease (see Table 1). For example, in the lumen of the mouse intestine, *S.* Typhimurium uses the PhoPQ and PmrAB two-component systems to respond to insult by CRAMP, which indicates the importance of PhoPQ and PmrAB in the pathogenesis of *S.* Typhimurium [148]. In further support of this idea, inactivation of the PhoPQ system of *S. typhimurium* leads to a decrease in survival in the *Caenorhabditis elegans* intestine. The loss of survival is abrogated when expression of the *C. elegans* AMP Spp-1 is reduced with RNAi treatment, suggesting that PhoPQ dependent resistance to AMPs is vital for survival in the host [149]. GAS mutants deficient in the transcriptional regulator RALP3 are more sensitive to mCRAMP and attenuated in a murine model of infection [150]. *S. aureus* strains lacking the AMP sensing protein ApsS (also known as GraS) are significantly attenuated for infection of the kidneys when intraperitoneally injected into the mouse [141]. Finally, *S. aureus* strains deficient in LytS of the LytSR two-component system are more susceptible to exogenous AMPs in a rabbit model of aortic infective endocarditis [140]. Together, these data suggest that bacterial responses to host defense are critical for the ability to withstand the host immune factors generated as a consequence of disease.

Table 1. *In vivo* Models of antimicrobial peptides (AMPs) in Disease.

Disease Site	Animal Model	Host AMP Neutralized	Bacteria	Gene	Source
Disseminated	*Drosophila*, Mouse	---	*S. aureus*	*dltA*	[39,40]
	Mouse	---	*Francisella tularensis*	*naxD*	[34]
	Mouse	---	*S.* Typhimurium	*pmrF*	[37]
	Mouse	---	*Y. pseudotuberculosis*	*pmrF*	[137]
	Mouse	---	GAS	*Ralp3*, *lsa*	[150]
	Rabbit	---	*S. aureus*	*lytS*	[140]
Intestine	Mouse	CRAMP	*E. coli*	---	[119]
		---	*S. aureus*	*dltA*	[38]
		α-defensins	---	---	[114]
		CRAMP	*C. rodentium*	---	[113]
		α-defensins	*C. trachomatis*	---	[115]
		CRAMP, α-defensins	*S.* Typhimurium	*phoPQ* and *pmrAB*	[148]
		---	*V. cholerae*	*msbB*	[27]
Kidney	Mouse	---	*S. aureus*	*apsS*	[141]
Lung	Mouse	CRAMP	*K. pneumoniae*, *P. aeruginosa*	---	[89]
Middle Ear	Chinchilla	CβD-1	NTHI	*sapA*	[50]
Nasopharynx	Chinchilla	CβD-1	NTHI	---	[21]
Skin	Human	---	NTHI	*sapBC*	[51]
	Mouse	CRAMP	GAS	---	[78]
Stomach	*C. elegans*	Spp1	*S. typhimurium*	*phoPQ*	[149]
Urogenital Tract	Mouse, Human	---	*N. gonorrhoeae*	*lptA*	[35–36]
	Mouse	CRAMP	*E. coli*	---	[121]

The interactions between bacteria and AMPs are complicated by the introduction of additional host factors as disease persists. AMPs induce immunomodulatory changes in the host in an effort to resist bacterial colonization. The stimulation of epithelial cells by LL-37 increases production of the chemokine IL-8, which leads to the recruitment of immune cells. This influx of immune cells leads to inflammation and the induction of further changes in the microenvironment. In the case of COPD, these inflammatory changes can become persistent and irreversible and thereby influence pathogenesis [90]. AMPs also provide a key function in immune cell recruitment as hBD2-4 and LL-37 induce human keratinocytes to produce the pro-inflammatory cytokine IL-18 [151]. In addition to pro-inflammatory cytokines, a diverse array of AMPs are produced as the disease progresses through bacterial stimulation of AMP release from epithelial cells and α-defensin release from the recruited immune cells. In healthy individuals, α-defensins are introduced into the environment primarily by neutrophils, but an alternative mechanism may be responsible for the increased levels of α-defensins in late stages of chronic disease [55]. Additionally, in both the non-infected human bladder and kidney, RNase7 provides one of the first lines of defense against

pathogens; however, expression of RNase6 significantly increases upon bacterial insult [152]. Furthermore, expression of RNase7 and hBD-2 increases early during oral bacterial biofilm formation on human gingival epithelial cells, but expression declines as biofilms mature [153]. AMPs that may prove to be important in the acute stages of infection may be less important in chronic infections, possibly due to bacterial adaptation to the environment. For example, as bacterial biofilms mature they release extracellular DNA (eDNA) that associate with AMPs and confer AMP resistance. AMPs that are effective at bacterial clearance during the early stages of disease, when the levels of eDNA are too low to have an impact, may lose effectiveness once the biofilm matures, primarily due to neutralization of AMP through eDNA association [67]. The chronology of AMP production during disease and consequence upon bacterial interaction in a rapidly changing inflammatory environment can significantly influence disease progression in the host. Understanding a role for AMP activity during acute and chronic disease states continues to be a significant challenge, but one that remains of importance to understand a therapeutic value for AMP interventions in disease.

5.3. Using What We Know About AMPs for Potential Therapeutic Use

It is clear that AMP molecules play a significant role in host–pathogen interactions that influence the establishment and course of disease progression. In addition to the bactericidal properties of AMPs, recent investigations support the use of AMPs as inflammatory modulators to reduce the pathological consequences associated with disease. For example, AMPs bind bacterial surface molecules to prevent interactions with host cell receptors and thus attenuate pro-inflammatory cytokine responses. LL-37 binds *N. meningitidis* capsular polysaccharides and blocks TLR2 and TLR4-MD2 dependent activation of pro-inflammatory cytokine release in human and murine macrophages [154]. In other studies, treatment of mice with CRAMP reduces intestinal inflammation in response to *Clostridium difficile* toxin A [155]. In addition, hBD-3 and LL-37 reduce IL-8 secretion from gingival fibroblast cells exposed to *Aggregatibacter actinomycetemcomitans* lipopolysaccharide [156]. The suppression of a pro-inflammatory cytokine response, through delivery of AMPs, also correlates with bacterial clearance and disease severity. In a mouse model of methicillin-resistant *S. aureus* (MRSA) induced pneumonia, LL-37 neutralization of lipoteichoic acid induces pro-inflammatory cytokine release resulting in clearance of bacterial infection [157,158]. Similarly, temporin B and royal jellein 1, secreted from the European red frog and honeybees, respectively, act synergistically to reduce inflammation through down regulation of the pro-inflammatory cytokines TNF-α and IFN-γ and upregulation of the anti-inflammatory cytokine IL-10. [159]. Topical application of the king cobra derived AMP OH-CATH30 significantly decreases release of the pro-inflammatory cytokines TNF-α and IL-1β coinciding with a marked reduction in corneal epithelial loss [160].

Whereas AMPs attenuate inflammation associated with disease, as described above, evidence also indicates that AMPs stimulate inflammatory responses in an effort to eradicate bacteria from the host. In a murine model of *P. aeruginosa* lung infection, exogenous LL-37 increases recruitment of neutrophils to the lung, which results in bacterial clearance [161]. Defensins and LL-37 can also induce secretion of the pro-inflammatory cytokine IL-31, as well as other pro-inflammatory cytokines involved in the innate immune response from human mast cells [162]. These immunomodulatory

activities of AMP molecules appear to differ based upon the concentration of peptide. When human epithelial cells are stimulated with LPS, low concentrations of LL-37 suppress IL-8 secretion while greater concentrations of LL-37 induce IL-8 secretion [163]. Furthermore, high concentrations of LL-37 either augment or antagonize inflammatory cytokine responses depending on the presence of specific immune mediators [164]. The immunomodulatory properties of AMPs highlight their multifunctional roles in the pathogenesis of disease independent of bacterial killing and may prove to be useful for potential therapeutic application.

In addition to the use of naturally derived AMPs or modification of existing AMPs, synthesis and utilization of entirely new AMP structures shows promise in treating bacterial infections. For instance, a truncated variant of LL-37 lacking the six C-terminal residues is more effective at killing planktonic and biofilm-associated *Burkholderia pseudomallei* than LL-37 or LL-37 derived fragments [165]. Furthermore, P60.4Ac and P10, synthetic derivatives of LL-37, are more efficient than LL-37 in the eradication of MRSA in a human skin model of infection [166]. Similarly, the synthetic peptides E2, E6, and CP26 are more effective than CRAMP and LL-37 at killing *Mycobacterium tuberculosis* and *P. aeruginosa in vitro* and importantly, are more efficacious in the control of *M. tuberculosis* in the mouse lung, which supports a therapeutic use for these peptides [167]. Synthetic AMPs may also demonstrate the capacity to act in an immunomodulatory manner similar to naturally produced AMPs. For example, the synthetically derived A3-APO decreases *S. aureus* load in a murine burn wound model and decreases the burden of *P. acnes* in an intradermal mouse ear model despite not directly killing the strains *in vitro*, suggesting that immunomodulation by A3-APO of the host microenvironment contributed to remediation of disease [168]. The efficacy of AMPs in disease treatment can be further improved through peptide modification. For example, the covalent linking of polyethylene glycol through the process of PEGylation, reduces the interactions of AMP molecules with both host cells and host enzymes, and so focus AMP interaction with the bacterial cell surface. In addition, PEGylation decreases the cytotoxicity of modified AMPs toward host cells. The PEGylation of the synthetic AMP CaLL, designed to incorporate regions from cecropin A and LL-37, decreases AMP elicited cytotoxicity of epithelial cells in an *ex vivo* perfused lung model [169]. When considering designing novel therapeutics based on LL-37, future studies should be cognizant that inflammatory environments contain the enzymes PAD2 and PAD4, which cause the deamination (citrullination) of LL-37 arginine residues. These residue changes impair the ability of LL-37 to bind LPS and fail to prevent septic shock in D-galactosamine-sensitized mice injected with LPS. A modification to prevent residue citrullination could potentially increase LL-37 efficacy in inflammatory environments [170]. Modification or synthesis of AMPs that select for improved killing and reduced host cell damage may prove to be a beneficial therapeutic tool for a variety of bacterial diseases where other therapies have failed.

The combination of AMPs with antibiotics or vitamins can enhance bactericidal activity. The oral administration of vitamin 25D3, a vitamin D precursor, increases cathelicidin production in bladder cells exposed to UPEC [171]. In addition to vitamin D, parathyroid hormone (PTH) can act in a synergistic manner to increase cathelicidin expression. The addition of PTH to mouse skin reduces susceptibility to GAS infection. Further, this effect was diminished in mice on a vitamin D

restricted diet, suggesting an important role for the combination of PTH and vitamin D in pathogenesis [172].

Susceptibility of bacteria to AMP molecules varies widely based on both the bacterial species and the microenvironment of the disease. As already discussed, in the cystic fibrosis lung, high salt concentrations can diminish activity of some AMPs. SMAP29 and CAP18, however, are not susceptible to high salt concentrations and therefore are most effective at killing *P. aeruginosa*, *E. coli* and *S. aureus* strains. These AMPs may thus be attractive therapeutics for the remediation of bacteria in cystic fibrosis patients [173].

As antibiotic resistance increases, novel therapies are needed to treat bacterial infections. The combinatorial use of AMPs and antibiotics has proven effective. When combined with antibiotics, hBD-3 and LL-37 act in a synergistic manner to kill toxinogenic and non-toxinogenic *C. difficile* strains *in vitro* [174]. Similarly, α-helical AMPs in combination with antibiotics demonstrate additive killing against Gram-negative and Gram-positive bacteria *in vitro* [175]. Finally, the combination of the synthetic AMP GL13K and tobramycin prove more effective in eradication of *in vitro* grown *P. aeruginosa* biofilms than either GL13K or tobramycin alone [176]. The AMP PL-5 likewise synergized with levofloxacin hydrochloride in a mouse model of *S. aureus* infection [175]. The use of combinatorial therapies to treat biofilm-related diseases has proven effective. Colistin targets metabolically inactive *P. aeruginosa* located deep within the biofilm; whereas the antibiotics ciprofloxacin and tetracycline target the outermost subpopulation of the *P. aeruginosa* biofilm that are more metabolically active [177]. As bactericidal mechanisms differ between AMP and antibiotics, the combinatorial use will likely decrease the emergence of antibiotic resistant strains. The continued study of AMP interactions in the host throughout pathogenesis is vital for continued design of implementation strategies or novel AMP structures, which may provide treatment for burdensome bacterial diseases that present recalcitrance to traditional therapies.

6. Conclusions

As bacteria navigate through the human host they are confronted with host AMPs that threaten their survival (Figure 1). By reliance on suppression of AMP activity or mediation of AMP resistance factors, bacteria can persist to establish residency in a new microenvironment. The colonization of a foreign invader disrupts environmental homeostasis which results in the increased expression of host defense factors, including AMPs. As bacteria sense the presence of AMPs, virulence gene expression is increased to potentiate survival. These changes in gene expression increase bacterial persistence and can modulate disease severity. As bacterial residence is maintained, the environment continues to evolve as new AMPs are produced in response to a changing inflammatory response. Inflammation, nutrient status, and the host microflora influence the interplay between pathogenic bacteria and host AMPs. This complex network of interdependent interactions is in constant flux with slight changes in variables dramatically impacting the course of disease. Here, we have summarized a sampling of the complex interactions between bacteria and host throughout pathogenesis. A better understanding of mechanisms of host defense, production of environmental cues during infection, and the evolving host response to maintain bacterial

homeostasis in the face of foreign invaders intent on dysbiosis, will direct design of therapies to alleviate disease.

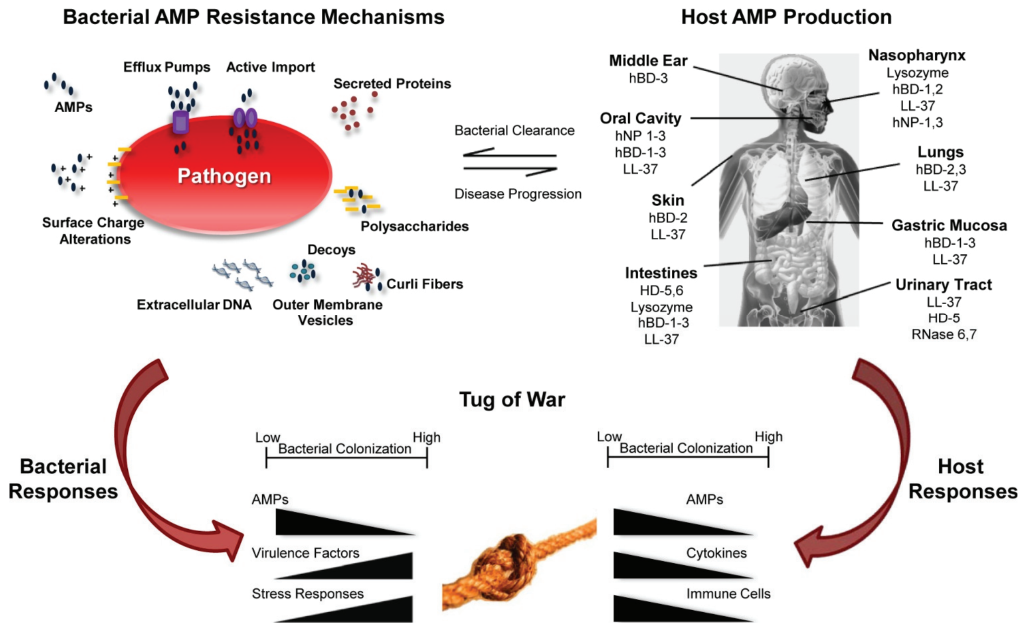

Figure 1. Summary of host-pathogen interaction as a consequence of AMP function. Bacterial AMP resistance mechanisms provide advantages to pathogens (**upper left**) leading to disease progression. The host produces AMPs to control bacterial growth leading to bacterial clearance (**upper right**). AMPs modulate gene expression by both host and pathogen resulting in a tug of war to shift the balance between health and disease.

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Author Contributions

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Conflicts of Interest

The authors declare no conflict of interest.

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The Role of Cationic Polypeptides in Modulating HIV-1 Infection of the Cervicovaginal Mucosa

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Abstract: The mucosa and overlying fluid of the female reproductive tract (FRT) are portals for the heterosexual transmission of HIV-1. Toward the ongoing development of topically applied microbicides and mucosal vaccines against HIV-1, it is evermore important to understand how the dynamic FRT mucosa is involved in controlling transmission and infection of HIV-1. Cationic peptides and proteins are the principal innate immune effector molecules of mucosal surfaces, and interact in a combinatorial fashion to modulate HIV-1 infection of the cervix and vagina. While cationic peptides and proteins have historically been categorized as antimicrobial or have other host-benefitting roles, an increasing number of these molecules have been found to augment HIV-1 infection and potentially antagonize host defense. Complex environmental factors such as hormonal fluctuations and/or bacterial and viral co-infections provide additional challenges to both experimentation and interpretation of results. In the context of heterosexual transmission of HIV-1, this review explores how various cationic peptides and proteins participate in modulating host defense against HIV-1 of the cervicovaginal mucosa.

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1. Introduction

According to the World Health Organization (www.who.int), by the end of 2013, HIV/AIDS had claimed nearly 40 million lives. Approximately 35 million individuals worldwide were living with HIV/AIDS, and 2.1 million people were newly infected in that year alone. Nearly three-quarters (24.7 million) of infected individuals reside in sub-Saharan Africa, an area that accounts for almost 70% of the global total of new HIV infections. Since the 1980s, the spread of HIV has shifted from male-to-male sexual contact and needle sharing, to a predominantly heterosexually transmitted disease with women becoming more likely to be infected than men. One-half of infected individuals worldwide are women, a percentage that rises to nearly 60% in sub-Saharan Africa and over 75% in the young population (<25 years of age) of that region. Antiretroviral therapy (ART) is on the rise, with 12.9 million people receiving ART globally. While the numbers of individuals receiving ART are encouraging, to fully control the pandemic it will be necessary to employ multiple tactics including pre-exposure prophylaxes, which have shown promise [1], and HIV vaccines, which even after three decades of research have been terribly elusive [2]. As the field endeavors to develop these strategies, it will be evermore important to understand how the female reproductive tract (FRT) immune system is involved in controlling heterosexually transmitted HIV-1.

Considering the number of individuals infected with HIV-1, the efficiency of heterosexual HIV-1 transmission is surprisingly low. In an updated, comprehensive analysis of aggregated primary data

regarding HIV transmission risk and modifying factors, Patel and colleagues estimated that the per-act HIV transmission risk for receptive uninfected females acquiring HIV-1 through penile-vaginal intercourse is 8 in 10,000 coital acts [3]. It is becoming evident that multiple physical, cellular and molecular mechanisms together contribute to keeping the incidence of transmission relatively low. The mucosal surfaces of the lower FRT, under healthy conditions are thought to act as efficient physical barriers to prevent cell-free and cell-associated HIV-1 from breaching the barrier and infecting underlying target $CD4^+$ immune cells within the FRT. Indeed, the vaginal mucosa is overlain by a non-keratinized, stratified squamous epithelium approximately 150–200 microns thick on average—nearly impenetrable by 0.12 micron HIV virions unless the barrier can be subverted or compromised (e.g., abrasion, trauma). Maturation and proliferation of the vaginal epithelium is under hormonal control, with the maximum thickness occurring during time periods that normally correspond with peak circulating levels of 17β-estradiol of the late follicular phase of the menstrual cycle [4]. This would therefore suggest that times of the menstrual cycle when circulating estradiol is lowest (e.g., end of luteal phase), and thus the vaginal epithelium is thinnest, might provide a window of opportunity for HIV-1 transmission.

The lower FRT is blanketed by commensal microbes, predominantly (but not exclusively [5]) Lactobacilli in healthy individuals, which are thought to play important roles in host defense of the vagina and ectocervix. Lactobacilli render the vaginal secretions acidic by metabolizing glycogen, released by vaginal epithelia, into lactic acid that exerts selective antimicrobial activity against nonresident microbiota [6]. Certain Lactobacilli also produce hydrogen peroxide, which is toxic to many microbes at the biological concentrations measured in vaginal secretions [7]. Less advantageous microbes, such as *Gardnerella vaginalis*, are also suppressed by natural antibiotic peptides produced by Lactobacilli, called “bacteriocins” [8–10]. As this review is focused on human-derived antibacterial peptides and proteins secreted into vaginal fluids, it should be noted that at least a portion of the intrinsic antimicrobial activity of this fluid is of microbial origin.

Ascending the FRT, the cervix transitions to a simple columnar epithelium, the pH of the overlying fluid normalizes, and very few microbes are present in healthy individuals. Using vaginal simian immunodeficiency virus (SIV) challenge in a rhesus macaque model [11], initial cervicovaginal infection was shown to occur in small clusters of susceptible target resting and activated T lymphocytes [12]. Clusters of SIV were routinely found in two primary regions of the FRT—the endocervix, and the cervical transformation zone between the endocervix and the ectocervix [12]. These regions are located in mucosal areas of rapid cellular turnover, have a single layer of columnar epithelium, and are populated with a high density of target $CD4^+$ cells, collectively providing evidence that the cervix is the primary site for initial HIV-1 infection [13]. Furthermore, from single genome amplification and sequencing of plasma virion RNA obtained from early stages of infection, it can be inferred that infection is acquired from a single founder virus in heterosexual transmission [14]. Innate processes that act as the first line of host defense against HIV-1 transmission are evermore important in preventing the establishment of this initial infection event. While other reviews and chapters have comprehensively described various aspects of innate immunity to HIV-1 infection and transmission in the FRT [15,16], this review specifically focuses on antimicrobial peptides and proteins and their role in preventing heterosexual HIV-1 infection and transmission.

2. Antiviral Peptides and Proteins of the FRT

Since the pioneering work of Sir Alexander Fleming in his discovery of lysozyme [17,18], and later work by James Hirsch on bactericidal histones [19], it has been known that humans have evolved various antimicrobial peptides and proteins as a first line of defense against microbial pathogens [20,21]. Most of these proteins and peptides are broad-spectrum antimicrobials, targeting gram-positive and gram-negative bacteria, fungi, and certain enveloped viruses such as HIV-1. Their mode of action can vary immensely, involving microbial membranolysis, enzymatic degradation of key microbial structural components, depletion of environmental nutrients essential for microbial growth, masking or down-regulation of receptors required for host cell entry, or modulating inflammation, adaptive immunity, and other functions related to host defense.

Although antimicrobial peptides and proteins can be structurally and evolutionarily diverse, there are common features that encompass most classes of molecules including overall net cationic charge at physiologic pH and amphipathic separation of polar and apolar residues [22]. These basic molecular features largely contribute to the membrane-active nature of most of these cationic peptides and proteins, with the cationic side groups binding to electronegative moieties on the microbial surface and the hydrophobic groups involved in membrane penetration, pore formation and lysis/dissolution. However, as is being increasingly recognized, other modes of action cannot be entirely explained by amphipathic sequestration and/or pore formation [23–26]. The canonical lytic pore mechanism is also in contrast to the anti-HIV-1 mechanism of action of many human-derived cationic peptides and proteins. Even though some directly affect the HIV-1 virion under certain conditions [27], many others interfere with one or more specific aspects of HIV-1's lifecycle. A number of antimicrobial peptides and proteins that are active against HIV-1 have been identified throughout the upper and lower FRT, which are discussed in the sections that follow. Importantly, while each peptide or protein has been shown to exert anti-HIV activity *in vitro* at supraphysiologic concentrations, within the cervicovaginal fluid the sum total of all of these components acting in concert is necessary for complete biological anti-HIV-1 activity [28]. Figure 1 provides a pictorial of major aspects of the HIV-1 lifecycle that are affected by cationic peptides and proteins of the FRT.

3. Defensins

Defensins comprise the most well-studied family of antimicrobial peptides, encompassing over 100 different peptides with a β-sheet structure, expressed by epithelia and leukocytes of many mammals and birds [29–31]. There are three main classes of defensins—α, β and θ—subcategorized primarily based on the disulfide bonding patterns of their six cysteine residues. In humans, α-defensins can be further divided into four peptides that are stored in neutrophil granules (human neutrophil peptides 1–4; HNP1–4), and two peptides that are inducible and mostly of epithelial origin (human defensins 5 and 6; HD5 and HD6).

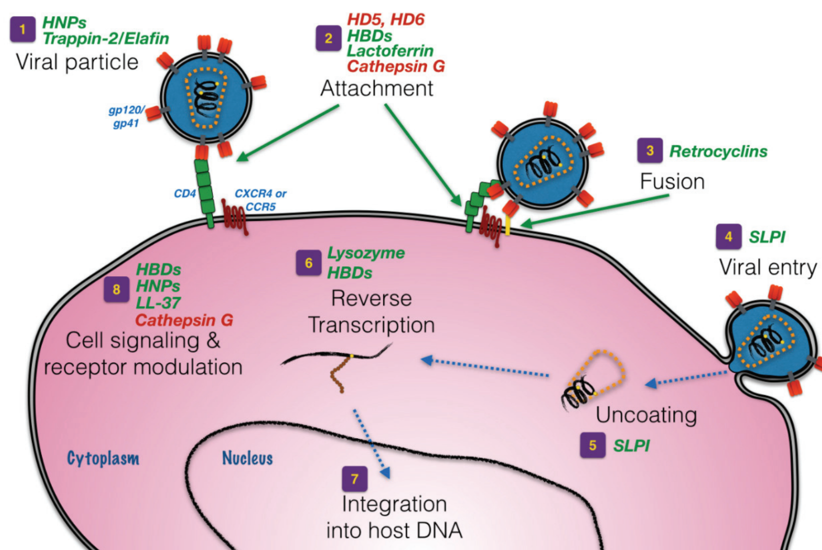

Figure 1. Anti-HIV-1 mechanisms of action of cationic peptides and peptides of the female reproductive tract (FRT). Depicted is the lifecycle of HIV-1 infecting a target CD4^{+} cell, beginning from a free virion ("1") to integration of viral cDNA into genomic DNA of the target cell ("7"); "8" indicates other aspects, including receptor downmodulation and cell signaling, that indirectly affect the ability of the virus to infect or propagate within host cells. Cationic peptides and proteins in green font are antiviral at the respective stage in the lifecycle, while peptides and proteins in red font promote HIV-1 infection. Viral envelope proteins (gp120, gp41), cellular receptor (CD4) and coreceptor (CXCR4 or CCR5) required for viral attachment and entry are provided. Events downstream of viral cDNA integration into host DNA are not depicted.

HNPs are synthesized as 93- to 94-residue prepropeptides, each of which is sequentially processed to liberate a signal peptide and an anionic propiece [32,33]. The active, mature peptides are packaged principally within azurophil granules of neutrophils, where they comprise nearly 30% of the granules' total protein content [34]. While most of the HNPs are discharged into phagocytic vacuoles where they reach millimolar concentrations, recent evidence suggests a potential extracellular arm of HNP-mediated host defense through constitutive exocytotic release of unprocessed pro-HNPs from neutrophils [35]. The first mention of the anti-HIV-1 activity of an antimicrobial peptide was reported in a short correspondence to the journal *AIDS* in 1993, whereby α-defensins from rats, guinea pigs and rabbits were shown to reduce HIV-induced cytopathogenicity of a CD4^{+} T lymphocytic cell line [36]. Monell and Strand then revealed similarities in the structure of the looped motifs from the fusogenic envelope protein gp41 of HIV-1 and α-defensins [37], pointing towards potential entry or fusion mechanisms of inhibition. Extending these findings, α-defensins were found to be directly virulytic as well as inhibit HIV-1 replication by interfering with the activity of protein kinase C [27]. In one study of healthy women, low levels (high nanogram/mL) of these α-defensins

have been found in the cervicovaginal fluids [6]. As α-defensins are a marker of neutrophil influx, even low concentrations of these peptides might reveal subclinical inflammation as a constitutive host defense mechanism. Conversely, inflammation-induced recruitment of additional target cells to the area might predispose to increased susceptibility to HIV-1 infection.

Originally isolated from the Paneth cells of the small intestinal Crypts of Lieberkuhn [38–40], the α-defensins HD5 and HD6 are stored in secretory granules as inactive peptide precursors until extracellularly released and proteolytically activated by trypsin [41]. HD5 and HD6 have classically been categorized as broad-spectrum antimicrobials; however, these two peptides play interesting and contrasting roles in the FRT. An elegant study that comprehensively explored HD5 in the FRT determined that this peptide immunolocalized to vaginal and ectocervical epithelium, the granules within the columnar epithelium of the endocervix as well as the surface of the endocervix [42]. Even though all other human defensins have been shown to inhibit HIV-1 infection, HD5 and HD6 instead promote infection by enhancing HIV-1 attachment to target cells [43]. In this light, one might speculate whether these peptides are (co-)determinants of the cervix being the initial site for primary HIV-1 infection. HD5 expression is also modulated during the menstrual cycle, with maximal expression during the secretory phase [42]. The relatively recent theme of hormonal regulation of peptides that augment HIV-1 infection might provide unique circumstances by which HIV-1 can subvert innate antiviral defenses of the FRT.

θ-Defensins are 18 residue peptides derived from two nonapeptide precursors, which are fused in a head-to-tail fashion and rendered macrocyclic through ligation of the resulting amino and carboxyl termini [44]. Humans and nonhuman primates produce α- and β-defensin peptides; however, only select nonhuman primates produce θ-defensin peptides [44–46]. In humans, although θ-defensin mRNA transcripts are produced within many cells and tissues, a premature termination codon near the end of the signal sequence precludes translation. Human θ-defensin genes, called retrocyclins, are nearly 90% identical at the nucleotide level as compared to the intact rhesus macaque θ-defensin genes [44–46]. It is remarkable that even though retrocyclin genes are located in areas of the genome that are highly polymorphic [46,47], aside from the premature termination codon, they have been so highly conserved evolutionarily over more than 35 million years. This begs questioning the potential contemporary role of such a gene, for example whether the premature termination codon is not a true “stop”, but rather a “yield” that can be activated by an unknown molecular process. In support of this conjecture is that while under normal circumstances retrocyclin peptides have not been recovered from human cells, promyelocytes and vaginal cells and organotypic tissue constructs can be chemically coaxed to produce bioactive retrocyclins, revealing that at least the cellular machinery necessary to process these cyclic peptides remains intact in humans [48].

What we understand about retrocyclin bioactivity has occurred through the analysis of retrocyclins produced by solid-phase and other chemical syntheses. Retrocyclins are remarkably active against a broad spectrum of microbes, and were found to be particularly antiviral against herpes simplex viruses [49], influenza [50], and HIV-1 strains representing most known groups and clades [45,51,52]. Retrocyclins inhibit the ability of HIV-1 to enter target $CD4^+$ cells regardless of coreceptor tropism [53], by interfering with the six-helix bundle fusogenic complex of the HIV-1 envelope glycoprotein gp41 [54]. Given its macrocyclic nature, retrocyclins are very stable peptides,

which are resistant to exoproteases, a wide pH range, high temperatures (e.g., boiling, 10 min), and other degradative environments ([55,56], and A.M.C. unpublished). Due to these beneficial properties and broad activity against primary HIV-1 isolates from many worldwide clades, retrocyclins are promising topical vaginal microbicides to prevent heterosexual transmission of HIV-1 [57].

Human β-defensin (HBD) peptides are predominantly produced by epithelia and although some are constitutively expressed, many are induced by inflammatory or microbial stimuli [29,58]. HBD1–3 are expressed ubiquitously by most epithelial surfaces, while HBD4–6 appear to be more restricted to the testes and gastric antrum (HBD4) and epididymis (HBD5–6). Although HBD1 is produced at constitutively low levels throughout the body (low nanograms/mL), the highest levels of HBD1 are found in tissues of the urogenital tract, including the kidney, vagina and cervix [59], at concentrations (low-to-mid micrograms/mL) likely sufficient to contribute to antimicrobial host defense [60]. HBD1–6 are broadly active against many bacteria, fungi and viruses, and in particular HBD1–3 have been show to inhibit HIV-1 infection [61]. HBD2 and HBD3 can inhibit HIV-1 replication by down-modulating expression of CXCR4 [62], or by HBD3 antagonizing CXCR4 [63], the cellular coreceptor required for entry of X4 tropic HIV-1 into $CD4^{+}$ cells. In vaginal fluid and cervical mucus plugs, HBD2 is present at concentrations (nanograms/mL) below the amount thought to be essential for effective direct anti-HIV-1 activity (low micrograms/mL) [6,60]. However, those concentrations are within the range that could impart other immunological functions. For example, HBD2 is a natural ligand for cells elaborating the chemokine receptor CCR6 such as a potential target of HIV-1, $CD45^{Ro+}$/$CD4^{+}$ T cells, as well as immature dendritic cells [64]. HBD2 and HBD3 have also been reported to chemoattract cells expressing CCR2, including macrophages, monocytes and neutrophils [65]. While unknown for cells within the FRT, as described for oral epithelial cells HIV-1 can induce the expression of HBD2 and HBD3, but not HBD1 [62]. Although β-defensins might not be directly participating in antiviral host defense, their presence and activation might attract additional cellular targets for HIV-1.

4. Whey Acidic Protein (WAP) Motif-Based Proteins

Secretory leukocyte protease inhibitor (SLPI) and Trappin-2/Elafin are members of the whey acidic protein (WAP) family [66,67], ascribed primary anti-inflammatory functions of inhibiting proteases including proteinase-3 and neutrophil elastase from neutrophils [68]. SLPI and Trappin-2/Elafin are secreted into overlying mucosal fluids, and both proteins exhibit antimicrobial activity (reviewed in [67]). Reports of the intrinsic anti-HIV-1 activity of SLPI have been mixed. High nanomolar concentrations of SLPI were reported to block HIV-1 entry or capsid uncoating independent of the protease inhibitor function of SLPI [69,70]. Another study suggested that the anti-HIV-1 activity of SLPI was likely due to artifact as even extremely high concentrations (1000 μg/mL) were not active against HIV-1 [71]. Evidence providing further support of an anti-HIV-1 role for SLPI has been through clinical correlative studies. Increased SLPI concentrations within vaginal fluid were associated with reduced rates of perinatal HIV-1 transmission [72], an association that was not observed for other cationic antimicrobial proteins or peptides. SLPI has also been shown to be decreased in women suffering from various sexually transmitted infections, and

these reduced levels may predispose women to HIV-1 and other infections [73]. Perhaps the anti-HIV-1 activity of SLPI is best realized in concert with other endogenous antivirals.

In an elegant study, Ghosh and colleagues revealed that epithelia of the upper and lower FRT produce constitutive amounts of Trappin-2/Elafin protein and mRNA [74], further supporting findings that Trappin-2/Elafin is produced by the cervical glandular epithelium during pregnancy [75]. Interestingly, only the uterine cells of the upper FRT could upregulate Trappin-2/Elafin when stimulated with a double-stranded RNA mimic, Poly(I:C). This group further explored the direct anti-HIV roles of Trappin-2/Elafin against X4 tropic and R5 tropic HIV-1, revealing dose-dependent direct activity against HIV-1 virions [74]. Additional studies provided further support for the role of Trappin-2/Elafin in innate anti-HIV-1 host defense. CVL from HIV-negative individuals contained higher amounts of Trappin-2/Elafin than HIV-infected patients. Similar to other cationic antimicrobial peptides and proteins, Trappin-2/Elafin expression is likely under hormonal control as the concentration of this protein in CVL was significantly higher during the secretory phase of the menstrual cycle as compared to the proliferative phase [74].

5. Other Anti-HIV Peptides and Proteins

Cathelicidins are a family of very diverse antimicrobial peptides that each share a common amino-terminal cathelin propiece, which is similar to the thiol protease inhibitor cystatin [76]. Even though pigs, cows, and other animals contain numerous different cathelicidins, humans are endowed with only one cathelicidin called human cationic protein of 18 kDa (hCAP18) [77,78]. Depending on the cellular or histological environment, hCAP18 can be preteolytically cleaved into the mature, active forms LL-37, ALL-38, and FALL-39. These three peptides are between 37 and 39 amino acids in length and differ only by their amino-terminal phenylalanine (F), alanine (A), and/or leucine residues (LL) [79–81]. LL-37, the most common mature form of hCAP18, is found in neutrophils and expressed by many epithelia including the mucosa and integument. Aside from direct antimicrobial mechanisms, LL-37 can also exhibit chemotactic, immunomodulatory and angiogenic effects that are all mediated by antagonistic binding of N-formyl peptide receptor 2 (FPR2), a G-protein coupled receptor. LL-37 was recently shown to inhibit HIV-1 replication using this mechanism, by binding to FPR2 which in turn down-regulated chemokine receptors necessary for HIV-1 entry in primary $CD4^+$ T cells [81]. In the FRT, hCAP18 has been immunolocalized to the upper epithelial layers of inflamed ectocervix in a band-like pattern [82]. Under healthy conditions, LL-37 is present in vaginal fluid at concentrations (mid-to-high nanograms/mL) [6] required to act on FPR2 and inhibit HIV-1 replication [81].

Due to structural and functional similarities to several antimicrobial peptides, peptide fragments of histones have also been implicated in the host defense of mucosal surfaces [83,84]. Histones and the related protamines are particularly well-endowed with basic amino acids, and thus their general microbicidal activities are likely related to electrostatic attraction to anionic microbial surfaces. However, the anti-HIV-1 activity of histones appears to be quite distinct from direct membranolytic action. Ubiquitinated histone 1B has been identified as an HIV-resistant factor, possibly regulating viral expression and secretion from $CD4^+$ T cells [85]. Although histones are present in the FRT [28], it remains to be determined whether histones have a true antiviral host defense role in this environment.

Larger cationic proteins are also components of human cervicovaginal fluids, and contribute to the collective anti-HIV-1 activity of the FRT. Lysozyme is a cationic 14.6 kDa enzyme whose primary bacteriolytic properties result from cleaving peptidoglycan between N-acetyl muramic acid and *N*-acetyl-D-glucosamine. Lysozyme also exhibits non-enzymatic properties that likely result from its electrostatically charged surface, which enable the protein to disrupt membranes and activate bacterially derived autolytic enzymes [86–88]. Alternative mechanisms of action extend to lysozyme's ability to inhibit HIV-1. Lysozyme purified from human neutrophils, breast milk, and β-core human chorionic gonadotropin preparations could lower the ability of HIV-1-infected primary T lymphocytes and monocytes to produce virus [89], potentially by directly binding to viral RNA [90]. Peptide fragmentation and activity mapping of human lysozyme revealed that a core nine-residue peptide derived from lysozyme exhibited much greater activity against HIV-1 (IC_{50} 50 nM) than the intact protein, and acted to prevent viral entry [91]. While the nonapeptide has not been isolated from biological cells or fluids, its cleavage sites suggest that trypsin or related human proteases could function to liberate this highly active lysozyme-derived anti-HIV-1 peptide *in vivo*.

Cathepsin G, a neutrophil-derived serine protease that is present in human CVF [28], has been reported to bind the HIV-1 envelope protein gp120 [92,93], and can promote HIV-1 infection of macrophages, but not $CD4^+$ T lymphocytes [94]. The mechanism of this antiviral activity likely requires G_i protein-mediated signal transduction, as treatment of cells with pertussis toxin abrogated the enhancement of HIV-1 infection of macrophages [94]. Interestingly, prolonged exposure of macrophages to cathepsin G suppressed HIV-1 infection, an effect that was neutralized by the addition of serine protease inhibitors [94]. Cathepsin G has also been reported to generate truncated variants of the chemokine RANTES, which exhibited lower binding to CCR5 and reduced antiviral activity [95]. Taken together, these studies suggest a multifactorial role for cathepsin G in enhancing HIV-1 infection.

Lactoferrin is an approximately 78 kDa basic protein, similar in structure and function to the iron-carrier protein transferrin. Lactoferrin can directly and indirectly inhibit HIV-1 by binding to the V3 loop of the HIV-1 envelope glycoprotein gp120, preventing adsorption of the virus to the surface of target cells [96,97]. Although the concentrations of lactoferrin and lysozyme are low in human vaginal fluid (1–13 μg/mL), they are extremely high (100–1000 μg/mL) in the cervical mucus plug [6,60]. Although the anti-HIV-1 activities for both lysozyme and lactoferrin are modest *in vitro*, it may be within the cervical mucus plug where their antiviral host defense properties are best realized.

6. Regulation of Cationic Peptides and Proteins in the FRT

Deficiencies in the production of antimicrobial peptides, including activation, release, and/or concentration, have been implicated in the pathogenesis of inflammatory or infectious conditions. Windows of opportunity likely arise in which HIV-1 transmission and infection in the FRT are increased due to mechanisms that enable the virus to subvert innate antiviral host defenses. While multiple components of innate and adaptive immunity are likely involved, this review is centered on how cationic antimicrobial peptides/proteins are modulated, and in the FRT, there are at least three principal strategies in which this regulation occurs: hormonal, microbial, and proteolytic. While earlier studies have suggested that there is little change in the expression of antimicrobial peptides

and proteins in the cervicovaginal fluid throughout the menstrual cycle [6], more recently it has been shown that concentrations of HNP1–3, SLPI, lysozyme, lactoferrin, and HBD-2 are all highest during the proliferative phase and to a lesser extent the secretory phase (reviewed in [98]). Oral contraceptives can also alter the expression of a number of peptides and proteins in cervical mucus, including lysozyme [99]. The regulation of defensin HD5, a cationic peptide that enhances HIV-1 infection, is under hormonal control, with maximal expression during the secretory phase [42].

For all studies that measure the concentration of antimicrobial peptides and proteins from lower FRT fluids, the method of collection (lavage, tampon, swab, diaphragm) has a large influence on the amounts and even types of recovered peptides and proteins. This is one reason (of many) why the field has only a coarse understanding of the regulation of antimicrobial peptides in the FRT, as each method of collection has its own merits and detractions. It has yet to be determined which fluid recovery technique would be best suited for the majority of applications and conditions, but it will be important that the field soon adopts a unified approach to reduce inter-study variability.

Sexually transmitted infections of the FRT, such as genital herpes, and microbial-shift conditions, including bacterial vaginosis, have been associated with an increase in the risk of acquiring HIV-1 [100], as well as modulating the expression of cationic peptides and proteins. For example, in HIV-exposed seronegative women in HIV-serodiscordant relationships, the levels of HNP1-3 and LL-37 were directly associated with the partner's viral load [101]. Selective depletion of cationic peptides and proteins from the cervicovaginal fluids rendered the remaining fractions inactive against HIV-1 [101], supporting the notion that these peptides are major components of innate antiviral host defense. *Neisseria gonorrhoeae*-induced HD5 and HD6 can increase HIV-1 Infectivity [102], which is not surprising since HD-5 is known to promote HIV-1 infection through increasing viral attachment to target cells [43]. Interestingly, in other co-infections, the presence of certain antiviral cationic peptides and proteins suggest roles that run counter to preconceived notions of antiviral defense. LL-37, produced by HSV-2-infected keratinocytes, was reported to upregulate the expression of HIV-1 receptors in monocyte-derived Langerhans cells, enhancing their HIV susceptibility—an effect that could be blocked by inhibiting LL-37 production [103]. While cervicovaginal levels of Trappin-2/elafin are diminished during BV [75], up to 200-fold greater concentration of α-defensins were found in the cervicovaginal fluids of women during frank BV [104]. In a study that collected cervicovaginal fluids from highly HIV-exposed, uninfected Kenyan sex workers, cervicovaginal levels of α-defensins and LL-37 were associated with increased HIV acquisition, which was likely due to sexually transmitted bacterial infections [105].

Proteolytic activation is now recognized as an important mechanism to regulate proteins in the FRT, which modulate HIV-1 infection. In an exciting study by Sorensen and colleagues, following heterosexual intercourse, the human cathelicidin hCAP18 was cleaved into the ALL-38, a peptide that retained complete biological activity as compared to LL-37 [80]. An interesting twist is that the enzyme responsible for this activation was the prostate-derived protease gastricsin, which is present in semen but not in vaginal fluid. Under the slightly basic pH of semen, gastricsin is not able to cleave hCAP18. However, upon incubation with low pH buffers *in vitro*, or contact with the acidic milieu of the vagina *in vivo*, gastricsin was activated and process hCAP18 into ALL-38. Although ALL-38 itself has not been tested against HIV-1, given that all biological tests performed confirm its

equivalent potency to LL-37 [80] and that LL-37 can inhibit HIV-1 replication [81], it reasons that gastricsin-mediated activation of hCAP18 represents a novel mechanism to prevent HIV-1 infection following sexual intercourse. As with all cationic antimicrobial peptides and proteins of the cervicovaginal mucosa, it is important to consider that antiviral activity of the FRT is highly dependent on the majority of these molecules acting together, and even slight dysregulation can result in increased susceptibility to HIV-1 transmission and infection [28].

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Amy Liese Cole and Alexander M. Cole wrote and edited this review article.

Conflicts of Interest

The authors declare no conflict of interest.

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NETs and CF Lung Disease: Current Status and Future Prospects

Robert D. Gray, Brian N. McCullagh and Paul B. McCray, Jr

Abstract: Cystic Fibrosis (CF) is the most common fatal monogenic disease among Caucasians. While CF affects multiple organ systems, the principle morbidity arises from progressive destruction of lung architecture due to chronic bacterial infection and inflammation. It is characterized by an innate immune defect that results in colonization of the airways with bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* from an early age. Within the airway microenvironment the innate immune cells including epithelial cells, neutrophils, and macrophages have all been implicated in the host defense defect. The neutrophil, however, is the principal effector cell facilitating bacterial killing, but also participates in lung damage. This is evidenced by a disproportionately elevated neutrophil burden in the airways and increased neutrophil products capable of tissue degradation, such as neutrophil elastase. The CF airways also contain an abundance of nuclear material that may be originating from neutrophils. Neutrophil extracellular traps (NETs) are the product of a novel neutrophil death process that involves the expulsion of nuclear material embedded with histones, proteases, and antimicrobial proteins and peptides. NETs have been postulated to contribute to the bacterial killing capacity of neutrophils, however they also function as a source of proteases and other neutrophil products that may contribute to lung injury. Targeting nuclear material with inhaled DNase therapy improves lung function and reduces exacerbations in CF and some of these effects may be due to the degradation of NETs. We critically discuss the evidence for an antimicrobial function of NETs and their potential to cause lung damage and inflammation. We propose that CF animal models that recapitulate the human CF phenotype such as the *CFTR*$^{-/-}$ pig may be useful in further elucidating a role for NETs.

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1. Introduction

Cystic fibrosis is a multisystem disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which codes for a phosphorylation and nucleotide activated anion channel, resulting in altered transcellular chloride and bicarbonate transport. In excess of 85% of people with CF will die prematurely from respiratory complications, in spite of recent therapeutic advances [1]. The exact mechanisms leading to lung destruction and progressive respiratory failure have not been fully elucidated but are in part the result of a host defense defect that impairs bacterial killing, abnormal mucociliary transport, and a dysfunctional innate immune response to infection [2]. These mechanisms contribute to airway colonization with bacteria such as *Staphylococcus aureus* and *Haemophilus influenzae* early in life and with *Pseudomonas aeruginosa* through later childhood and adolescence [3].

2. Investigating Innate Immunity in CF Using Animal Models

The lung innate immune system includes airway epithelial cells, neutrophils, macrophages, and a vast array of proteins and peptides produced by these cells. Understanding how innate immunity is altered in CF is key to understanding the onset and progression of lung disease in early childhood. Progress has been hampered by the lack of an animal model that mirrors the lung disease features of people with CF. For example, *cftr*$^{-/-}$ mice developed following the discovery of CFTR lack key features of lung disease seen in the human [4]. The advent of the CF pig has facilitated advancements in our understanding of CF lung disease as, like man, it develops a spontaneous lung disease phenotype with spontaneous bacterial colonization and associated innate immune dysfunction when CFTR function is lost [5,6]. CF pigs also display defective mucociliary transport and abnormal mucous properties [7,8], both features of early CF lung disease in humans. Furthermore, ferrets with loss of CFTR function also develop lung disease with similarities to the human disease [9].

3. Airways Surface Liquid (ASL) Antimicrobial Peptides in CF

ASL is a first line of defense against potential pathogens and contains secreted products of surface and submucosal gland epithelia. ASL is comprised of a mucous layer that traps inhaled or aspirated organisms and an aqueous layer in which cilia beat. ASL acts as a physical barrier to microorganisms but also contains numerous antimicrobial proteins and peptides (AMPs) to combat potentially harmful bacteria, fungi, and viruses [10]. These include lysozyme, lactoferrin, cathelicidins, β-defensins, secretory leukocyte protease inhibitor (SLPI), and the collectins surfactant proteins A and D (SP-A and SP-D). These multiple AMPs interact in an additive or synergistic fashion to inactivate pathogens [11]. ASL pH is in part regulated by HCO_3^- secretion via CFTR; loss of CFTR function leads to acidification of ASL, a feature reported in primary cell culture of epithelial cells [12] and submucosal glands [13], as well as airways breath condensate from CF patients [14], and more recently in the CF pig [15]. Low (acidic) pH impairs the function of several antimicrobial peptides such as lysozyme, lactoferrin, cathelicidin LL37, SP-A and -D, and SLPI [10]. ASL acidification in the CF pig impairs bacterial killing at birth, and raising ASL pH in CF pig airways reverses this defect. Conversely, the antimicrobial activity of ASL of non-CF pigs was impaired by lowering the pH. *In vitro* studies with individual AMPs, including lactoferrin and lysozyme, demonstrated that the factors antibacterial properties were pH dependent. Together, these studies provide *in vivo* evidence of a primary bacterial killing defect due to impaired AMP function in a low pH environment [15].

4. Neutrophils and Antimicrobial Defense in CF

ASL also contains resident immune cells that act as a first line of host defense against inhaled pathogens, with the neutrophil being of primary importance as a phagocyte. Neutrophils are recruited to the lungs as part of the innate immune response to microbes (Figure 1). The airways of infants and children with CF are characterized by a disproportionately elevated neutrophil burden either attributed to early microbial challenge [16–18] or, in the absence of microbes, to dysfunctional CFTR trafficking [19–21]. In spite of this response, bacteria such as *Staphylococcus aureus*, *Haemophilus*

influenzae, and *Pseudomonas aeruginosa* persist in the airways and establish chronic infection. Neutrophils have granules that contain a wide array of products such as proteolytic enzymes, AMPs, myeloperoxidase (MPO), and peptides that degrade bacteria following phagocytosis. These products may also be released by degranulation to assist in the extracellular killing of organisms [22]. Phagocytosis is a primary function of neutrophils and is composed of three processes: (1) receptor mediated pathogen uptake into vacuoles (phagosomes); (2) production of reactive oxygen species in the vacuole (later utilized by MPO to form hypochlorous acid); and (3) the fusion of granules containing proteases and antimicrobial mediators to these vacuoles (the phagolysosome) resulting in destruction and digestion of pathogens [22]. There is evidence to suggest that microbicidal function of neutrophils is impaired in CF as a result of reduced chloride anion availability for the production of hypochlorous acid, an essential mediator in the destruction of pathogens in the phagolysosome [23]. Phagocytosis typically accelerates neutrophil apoptosis; in turn, macrophages recognize and phagocytose the aging neutrophil which ultimately promotes an orderly resolution of inflammation [24,25]. Neutrophils also release proteases and AMPs by degranulation with extracellular levels of these proteins increasing as the neutrophil burden increases in the lung [26]. AMPs produced by neutrophils include lysozyme, phospholipase A2, bacterial permeability increasing protein (BPI), lactoferrin, cathelicidins such as LL37, and the α-defensins or human neutrophil defense peptides 1–4 (HNP). In CF the proteolytic degradation of AMPs can further reduce their effectiveness and exacerbate local tissue damage [27,28].

Neutrophil proteases have the potential to damage the host if present extracellularly instead of targeted to the phagolysosome. Neutrophil elastase (NE), a serine protease contained in azurophilic (primary) granules, can degrade nearly all structural proteins of the lung [29], as well as reduce ciliary motility and facilitate bacterial colonization [16]. NE is present early on in the airways of infants and young children with CF, and serves as a predictor of disease progression and is associated with lung function decline, as reported in the ARREST CF study [30]. The quantity of NE released into CF airways overwhelms the anti-protease capacity, leading to detectable NE activity and local architectural destruction. Therefore, a paradox exists in CF whereby an abundance of resident antimicrobial material, including AMPs and proteases, has the potential to damage the host. Moreover, in spite of the abundance of antimicrobial products, chronic lung infection is the characteristic phenotype in people with CF.

5. Neutrophil Extracellular Traps and CF

Neutrophil extracellular trap (NET) formation was described in 2004 by Brinkmann *et al*. [31] and has rapidly gained prominence in the literature as an antibacterial defense mechanism employed by neutrophils to kill microorganisms (by a mechanism distinct from phagocytosis or degranulation). Now termed NETosis, the process of NET formation is characterized by neutrophils undergoing an oxidative burst and releasing webs of decondensed DNA complexed to peptides and proteins, accompanied by cell death [32]. NETs have been described in CF sputum by a number of investigators [33–36], and recently it has been suggested that the majority of extracellular DNA in the CF lung is derived from NETs [33]. Oxidative burst and NADPH oxidase activation are thought to be central mechanisms in NET formation [32] and both MPO and NE are deemed essential

co-factors [37,38]. The oxidative burst ultimately leads to downstream activation of peptidyl dearginase 4 (PAD4) which translocates to the nucleus and modifies histones (H3 and H4) by (hyper)citrinulation which contributes to nuclear decondensation and NET formation [39]. This is supported by evidence that neutrophils from PAD4$^{-/-}$ mice do not make NETs, and furthermore PAD4$^{-/-}$ mice are more susceptible to challenge with bacteria [40]. Neutrophil elastase complexes are involved in the decondensation of chromatin and consequently NET formation can be inhibited by neutrophil elastase inhibitors [38]. Furthermore, recent evidence suggests that MPO is an important co-factor in elastase release from granules and its proteolytic activity during NET formation [41]. Additional evidence implicates autophagic activity in NETosis as an essential partner to the oxidative burst demonstrating that inhibition of either system stopped NETosis. Indeed the promotion and inhbition of autophagy have now been demonstrated to alter NET formation in a number of studies [42,43]. The normal fate of a neutrophil is to perform its role in host defense and die by apoptosis. Why neutrophils (responding to infection) may favor a radically different form of cell death than apoptosis is intriguing and an additional hypothesis proposes that CF neutrophils do not undergo apoptosis or NET formation but rather switch to an alternate "hyperexocytosing" phenotype and release their primary granule content including MPO and NE into the airway lumen by enhanced exocytosis [44–46]. Therefore, further investigation of the process and relevance of NET formation in CF is required.

Initial studies demonstrated NE, MPO, and histones as the main proteins associated with NETs [31,32,47]. Further proteomic studies have revealed a major presence of AMPs on NETs with lysozyme, lactoferrin, HNPs, and calprotectin all demonstrated as NET constituents [48,49], adding to the hypothesis that NETs play a role in bacterial killing. Histones are also believed to have a major role as an antimicrobial on NETs [47]. Cathelicidins, although not identified in proteomic studies of NET formation have nevertheless been described as NET proteins [50,51]. Therefore, NETs could be a rich source of AMPs in the CF airway, but the functional significance of this has yet to be elucidated, particularly in the context of chronic infection.

6. NET Formation and Killing in Response to Bacteria

A number of groups have demonstrated the ability of *Pseudomonas aeruginosa* to induce NET formation, with reference to CF lung disease [33,36,52–54]. Interestingly, two groups independently demonstrated that NET induction using clinical isolates of *Pseudomonas* results in a divergent response, namely isolates from early disease induce NETs but isolates from the same patients in later disease do not [36,53]. Furthermore pyocyanin, a bacterial toxin produced by *Pseudomonas* can induce NET formation [55] but can also induce neutrophil apoptosis [56]. These observations require further investigation to help us understand the temporal relationship between *Pseudomonas* and NET production and why certain toxin challenges such as pyocyanin can result in disparate outcomes for neutrophils.

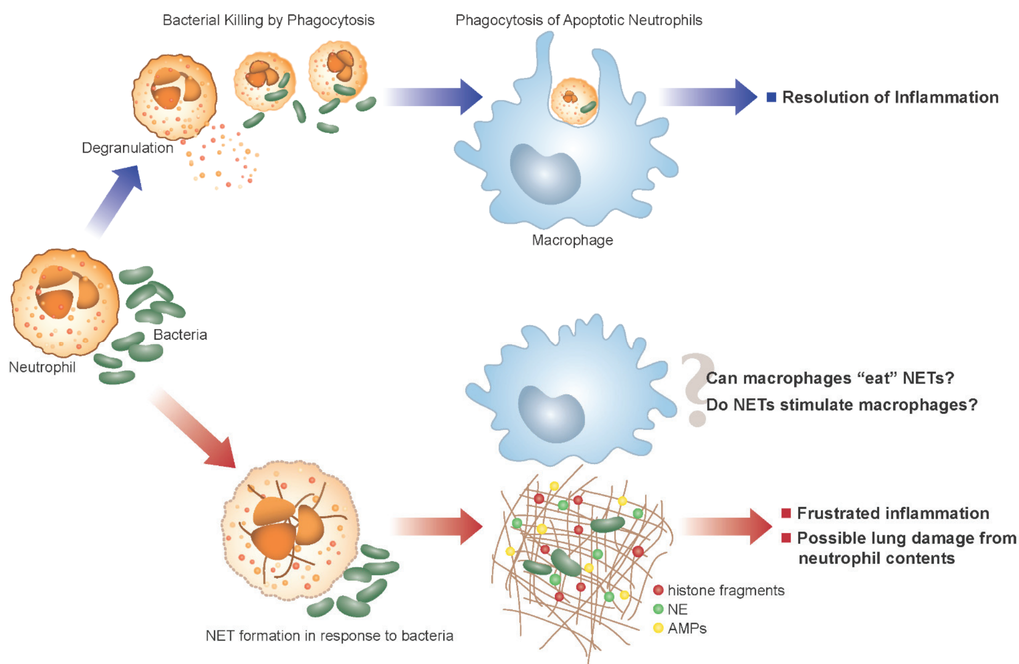

Figure 1. Alternative fates of Neutrophils in the Cystic Fibrosis (CF) lung. In the normal lung (purple arrows/top panel), neutrophils encounter and phagocytose bacteria. Following phagocytosis neutrophils rapidly undergo apoptosis and clearance by macrophages thus promoting resolution of inflammation. Alternatively, the excess of bacteria in the CF airway (red arrows/bottom panel) may lead to neutrophils forming neutrophil extracellular traps (NETs) in addition to normal phagocytosis. NETs may contribute to host defense, but also allow the release of toxic components into the airway that can damage the host lung. We can postulate that NETs contribute to the failed resolution of inflammation in the CF lung, and the clearance of NETs by macrophages may not be as anti-inflammatory as the clearance of apoptotic neutrophils.

The ability of NETs to kill bacteria following their induction remains controversial. Early reports demonstrated that neutrophils, pre-activated with cytokines or phorbol ester to form NETs, could subsequently trap and kill pathogens in a manner independent of phagocytosis [31,57]. Young and colleagues demonstrated that killing of *Pseudomonas* did occur, even without the pre-activation of neutrophils. However, this NET-mediated killing only accounted for a small proportion of total bacterial killing compared to phagocytosis, unless the experiments were performed with neutrophils in suspension; the effect was lost if neutrophils were cultured under standard conditions [36]. Additionally, they demonstrated that the ability to kill *Pseudomonas* was lost when patient isolates from later stages in disease were used, suggesting this pathogen develops resistance to NET-mediated killing as CF lung disease advances. This finding was further reinforced by Dwyer and colleagues who demonstrated a decreased ability of NETs to kill mucoid strains of

*Pseudomona*s [33]. Other reports suggest that NETs may not be as potent in killing as initially suggested. Mengazzi et al reported that NETs trap rather than kill microbes, with the majority of NET bound bacteria being live when liberated by DNase treatment [58], which is in keeping with a previous observation that NETs alone were not sufficient to kill bacteria and needed the addition of exogenous hydrogen peroxide to be truly bacteriocidal [59]. Nevertheless the interaction of *Pseudomonas* and NETs is an area of great interest in CF and whether this process is central to the development of lung disease or indeed involved the pathoadaptation of *Pseudomonas* in the CF airway will require further investigation as reviewed elsewhere recently [60].

To date, the majority of studies linking NETs to CF have concentrated on the interaction of neutrophils with *Pseudomonas*, however the ability of *S. aureus* to induce NET formation has been studied and confirmed [52,61–63]. *S. aureus* is a major pathogen in early CF lung disease, with colonization often pre-dating *Pseudomonas* acquisition by years. Furthermore, the ability of *S. aureus* to degrade NETs and escape specific protease activity has been described [61,64]. This could be significant in early CF lung disease if indeed killing of S. *aureus* by NETs was inefficient. Thus, further work utilizing CF isolates of *S. aureus* to induce NETs and assess killing capacity are required, particularly in models of early disease.

7. Potential Damaging Consequences of NETs in CF

CF sputum contains large amounts of NETs when measured by biochemical assays, and these are decorated with antimicrobial peptides, neutrophil proteins, and proteases [33]. Therefore, it is somewhat surprising that (in spite of these structures) chronic infection persists. This observation suggests that the innate antimicrobial properties of NETs may be over emphasized in the context of CF lung disease. The presence of large amounts of DNA in the CF airways and its potential contribution to CF pathogenesis was first acknowledged in the 1950s [65]. However, it was not until several decades later that targeting DNA degradation as a therapy was proved to be efficacious in reducing sputum viscosity, improving pulmonary function, and reducing the number of pulmonary exacerbations [66]. These data suggest that clearing NETs from the airway in CF is beneficial and this may account for some of the effects of DNase therapy. As previously stated NE (a key NET-associated protein) is present in high concentrations in CF, contributing to a protease/anti-protease imbalance in the lung. Cathepsin G and proteinase 3 are also released in NETs and likely add to the protease excess in the CF lung and damage tissue [52]. These data suggest that NETs may also represent a potentially deleterious source of proteases in the CF lung.

In addition to possibly contributing to protease-induced lung damage in CF, NETs may also be an important pro-inflammatory stimulus in the CF lung. NETs are a feature of autoimmune diseases characterized by significant levels of inflammation, and are implicated in anti-neutrophil cyoplasmic antibody (ANCA) vasculitis and associated kidney disease [67], and the pathogenesis of systemic lupus erythematosis (SLE). An impairment of DNAse degradation of NETs has been linked with the development of lupus nephritis [68], and further studies have demonstrated this inability to degrade NETs as more prominent in patients with other severe complications such as alopecia [69]. Un-degraded NET fragments activate complement as a central disease mechanism [70] and NETs in association with LL37 have been described as a source of extracellular antigen (acting via

TLR-9) in SLE, which may also drive the disease process [71]. The presence of the cathelicidin on NETs has also been shown to promote atherosclerosis in mice by activating plasmacytoid dendritic cells [50] further suggesting that AMPs in association with DNA may promote inflammation. This feature may be highly relevant to CF lung disease, particularly when we consider that DNase is already employed as a successful therapy in CF.

8. Outstanding Questions in CF

Despite the presence of NETs (and associated AMPs, proteases, and other key neutrophil proteins) in the CF airway, there is unremitting bacterial infection. Thus we can infer that either NETs are inefficient in killing bacteria in CF lungs (possibly because the bacteria have evolved mechanisms to evade NET-based killing) or that NETs are simply trapping bugs rather than killing them. In fact, the process of trapping bacteria could be an important factor in stopping bacterial dissemination in CF and may be similar to extracellular trap encapsulation, a process seen in invertebrates, which possess only an innate immune system [72]. A further consideration is that phagocytosis and NET-mediated killing are overwhelmed by the volume of bacteria in the CF lung and simply attempt to hold infection in check. This is an attractive theory, particularly if we consider that low ASL pH, poor mucociliary transport, and other features of the environment of the CF lung could dampen the antimicrobial functions of NET-bound AMPs. Therefore, NETs in the CF lung may predominantly act as a source of damaging neutrophil products while being relatively impotent in antimicrobial activity. A further, as yet unanswered question, is whether the abundance of NETs in the CF lung is related to overproduction by CFTR-deficient neutrophils or a lack of clearance due to the reduced mucociliary transport in CF. Finally, there is a paucity of data on the mechanism of clearance of NETs with some evidence suggesting that NETs can be taken up by macrophages and degraded in the lysosome in an immunologically silent process [73]. However, further work is required to determine the mechanism for macrophage mediated clearance of NETs and how it affects the resolution of inflammation, which is normally driven by the efferocytosis of apoptotic neutrophils [25]. We propose that many of these questions could be answered by carefully designed experiments in appropriate systems and animal models such as the CF pig.

9. NETs Research in the CF Pig Model

The CF pig, like the human, develops significant gut and lung disease, the latter being characterized by neutrophil infiltration and lung destruction [6]. Human CF blood neutrophils demonstrate no impairment in releasing NETs when compared to non-CF neutrophils [36]. NET formation and the associated release of proteases have been demonstrated in wild type porcine neutrophils following calcium ionophore treatment [74]. We can demonstrate NET formation by neutrophils from *CFTR*$^{-/-}$ animals following lipopolysaccharide (LPS) treatment (Figure 2). These porcine NETs show characteristic extracellular DNA release in *ex vivo* cell culture and associated features of NETosis by scanning electron microscopy. Thus, we can study the process of NETosis in neutrophils from a relevant large animal model of CF and potentially address many of the questions posed above. Furthermore by monitoring the development of lung disease in the CF pig

model it may be possible to delineate the role of NET formation in this process. By measuring NET abundance in this model at different time points both longitudinally and following challenge with pathogens, we will be able to assess the contribution of NETs to the early pathogenesis of CF lung disease. Furthermore, therapeutic approaches to modulate NET production may be studied in an animal where repeated sampling and biopsy of the airway with standard techniques is possible.

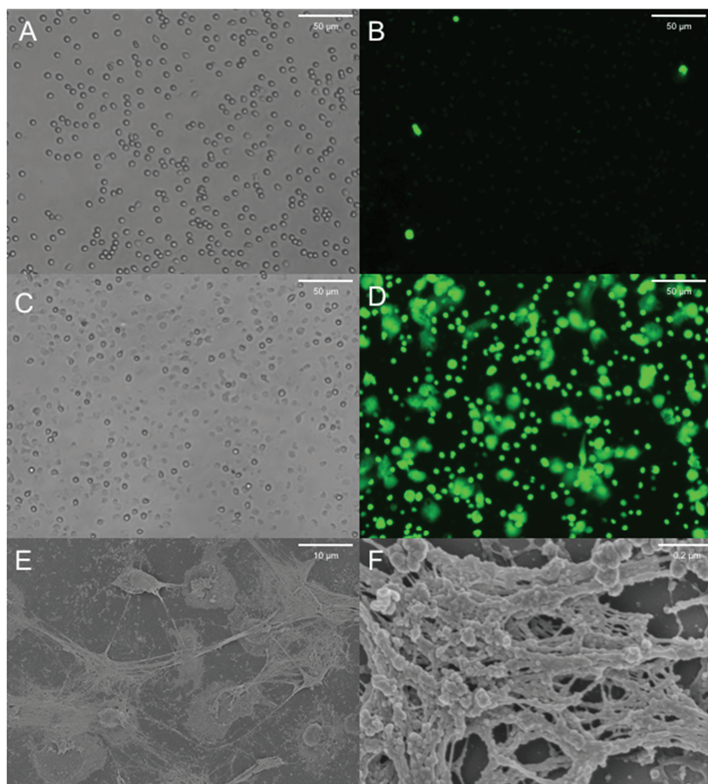

Figure 2. Porcine *CFTR*$^{-/-}$ neutrophils stimulated with LPS to produce NETs. Cells were plated at a density of 50,000 cells per well in 24 well plates and treated for 6–8 h with LPS 100 µg/mL or control. Cells were then stained with Sytox green, which preferentially stains extracellular DNA and is excluded from live cells. (**A**) Brightfield image of untreated neutrophils showing normal morphology; (**B**) Sytox staining of untreated neutrophils demonstrates minimal staining for extracellular DNA; (**C**) Brightfield image of LPS treated neutrophils showing flattened and activated cells; (**D**) Sytox staining of LPS treated neutrophils showing characteristic NET structures; (**E**,**F**) Scanning electron microscopy (SEM) of fixed preparations at low and high magnification demonstrating characteristic mesh-like structures of NETs following LPS treatment.

10. Conclusions

NET formation is a potentially important mechanism in the development of CF lung disease with results to date showing that it likely occurs in response to bacterial infection. The evidence that NETs are a major antimicrobial mechanism in CF is at present incomplete. Further work is required to determine whether NETs in CF contribute to host defense (which is knowingly impaired in CF) or whether their main contribution is to inflammation and lung damage.

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Author Contributions

Robert D. Gray, Brian N. McCullagh and Paul B. McCray, Jr. conceived and wrote the article.

Conflicts of Interest

The authors declare no conflict of interest.

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Anti-Biofilm and Immunomodulatory Activities of Peptides That Inhibit Biofilms Formed by Pathogens Isolated from Cystic Fibrosis Patients

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Abstract: Cystic fibrosis (CF) patients often acquire chronic respiratory tract infections due to *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex (Bcc) species. In the CF lung, these bacteria grow as multicellular aggregates termed biofilms. Biofilms demonstrate increased (adaptive) resistance to conventional antibiotics, and there are currently no available biofilm-specific therapies. Using plastic adherent, hydroxyapatite and flow cell biofilm models coupled with confocal and scanning electron microscopy, it was demonstrated that an anti-biofilm peptide 1018 prevented biofilm formation, eradicated mature biofilms and killed biofilms formed by a wide range of *P. aeruginosa* and *B. cenocepacia* clinical isolates. New peptide derivatives were designed that, compared to their parent peptide 1018, showed similar or decreased anti-biofilm activity against *P. aeruginosa* biofilms, but increased activity against biofilms formed by the Gram-positive bacterium methicillin resistant *Staphylococcus aureus*. In addition, some of these new peptide derivatives retained the immunomodulatory activity of 1018 since they induced the production of the chemokine monocyte chemotactic protein-1 (MCP-1) and suppressed lipopolysaccharide-mediated tumor necrosis factor-α (TNF-α) production by human peripheral blood mononuclear cells (PBMC) and were non-toxic towards these cells. Peptide 1018 and its derivatives provide promising leads for the treatment of chronic biofilm infections and hyperinflammatory lung disease in CF patients.

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1. Introduction

Bacteria form biofilms when growing on surfaces or air-liquid interfaces. Biofilms are structured aggregates of bacteria encased in a protective extracellular matrix that can contain polysaccharides, proteins, extracellular DNA, and lipids [1]. The switch from a free swimming, planktonic to an adherent biofilm lifestyle results in increased adaptive resistance to antimicrobial agents making biofilm-related infections inherently difficult to treat [1,2]. Apart from colonizing inert materials such as catheters and medical implants, bacterial biofilms are prevalent in chronic infections, such as those that develop in the lungs of cystic fibrosis (CF) patients [3,4]. CF is the most common eventually fatal autosomal-recessive disorder in Caucasian populations and is caused by mutations in

the CF transmembrane conductance regulator (CFTR) chloride-channel, as well as various modifier genes that influence severity. CF patients rapidly acquire lifelong chronic respiratory infections that lead to hyper-inflammation and progressive destruction of lung function [5].

The opportunistic pathogen *P. aeruginosa* is the most prevalent pathogen associated with lung deterioration in patients with CF [3,5]. Its high intrinsic resistance and ability to develop biofilms in the CF lung, combined with the lack of specific anti-biofilm therapeutics, seriously hinders the treatment of chronically-infected CF patients, and although *P. aeruginosa* can initially be transiently suppressed by aggressive antibiotic therapy, the same strain can re-emerge and predominate for years. In fact, it has been suggested that treatment with antibiotics such as tobramycin (used in the treatment of CF), tetracycline, and norfloxacin might contribute to infection chronicity by inducing biofilm formation [6,7]. During its long-term colonization of the CF lung, *P. aeruginosa* undergoes several genetic adaptations leading to different colony morphologies [8], some of which are linked to bacterial persistence. For example, mucoid *P. aeruginosa* strains, which result from mutations within the anti-sigma factor *mucA*, have been associated with biofilm formation [9] and antibiotic resistance [10]. Likewise, small colony variant (SCV) colonies have been shown to be hyper-adherent with reduced susceptibility to antibiotics [11–13]. Another major factor is the emergence of mutator strains that enable the rapid evolution of resistant strains in the CF lung. The biofilm mode is also associated with resistance to phagocytic killing, which is one of the major mechanisms of clearance in the lung. In addition to *P. aeruginosa*, infection with bacterial species from the *Burkholderia cepacia* complex (Bcc), a collection of nine genotypically distinct but phenotypically similar species, has been associated with a poor prognosis in CF patients [14,15].

Cationic host defense (antimicrobial) peptides represent a novel alternative to conventional antibiotics. These peptides, e.g., the human cathelicidin LL-37, often have modest direct antimicrobial activities for planktonic cells [16] and are known to possess immunomodulatory properties [17]. More recently, it has been demonstrated that LL-37 has excellent anti-biofilm activity *vs.* *P. aeruginosa* causing a 50% decrease in biofilm formation at one sixteenth the *minimum inhibitory concentration* (MIC) [18]. Similar results were subsequently shown by other groups [19,20]. This encouraged the development of improved synthetic cationic peptides with activity against bacterial biofilms [21–27].

Anti-biofilm peptides are biochemically similar to antimicrobial peptides in that they are short (12–50 amino acids long) and amphipathic, having two to nine basic residues (R or K) and ~50% hydrophobic residues [18–22]; however they exhibit specific activity against biofilms at concentrations often well below their MIC for planktonic cells [18–22], and there is no correlation between anti-biofilm activity and anti-planktonic cell activity [18]. Previously identified anti-biofilm peptides include peptide 1037 that was shown to inhibit biofilm formation by both Gram-negative and Gram-positive bacteria [21]. More recently, peptide 1018 (VRLIVAVRIWRR-NH_2; also called IDR-1018), developed initially as an immune modulator [28–30], was demonstrated to have potent broad-spectrum anti-biofilm activity that mechanistically involved the binding and degradation of (p)ppGpp nucleotides, which are involved in biofilm formation and maintenance [22]. Peptide 1018 has also been shown to synergize with conventional antibiotics to treat biofilms formed by multiple

bacterial species [27]. These properties indicate that 1018 might be a useful lead for the treatment of hyperinflammatory chronic biofilm infections in the context of CF.

Here we tested the anti-biofilm activity of peptide 1018 against a variety of *P. aeruginosa* and *Burkholderia* strains isolated from CF patients. Further, we designed a variety of 1018 derivatives and assessed their anti-biofilm and immunomodulatory properties. Since persistent lower airway infection and inflammation is a significant cause of mortality and morbidity in cystic fibrosis [31], the combined immunomodulatory and anti-biofilm effects of the given peptides provide promising leads for decreasing infections and improving lung pathology and the overall course of the disease.

2. Results

2.1. MICs vs. Cystic Fibrosis Isolates

Peptide 1018 has previously been shown to have activity both as an immune modulator [28–30] and as an anti-biofilm agent [22,27] against individual strains from several species of bacteria including *P. aeruginosa* and *Burkholderia cenocepacia*. The latter observation was particularly surprising since planktonic *B. cepacia* complex strains are known to be completely resistant to the effects of cationic agents like polymyxin B and cationic antimicrobial peptides by virtue of their lack of a self-promoted uptake system across the outer membrane [32]. Here we extended our observations on the anti-biofilm activity *vs. Burkholderia* using a broad variety of *B. cepacia* complex species from CF patients and also examined activity *vs.* a broad range of *P. aeruginosa* chronic pulmonary infection CF isolates, since these are known to form biofilms in the CF lung. MIC assays were performed to determine the direct antimicrobial activity of peptide 1018 against planktonic cells. Peptide 1018 exhibited somewhat better planktonic antimicrobial activity *vs. Pseudomonas* compared to human host defense peptide LL-37 [18] and synthetic cationic peptide 1037 [21] (Table 1), but was still quite weak with MICs ranging from 16 to 32 µg/mL. Interestingly, all *P. aeruginosa* clinical isolates showed higher MICs for human cathelicidin LL-37 compared to reference strains PAO1 and PA14, confirming the idea that this peptide has little useful activity *vs.* planktonic strains (Table 1). In contrast cationic antibiotic polymyxin B had MICs of 2–4 µg/mL indicating that the low activity did not *per se* reflect resistance to cationic peptides.

Consistent with previous studies [22,32], *Burkholderia* was confirmed to be intrinsically resistant to antimicrobial peptides and polymyxin B by the MIC assays (Table 1). Only peptide 1018 was able to affect any *Burkholderia* planktonic cells, demonstrating relatively high MICs of 64–128 µg/mL *vs.* five out of the total of 11 known genomovars. Even peptides known to exhibit very potent direct planktonic antimicrobial activity (HHC-10 and HHC-36) [33] were found to be inactive against planktonic cells of *Burkholderia* strains (MIC > 256 µg/mL).

2.2. Anti-Biofilm Activity vs. Cystic Fibrosis Isolates

To assess the biofilm-forming ability of the different clinical isolates, dilutions (1/100) of overnight cultures were incubated in 96-well polypropylene plates using BM2 glucose medium, as previously described [22]. No apparent correlation was observed between the different bacterial morphologies and the formation of biofilm biomass of *P. aeruginosa* isolates (Table 2). Three

P. aeruginosa strains with classical morphology C2773, 3195, and 1172, and mucoid strain 2639 exhibited increased biofilm formation when compared to reference strains PA01 and PA14 (Table 2). In contrast, strains *B. cenocepacia* CEP0055 and *B. ambifaria* CEP0996 produced about a third of the biofilm compared to the previously-studied strain *B. cenocepacia* 4813 (Table 2). Overall, five of the eight tested *Burkholderia* strains showed relatively weak biofilm formation on the surface of the microtiter wells (Table 2).

Table 1. Peptide 1018 demonstrated weak activity *vs.* planktonically-grown *P. aeruginosa* cystic fibrosis strains and no activity *vs. B. cepacia* complex cystic fibrosis isolates. All *minimum inhibitory concentration* (MIC) assays were performed at least three times. For comparison we included the previously studied *P. aeruginosa* (non-cystic fibrosis) wild type strains PAO1 and PA14 and *B. cenocepacia* 4813 [22], as well as showing results for two previously described anti-biofilm peptides (LL-37 [18] and 1037 [21]) and the cationic lipopeptide polymyxin B. Different *Burkholderia* genomovars denote strains which are phylogenetically differentiable but phenotypically indistinguishable. *Burkholderia multivorans* (genomovar II), genomovar III (divided into two *recA* clusters, III-a and III-b, *Burkholderia stabilis* (genomovar IV), *Burkholderia dolosa* (genomovar VI), *Burkholderia ambifaria* (genomovar VII).

Strains	**MIC (µg/mL)**			
(Colonial Morphology or Genomovar)	**1018**	**LL-37**	**1037**	**Polymyxin B**
P. aeruginosa PA01	16	32	304	2
P. aeruginosa PA14	16	32	304	2
P. aeruginosa 3195 (Classic)	16	64	304	2
P. aeruginosa C2773 (Classic)	16	256	152	4
P. aeruginosa 1172 (Classic)	16	>128	304	2
P. aeruginosa 7632 (Classic)	16	256	304	2
P. aeruginosa 3330 (Classic)	16	>128	304	2
P. aeruginosa 4020 (Classic)	16	>128	304	2
P. aeruginosa C4276 (Classic)	32	256	608	2
P. aeruginosa 2631 (Classic)	32	>256	608	4
P. aeruginosa C4278 (Mucoid)	16	>256	304	2
P. aeruginosa 4608 (Mucoid)	32	>128	304	2
P. aeruginosa 2639 (Mucoid)	32	>128	608	2
P. aeruginosa 7633 (Entire)	16	>128	304	2
P. aeruginosa 2955 (Dwarf)	50	- [a]	-	-
B. multivorans D2661 (Genomovar II)	>512	>512	>608	>128
B. cenocepacia 4813 (IIIa)	>256	>256	>608	>128
B. cenocepacia C5424 (IIIa)	128	>256	>608	>128
B. cenocepacia CEP0055 (IIIb)	128	>512	>608	>128
B. cenocepacia CEP509 (IIIb)	>256	>512	>608	>128
B. stabilis C6061(IV)	64	>512	304	>128
B. dolosa CEP0021(VI)	>256	>512	>608	>128
B. ambifaria CEP0996 (VII)	64	>512	>608	>128

[a] Means not done.

Table 2. Activity of peptide 1018 against biofilms formed by *P. aeruginosa* (PA) and *Burkholderia* (B) clinical isolates from cystic fibrosis (CF) patients. Usually three, but at least two independent experiments and eighteen replicates were performed per condition. The differentiating colony morphology or genomovar is given in brackets in the first column. Strains PAO1 and PA14 are reference strains that have been fully sequenced.

Strains (Colonial Morphology or Genomovar)	**Biofilm Formation [a]**	**Inhibition by 10 µg/mL 1018**
P. aeruginosa PA01	0.79 ± 0.22	51%
P. aeruginosa PA14	0.82 ± 0.21	49%
P. aeruginosa 3195 (Classic)	0.99 ± 0.16	37%
P. aeruginosa C2773 (Classic)	0.89 ± 0.17	60%
P. aeruginosa 1172 (Classic)	0.92 ± 0.27	37%
P. aeruginosa 7632 (Classic)	0.59 ± 0.12	19%
P. aeruginosa 3330 (Classic)	0.84 ± 0.20	33%
P. aeruginosa 4020 (Classic)	0.62 ± 0.16	53%
P. aeruginosa C4276 (Classic)	0.62 ± 0.28	53%
P. aeruginosa 2631 (Classic)	0.74 ± 0.27	44%
P. aeruginosa C4278 (Mucoid)	0.73 ± 0.20	58%
P. aeruginosa 4608 (Mucoid)	0.62 ± 0.09	67%
P. aeruginosa 2639 (Mucoid)	0.93 ± 0.20	44%
P. aeruginosa 7633 (Entire)	0.72 ± 0.16	49%
P. aeruginosa 2955 (Dwarf)	0.64 ± 0.19	41%
B. multivorans D2661 (Genomovar II)	0.75 ± 0.07	75%
B. cenocepacia 4813 (IIIa)	0.94 ± 0.18	52%
B. cenocepacia C5424 (IIIa)	0.75 ± 0.18	59%
B. cenocepacia CEP0055 (IIIb)	0.33 ± 0.11	58%
B. cenocepacia CEP509 (IIIb)	0.42 ± 0.08	61%
B. stabilis C6061 (IV)	0.39 ± 0.07	53%
B. dolosa CEP0021 (VI)	0.41 ± 0.06	55%
B. ambifaria CEP0996 (VII)	0.29 ± 0.10	62%

[a] Values shown correspond to mean ± SD $absorbance_{595}$ readings after crystal violet staining of biofilms adherent to the wells of microtiter growth trays.

These strains were then challenged with sub-inhibitory levels of anti-biofilm peptide 1018. At 10 µg/mL, the peptide was able to reduce PA01 and PA14 biofilm formation by ~50% ($p < 0.05$; Table 2). Similarly, biofilms formed by different *P. aeruginosa* clinical isolates were also susceptible to the peptide (Table 2). Biofilms formed by classic morphology strains C2773, C4276, and 4020, and mucoid strains C4278 and 4608 were inhibited by at least 50% by the peptide (Table 2). On the other hand, biofilms produced by with classic strains 3195, 7632, 3330, 1172, and 2631, mucoid strain 2639, and dwarf (*i.e.*, small-colony variant) strain 2955 were somewhat less susceptible to the peptide (Table 2). Biofilm biomass was also significantly reduced by 52%–75%, upon peptide challenge in *Burkholderia* strains belonging to different genomovars, and this appeared to be independent of the relative ability of each strain to form biofilms (Table 2), as well as the resistance of planktonic cells to treatment with peptide 1018 (Table 1).

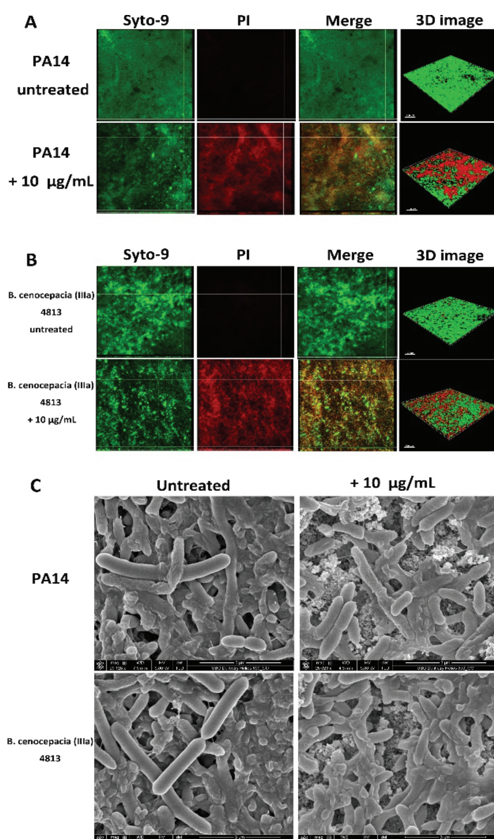

Figure 1. Sub-MIC concentrations of anti-biofilm peptide 1018 triggered biofilm cell death in the hydroxyapatite biofilm model. (A and B) Biofilms were stained using SYTO-9 to stain live cells green and propidium iodide (PI) to stain dead cells red. Merged images showing bacteria stained with both SYTO-9 and PI are also shown, and a yellow or red merged color was observed for dead cells. Samples were then examined using *confocal laser scanning microscopy* (CLSM). Each panel shows reconstructions from the top in the large panel and sides in the right and bottom panels (xy, yz, and xz dimensions). Three-dimensional reconstructions of biofilms are shown on the far right panel (3D image). The scale bar represents 100 µm in length. (**A**) *P. aeruginosa* PA14 untreated and treated with 10 µg/mL of peptide; (**B**) *B. cenocepacia* (IIIa) 4813 untreated and treated with peptide. Two independent experiments were performed for each condition; (**C**) SEM images showing the effect of peptide treatment on biofilm cells. *P. aeruginosa* and *B. cenocepacia* biofilms were developed on hydroxyapatite (HA) discs for 3 days and treated three times (every 24 h) with 10 µg/mL of peptide 1018. Biofilms were observed using SEM at a magnification of 20,000× operating at 5 kV. Untreated biofilms grown using the HA biofilm model were typically 30–40 µm thick, while biofilm thickness was reduced to 10–20 µm in peptide-treated samples.

2.3. Microscopic Imaging of Anti-Biofilm Activity

The ability of peptide 1018 to inhibit biofilms was further investigated using hydroxyapatite (HA) discs as a biologically-relevant biofilm substratum [34]. *P. aeruginosa* PA14 (Figure 1A) and *B. cenocepacia* 4813 (Figure 1B) were resuspended in brain-heart infusion broth (BHI) and grown for 3 days on HA disks. Subsequently the biofilm coated HA disks were treated or not with 10 µg/mL of peptide. Fresh BHI medium and peptide were added every 24 h for a total of 3 days, and the effect of the peptide was evaluated using confocal microscopy and scanning electron microscopy. Samples for confocal microscopy were rinsed in 0.85% physiological saline for 2 min to remove the culture broth. After live/dead cell staining, five random areas of the biofilm on each disk were examined. Treatment with peptide 1018 significantly increased the number of dead biofilm cells to around 50% for both pathogens (Table 3, Figure 1). Interestingly, peptide-induced cell death was observed in *B. cenocepacia* biofilms grown on hydroxyapatite discs, which differed from previous results obtained using the flow cell methodology in which no major increase in cell death was recorded [20]. Additional experiments using scanning electron microscopy further supported the notion that the peptide killed biofilm cells, as cell death was apparent from the substantial amount of extracellular debris (presumably from compromised cells) accumulated within the biofilm (Figure 1C). Also, biofilm cells exhibited disrupted morphologies and were smaller in size in the treated samples (Figure 1C).

Table 3. Proportion of dead biofilm cells in hydroxyapatite plates after treatment with sub-MIC levels of peptide 1018. Biofilms were grown in HA discs for 3 days and treated with peptide 1018 every 24 h. After scanning confocal microscopy the volume ratio of red fluorescence (due to the dead cell stain propidium iodide to green (all bacteria stain Syto-9) plus red fluorescence indicated the proportion of killed cells. Three independent assays were performed. Different superscript letters indicate statistically significant differences between groups ($p < 0.05$).

Bacterial Strains	Proportion of Dead Cells (%)	
	Untreated	**10 µg/mL 1018**
P. aeruginosa PA14	7 ± 4	48 ± 17 [a]
B. cenocepacia (IIIa) 4813	6 ± 3	42 ± 13 [a]

[a] Statistically significant differences ($p < 0.05$) were found between untreated and 1018-treated *P. aeruginosa* PA14 and *B. cenocepacia* (IIIa) 4813.

2.4. Derivatives of 1018: Anti-Biofilm and Immunomodulatory Activity

As peptide 1018 has demonstrated potent anti-biofilm activity against diverse bacterial species [22] and excellent immune modulatory activity [28–30], we attempted to investigate structure-activity relationships by designing peptides based on the amino acid sequence of 1018 but with various amino acid substitutions (Table 4), designed to test the effects of sequence inversion and especially altering the five amino acid hydrophobic stretch L_3IVAV_7. Interestingly, inverting the sequence of 1018 in peptide HE1 maintained the anti-biofilm activity *vs. P. aeruginosa* and only slightly reduced it against *B. cenocepacia*. In contrast, altering three amino acids including switching an R and V

residue in the sequence substantially reduced activity, which was also true for the reversed sequence in peptide HE2. These initial screens also identified peptides HE4 and HE10 as two of the more active anti-biofilm peptides, and a scrambled shorter peptide HE12 as a virtually inactive peptide (Figure 2) that we subsequently used as a negative control.

Table 4. Anti-biofilm activity of peptides derived from parent peptide 1018. In bold are shown the different amino acid substitutions made to parent peptide 1018. Hyphens indicate amino acid residues that were deleted in relation to peptide 1018. For peptides 1018, HE4, HE10, and HE12 the concentration used was 10 µg/mL. These studies were performed with a Bioflux apparatus resulting in the higher activity *vs. Pseudomonas*. For peptides HE1, HE2, and HE3, 15 µg/mL of peptide was used since 10 µg/mL of peptide showed low activity. The concentration of peptide used in each case was lower than the MIC for both *P. aeruginosa* (e.g., MICs for HE-4 and HE-12 *vs*. PA14 were 80 µg/mL) and *B. cenocepacia* (MICs > 256 µg/mL).

Peptide	Sequence [a]	Modification	Anti-Biofilm Activity (% Decrease in Biofilm)	
			***P. aeruginosa* PA14**	***B. cenocepacia* 4813**
1018	VRLIVAVRIWRR	Parent	99%	52%
HE1	RRWIRVAVILRV	Reverse sequence	93%	73%
HE2	VRLI**R**AVR**A**WR**V**	Break hydrophobicity; maintain charge	42%	24%
HE3	**V**RW**A**RVA**R**ILRV	Reverse of HE2	31%	21%
HE4	VRLI**W**AVRIWRR	Substitute in another W for V	99%	- [b]
HE10	VRLI--VRIWRR	Truncate to remove hydrophobic patch	39%	-
HE12	RFKRVARVIW	Scrambled smaller peptide	10%	-

[a] Single amino acids sequence. In bold are shown the different amino acid substitutions made to parent peptide 1018; [b] Means not done.

The three most effective 1018 derivatives were then further evaluated for anti-biofilm activity against *P. aeruginosa* PA14 using the flow cell method (Figure 2). Peptides HE4 and HE10 demonstrated strong anti-biofilm activity comparable with that of 1018, while HE12 was substantially less active against *P. aeruginosa* biofilms, both under inhibition and eradication conditions (Figure 2). We then considered whether these peptide derivatives had conserved the ability of peptide 1018 to inhibit biofilms formed by Gram-positive bacteria. Interestingly, HE4 and HE10 were notably more active against *methicillin-resistant Staphylococcus aureus* (MRSA) compared to 1018 under static conditions, while HE12 showed less activity (Figure 3). These results demonstrate that the broad-spectrum activity displayed by 1018 [22] is generally conserved in these 1018 derivatives.

We previously identified 1018 as a potent immunomodulatory peptide with excellent *in vivo* protective and anti-inflammatory activity [28–30]. The identification here of 1018 derivatives enabled us to see if the two properties of anti-biofilm and immunomodulatory activity were conserved in the new 1018 derivatives. Therefore, we assessed the relative ability of HE1, HE4, HE10, and HE12 *cf.* 1018 to induce monocyte chemotactic protein-1 (MCP-1), a key chemokine that promotes recruitment and infiltration of monocytes and macrophages [17]. At 20 and 100 µg/mL, peptides HE1, HE4, and

HE10 significantly induced MCP-1 in human peripheral blood mononuclear cells (PBMCs) to the same (or greater) extent as 1018 *cf.* the untreated control (Figure 4A). In contrast, peptide HE12 was virtually inactive. None of these peptides showed substantial cytotoxicity as assessed by the release of cytosolic lactate dehydrogenase (LDH) (Figure 4C). As persistent lower airway infection contributes to the lung pathology exhibited in cystic fibrosis, the peptides were also assessed for their ability to reduce the production of tumor necrosis factor-alpha (TNF-α) in response to pro-inflammatory bacterial lipopolysaccharides (LPS). Interestingly all HE peptides substantially reduced—by more than 95%—the levels of LPS-induced TNF-α (Figure 4B). These results indicate that peptides HE4 and HE10, like 1018, are not only potent anti-biofilm agents, but also possess notable immunomodulatory potential by selectively enhancing chemokine production while balancing the pro-inflammatory response.

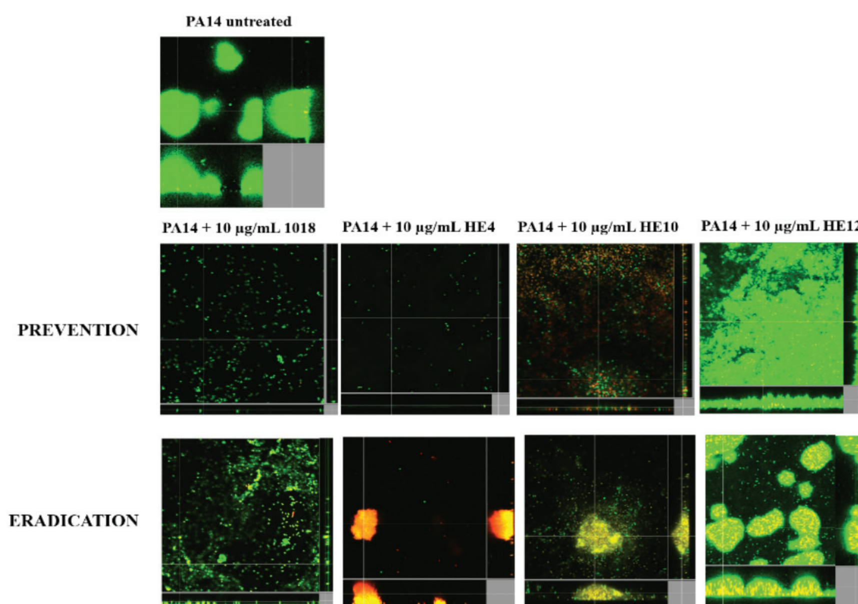

Figure 2. Anti-biofilm activity of peptide 1018 derivatives against *P. aeruginosa* PA14 in flow cells. For biofilm prevention conditions, the peptides were added to the medium from the beginning of the experiment. For eradication experiments, bacteria were allowed to develop structured 2-day-old biofilms prior to peptide and antibiotics treatment for the following 24 h. After 3 days, bacteria were stained green with the all bacteria stain Syto-9 and red with the dead-bacteria stain propidium iodide (merge shows as yellow to red) prior to confocal imaging. Each panel shows reconstructions from the top in the large panel and sides in the right and bottom panels (xy, yz, and xz dimensions).

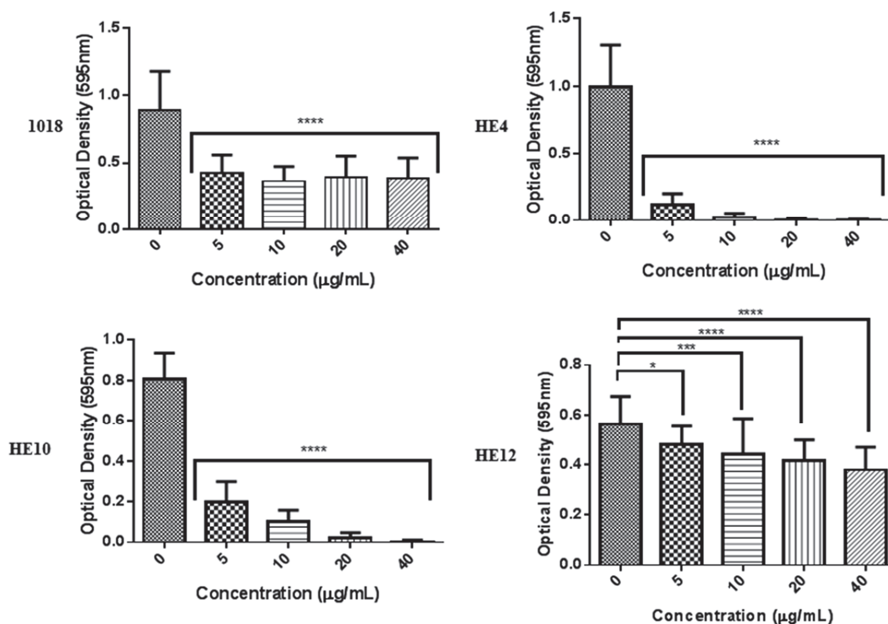

Figure 3. Biofilm inhibitory activity against methicillin resistant *Staphylococcus aureus* (MRSA) in 96-well plates. Dilutions (1/100) of overnight cultures were incubated in BM2 biofilm-adjusted in polypropylene microtiter plates (Fisher Scientific, Waltham, MA, USA) in the presence of the different peptides for 24 h at 37 °C. Planktonic cells were removed, biofilm cells adhering to the side of the tubes were stained with crystal violet, and absorbance at 595 nm was measured using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Statistical significance was assessed using one-way ANOVA (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$).

3. Discussion

Currently, there are no clinically approved antimicrobial agents that target bacterial biofilms. Previously, we described that the mechanism of action of broad-spectrum anti-biofilm peptide 1018 involved binding and degradation of the second messenger nucleotides (p)ppGpp, which are involved in biofilm development [22]. In this report, we extended these studies on anti-biofilm peptide 1018 to examine whether this peptide inhibited biofilms formed by clinical strains isolated from CF patients. In addition, we examined if the dual properties of immunomodulatory and anti-biofilm activity were conserved in 1018 derivatives, since both of these activities have been reported to be independent of antimicrobial activity *vs.* planktonic cells [17,21,30].

It was demonstrated that 10 µg/mL of 1018 inhibited biofilm formation by most strains screened (Table 1), which was generally well below the MIC *vs.* planktonic cells (Table 1). Indeed, consistent with previous studies [21], all *Burkholderia* genomovars were found to be highly resistant to peptides 1018, LL-37, and 1037, as well as to the cationic antibiotic polymyxin B (Table 1). Against

P. aeruginosa clinical strains, the peptides showed modest to very weak antimicrobial activity in the order 1018 > LL-37 > 1037.

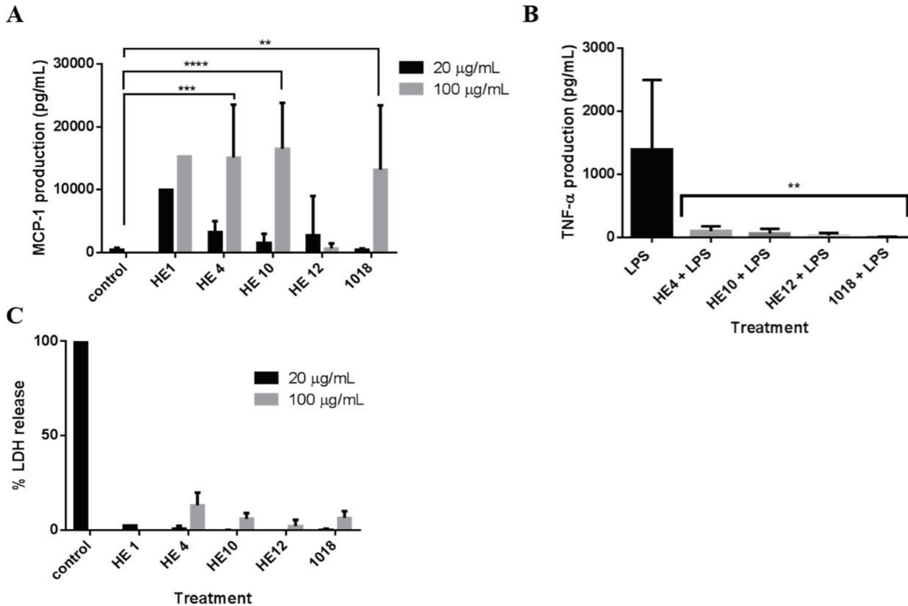

Figure 4. Immunomodulatory activity and cytotoxicity of peptide 1018 and its derivatives HE1, HE4, HE10, HE12. (**A**) Human peripheral blood mononuclear cells were subjected to 20 μg/mL and 100 μg/mL of HE1, HE4, HE10, HE12, and 1018 for 24 h. Monocyte chemotactic protein-1 (MCP-1) production was assessed using sandwich enzyme linked immunosorbent assay (ELISA). The level of MCP-1 induction was similar for HE1, HE4, HE10, and 1018 at 100 μg/mL; (**B**) LPS (20 ng/mL) was co-administered with 20 μg/mL of each of the aforementioned peptides for 24 h. The tumor necrosis factor-alpha (TNF-α) production in response to both stimulants was assessed using enzyme linked immunosorbent assay (ELISA) and compared to lipopolysaccharides (LPS) alone. (The peptides themselves did not induce any significant TNF-α production). In both A and B, statistical significance was assessed using one-way ANOVA (**, $p < 0.01$; ***, $p < 0.001$); (**C**) Peptide 1018 and its derivatives were not cytotoxic. Lactate dehydrogenase release from peripheral blood mononuclear cells (PBMCs) was measured to assess the toxicity of 20 μg/mL and 100 μg/mL of 1018 and its derivatives.

Biofilm formation by most *P. aeruginosa* isolates (Table 2) was substantially inhibited by 10 μg/mL of 1018, except strain 7632C, for which biofilm formation was only inhibited by 19% (Table 2). Biofilm formation by *P. aeruginosa* mucoid strain 4608 was affected the most by peptide action (67% inhibition). Peptide 1018 also inhibited biofilm formation by at least 52% in all *Burkholderia* CF isolates tested, with *B. multivorans* D2661 being the most susceptible strain (75% inhibition) (Table 2). Additional experiments using a hydroxyapatite disc biofilm model revealed that the

peptide (10 μg/mL) triggered cell death in biofilms formed by both *P. aeruginosa* PA14 and *B. cenocepacia* (IIIa) 4813 (Figure 1 and Table 3), although interestingly, 1018-induced death of *B. cenocepacia* biofilms had not been previously observed using the flow cell biofilm assay [22]. However, *B. cenocepacia* cell death, as observed here, might reflect the different model systems. Killing was further confirmed using scanning electron microscopy (Figure 1C). Thus, the biofilm inhibitory activity of peptide 1018 (Table 2) might be due in part to the ability of 1018 to induce biofilm cell death (Figures 1 and 2; Table 3).

Peptide 1018 has two independent activities, as an immunomodulator [28–30] and as an antibiofilm agent [22]. To understand if these properties were evident in related peptides we designed a series of 1018 derivatives. The best of these showed similar (HE4) or reduced (HE10) anti-biofilm activity against *P. aeruginosa* (Figure 2) but enhanced activity *vs.* the Gram-positive organism *S. aureus* (Figure 3), and generally conserved the broad-spectrum activity of the parent peptide 1018. Most intriguing was the effect of deletion of the hydrophobic residues valine and alanine in peptide HE10 which decreased anti-*Pseudomonas* activity but increased anti-MRSA activity observed. Peptide HE12, which was the same length as HE10 and had the same balance of charged and hydrophobic amino acids, lacked anti-biofilm activity against both the tested Gram-negative and Gram-positive bacteria (Figures 2 and 3), demonstrating the importance of the amino acid sequence rather than composition. Interestingly, peptides HE4 and HE10 retained the ability of 1018 to modulate the immune response, as they enhanced the production of chemokine MCP-1 (Figure 4A) while strongly suppressing the production of TNF-α (Figure 4B), an anti-inflammatory property. Conversely, peptide HE12 did not significantly stimulate MCP-1 production (Figure 4A), but did inhibit the release of TNF-α from PBMCs (Figure 4B). This strongly suggests that chemokine induction and suppression of pro-inflammatory cytokines is independently determined, as proposed earlier [35]. Importantly, none of the amino acid modifications tested led to increased cytotoxicity of the peptides (Figure 4C).

In conclusion, we report that anti-biofilm peptide 1018 inhibited biofilm formation by multiple clinical isolates from CF patients. Moreover, 1018 derivative HE4 showed equivalent activity *vs. Pseudomonas* and enhanced anti-biofilm activity against MRSA while retaining the immunomodulatory properties of parent peptide 1018. On the other hand, peptide HE12 provided a potential negative control since it lacked anti-biofilm activity and did not substantially stimulate MCP-1 production. Since *P. aeruginosa* and *Burkholderia* strains are the most prevalent pathogens in the CF lung and are known to form biofilms, our findings suggest that peptide 1018 and its derivatives may be used in combination with conventional antibiotics [27] against CF-related inflammatory lung infections.

4. Experimental

4.1. Peptide Synthesis

Peptides used in this study were synthesized by GenScript (Piscataway, NJ, USA) using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and made >95% pure using reverse-phase high-performance liquid chromatography (HPLC). Peptide mass was confirmed by mass spectrometry.

4.2. Bacteria, Growth, and Assessment of Minimal Inhibitory Concentrations (MIC)

In addition to the wild type lab isolates *Pseudomonas aeruginosa* strains PAO1 and PA14, a variety of cystic fibrosis clinical isolates of *P. aeruginosa* and *Burkholderia cepacia* complex strains were utilized (Table 1). Planktonic cells from each of the *P. aeruginosa* and *B. cepacia* strains were grown in BM2 glucose or cation-adjusted Mueller-Hinton. The minimal inhibitory concentration (MIC) for peptides in brain-heart infusion (BHI) was assessed by the broth microdilution method with minor modifications for cationic peptides [21]. Peptide 1018 was dissolved in water and stored in glass vials at −20 °C. MIC assays were performed in sterile 96-well polypropylene microtiter plates. Peptide 1018 was added to the plate at increasing concentrations and *P. aeruginosa* or *B. cepacia* bacteria were inoculated to a final concentration of 5×10^5 colony forming units (CFU)/mL per well. The plates were incubated at 37 °C for 18 h. The MIC was defined as the lowest concentration of peptide at which no planktonic growth was observed.

4.3. Biofilm 96-Well Plate Assays

Biofilm formation was initially analyzed using a static abiotic solid surface assay as described elsewhere [21]. Dilutions (1/100) of overnight cultures were incubated in BM2 glucose medium (62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids) in polypropylene microtiter plates (Fisher Scientific, Waltham, MA, USA) in the presence of the different peptides for 22 h at 37 °C. Planktonic cells were removed and biofilm cells adhering to the side of the tubes were stained with crystal violet, extracted into ethanol, and the optical density at 595 nm (OD_{595}) measured using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Peptides were added at time zero (prior to adding the diluted, overnight cultures) at various concentrations, and the decrease in biofilm formation was recorded at 22 h for *P. aeruginosa*, *Burkholderia* and Methicillin-resistant *Staphylococcus aureus* isolates. Peptides 1018, HE4, HE10 and HE12 at 10 μg/mL, and peptides HE1, HE2 and HE3 at 15 μg/mL (due to their lesser activity), were investigated using the BioFlux apparatus [36].

4.4. Culturing Biofilms Using the Hydroxyapatite Biofilm Model

Sterile hydroxyapatite (HA) disks (0.38-inch diameter by 0.06-inch thickness; Clarkson Chromatography Products, Williamsport, PA, USA) were used as a biofilm substrate. *P. aeruginosa* or *B. cepacia* strains isolated from cystic fibrosis (CF) patients were used as the test organisms and grown at 37 °C overnight on BHI agar (Becton-Dickinson, Sparks, MD, USA) plates and then suspended in BHI broth at a spectrophotometrically-standardized $OD_{405} = 0.10$ (3.0×10^7 CFU/mL). The HA disks were placed in the wells of a 24-well tissue culture plate containing 1.8 mL of BHI. Each well was inoculated with 0.2 mL of the above *P. aeruginosa* or *B. cepacia* suspensions. The discs were then incubated at 37 °C for 3 days to allow biofilm growth prior to peptide treatment. The HA disk is a well-established platform for biofilm growth [37]. Previous studies have shown that HA does not affect the viability of bacteria within biofilms [38] and indeed most of these bacteria remain viable for months [38]. Further, HA is the component of human bone tissue, which makes it more

biocompatible and less cytotoxic compared to other surfaces such as glass. Thus, HA is less likely to affect the susceptibility to peptides.

4.5. Anti-Biofilm Effect of Peptide 1018 Evaluated Using the Hydroxyapatite Biofilm Model

After the formation of a 3-day-old biofilm, the culture medium of each well was replaced by 1.98 mL of fresh BHI. The biofilm-covered HA disks were subjected to peptide 1018 treatment by adding 20 µL of 1.0 mg/mL peptide (or 20 µL of water as a negative control) into each well (1.98 mL BHI) to achieve a final concentration of 10 µg/mL. HA disks were treated for 72 h at 37 °C (the same concentration of peptide solution was replenished every 24 h).

4.6. Confocal Laser Scanning Microscopy

P. aeruginosa or *B. cepacia* biofilms on HA disks, including peptide exposed and control disks, were rinsed in 0.85% physiological saline for 1 min and subjected to bacterial viability staining and confocal laser scanning microscopy as previously described [22,34].

4.7. Scanning Electron Microscopy Examination of Hydroxyapatite-Grown Biofilms

Three additional *P. aeruginosa* and *B. cepacia* biofilms treated with 10 µg/mL peptide 1018 for 72 h, or the equivalent amount of water as a negative control, were collected for scanning electron microscopy examination. Samples were prefixed with phosphate-buffered 2.5% glutaraldehyde for 10 min before further fixation in 1% osmium tetroxide for 1 h. The specimens were then subjected to increasing concentrations of ethanol (50%, 70%, 80%, and 100%) for dehydration. The dehydrated specimens were dried by using a critical point drier (Samdri-795; Tousimis Research Corporation, Rockville, MD, USA), sputter-coated with gold palladium (Hummer VI; Technic Inc, Anaheim, CA, USA), and examined by SEM (Helios Nanolab 650, FEI, Eindhoven, The Netherlands) at an accelerating voltage of 5 kV using 20,000× magnification.

4.8. Biofilm Cultivation in Flow Cell Chambers and Microscopy

Biofilms were grown for 72 h, in the absence or presence of the desired concentration of the different peptides, at 37 °C in flow chambers with channel dimensions of 1 by 4 by 40 mm, and then stained and examined by confocal microscopy, as described previously [22]. For biofilm prevention studies the peptide was present in the flow-through medium throughout the biofilm growth while for eradication experiments, biofilms were allowed to grow for 2 days, before treating them with peptide for the last 24 h of the experiment.

4.9. Immune Modulation Studies

These studies were done as described previously [28]. Briefly, human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy individuals obtained under University of British Columbia (UBC) human ethics approval. PBMCs were diluted to 2×10^6 cells/mL in RPMI-1640 medium (Gibco®, Grand Island, NY, USA) supplemented with 10% fetal bovine

serum (FBS). Subsequently, 250 µL of cells were seeded in each well of a 48-well dish. PBMCs were allowed to incubate for one hour at 37 °C in 5% CO_2, and then ether-washed peptides HE4, HE10, HE12 or 1018 were subsequently added to give a final peptide concentration of 20 µg/mL or 100 µg/mL. To assess chemokine induction after 24 h, the levels of chemokine MCP-1 (CCL2) were assessed in the supernatants three times independently using a sandwich ELISA kit (BioSource International, Grand Island, NY, USA). To look at the anti-inflammatory activity of the peptides in suppressing LPS-induced TNFα, *P. aeruginosa* LPS was added at a final concentration of 20 ng/mL as well as peptides at 20 µg/mL or 100 µg/mL. Secretion of TNF-α was monitored by capture ELISA (eBiosciences, San Diego, CA, USA) after 24 h.

4.10. Cytotoxicity Assessment

To assess peptide-mediated cytotoxicity, human PBMCs (1×10^5) were seeded into 96-well plates (Sarstedt, Newton, NC, USA) in RPMI-1640 medium and incubated for one hour at 37 °C in 5% CO_2 before peptide treatment. The release of the cytosolic enzyme lactate dehydrogenase (LDH) was then measured after 24 h. Untreated control cells were used as a reference for normalization. All experiments were performed in triplicate.

5. Conclusions

Our study extends our research on anti-biofilm peptide 1018. Here we show that this peptide prevents biofilm formation, eradicates mature biofilms and kills biofilms formed by a wide range of *P. aeruginosa* and *B. cenocepacia* strains isolated from cystic fibrosis patients. New peptides derived from 1018 showed similar or decreased anti-biofilm activity against *P. aeruginosa* biofilms, but increased activity against biofilms formed by the Gram-positive bacterium methicillin resistant *Staphylococcus aureus*. In addition, some of these new peptide derivatives retained the immunomodulatory activity of 1018 and were non-toxic towards human PBMCs. The combined anti-biofilm and immunomodulatory activities of the peptides described here make them promising therapeutic candidates for the treatment of antibiotic-resistant infections.

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Author Contributions

C.D.L.F.-N. and R.E.W.H. conceived and designed the project; C.D.L.F.-N., S.C.M., Z.W., L.J., E.B.M.B., M.E., F.R. and S.L.R-Z performed and analyzed studies; C.D.L.F.-N., S.C.M., Z.W., L.J., E.B.M.B., M.E., D.P.S., S.L.R.-Z., Y.S., M.H. and R.E.W.H. supplied reagents/materials/analysis tools. C.D.L.F.-N. and R.E.W.H. wrote the paper; S.L.R.-Z., Y.S., M.H. and R.E.W.H. supervised all studies.

Conflicts of Interests

C.D.L.F.-N and R.E.W.H. are co-inventors of a provisional patent application on the use of cationic anti-biofilm and innate defense regulator (IDR) peptides (U.S. Patent Application No. 61/870,655).

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Host Defense Peptides from Asian Frogs as Potential Clinical Therapies

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Abstract: Host defense peptides (HDPs) are currently major focal points of medical research as infectious microbes are gaining resistance to existing drugs. They are effective against multi-drug resistant pathogens due to their unique primary target, biological membranes, and their peculiar mode of action. Even though HDPs from 60 Asian frog species belonging to 15 genera have been characterized, research into these peptides is at a very early stage. The purpose of this review is to showcase the status of peptide research in Asia. Here we provide a summary of HDPs from Asian frogs.

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1. Introduction

The origins of the study into host defense peptides began with the pioneering studies of Vittorio Erspamer, who discovered biogenic amines and peptides secreted by the amphibian skin. The field later diversified when Zasloff isolated magainin from *Xenopus laevis* [1,2]. In our current era of multi-drug resistant bacterial strains, discovering new and effective treatments to replace traditional antibiotics is a critical area of research. Host defense peptides (HDPs), such as those being found in the skin of Asian frogs, have shown effectiveness in killing both gram-negative and gram-positive bacteria, as well as some viruses and cancers, without any of the drawbacks of antibiotic resistance [3]. These properties make them viable candidates as the next wave of therapies against infectious and non-infectious diseases that are the leading cause of death in developing countries.

Research on how these anti-microbial peptides function is still in the early stages. Most of the HDPs are typically cationic, a feature which allows them to interact with the negatively charged bacterial and cancer cell membranes. This cationic nature allows some peptides to actually penetrate the surface of pathogens as a means of killing them [4]. Asia is well known for its diverse amphibian populations and has been a resource for novel HDPs. Recently, potent HDPs have been isolated from amphibians located in temperate regions (traditionally believed to have low biodiversity) and have proven to be efficient in combating many clinical pathogens, though research into these peptides is still at a very early stage. We hope as we continue to discover new peptides in this region we will also find more potential candidates for novel therapeutics.

2. Diversity of HDPs Found in the Skin Secretion of Asian Frogs

In the tropics, especially in the rain forests of Asia, effective host defense peptides have helped the frogs to survive within their environment and attain a rich diversity. However with the alarming rate of decline in amphibians globally, many identified and unidentified species have become extinct

before researchers ever had a chance to isolate their HDPs for study [5]. Those peptides that have been isolated show a great degree of variability—even within a single genus, different species produce varied repertoires. These differences can be correlated to habitat specific challenges endured by each species, and how these frogs uniquely evolved alongside their environment [6]. Even within the same frog, expression of several members of a particular family of HDP is observed. The variation in these peptide sequences is suggestive evidence for ancestral gene duplication [7]. No two peptides with identical amino acid sequences have been reported even from closely related species. Such diversity provides broad-spectrum protection against the large pool of microbes/predators in their habitat (Figure A1–A4) [8]. Transcriptomic and peptidomic analysis revealed that genera will have HDP families in common, apart from this, some unique peptides, such as neuroendocrine and smooth muscle active, are reported that serve a particular purpose in that habitat e.g., bradykinin related peptides (BRPs) and Trypsin inhibitors from Odorrana genus. Here we review the HDP families reported from Asian frogs. Despite the wealth of biodiversity, attempts to characterize HDPs from Asian frogs have been limited. In the present medical scenario there is an urgent need to uncover the hidden peptides that may lead to developing new and potentially crucial therapeutic agents.

2.1. Amolops

Genus Amolops, which belongs to the family Ranidae, is endemic to China. Eight species of frogs from this genus have been studied and their HDPs isolated (Tables 1 and 2). The most common peptides isolated are of the Brevenin 1 and 2, Esculentin 2, Palustrin 2, and Temporin families, and all of them have been shown to be active against gram-positive and -negative bacteria, fungi, and cancer cells [9–14]. Cytotoxicity analysis against K562 and HT29 of Palustrin 2 from *A. jindongensis* skin revealed IC_{50} values between 57–58 μM [11].

Amolopins, a novel family of two peptides (P1 and P2), were isolated from *A. loloesis* and have shown potent antimicrobial activity [15]. Both peptides were effective against the tested gram-positive bacteria over a range of 37–75 μg/mL, though they were found not to be effective against the tested range of gram-negative bacteria. Amolopins also showed 3% hemolysis at 200 μg/mL [15]. Additionally, Amolopin 1 and Amolopin 6 were isolated from *A. hainanensis* [10], and Jindongenin—a novel family characterized from *A. jindongensis*—were found to be effective against gram positive (Minimal inhibitory concentration [MIC]: 17–60 μM), gram-negative bacteria (MIC: 10–40 μM) and fungi (*Candida albicans*: MIC 60 μM). Cytotoxicity analysis of Jindongenin from *A. jindongensis* against K562 and HT29 revealed that IC_{50} values ranged between 40–46 μM [11].

Hainanenins 1–5 were isolated from *A. hainanensis* [10] and also showed potent antimicrobial function against gram-positive bacteria, gram negative bacteria and fungi in the ranges 4–40 μg/mL, 4–75 μg/mL, and 2–75 μg/mL respectively. Three Bradykinin related peptides (BRPs) named Amolopkinins were found in *A. loloesis* and *A. wuyiensis* [16,17]. Amylopkinin from *A. loloesis* exhibited dose dependent contractile activity in guinea pig ileum [16]. The function of BRPs in frog skin secretion is thought to relate to smooth muscle contraction activity—if a predator swallows the frog, these peptides induce smooth muscle contractions that will result in a vomiting sensation, thereby allowing the frog to escape predation. Two other Amylopkinins from *A. wuyiensis* inhibited Bradykinin induced contractile effects on isolated rat ileum smooth muscle preparations [17].

Table 1. List of peptides identified from the skin secretion of Asian frogs.

		Peptides Isolated from the Respective Frog Species: Numbers Indicate Paralogs of Each Peptide Family						
Genus: Amolops		**Brevinin-1**	**Brevinin-2**	**Esculentin-2**	**Palustrin-2**	**Temporin**	**Novel family of Peptides**	**Ref**
1	*A. chunganensis*	5	1	1	1	5		[9–17]
2	*A. hainanensis*		2		1		Amylopin-1: 3 Amylopin-6: 1 Hainanenin 1-5,5 families: 5	
3	*A. jindongenensis*	1		2	2		Jindongnenin: 1	
4	*A. loloensis*	4		2		11	Amylopin 1-2,2 families: 2 Amylopkinin: 1	
5	*A. lifanensis*	3		1				
6	*A. ricketti*	3	2					
7	*A. torrentis*		1					
8	*A. wuyiensis*						Amylopkinin: 2	
Genus: Glandirana		**Brevinin-1**	**Brevinin-2**	**Esculentin-2**				**Ref**
1	*G. rugosa*	2 (Gaegurin 5–6)	6 (Gaegurin 1–3 Rugosin A,B,C)					[13,18–20]
2	*G. emeljanovi*	2 (Gaegurin 5–6)		1 (Gaegurin 5–6)				
Genus: Hylarana		**Brevinin-1**	**Brevinin-2**	**Esculentin-1**	**Esculentin-2**	**Temporin**	**Novel family of Peptides**	**Ref**
1	*H. erythrea*	3	2		4	1		[8,21–36]
2	*H. guentheri*	2	6			6	Guentherin: 1 Bradykinin BRP: 12 *	
3	*H. latuouchii*	4	3	2	2	6	Palustrin-2	
4	*H. luctiosa*		4	1	1	2	Palustrin-2: 2	
5	*H. nigrovittata*	7 (7 **Gaegurins**)	9 (9 **Rugosins**)			3	Nigroain: 15 Ranakinin N: 1 Cholycytokinin: 1	
6	*H. picturata*	2	5			1		
7	*H. signata*	5	4			2	Palustrin-2	
8	*H. spinulosa*	2	4	1	2	5	Spinulosain: 1 Ranatuerin: 1 Nigroain: 5 Odorranain: 1 Ranacyclin: 1	
9	*H. temporalis*	1	2		3		Hylaranakinin: 2	

Table 1. *Cont.*

		Peptides Isolated from the Respective Frog Species: Numbers Indicate Paralogs of Each Peptide Family						
Genus: Odorrana		**Brevinin-1**	**Brevinin-2**	**Esculentin-1**	**Esculentin-2**	**Nigrocin-2**	**Novel family of Peptides**	**Ref**
1	*O. grahamii*	2	4	2	4	4	Takykinin: 2 TrypsinInhibitor: 1, BRP: 3 * BLP: 5 * Odorrnalectin: 1 Palustrin-2: 1 Grahamin 1–2: 2 families: 2 Odorranain: 27	[14,28,31, 37–58]
2	*O. hananensis*	2	2				Odorrnain: 2 Temporin: 2	
3	*O. hejiangensis*						TrypsinInhibitor: 1	
4	*O. hossi*	2	2	1	1	2		
5	*O. ishikawae*	1	3	2	1	5	Ishikawain 1–8, 8 families: 8 Palustrin 2: 3 Odorranain: 2	
6	*O. jingdongensis*	3	1		2	2		
7	*O. livida*						Lividin 1–4, 4 families: 4	
8	*O. schmakeri*	3	3	1	1		BRP: 7 *	
9	*O. tiannanensis*	3		1	2	1	Mararetaein: 2 Pleurain: 1 Tiannenensin: 1 Odorranain: 10	
10	*O. versablis*	2		2	2		Ranatuerins: 2 Temporin: 1 TrypsinInhibitor: 1	
Genus: Pelophylax		**Brevinin-1**	**Brevinin-2**	**Esculentin-1**	**Esculentin-2**		**Novel family of Peptides**	**Ref**
1	*P. plancyi*		3					
2	*P. porosus*		1					
3	*P. chosenicus*		1					[13,20,56,59,60]
4	*P. fukienensis*	1		1	1		Pelophylaxin 1–4, 4 families Ranakinestatin: 1	
5	*P. nigromaculata*		1 (Nigrocin-1)	2			Nigocin-2: 1	

Table 1. *Cont.*

		Peptides Isolated from the Respective Frog Species: Numbers Indicate Paralogs of Each Peptide Family					
Genus: Rana		**Brevinin-1**	**Brevinin-2**	**Temporin-1**	**Ranatuerin-2**	**Novel family of Peptides**	**Ref**
1	*R. amurensis*	3 (Amurin 1–3:3 families)			2		
2	*R brevipoda porsa*	1	1				
3	*R. chaochioensis*					Japonicin-2: 4	
4	*R. chensinensis*	6	3	11		RCSK 1–4, 4 families: 4 * Chensinin 1–4, 4 families: 7 Japonicin-1: 1 D-1CDYa: 1 **	
5	*R. dybowskii*	18 (9 Dybowskins)	7	3		Japonicin-1: 1	
6	*R. japonica*			1		Japonicin-1: 1 Japonicin-2: 1	
7	*R. okinavana*	4			1		[20,61–80]
8	*R. ornativentris*		2	7	4	Palustrin: 1	
9	*R. pirica*	1	5	2	1		
10	*R. pleuradan*					Pleurain: 2	
11	*R. sakurai*		2	4	1	MRP: 1 *, BRP: 1 *	
12	*R. shuchinae*					Shuchin 1–5, 5 families: 5	
13	*R. tagoi*	1		1		MRP: 1 *	
14	*R. tagoiokiensis*	2		2	2		
15	*R. tsushimensis*	1	1	4			
Genus: Clinotarsus							**Ref**
1	*C. curtipes*	Brevinin-1: 5					[81]
Genus: Fejervaria							
1	*F. carcrivora*	Tigerinin: 2					[82]
Genus: Hoplobatracus							
1	*H. rugulosus*	Tigerinin-1: 1					[83–85]
2	*H. tigerinus*	Tigerinin 1–4, 4 families: 4					

Table 1. *Cont.*

		Peptides Isolated from the Respective Frog Species: Numbers Indicate Paralogs of Each Peptide Family	
Genus: Hyla			**Ref**
1	*H. annectans*	Annotoxin: 1	[86]
Genus: Limnonectes			
1	*L. fujianensis*	Limnonectins: 2	[87]
Genus: Nanorana			
1	*N. parkeri*	Japonicin: 2 Parkerin: 1	[88]
Genus: Rhacophorus			
1	*R. duboisi*	Polypedarelaxin: 1 Polypedatein: 1	[89,90]
2	*R. scheglii*	Histone 2B	
Genus: Sanguirana			
1	*S. varians*	BLP: 1 *	[91]
Genus: Euphlyctis			
1	*E. hexadactylus*	Crude skin extract: peptidesnot characterized	[92]

* Abbreviations used in the table: BRP—Bradykinin like peptide, BLP—Bombesisn like peptide, RCSK—*R. chensinensis* skin kinogen, MRP—Mellitin related peptide. Paralogs: Peptides of a particular family with little sequence homology found within a species, Orthologs: Particular family of peptides found withi two different species or genuses. ** CDY in the peptide D-1CDYa was used to indicate that the peptides were obtained from Chinese frog (C) found in Dongbei Region (D, northeast), city of Xifeng (Y).

Table 2. List of peptides identified from the skin secretion of Asian frogs with their corresponding MIC and IC_{50}/LD_{50} values.

		MIC and IC_{50}/LD_{50} Values					
	Genus: Amolops	**Brevinin-1**	**Brevinin-2**	**Esculentin-2**	**Palustrin-2**	**Temporin**	**Novel family of Peptides**
1	*A. chunganensis*	G+: 0.5–75 μM G−: 4–150 μM F: 4–150 μM LD50: 15–150 μM	G+: 2–75 μM G−: 2–150 μM F: 9 μM LD50: 75 μM	G+: 1–19 μM G−: 75–150 μM F: 4.5 μM LD50: 75 μM	G+: 2–150 μM G−: 4–10 μM F: 4.5 μM LD50: 75 μM	G+: 4–150 μM G−: 75 μM F: 9–150 μM LD50: 150–200 μM	
2	*A. hainanensis*		Unknown		Unknown		Amylopins: G+: 37–75 μg/mL G−: no active Hainanenin: G+: 4–40 μM G−: 4–75 μM F: 2–75 μM
3	*A. jindongenensis*	Unknown		Unknown	G+: 20 μM G−: 13–50 μM F: not active IC50: 57–58 μM (K562 & HT29 cell lines)		Jindongnenin:G+: 17–60 μM G−: 10–40 μM F: 60 μM
4	*A. loloensis*	G+: 5 μg/mL G−: 2–7 μg/mL F: not active IC50: 58 μg/mL (HepG2 cell line)		G+: 1–8 μg/mL G−: 7–50 μg/mL F: 2–22 μg/mL		G+: 1–75 μg/mL G−: 1–75 μg/mL F: 1–25 μg/mL IC50: 77 μg/mL (HepG2 cell line)	Amylopin: G+: 37–75 μg/mL G−: no active Amylopkinin: Smooth muscle active peptide
5	*A. lifanensis*	Unknown		Unknown			
6	*A. ricketti*	G+: 3–25 μg/mL G−: 12.5 μg/mL F: 100–200 μg/mL LD50: 100–200 μg/mL	G+: 1–200 μg/mL G−: 6–15 μg/mL F: 200 μg/mL				
7	*A. torrentis*		Unknown				
8	*A. wuyiensis*						Amylopkinin: Smooth muscle active peptide
	Genus: Glandirana	**Brevinin-1**	**Brevinin-2**	**Esculentin-2**			
1	*G. rugosa*	Unknown	G+: 6–50 μg/mL G−: 12.5–100 μg/mL				
2	*G. emeljanovi*	Unknown		Unknown			

Table 2. *Cont.*

		MIC and IC_{50}/LD_{50} Values					
	Genus: Hylarana	Brevinin-1	Brevinin-2	Esculentin-1	Esculentin-2	Temporin	Novel family of Peptides
1	*H. erythrea*	Unknown	G+: 12.5 µM G−: 12.5 µM F: 50 µM LD50: 280 µM		Unknown	Unknown	
2	*H. guentheri*	Unknown	G+:3–6µMG−:2–6µM F: not active LD50: 280 µM			G+: 30–50 µM G−: not active F: not active	Guentherin G+: 33.5 µg/mL Bradykinin, BRP: smooth muscle active peptide
3	*H. latuouchii*	G+: 6–10 µg/mL G−: 12.5 µg/mL F: 100–200 µg/mL LD50: 100–200 µg/mL	G+: 0.5–8 µg/mL G−: 0.5–130 µg/mL F: not active LD50: 400–600 µg/mL	G+: 0.6–10 µg/mL G−: 0.6–10 µg/mL F: 80 µg/mL LD50: 500 µg/mL	G+: 30–60 µg/mL G−: 6–15 µg/mL F: not active LD50: 500 µg/mL	G+: 6–10 µg/mL G−: not active F: not active LD50: 40 µg/mL	Palustrin: G+: 1–14 µg/mL G−: not active F: not active LD50: 220 µg/mL
4	*H. luctiosa*		Unknown	Unknown	G+: 4 µM G−: 32 µM	G+: 32 µM G−: 128 µM	Palustrin: G+: 1–14 µg/mL G−: not active F: not active LD50: 220 µg/mL
5	*H. nigrovittata*	G+: 1–65 µg/mL G−: 18–40 µg/mL F: 2–5 µg/mL	G+: 4–20 µg/mL G−: 25–100 µg/mL F: 5–20 µg/mL			G+: 3–9 µg/mL G−: 4–15 µg/mL F: 3–9 µg/mL	Nigroain:G+: 9–50 µg/mL G−: 25–110 µg/mL F: 2–4 µg/mL Ranakinin N, Cholycytokinin: smooth muscle active peptides
6	*H. picturata*	G+: 3 µM G−: 24 µM	G+: 9–18 µM G−: 9–72 µM			Unknown	
7	*H. signata*	Unknown	Unknown			Unknown	Palustrin G+: 1–14 µM
8	*H. spinulosa*	G+: 3–100 µM G−: 100–400 µM F: 12.5 µM	G+: 3–200 µM G−: 3–400 µM F: 100–400 µM	Unknown	G+: 6–200 µM G−: 12–400 µM F: not active	G+: 6–25 µM G−: not active F: 100–400 µM	Spinulosain,Ranatuerin,Nigroain,Odorranain, Ranacyclin:Unknown
9	*H. temporalis*	G+: 100–150 µg/mL G−: 30–150 µg/mL	G+: 40–150 µg/mL G−: 20–150 µg/mL		Unknown		Hylaranakinin:Unknown

Table 2. *Cont.*

		MIC and IC_{50}/LD_{50} Values					
Genus: Odorrana		**Brevinin-1**	**Brevinin-2**	**Esculentin-1**	**Esculentin-2**	**Nigrocin-2**	**Novel family of Peptides**
1	*O. grahamii*	Unknown	Unknown	Unknown	Unknown	G+: 9–100 µg/mL G−: 4–100 µg/mL F: 1–10 µg/mL	Takykinin,TrypsinInhibitor, BRP,BLP: smooth muscle active peptides Odormalectin: drug targeting Grahamin: G+: 2.5 µg/mL G−: 1–8 µg/mL F: 7.5 µg/mL Palustrin: G+: 12–100 µM G−: 100 µM F: 100 µM Odorranain: G+: 2–90 µg/mL G−: 3–50 µg/mL F: 1–50 µg/mL
2	*O. hananensis*	G+: 1–150 µM G−: 9–150 µM F: 1–10 µM LD50: 75 µM	G+: 9–150 µM G−: 9–10 µM F: 19–40 µM LD50: 300 µM				Odorranain: Unknown Temporin: G+: 2–150 µM G−: 30–75 µM F: 9–75 µM LD50: 75–300 µM
3	*O. hejiangensis*						Trypsin Inhibitor: smooth muscle active peptide
4	*O. hossi*	G+: 3 µM G−: 24–50 µM	G+: 18 µM G−: 36 µM	G+: 12 µM G−: 12 µM	G+: 16 µM G−: 32 µM	G+: 25–60 µM G−: 10–30 µM	
5	*O. ishikawae*	G+: 6–100 µM G−: not active F: 50 µM	G+: 6–100 µM G−: 12–50 µM F: not active	G+: 3–25 µM G−: 3–12 µM F: 50 µM	G+: 3–25 µM G−: 12.5 µM F: 100 µM	G+: 3–15 µM G−: 25–50 µM F: 50 µM	Ishikawain: Unknown Palustrin-2: G+: 12–100 µM G−: 100 µM F: 100 µM Odorranain: Unknown
6	*O. jingdongensis*	G+: 6–15 µM G−: 25–50 µM F: 50µM	G+: 19 µM G−: 38 µM F: 19 µM		G+: 8 µM G−: 34 µM F: not active	G+: 8–16 µM G−: 15–16 µM F: 30–70 µM	
7	*O. livida*						Lividin 1–4: Unknown
8	*O. schmakeri*	Unknown	Unknown	Unknown	Unknown		BRP: 7
9	*O. tiannanensis*	Unknown		Unknown	Unknown	Unknown	Mararetaein, Pleurain, Odorranain: Unknown Tiannenensin: G+: 75 µM F:>100 µM
10	*O. versablis*	Unknown		Unknown	Unknown		Ranatuerins, Temporin, Trypsininhibitor: Unknown
Genus: Pelophylax		**Brevinin-1**	**Brevinin-2**	**Esculentin-1**	**Esculentin-2**		**Novel family of Peptides**
1	*P. plancyi*		Unknown				
2	*P. porosus*		Unknown				
3	*P. chosenicus*		Unknown				
4	*P. fukienensis*	Unknown		Unknown	Unknown		Pelophylaxin: Unknown Ranakinestatin: bradykinin antagonist
5	*P. nigromaculata*		Unknown				Nigocin-2 G+: 2.5 µg/mL G−: 10–100 µg/mL F: 150 µg/mL

Table 2. *Cont.*

		MIC and IC_{50}/LD_{50} Values				
	Genus: Rana	**Brevinin-1**	**Brevinin-2**	**Temporin-1**	**Ranatuerin-2**	**Novel family of Peptides**
1	*R. amurensis*	Unknown			2	
2	*R. brevipoda porsa*	G+: 8 µg/mL G−: 34 µg/mL	G+: 8 µg/mL G−: 4 µg/mL			
3	*R. chaochioensis*					JaponicinG+: 25–100 µg/mL G−: 12–100 µg/mL
4	*R. chensinensis*	G+: 12.5 µM G−: 25 µM HC50: 180–200 µM	3	G+: 100 µM G−: 100 µM IC50: 30–60 µM (Mcf 7 breast cancer cell line) LD50: 100 µM		RCSK 1–4, Chensinin 1–4, Japonicin-1, D-1CDYa: G+: 6–8 µM G−: 3–5 µM HC50: 450 µM
5	*R. dybowskii*	G+: 12.5 µM G−: 25 µM HC50: 125 µM	G+: 15–30 µM G−: 15–30 µM	G+: 60–100 µM G−: 60–100 µM HC50: 180 µM		Japonicin-1:G+: 100 µM G−: 25 µM HC50: 300 µM
6	*R. japonica*			G+: >100 µM G−: >100 µM		Japonicin-1:G+: >100 µM G−: 30 µM Japonicin-2: G+: 20 µM G−: 12 µM
7	*R. okinavana*	G+: 12.5 µM G−: 6–12.5 µM F: not active			G+: 50 µM G−: 12.5 µM F: 100 µM	
8	*R. ornativentris*		Unknown	G+: 200 µM F: 200 µM	Unknown	Palustrin: Unknown
9	*R. pirica*	G+: 13µM G−: not active F: 100 µM HC50: 7 µM	G+: 25 µM G−: 3–12 µM F: 100 µM HC50: 50 µM	G+: 100 µM G−: not active F: 100 µM HC50: 300 µM	G+: 100 µM G−: not active F: 100 µM HC50: 150 µM	
10	*R. pleuradan*					Pleurain: G+: 15–30 µg/mL G−: 60–120 µg/mL F: 30 µg/mL
11	*R. sakurai*		G+: >50 µM G−: 3 µM F: not active	G+: 25 µM G−:>50 µM F: >50 µM	G+: >50 µM G−: 50 µM F: >50 µM	MRP (AR 23), BRP: Smooth muscle active peptides
12	*R. shuchinae*					Shuchin G+: 6–15 µg/mL G−: 3–50 µg/mL F: 6.25 µg/mL
13	*R. tagoi*	Unknown		G+: 10–40 µM		MRP (AR23): G+: 2–20 µM
14	*R. tagoiokiensis*	G+: 5 µM G−: 20 µM F: 20 µM		G+: 10 µM G−: 160 µM F: 80µM	G+: 160 µM G−: 80 µM F: 160 µM	
15	*R. tsushimensis*	G+: 12–25 µM G−: 25–100 µM F: 50 µM LD50: 12 µM	G+: 5 µM G−: 20 µM F: 20 µM LD50: 100 µM	Unknown		

Table 2. *Cont.*

		MIC and IC_{50}/LD_{50} Values
Genus: Clinotarsus		
1	*C. curtipes*	Brevinin-1 G+: 6–100 µg/mL G−: 7–60 µg/mL
Genus: Fejervaria		
1	*F. carcrivora*	Tigerinin 2G+: 20–80 µg/mL G−: 10–40 µg/mL F: 80–180 µg/mL
Genus: Hoplobatracus		
1	*H. rugulosus*	Tigerinin 1: Insulin releasing peptide
2	*H. tigerinus*	Tigerinin 1: G+: 20–50 µg/mL G−: 20–100 µg/mL
Genus: Hyla		
1	*H. annectans*	Annotoxin-1: Inhibitor of tetradotoxin sensitive sodium channel
Genus: Limnonectes		
1	*L. fujianensis*	Limnonectin-2: G+: not active G−: 35–70 µM LD50: 160 µM
Genus: Nanorana		
1	*N. parkeri*	Japonicin:G+: 9–40 µg/mL G−: >100 µg/mL Parkerin: G+: 37.5 µg/mL G−: 37–100 µg/mL
Genus: Rhacophorus		
1	*R. duboisi*	Polypedarelaxin: Smooth muscle active peptide Polypedatein: Unknown
2	*R. Scheglii*	Histone 2B: Unknown
Genus: Sanguirana		
1	*S. varians*	BLP: Smooth muscle active peptide
Genus: Euphlyctis		
	E. hexadactylus	Crude skin extract: G+: 120–260 µg/mL G−: 120–520 µg/mL F: 32–64 µg/mL

G+: gram positive bacteria; G−: gram negative bacteria; F: fungi; LD_{50}: mean lethal dose against RBC; IC_{50}: drug concentration causing 50% inhibition against cancer cell lines.

2.2. *Clinotarsus*

The Clinotarsus genus is endemic to the Western Ghats of India where new HDPs are being discovered. The peptidomic approach revealed the presence of five novel peptide amides homologous to the Brevinin 1 family from a single species, *C. curtipes*, that all show promising effectiveness against gram-positive and gram-negative bacteria [81]. The MICs against the tested pathogenic bacteria were reported to be between 6–25 µg/mL (Tables 1 and 2). It was previously reported that bacterial membranes depolarize during pore formation and bacterial killing, but in the case of Brevinin 1 from *C. curtipes*, it was shown that depolarization and bacterial killing are independent events. We are slowly beginning to understand the methods by which these HDPs effectively fight disease.

2.3. *Euphlyctis*

Ramesh B. *et al.* [92] reported on the potential of HDPs discovered on the skin of the Indian green frog, *Euphlyctis hexadactylus*. They showed that the lyophilized crude skin secretion was inhibiting the growth of pathogenic gram-positive and gram-negative bacteria, as well as fungal pathogens—which included plant fungal pathogens. The MIC of the peptide against several human-relevant bacteria ranged from 128 to 512 µg/mL and for fungus it ranged between 32 to 64 µg/mL (Tables 1 and 2). However it has not yet been reported what the composition of these skin secretions might be.

2.4. *Fejervarya*

Two Tigerinin peptides were identified from *Fejervarya cancrivora* [82], similar to the Tigerinins originally isolated from the Indian frog *Hoplobatrachus tigrinus*. The effects of a *C*-terminal amidation on both peptides include a decrease in MIC against pathogens compared to their non-amidated counterparts. MICs of non-amidated Tigerinins against gram-positive bacteria, gram-negative bacteria and fungi ranged from 20–80 µg/mL, 10–40 µg/mL and 80–180 µg/mL respectively (Tables 1 and 2) and that of *C* terminally amidated peptides were 10–40 µg/mL, 5–40 µg/mL, and 15 µg/mL respectively. They also exhibited 10%–12% hemolysis against rabbit red blood cells (RBCs) at 100 µg/mL [82]. With this data we can better understand the influence of the peptide's sequence on its function.

2.5. *Glandirana*

HDPs with significant antimicrobial activity have been reported from two species of genus Glandirana—*G. rugosus* [13,18] and *G. emeljanovi* [19] (Table 1). Novel peptides such as Rugocin A–C and Gaegurins 1–3 (6 peptides) were characterized from *G. rugosus*, which is now reclassified as the Brevinin 2 family [20]. Gaegurins 5–6 from *G. emeljanovi* have been reclassified as the Brevinin 1 family [20], and Gaegurin 4 as Esculentin 2 [19]. MICs of Brevinin-2 (Rugosin A and B) from *G. rugosus* were found to be 6–50 µg/mL for gram-positive bacteria and 12.5–100 µg/mL for gram-negative bacteria (Tables 1 and 2) [18].

2.6. Hoplobatrachus

Tigerinins, first reported in *H. tigerinus*, [83] are a family of 4 peptides identified in *H. rugulosus* that are characterized by potent antimicrobial activity against gram-positive bacteria (MICs: 20–50 µg/mL) and gram-negative bacteria (MICs: 20–100 µg/mL) (Tables 1 and 2). Tigerinins isolated from *H. rugulosus* have also been found to stimulate insulin release from rat BRIN-BD11 clonal β cell line [84,85]. Ojo *et al*. [84] also reported that C-terminal amidation was crucial for its activity.

2.7. Hyla

Anntoxin, a peptide that inhibits tetradotoxin-sensitive voltage-gated sodium channels, was identified in the skin secretions of *Hyla annectans*. This peptide is believed to have a great therapeutic potential as an anti-nociceptive and anti-inflammatory agent [86] (Tables 1 and 2).

2.8. Hylarana

Many of the peptides isolated from the different Hylarana species belong to the Brevinin 1 and 2, Esculentin 2, and Temporin families, and have showed activity against gram-positive and -negative bacteria, fungi and cancer cells (Tables 1 and 2) [21–30]. Gaegurins (7 peptides) and Rugosins (9 peptides) from *H. nigrovittata* have been reclassified to the families Brevinin 1 and 2, respectively [27]. Brevinin 2 from *H. guentherii* was also able to stimulate insulin secretion from the rat BRIN-BDII clonal β cell line [25].

H. nigrovittata and *H. spinulosa* also produce a novel family of antimicrobial peptides [27,30] called the Nigroain family, which exhibited activity against gram-positive bacteria (MICs: 9–50 µg/mL), gram-negative bacteria (MICs: 25–110 µg/mL) and fungi (MICs: 2–4 µg/mL). Bradykinin-like peptides identified from this genus include Ranakinin N and Cholycytokinin from *H. nigrovittata*, as well as BRPs from *H. guentherii* [31–33]. Ranakinin N induced dose-dependent contractile effects on guinea pig ileum [32] and BRPs from *H. guentherii* revealed dose-dependent contraction of intestinal smooth muscle but did not have any effect on rat arterial smooth muscle [33].

Antimicrobial peptide Guentherin was first reported from *H. guentherii* and showed potent activity only against gram-positive bacteria (MIC: 33.5 µg/mL) [33]. Odorranain, another novel peptide of Odorrana genus, was identified from *H. spinulosa* [30]. Antimicrobial peptide Esculentin 1 was identified in three Hylarana species: *H. latouchii, H. spinulosa* and *H. luctuosa* [8,24,30]. Palustrin 2 was reported from *H. latouchii*, *H. signata* and *H. luctuosa* [24,29]. Palustrin 2, found on *H. latouchii,* was active only against gram-positive bacteria (MICs: 1–14 µM). Three peptide familes, Ranateurin, Ranacyclin, and Spinulosain were uniquely found in *H. spinulosa* [30]. These three peptides did not show any antimicrobial activity against the tested pathogens but Ranatuerin exhibited very low hemolysis of 10% at 200 µM against rabbit RBCs.

H.temporalis is the first among the genus to be reported from Western Ghats, India. Seven novel peptides are reported from *H.temporalis,* which include one Brevinin 1, two Brevinin 2 peptides, three Esculentin 2 peptides, and one Hylaranakinin peptide [34–36]. The MICs of Brevinin and Esculentin peptides ranges from 30 to 150 µg/mL.

2.9. Limnonectus

Limnonectins (2 peptides) from *L. fujianensis* are the only family of HDPs reported from the genus Limnonectus thus far [87] (Tables 1 and 2). These peptides were reported to have no antimicrobial activity towards gram-positive bacteria and fungi, but MICs against gram-negative bacteria range between 35–70 µM. They exhibited no hemolysis up to a concentration of 160 µM.

2.10. Nanorana

Genus Nanorana has provided the antimicrobial peptides Japonicin 1 (2 peptides) and a novel family named Parkerin, both isolated from *N. parkeri* [88] (Tables 1 and 2). Japonicin 1 and Parkerin peptides were reported to be effective only against gram-positive bacteria with MICs in the range 9–37 µg/mL and 37–40 µg/mL respectively. 1%–3% hemolysis was observed for both peptides at 80 µg/mL. Mast cell degranulation, which is thought to mediate antimicrobial activity of HDPs, was exhibited by both the peptides.

2.11. Odorrana

HDPs have been identified from ten species of Odorrana (Table 1). *O. grahamii* is one of the well-studied species in Asia. Common peptide families include Brevinin 1 and 2, Esculentin 1 and 2, and Nigrocin 2 [28,31,37–47], all of which have been shown to be effective against gram-positive and -negative bacteria, as well as fungi. A novel antimicrobial peptide family, Odorranain, was reported from four species of the genus [14,37,41,42,48,49]. MICs of Odorranain peptides against gram-positive bacteria, gram-negative bacteria and fungi were in the ranges 2–90 µg/mL, 3–50 µg/mL and 1–50 µg/mL, respectively. MIC of Odorranain towards *Helicobacter pylori* was reported to be 20 µg/mL (Tables 1 and 2).

Many novel peptide families were reported from *O. grahamii* such as Tachykinin (2 peptides), Odorranalectin (the smallest lectin reported so far), 5 Bombesin-like peptide, and 2 Grahamins [37,48–52]. Odorranalectin coupled with a nanoparticle complex improved the therapeutic effects of UCN-loaded nanoparticle in treating Parkinson's disease. Results also suggested the use of Odorranalactin as a carrier in nasal delivery of macromolecular drugs to the brain [53]. Antimicrobial activity of Grahamins was reported against gram-positive bacteria (MIC: 2.5 µg/mL), gram-negative bacteria (MICs: 1–8 µg/mL) and fungi (MIC: 7.5 µg/mL). Trypsin inhibitor peptide was isolated from *O.grahamii*, *O. versablis*, and *O. hejiangensis*. These inhibitors are thought to protect the host from a range of proteases produced by invading pathogens [47,54,55].

Studies of the skin of *O. tiannanensis* also revealed a set of novel HDP families—Margaretaein (2 peptides), Pleurain, and Tiannanensin [42]. Tiannanensin, an antimicrobial peptide, showed activity against gram-positive bacteria and fungi with MICs 75 and >100 µM, respectively. In addition to these there are other novel HDP families including Iksikawain, Lividin (first report), and Ranateurin, found in *O. ishikawae*, *O. livida*, *and O. versablis*, respectively [41,43,56]. Bradykinin related peptides were also isolated from *O. grahamii* (3 peptides) and *O. schmakeri* (7 peptides) [57,58]. Three of the five Bombesin-like peptides from *O. grahamii* induced contraction of the stomach

muscle tissue. The other two BLPs, which have a C terminal octapeptide, antagonized stomach muscle contraction. Inhibition of contraction is thought to be due to the presence of the octapeptide [37].

2.12. *Pelophylax*

Five species of the genus Pelophylax (*P. plancyi*, *P. porosus*, *P. chosensicus*, *P. nigromaculata*, and *P. fukienensis*) are well-known for their HDPs (Tables 1 and 2). Pelophylaxins 1–4 are a novel peptide group with no orthologs, and are found in *P. fukienensis* [56]. Two other novel peptides, Nigrocin 1 and 2, were isolated from *P. nigromaculata* [13,59] and later reclassified to the Brevinin 2 family [20]. Brevinin 2 (Nigrocin 1) was reported to have antimicrobial activity towards gram-positive bacteria (MIC: 2.5 µg/mL), gram-negative bacteria (MIC: 10–100 µg/mL), and fungi (MIC: 100 µg/mL). Nigrocin 2 was also reported to be effective against gram-positive and gram-negative bacteria as well as fungi in the ranges 2.5 µg/mL, 10–200 µg/mL, and 150 µg/mL, respectively. Both of the peptides from *P. nigroemaculata* showed low hemolysis of 0.9%–1% at 100 µg/mL. A Bradykinin antagonist named Ranakenestatin was recently reported from *P. fukienensis*—though the exact function of this peptide in frogs remains controversial, it is expected to have great potential in therapeutic applications [60].

2.13. *Rana*

Rana is one of the most diverse and well-studied of the amphibian genera in Asia with respect to their host defense peptides. HDPs have been isolated from 16 different species (Tables 1 and 2). Common antimicrobial peptide families that were identified from this genus include Brevinin 1 and 2, Ranateurin, and Temporin 1 [61–75]. Temporin 1 from *R. chensinensis* exhibited cytotoxic effects against 12 tested carcinoma cell lines. They also showed low hemolytic effect against human RBCs and no considerable cytotoxic activity against normal human umbilical vein smooth muscle cells (HUVSMCs).

Japonicin 1 and 2, first isolated from *R. japonica*, were isolated from three other species of the genus [62,64,65,74]. Japonicins were found to be effective against gram-positive and gram-negative bacteria in the range of 25–100 µg/mL and 12–100 µg/mL respectively. They were also reported to be strongly hemolytic against mammalian RBCs.

Amurins reported from *R. amurensis* and Dybowskins from *R. Dybowsskii* (1–6: 6 peptides) [62] have been reclassified to the Brevinin 1 family [20]. Palustrin 1, first identified from *R. palustris*, was also reported from *R. ornativentris* [67]. Mellitin related peptides (MRPs) were isolated from *R. tagoi* and *R. sakuraii* [70,76]. MRPs are active against gram-positive bacteria, gram-negative bacteria, and fungi. Bradykinin related peptides were reported from *R. okienensis*, *R. tagoi*, *R. chensinensis* and *R. sakuraii* [69,71,72,77]. Shuchin (5 peptides) and Pleuran, two novel peptide families, were identified from *R. shuchinae* and *R. pleuridan*, respectively, both of which are potent against gram-positive and -negative bacteria, as well as fungi [78–80].

2.14. Rhacophorus

HDPs from two species—*Rhacophorus duboisi* (previously *Polypedates pingbianensis*) and *Rhacophorus schegelii*—have recently been isolated (Tables 1 and 2). Polypedarelaxin from *Rhacophorus duboisi* effectively relaxes smooth muscle contractions [89]. This peptide exhibited concentration-dependent relaxation effects on isolated rat ileum but did not show antimicrobial and serine protease activity. Cationic antimicrobial peptides do not appear in the dermal immune system of *Rhacophorus schegelii* [90]. However, Histone 2B was reported from *Rhacophorus schegelii*, [90] which is evidence for the role of histones as antimicrobials in addition to their ability to remodel chromatin. This was the first report of histones from frog skin secretion.

2.15. Sanguirana

HDPs isolated from *Sanguirana varians* (Tables 1 and 2) of the genus Sanguirana revealed homology to the neuroendocrine peptide Bombesin [91]. These BLPs revealed dose dependent contractile effects of stomach tissue.

3. Conclusions

With the increasing emergence of multi-drug resistant pathogens, as well as the constant struggle against non-infectious illnesses such as cancer, there is a critical need to develop new therapies where old treatments have failed. Amphibian-derived antimicrobial peptides offer us a potential solution. We are now just beginning to understand the diversity and functions of these peptides, but this review outlines the ways in which we already see the potential clinical uses of these HBPs. The potent antimicrobial peptides in Asian frogs belong to the Brevinin and Esculentin. They are very effective against gram-positive and negative-bacteria as well as fungi. The only problem with these peptides is their high cytotoxicity, which would be eliminated by effective modification. The Temporin family of peptides are potent anticancer agents which are highly toxic against cancer cells, especially breast cancer cell lines and demonstrate very low toxicity towards normal cells (e.g., temporin from *R. chensinensis*). Hopefully further study of these animals and their skin peptides will reveal novel therapies that may potentially fill the gap left by antibiotic resistance and the failure of many of our current cancer treatments. There is therefore a need to turn our attention to this region and its biodiversity as a key medical resource.

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Author Contributions

Vineeth T.V. Kumar, David Holthausen, Joshy Jacob and Sanil George wrote the paper.

Appendix

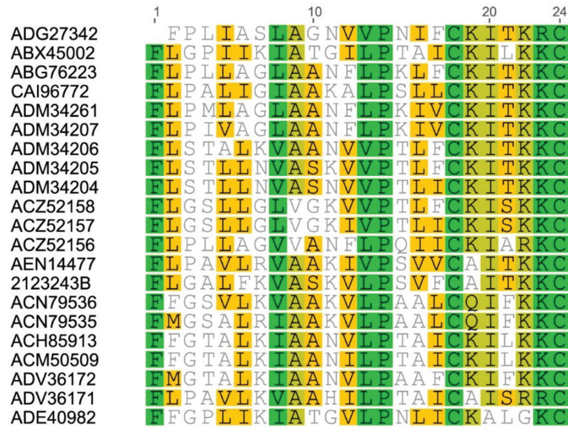

Figure A1. Multiple sequence alignment of Brevinin-1 from Asian frogs.

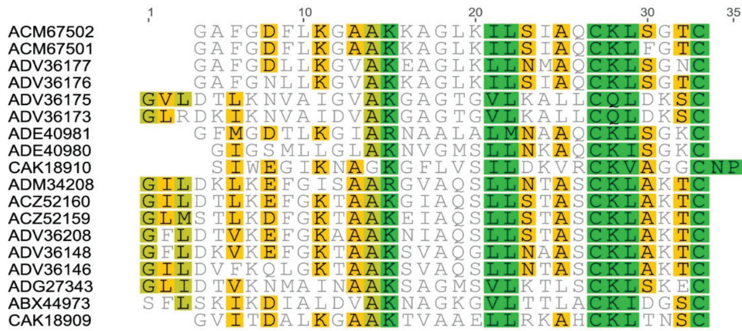

Figure A2. Multiple sequence alignment of Brevinin-2 from Asian frogs.

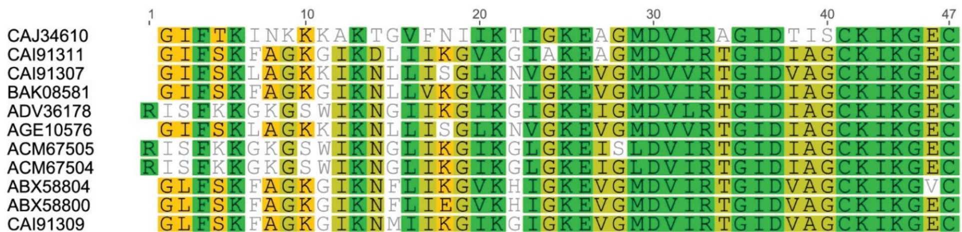

Figure A3. Multiple sequence alignment of Esculetin-1 from Asian frogs.

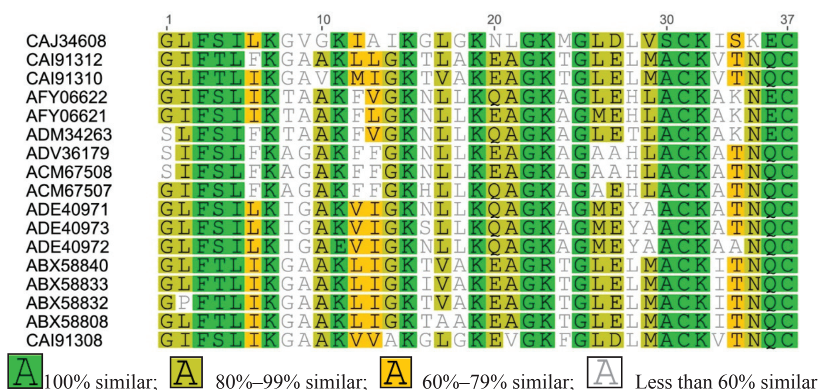

Figure A4. Multiple sequence alignment of Esculetin-2 from Asian frogs.

Conflicts of Interest

The authors declare no conflict of interest.

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Killing of Staphylococci by θ-Defensins Involves Membrane Impairment and Activation of Autolytic Enzymes

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Abstract: θ-Defensins are cyclic antimicrobial peptides expressed in leukocytes of Old world monkeys. To get insight into their antibacterial mode of action, we studied the activity of RTDs (rhesus macaque θ-defensins) against staphylococci. We found that in contrast to other defensins, RTDs do not interfere with peptidoglycan biosynthesis, but rather induce bacterial lysis in staphylococci by interaction with the bacterial membrane and/or release of cell wall lytic enzymes. Potassium efflux experiments and membrane potential measurements revealed that the membrane impairment by RTDs strongly depends on the energization of the membrane. In addition, RTD treatment caused the release of Atl-derived cell wall lytic enzymes probably by interaction with membrane-bound lipoteichoic acid. Thus, the premature and uncontrolled activity of these enzymes contributes strongly to the overall killing by θ-defensins. Interestingly, a similar mode of action has been described for Pep5, an antimicrobial peptide of bacterial origin.

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1. Introduction

Host defense peptides (HDPs) are important effector molecules of the ancient, non-specific innate immune system displaying multiple functions involved in microbial clearance. They may be constitutively expressed or be induced in response to infection or injury, e.g., through activation of Toll-like receptors or pro-inflammatory cytokines. Most peptides exhibit direct antimicrobial activity in the low micromolar concentration range against a large number of microorganisms, including multidrug resistant bacteria [1–3]. In higher organisms, HDPs have also been recognized as important immune regulators affecting either stimulation or suppression of immune cell activity [4,5]. Thus, the gene copy number or dysregulated expression of certain HDPs predisposes to various infectious and inflammatory diseases, underlining the importance of these peptides in controlling microbial pathogens [6,7].

Despite the great diversity in their primary structure and amino acid composition, HDPs are typically small (12–50 amino acids), positively charged and able to adopt an amphiphilic structure in solution [1,3,8]. One conserved group of HDPs comprises defensins *sensu stricto*, which were first discovered in mammals and subsequently found in invertebrates, plants and fungi. These peptides are characterized by a disulfide-stabilized β-sheet structure and primarily expressed in epithelial tissues or phagocytic cells [9,10]. Due to structural and functional similarity, it has been

proposed that all defensins evolved from a single precursor that can be traced back to prokaryotic origin [11,12].

Mammalian defensins possess potent broad-spectrum activity against both Gram-positive and Gram-negative bacteria, fungi and certain viruses. They are further divided into three groups, α-, β- and θ-defensins, based on their gene structure as well as spacing and pairing of their six conserved cysteine residues. α- And β-defensins are widely expressed in mammals and share a three-stranded antiparallel β-sheet fold, whereas the cyclic θ-defensins have only been isolated from the leukocytes of Old World monkeys [13–15]. θ-Defensins arose from a mutated α-defensin gene containing a premature stop codon in its defensin domain. During biogenesis, a nine amino acid segment is excised from the truncated α-defensin precursor and subsequently ligated head to tail to a similar or identical nonapeptide [15].

Rhesus macaques express three θ-defensin precursors, which can pair to generate six different homodimeric and heterodimeric isoforms (rhesus macaque θ-defensins 1 to 6; RTDs). Their concentration in the PMNs differs greatly, with RTD-1 being the most abundant [16]. Interestingly, the cyclic structure seems to be crucial for antimicrobial activity and confers salt resistance up to 150 mM NaCl [14]. In addition, θ-defensins possess potent anti-inflammatory properties *in vitro* and *in vivo* mediated by the suppression of numerous pro-inflammatory cytokines and blockade of TNF-α release [17]. Noteworthy, there are six θ-defensin genes present in the human genome, but a stop codon in the signal sequence blocks their translation. Synthetic or restored products of these pseudogenes termed retrocyclins show remarkable anti-HIV activity by inhibiting virus attachment and entry [18–20].

Mode of action studies with defensins from different eukaryotic kingdoms demonstrated that conserved molecules of the microbial cell envelope, which are readily accessible, such as the bacterial cell wall precursor lipid II or fungal sphingolipids, are targets of defensins and an important component of the killing mechanism [21–27]. Here, we have investigated the mechanism of action of θ-defensin bactericidal activity. Interestingly, RTDs do not interfere with peptidoglycan biosynthesis, but rather induce bacterial lysis in staphylococci by interaction with the bacterial membrane and/or release of autolytic enzymes similar to the lantibiotic Pep5.

2. Results and Discussion

The two θ-defensins, RTD-1 and RTD-2 (Figure 1), were initially tested for their activities against different staphylococcal species in a standard broth microdilution assay. Both peptides exhibited potent antimicrobial activity and inhibited growth of the three strains tested at concentrations ranging from 0.5 to 6 μg/mL (Table 1).

2.1. Impact on Bacterial Cell Wall Biosynthesis

Fungal [22,25] and invertebrate defensins [24] bind with high affinity to the cell wall building block lipid II, thereby specifically inhibiting peptidoglycan biosynthesis in Gram-positive bacteria. Similarly, lipid II targeting has also been reported for two mammalian defensins, the human α-defensins HNP-1 and β-defensin hBD3, which are both broad-spectrum antimicrobials [21,23].

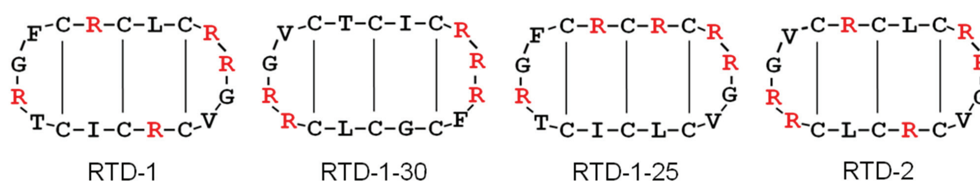

Figure 1. Amino acid sequences of heterodimeric RTD-1 and its two variants and of homodimeric RTD-2. Positively charged residues are marked in red.

Table 1. Antimicrobial activity of RTDs against staphylococci in half-concentrated MHB. MIC values (µg/mL) * are expressed as the lowest concentration that caused visible growth inhibition.

	RTD-1	**RTD-2**	**RTD-1-30**	**RTD-1-25**
S. aureus SG511-Berlin	6 ± 2	4 ± 0	6 ± 2	8 ± 4
S. simulans 22	1.5 ± 0.5	1.5 ± 0.5	3 ± 1	3 ± 1
S. carnosus TM300	0.75 ± 0.25	0.5 ± 0	ND	ND

ND, not determined; * Average values obtained from two or more independent experiments (±SD).

In order to verify whether θ-defensins also interfere with peptidoglycan biosynthesis, the cytoplasmic level of the cell wall precursor UDP-MurNAc-pentapeptide in *Staphylococcus simulans* 22 treated with RTDs was determined. Accumulation of UDP-MurNAc-pentapeptide is typically induced by antibiotics which inhibit the late, membrane-bound steps of cell wall biosynthesis and has also been demonstrated for hBD3 [23]. As shown in Figure 2, θ-defensins did not cause a significant accumulation of the cell wall precursor compared to vancomycin-treated control cells indicating that the antibiotic action of RTDs differs from that of defensins mentioned above.

2.2. Impact on Membrane Integrity

Due to their cationic and amphiphilic nature, it is widely believed that the killing activity of HDPs is based on the disruption of the membrane barrier function. To assess the membrane impairment by θ-defensins, the potassium release of whole cells was monitored over a period of 5 min by growing *S. simulans 22* in half-concentrated Mueller-Hinton broth (MHB) and subsequently diluting the cells in choline buffer (see Experimental). Under these conditions, with cells suspended in buffer without an energy source, significant potassium efflux could not be detected in response to RTDs at 5× and 10× MIC (Figure 3A). However, when cells were energized by addition of 10 mM glucose, rapid concentration-dependent ion release occurred after peptide treatment (Figure 3B). In contrast, the activity of the pore-forming lantibiotic nisin—used here as a positive control—was independent of the presence of glucose (Figure 3).

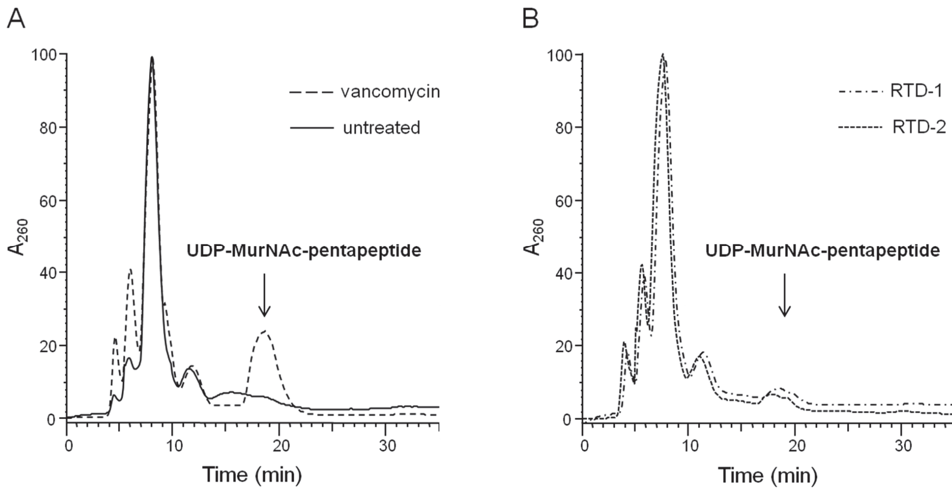

Figure 2. Intracellular accumulation of the final soluble cell wall precursor UDP-MurNAc-pentapeptide in *S. simulans* 22 exposed to θ-defensins. Cells were treated with 10× MIC vancomycin (positive control) (**A**) or RTDs (**B**), incubated for 30 min, and subsequently extracted with boiling water. The cytoplasmic pool of UDP-linked cell wall precursors was analyzed by RP-HPLC.

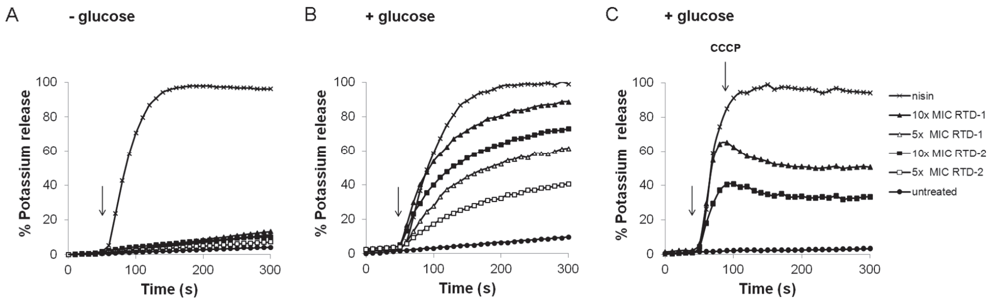

Figure 3. Impact on the membrane integrity of RTD-treated *S. simulans* 22 cells. Potassium efflux was monitored with a potassium-sensitive electrode in absence (**A**) and presence (**B**) of 10 mM glucose. RTD-induced potassium release of energized cells could be blocked by the addition of 5 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone; (**C**). Ion leakage was expressed relative to the total amount of potassium released after addition of 1 μM of the pore-forming lantibiotic nisin (100% efflux). RTDs were added at 5× and 10× MIC; controls were incubated without peptide. The arrows indicate the moment of peptide addition.

These results suggest that the membrane activity of RTDs depends on the bacterial membrane potential. To investigate this hypothesis further, 5 μM of the ionophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) were added to energized cells shortly after the peptides. CCCP uncouples the proton gradient across the cytoplasmic membrane leading to fast membrane depolarization. Indeed, RTD-induced ion leakage was blocked immediately after CCCP addition (Figure 3C).

Moreover, the membrane potential of *S. simulans* 22 in choline buffer (used for the potassium efflux experiments) was estimated by the distribution of the lipophilic cation TPP^+ inside and outside the cells. As expected, membrane potential increased by 15–20 mV after incubation with 10 mM glucose (data not shown [28]). Thus, a membrane potential of sufficient magnitude—as builds up after addition of glucose—seems to be essential for the membrane-disrupting activity of RTDs.

Similarly, the membrane potential of *S. aureus* SG511-Berlin was monitored in half-concentrated MHB, which was routinely used for MIC determinations and mode of action studies. In absence of glucose, the peptides did not cause any change of the membrane potential when added at 10× MIC. In contrast, in presence of glucose RTDs caused a reduction of the membrane potential of about 15 mV, which was, however, slowly restored within 20 min of treatment (Supplementary Figure S1).

The requirement for an energized membrane for antibacterial activity has also been described for the cationic antimicrobial peptide Pep5 produced by *S. epidermidis* 5 [29]. Further, Pep5 induces autolysis in *S. simulans* 22 by releasing cell wall lytic enzymes [30,31]. Thus, the question was raised if the membrane impairment alone is sufficient for killing by RTDs or if additional activities are involved in the killing mechanism—such as activation of autolytic enzymes—as described not only for Pep5 [30,31] but also for cationic peptides and proteins in general [32,33].

2.3. Impact on Autolytic Enzymes

RTD-treated cells of *S. aureus* SG511-Berlin grown in half-concentrated MHB were inspected by transmission electron microscopy. After 30 min treatment, additional membranous structures could be observed in many cells (Figure 4A–C), indicating the loss of cytoplasmic content. After 60 min exposure to RTD-2, cells showed evidence of cell wall degradation, particularly in the septum area between two daughter cells (Figure 4D,E). Moreover, in some cells, the cell wall was completely peeled off (Figure 4F). These morphological changes might indicate a premature activation and release of peptidoglycan lytic enzymes (referred to as autolysins) involved in cell separation as has been described for Pep5-treated cells [34]. These results suggest that the release of autolysins, which hydrolyze the glycan chains and peptide bridges of murein, also contributes to the killing activity of RTDs.

To test for release of cell wall hydrolases as a relevant component of the antistaphylococcal activity of RTDs, the supernatant of RTD-treated cells was analyzed for autolytic activity. Hence, *S. aureus* SG511-Berlin was incubated in the presence of RTDs at 10× MIC for 30 or 60 min, harvested and the concentrated supernatants were subjected to SDS-PAGE containing heat-inactivated *M. luteus* cells as substrate. Clear bands indicated the cell wall lytic activity of released enzymes. Cells exposed to Pep5 were included in the study and served as a positive control.

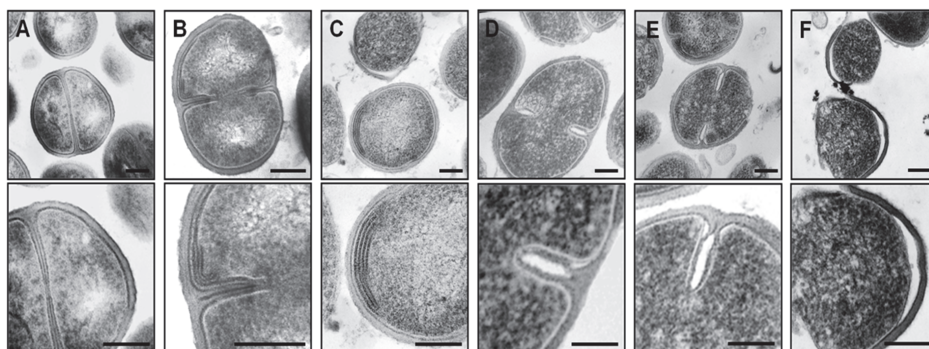

Figure 4. Transmission electron microscopy of *S. aureus* SG511-Berlin treated with 10× MIC RTD-2. (**A**) Untreated control cells. (**B**,**C**) Cells treated for 30 min. Additional membranous structures could be observed. (**D**,**E**,**F**) Cells treated for 60 min. Dividing cells showed degradation of the cell wall in the septum area between two daughter cells (**D**,**E**) or peeling of the cell wall (**F**). Scale bar: 0.2 µm.

Autolysins could be observed in all peptide treated samples, whereas hardly any activity was detectable in the untreated control (Figure 5A). Interestingly, all detected bands represent different processed forms of the autolysin Atl since in an *atl* deletion mutant (*S. aureus* SA113 *Δatl*; Figure 5B) corresponding bands were missing after treatment with RTD-2. Atl is a bifunctional autolysin that plays a key role in separating cells after cell division and is highly conserved among staphylococci [35]. Proteolytic processing of the Atl precursor protein of *S. aureus* generates two catalytically active enzymes fused to repeat units, an amidase (AM, 62 kDa; cleaves the amide bond between MurNAc and L-alanine) and a glucosaminidase (GL, 51 kDa; cleaves the β-1,4-glycosidic bond between GlcNAc and adjacent monosaccharides), and both components bind to the septum site of dividing cells [36,37]. In addition to the AM and GL bands, three additional bands with molecular masses of 138 kDa, 113 kDa and 87 kDa could be detected. The 138 kDa band corresponded to the full length protein (Pro-Atl). The 113 kDa and 87 kDa bands presumably represented the unprocessed amidase and glucosaminidase domains after proteolytic cleavage of the signal and propeptide (Atl) and the amidase with the propeptide (PP-AM), respectively (according to Schlag *et al*. [36]).

Consistent with the release of the autolysin Atl by RTDs, θ-defensin-mediated killing was diminished in *S. aureus* SA113 *Δatl*. Killing kinetics of the *atl* deletion mutant and its wild-type strain (WT) showed that the mutant was significantly more resistant to the action of RTD-2 (Figure 6). In the course of the experiment, the number of colony forming units (CFU) of the WT was reduced by several log after addition of the peptide, whereas the *atl* deletion mutant was only slightly affected by RTD-2 even at 80 µg/mL (Figure 6).

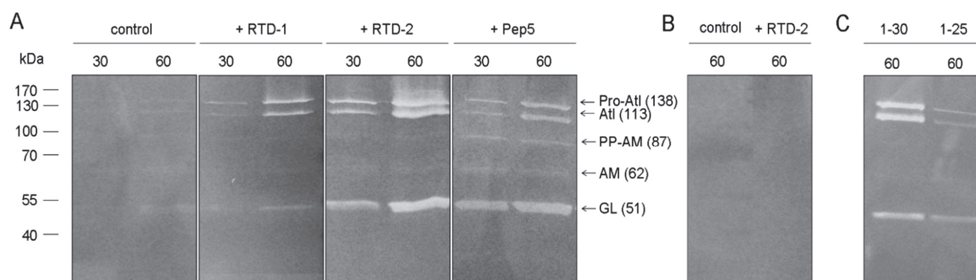

Figure 5. Detection of cell wall lytic enzymes in the supernatant of RTD-treated cells. (**A**) *S. aureus* SG511-Berlin was exposed to 10× MIC of RTDs or Pep5, respectively, for 30 and 60 min. Equal amounts of the concentrated culture supernatant (containing autolysins released from the cell surface) were separated on a 12% SDS-PAGE containing heat-inactivated *M. luteus* cells as substrate. Bands represent cell lysis zones; (**B**) *S. aureus* SA113 Δ*atl* treated for 60 min with 10× MIC RTD-2; (**C**) *S. aureus* SG511-Berlin treated for 60 min with two RTD-1 variants (RTD-1-30 and RTD-1-25) which differ in the distribution of positively charged residues. All bands represent differently processed forms of the autolysin Atl as in the *atl* deletion mutant (Δ*atl)* the corresponding autolysis bands were missing (**B**). Pro-Atl: Atl with full-length propeptide, Atl: amidase and glucosaminidase, PP-AM: amidase with propeptide, AM: amidase, GL: glucosaminidase.

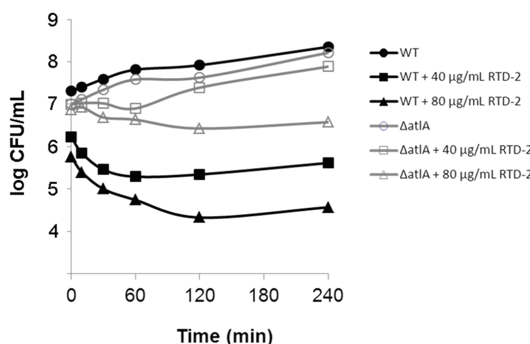

Figure 6. Killing kinetic of *S. aureus* SA113 (WT) and its *atl* deletion mutant (Δ*atl*) in presence of 40 and 80 µg/mL RTD-2 (corresponding to 5× and 10× MIC, respectively) over a period of 4 h. The first sample was taken immediately after peptide addition (time point 0). The results given are mean values of at least two independent experiments.

Recently, it has been reported that the Atl amidase is directed to the septal region by its repeat domains. In this process, *S. aureus* wall teichoic acid (WTA)—present in the old cell wall—acts as a repellent for the repeats, thereby directing the enzymes to the septum, where they bind to and are controlled by lipoteichoic acid (LTA) [36,38]. This suggests that highly cationic molecules such as RTDs and Pep5 bind to the polyanionic LTA, thereby liberating the enzymes such that they can degrade the cell wall in an uncontrolled manner. This view is supported by the fact that only in the

WT strain the colony count dropped by 1 to 2 log immediately after RTD addition (data at time point 0, Figure 6). Apparently, the displacement of Atl-derived enzymes from LTA occurs rapidly and is irreversible, such that autolysis can proceed after plating and prevent colony formation. Similar rapid effects described as "contact killing" have been observed with other highly cationic peptides, e.g., hBD3 [39]. How the activity of the Atl enzymes is controlled under coordinated physiological conditions remains to be elucidated.

Consistently, addition of LTA in a 4-fold molar excess in respect to the peptide antagonized the antimicrobial activity of RTDs and resulted in unhindered growth (Figure 7). Moreover, fluorescence microscopy of *S. aureus* SA113 treated with Pep5-Cy3 demonstrated that the peptide localizes preferentially in the septal region of dividing cells (Supplementary Figure S2) similar to Atl repeats [36]. Unfortunately, this experiment could not be performed with RTDs as they do not harbor any free amine group for labeling.

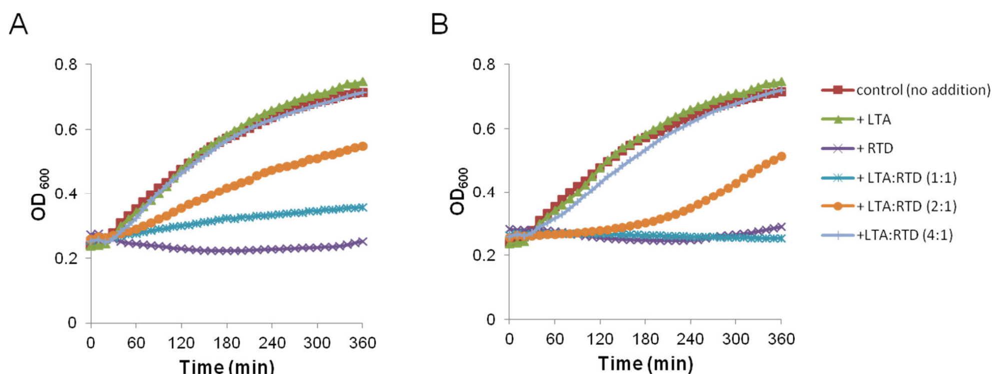

Figure 7. Growth kinetic measurement of *S. aureus* SG511-Berlin in half-concentrated MHB in presence of RTD-1 (**A**) or RTD-2 (**B**) and different molar ratios of LTA. Peptides were added at 10× MIC (corresponding to 28.8 µM and 19.1 µM, respectively).

Interestingly, remarkable differences could be revealed between cells treated with RTD-1, its two variants (namely RTD-1-30 and RTD-1-25) and RTD-2 indicating that the number and distribution of positive charges of a given peptide might be of particular relevance for the interaction with LTA and thereby for the release of autolysins. A higher autolysin activity could be detected in the supernatant of cells exposed to RTD-2 (net charge +6) as well as in RTD-1-30 (net charge: +5; Figure 5C) in which the charged residues are clustered on both sides of the molecule (compare Figure 1).

As θ-defensins and Pep5 both interact with membrane-bound lipoteichoic acid, we investigated whether this interaction also facilitates the pore-formation process. For example, the lantibiotic nisin uses lipid II as docking molecule to subsequently form pores in the membrane of susceptible strains [40]. Thus, carboxyfluorescein-loaded liposomes were made of DOPC or DOPC supplemented with 0.5 mol% purified LTA to monitor the efflux of the fluorescent dye after addition of 1 µM of each peptide. However, only minor marker release was observed with pure DOPC vesicles and DOPC

doped with LTA indicating that LTA are not involved in the membrane-disrupting activity of RTDs and Pep5 (Supplementary Figure S3).

2.4. Activity against Gram-Negative Bacteria

In contrast to Pep5, RTDs also exhibit activity against Gram-negative bacteria over a similar concentration range as against Gram-positives [14,15,41]. Tran *et al*. [41] reported that RTD-1 and RTD-2 effectively permeabilize the outer and inner membrane of *E. coli* ML35-pYC. Consistent with these results, a significant loss of cytoplasmic content could be observed in RTD-2 treated *E. coli* BW25113 cells (Supplementary Figure S4). Moreover, the interaction with the outer membrane seems to differ from hBD3 [42] as blebbing of the outer membrane which is indicative of LPS released from the surface could not be observed (Supplementary Figure S4).

3. Experimental Section

3.1. Peptide Synthesis and Purification

RTDs were assembled by solid-phase synthesis as described previously [14,15]. Purification of Pep5 and nisin was performed according to the protocol of Sahl and Brandis [43] and Burianek and Yousef [44], respectively.

3.2. Determination of Minimal Inhibitory Concentration (MIC)

MIC determinations were carried out in 96-well polypropylene microtiter plates (Nunc^{TM}; Thermo Fisher Scientific, Schwerte, Germany) by standard broth microdilution using half-concentrated Mueller-Hinton broth (MHB). Test strains were grown to an optical density at 600 nm (OD_{600}) of 1 and subsequently diluted to 1–2 × 10^5 cells/mL. Then, 50 µL of the bacterial suspension were mixed with 50 µL of the peptide solution. MICs were read after 24 h of incubation at 37 °C without agitation. The results given are mean values of at least two independent experiments performed in duplicate.

3.3. Bacterial Killing Kinetics

Cells were grown in half-concentrated MHB to an OD_{600} of 0.1. Defensins were added in concentrations corresponding to 5× or 10× MIC (as determined after 24 h). At defined time intervals, 40 µL aliquots of the culture were taken, diluted in 360 µL 10 mM potassium phosphate buffer (pH 7) and 100 µL of appropriate dilutions were plated in triplicate on Mueller-Hinton II agar plates (Becton Dickinson GmbH). The plates were incubated overnight at 37 °C and the number of colony forming units (CFU) was calculated based on the respective dilution factor. An untreated culture was run as a control. The results given are mean values of at least two independent experiments.

3.4. Intracellular Accumulation of the Final Soluble Cell Wall Precursor UDP-MurNAc-Pentapeptide

Antibiotics that interfere with the late, membrane-bound steps of peptidoglycan biosynthesis, trigger the accumulation of UDP-MurNAc-pentapeptide in the cytoplasm which can be isolated and detected by HPLC. Analysis of the cytoplasmic peptidoglycan precursor pool was performed as described previously [23,24]. Briefly, *S. simulans* 22 was grown to an OD_{600} of 0.5 in half-concentrated MHB and supplemented with 130 µg/mL of chloramphenicol. After 15 min of incubation, defensins or vancomycin, respectively, were added at 10× MIC and the samples were further incubated for 30 min. Then, cells were harvested and treated with two volumes boiling water for 15 min. Insoluble components were removed by centrifugation and the supernatants analyzed by reversed-phase high pressure liquid chromatography (RP-HPLC) in 50 mM sodium phosphate buffer (pH 5.2) under isocratic conditions on a Nucleosil 100-C18 column (Schambeck SFD GmbH, Bad Honnef, Germany).

3.5. Potassium Release from Whole Cells

Potassium efflux from whole cells was monitored with a MI-442 potassium electrode and a MI-409F reference electrode (Microelectrodes Inc., Bedford, MA, USA) connected to a microprocessor pH meter (pH 213; HANNA® Instruments, Kehl, Germany).

S. simulans 22 was grown in half-concentrated MHB (±10 mM glucose) at 37 °C to an OD_{600} of 1 to 1.5. Then, cells were harvested by centrifugation (4000 rpm, 3 min, 4 °C), washed with prechilled choline buffer (300 mM choline chloride, 30 mM MES, 20 mM Tris; pH 6.5) and resuspended in the same buffer (±10 mM glucose) to a final OD_{600} of 30. For each measurement, cells were diluted in choline buffer (±10 mM glucose) to an OD_{600} of 3, and the potassium release was monitored for 5 min at room temperature. Peptides were added at 5× and 10× MIC. Potassium concentrations were calculated from the measured voltage according to Orlov *et al*. [45] and expressed relative to the total amount of potassium released after addition of 1 µM of the pore-forming lantibiotic nisin (100% efflux). Results given are mean values of three independent experiments.

3.6. Estimation of Membrane Potential

Cells were grown in half-concentrated MHB (±10 mM glucose) to an OD_{600} of 0.5 to 0.6. To monitor the membrane potential, 1 µCi/mL of [^{3}H]tetraphenylphosphonium bromide (TPP^+; 26 Ci/mM; Hartmann Analytic GmbH, Braunschweig, Germany) was added (the lipophilic TPP^+ diffuses across the bacterial membrane in response to a trans-negative membrane potential). The culture was treated with defensins at 10× MIC, sample aliquots of 100 µL were filtered through cellulose acetate filters (pore size 0.2 µm; Whatman™, Dassel, Germany) and washed twice with 5 mL of 50 mM potassium phosphate buffer (pH 7). The filters were dried, placed into 5 mL scintillation fluid and the radioactivity was measured with a liquid scintillation counter for 5 min per filter. Non specific TPP^+ binding was determined by measuring the TPP^+ incorporation into cells treated with 10% butanol (v/v); the total radioactivity was measured using unfiltered 100 µL sample aliquots. The pore-forming lantibiotic nisin was used as a control. For calculation of the membrane potential ($\Delta\psi$), the TPP^+ concentrations were applied into the Nernst equation $\Delta\psi = (2.3 \times R \times T/F) \times \log [(TPP^+ \text{ inside})/(TPP^+ \text{ outside})]$, where

T is the absolute temperature, *R* is the universal gas constant and *F* is the Faraday constant. A mean $\Delta\psi$ was calculated from at least two independent experiments.

3.7. Zymogram Analysis

Cell wall lytic enzymes in the supernatant of RTD- and Pep5-treated cells were analyzed by zymograms. For this, *S. aureus* SG511-Berlin was grown in half-concentrated MHB to an OD_{600} of 0.6. Then, cells were harvested by centrifugation (4000 rpm, 5 min, 4 °C) and washed with 10 mM sodium phosphate buffer (SPB; pH 7.4). Finally, cells were resuspended in 10% MHB (in 10 mM SPB; pH 7.4) and aliquots of 2.5 mL were incubated with peptides corresponding to 10× MIC. After incubation for 30 or 60 min, cells were pelleted (10,000 rpm, 5 min, 4 °C). The supernatant containing released proteins was concentrated to a volume of 50 µL using VivaSpin-columns (Sartorius AG, Göttingen, Germany) according to the manufacturer's instructions. Equal amounts of the enzyme extract were loaded onto a polyacrylamide gel containing heat-killed *M. luteus* DSM 1790 cells as substrate. After the run, the gel was washed three times with distilled water for 15 min before overnight incubation in buffer (50 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1% Triton X-100, v/v) at 37 °C. Lytic activity was observed as clear zones against an opaque background. To gain a higher contrast, gels were stained with 0.1% methylene blue (w/v) for 15 min and washed with distilled water until clear bands became visible.

3.8. Transmission Electron Microscopy

Cells were grown in half-concentrated MHB to an OD_{600} of 0.6. Aliquots of 5 mL were exposed to RTDs (at 10× MIC) for 30 min or 60 min at 37 °C. Afterwards, the bacteria were harvested by centrifugation (5000 rpm, 5 min, 4 °C), resuspended in 0.1 M SPB (pH 7.4) containing 3% glutaraldehyde (v/v) and fixed overnight at 4 °C. After washing the cells three times for 10 min with 0.1 M SPB (pH 7.4), they were postfixed in 2% phosphate-buffered osmium tetroxide (v/v) at 4 °C for 2 h. Subsequently, the samples were dehydrated with increasing concentrations of ethanol beginning with 30%. The dehydrated cells were incubated three times in propylene oxide for 5 min, followed by a treatment with a 1:1 mixture of polypropylene oxide and Epon-812 (v/v; Science Services, München, Germany) overnight at RT. Finally, cells were embedded in Epon-812 and incubated for polymerization at 60 °C for 48 h. Thin sections (60 nm) were contrasted with 3% uranyl acetate and 0.3% lead citrate and subsequently examined with an EM900 electron microscope (Zeiss; Oberkochen, Germany) at 50 kV.

3.9. Growth Kinetic in Presence of LTA

LTA isolated from *S. aureus* (Sigma-Aldrich, Taufkirchen, Germany) was tested for antagonization of antimicrobial activity. Therefore, *S. aureus* SG511-Berlin was grown in half-concentrated MHB to an OD_{600} of 0.5 to 0.6. Then, 100 µL of the cell suspension were added to 100 µL of RTD-1 or RTD-2 (at 10× MIC) preincubated with LTA in a molar ratios of 1:1, 1:2 and 1:4 in respect to the peptide. Cell growth was measured on a microplate reader (Sunrise™; Tecan, Crailsheim, Germany) over a period of 6 h at 37 °C and obtained data were analyzed by Magellan™ data analysis

software [46]. Results given are mean values of at least two independent experiments performed in triplicate.

4. Conclusions

Here we report that RTDs do not only impair the membrane barrier function, but also liberate the staphylococcal autolysin Atl from LTA in a similar way as the lantibiotic Pep5. These peptides share some features such as the regular spacing of positively charged residues and a structure stabilized by disulfide bridges or lanthionines, respectively. Thus, the antimicrobial activity may rather depend on the overall conformation and charge distribution than on the primary sequence. However, Pep5 and RTDs differ in their activity spectrum, in particular in their efficacy against Gram-negative bacteria. This demonstrates that the physico-chemical properties which provide the basis for the activity against Gram-positive bacteria are not relevant for the killing of Gram-negative bacteria where—in case of RTDs—membrane permeabilization may be the dominant killing activity.

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Author Contributions

Miriam Wilmes, Marina Stockem, Gabriele Bierbaum and Hans-Georg Sahl conceived and designed the experiments. Miriam Wilmes and Marina Stockem performed the laboratory experiments. André J. Ouellette, Dat Q. Tran, Justin B. Schaal, Michael E. Selsted, Martin Schlag and Friedrich Götz supplied reagents/material/analysis tools. Miriam Wilmes and Hans-Georg Sahl drafted the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Position-Dependent Influence of the Three Trp Residues on the Membrane Activity of the Antimicrobial Peptide, Tritrpticin

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Abstract: Antimicrobial peptides (AMPs) constitute promising candidates for the development of new antibiotics. Among the ever-expanding family of AMPs, tritrpticin has strong antimicrobial activity against a broad range of pathogens. This 13-residue peptide has an unusual amino acid sequence that is almost symmetrical and features three central Trp residues with two Arg residues near each end of the peptide. In this work, the role of the three sequential Trp residues in tritrpticin was studied in a systematic fashion by making a series of synthetic peptides with single-, double- and triple-Trp substitutions to Tyr or Ala. ^{1}H NMR and fluorescence spectroscopy demonstrated the ability of all of the tritrpticin-analog peptides to interact with negatively-charged membranes. Consequently, most tritrpticin analogs exhibited the ability to permeabilize synthetic ePC:ePG (egg-yolk phosphatidylcholine (ePC), egg-yolk phosphatidylglycerol (ePG)) vesicles and live *Escherichia coli* bacteria. The membrane perturbation characteristics were highly dependent on the location of the Trp residue substitution, with Trp6 being the most important residue and Trp8 the least. The membrane permeabilization activity of the peptides in synthetic and biological membranes was directly correlated with the antimicrobial potency of the peptides against *E. coli*. These results contribute to the understanding of the role of each of the three Trp residues to the antimicrobial activity of tritrpticin.

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1. Introduction

The growing number of bacterial strains that are resistant to currently used antibiotics has been recognized as a major threat to public health by the World Health Organization [1]. The inefficacy of current antibiotics has triggered a crucial search for new sources and types of antimicrobial agents. One potential source for novel antibiotics could be found among the many antimicrobial peptides (AMPs) [2–5]. AMPs have been intensively studied for the last three decades, as they constitute a crucial element of the innate immune system of higher organisms [6–8], highlighting their value as potential therapeutic agents. Additionally, AMPs have been found in virtually every living organism on Earth, from bacteria to humans [8,9]. Their ability to efficiently kill microbial invaders by a direct mechanism or by modulation of the host immune systems represents a novel mode of action compared to currently used therapeutic agents against infections [10–12]. A multidisciplinary approach in the study and discovery of AMPs has already resulted in databases containing the amino

acid sequences of more than 5000 antimicrobial peptides, encompassing naturally-derived peptides sequences, as well as peptide analogs and predicted sequences [13,14]. Despite all of the efforts studying these peptides, the mechanism of action of many AMPs is not yet completely understood.

Tritrpticin is a relatively short 13-amino acid residue peptide that is part of the cathelicidin family of AMPs. Originally, it was discovered in porcine neutrophils and was shown to exhibit a strong antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as fungi [15]. Due to its amino acid composition, containing three Trp and four Arg residues, it is also a member of the Arg/Trp-rich family of AMPs [16]. In a previous study, we already investigated the effects of the substitution of the Arg residues with various natural and unnatural amino acids on the antimicrobial activity [17]. Here, we will focus on the Trp residues, because these have been identified as key elements for several Trp-rich AMPs in terms of regulating their antimicrobial activities and mechanism of action [18–22]. Substitutions of all three Trp residues simultaneously in tritrpticin with Ala, Phe and Tyr have already been reported. Mutation of all Trp residues to Phe resulted in a more potent antimicrobial peptide, while substitution to Tyr and Ala had a negative effect on tritrpticin's antimicrobial activity. The preferential localization of the Trp indole rings in the water-lipid interface when these peptides are bound to the membrane appears to be directly related to the potent activity of tritrpticin and related cationic Trp-rich AMPs [21–23]. Interestingly, substitutions of Trp with the more polar hydroxy-Trp did not alter the antimicrobial activity, although it caused changes in the mode of action of the peptide [24].

While tritrpticin has been extensively studied by making complete amino acid substitutions of all three Trp residues simultaneously, a systematic study of the importance of each individual Trp residue, from the central Trp-cluster in tritrpticin, has not yet been carried out. In this study, the *C*-terminally amidated version of tritrpticin, Tritrp1, was used as the reference. Tritrp1 exhibits a slightly higher antimicrobial activity in comparison to its precursor [17,22]. Although *C*-terminal amidation of tritrpticin has not been established *in vivo*, the related Trp/Arg-rich peptide, indolicidin, is naturally *C*-terminally amidated [25]. Synthetic peptides with single, double and triple substitutions of the Trp residues in Tritrp1 were prepared using Tyr and Ala. The Tyr substitutions were included in this work to preserve the preference of the amino acid side chain for localization in the membrane interface [26,27]. On the other hand, Ala substitution would completely remove the side chains that contribute to preferential membrane interface binding. Our results show that the three Trp residues of Tritrp1 exert a position-dependent influence on the antimicrobial activity. This effect could be correlated with the altered *in vitro* and *in vivo* membrane permeabilization properties of the peptides in this study.

2. Results and Discussion

2.1. Peptide Design

The amino acid sequences of the peptides that were used in this study are shown in Table 1. Tyr or Ala residues were used in a systematic manner to substitute for the three Trp residues to introduce moderate or major changes, respectively, compared to the Tritrp1 parent peptide.

Table 1. The amino acid sequences and antimicrobial activities reported as minimal inhibitory (MIC) and bactericidal concentrations (MBC) for Tritrp1 and its analogs against *E. coli* ATCC 25922.

Peptide	Sequence	MIC (μM)	MBC (μM)
Tritrp1	VRRFPWWWPFLRR-NH_2	4	4
Trp-to-Tyr analogs			
W6Y	VRRFP**Y**WWPFLRR-NH_2	16	16
W7Y	VRRFPW**Y**WPFLRR-NH_2	8	8
W8Y	VRRFPWW**Y**PFLRR-NH_2	4	4
W67Y	VRRFP**YY**WPFLRR-NH_2	32	32
W78Y	VRRFPW**YY**PFLRR-NH_2	16	16
W68Y	VRRFP**Y**W**Y**PFLRR-NH_2	16	16
Y-Tritrp	VRRFP**YYY**PFLRR-NH_2	16–32	16–32
Trp-to-Ala analogs			
W6A	VRRFP**A**WWPFLRR-NH_2	32–64	32–64
W7A	VRRFPW**A**WPFLRR-NH_2	16	16
W8A	VRRFPWW**A**PFLRR-NH_2	8	8
W67A	VRRFP**AA**WPFLRR-NH_2	64–128	64–128
W78A	VRRFPW**AA**PFLRR-NH_2	64–128	64–128
W68A	VRRFP**A**W**A**PFLRR-NH_2	64–128	64–128
A-Tritrp	VRRFP**AAA**PFLRR-NH_2	>128	>128

2.2. Antibacterial Activity

The antimicrobial activities of all of the peptides are shown in Table 1. Many of the peptides lost some activity against *E. coli* ATCC25922 compared to the parent peptide, with the triple-Trp-to-Ala substitution rendering the peptide completely inactive in the concentration range tested. The loss of antimicrobial activity for A-Tritrp was also observed in studies involving other bacterial strains [21]. For the remaining peptides, the antimicrobial activity was bactericidal rather than bacteriostatic, as indicated by the matching values for the MICs and minimal bactericidal concentrations (MBCs).

The Tyr-derived peptides in general exhibited higher antimicrobial activities than their Ala-derived counterparts. These results indicate that at the center of Tritrp1, where the Trp residues are located, a certain degree of polarity and aromaticity is preferred, but not mandatory in order to exhibit antimicrobial activity. However, a clear pattern emerges when considering the single-substituted peptides. The Trp located at position 6 (Trp6) appears to be very important for the antimicrobial activity. Substitution of this residue by either Tyr or Ala induced a substantially higher loss of antimicrobial activity. In comparison, the substitution of Trp8 with Tyr did not affect the activity of the peptide, while the substitution of Trp7 plays an intermediate role for activity.

Double substitutions with Ala residues reduced the antimicrobial activity of the peptides considerably, with no apparent difference between the positions of the substituted Trp residues. Nevertheless, the double-Trp-to-Tyr-substituted peptides' activities enforce the notion of a dominant role for Trp6 and Trp7 for the antimicrobial activity, with W67Y being less antimicrobial than

W68Y and W78Y. Interestingly, triple substitutions with Tyr reduced the bactericidal potency of the peptide, similarly as described by Schibli *et al.* [22]. However, this potency was close to the potency of the W67Y peptide. Likewise, several other antimicrobial peptides have been reported to lose bactericidal activity due to Trp-to-Tyr mutations, e.g., indolicidin [28], synthetic hexapeptides [29], as well as lactoferricin- and lysozyme-derived peptides [30,31].

Previous work established that the substitution of all three Trp residues with Phe increased the antimicrobial activity of Tritrp1 [21,22]. Since Phe, unlike Trp and Tyr, does not have a preference for the membrane interface [32,33], this effect was thought to be related to the increased hydrophobic character of the peptide. These results reinforce the importance of the hydrophobic and/or aromatic character of the residues located at the core of the peptide.

2.3. ^{1}H NMR Spectroscopy

The binding of linear AMPs to lipid bilayers is normally coupled with changes in the conformation of the peptides. In many cases, most peptides do not adopt a single conformation in buffer, but they usually do so when bound to a membrane or membrane-mimetic surface [34]. In the case of Tritrp1, it has been shown that the two Pro residues flanking the Trp residues are responsible for a high conformational heterogeneity, which is caused by *cis-trans* isomerization around the X-Pro (with X representing any amino acid residue that precedes the proline residue) bonds in aqueous environments. Upon binding to lipid micelles, the peptide acquired a more rigid and defined conformation, as shown by Schibli *et al.* [22,35]. In order to study the effects of membrane binding on the heterogeneity of the Tyr- and Ala-substituted Tritrp1 peptides, ^{1}H NMR spectra were acquired. Each peptide was studied in aqueous solution and in the presence of SDS (sodium dodecyl sulfate) micelles, as depicted in Figures 1 and 2. SDS micelles, while not a perfect membrane mimetic, have been widely used for the NMR structure determination of AMPs in solution [36–38]. The size of these micelles allows for regular high resolution solution state ^{1}H NMR studies to be performed [39,40]. In addition, the negatively-charged nature of the SDS detergent emulates the negatively-charged surface of the bacterial membrane [36–38,41]. The spectral region between 11.0 and 9.0 ppm in the ^{1}H NMR spectra exclusively shows the resonances corresponding to the Hε1 protons of the Trp indole rings, as shown in Figures 1 and 2. This region is far removed from the highly crowded upfield regions of the spectra, allowing a direct interpretation of the conformational status of the Trp residues in the peptide samples.

In aqueous solution, all of the single-substituted peptides (Figures 1a and 2a) were characterized by the presence of two strong and several weaker resonances. This reflects the existence of different conformers in aqueous solution. The *cis-trans* isomerization of the two X-Pro bonds in all of our peptides is likely responsible for the multiple conformation of the peptides, as was previously described for Tritrp1 [22]. Upon binding to the SDS micelles, the number of Hε1 resonances is significantly reduced. In most cases, only two strong peaks are observed, although in some cases, a weaker pair of resonances is still detected. These spectra indicate that all of the single-substituted peptides interacting with the micelles acquired a main and stable conformation. However a minor conformation was still present, although it represents only a small fraction of the peptides.

For the double-substituted peptides, a similar pattern was observed (Figures 1b and 2b). Due to the presence of only one Trp residue in the peptides, one strong Hε1 peak was expected. The transition from the aqueous environment to SDS micelles was again accompanied by a considerable reduction in the number of peaks, again indicating a transition from several conformers in the aqueous environment to only one major micelle-bound conformer (Figures 1b and 2b).

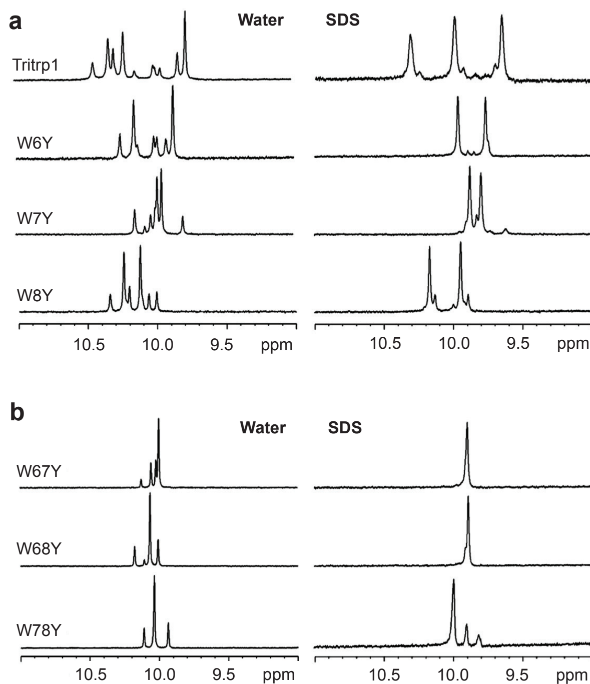

Figure 1. One-dimensional proton NMR spectra recorded for the Hε1-tryptophan region of single- (**a**) and double- (**b**) Trp-substituted Tritrp1 peptides with Tyr in aqueous solution (**left**) and in the presence of d_{25}-SDS micelles (**right**) at 37 °C.

2.4. Tryptophan Fluorescence Spectroscopy

The fluorescence emission of the Trp indole side-chain is highly sensitive to the polarity of its environment, which allows for the study of the interaction between Trp-containing peptides and lipid bilayers. It is expected that upon binding of the peptides to membrane mimetic surfaces, the hydrophobicity of the environment surrounding the Trp residues would increase, leading to a shift of the maximum emission wavelengths to lower values, commonly referred to as the blue shift [42]. The blue shifts induced upon the interaction of Tritrp1 and its Trp-substituted peptides with ePC:ePG (egg-yolk phosphatidylcholine (ePC), egg-yolk phosphatidylglycerol (ePG)) and ePC:Chol (egg-yolk phosphatidylcholine (ePC), cholesterol (Chol)) vesicles are depicted in Table 2. The use of a zwitterionic phospholipid (PC) and cholesterol simulated the electrically neutral and fluidity/rigidity characteristics of a eukaryotic membrane [22,43], while the negatively-charged phospholipid (PG)

with PC can be used to emulate the negatively-charged surface of bacterial cell membranes, as previously indicated [22].

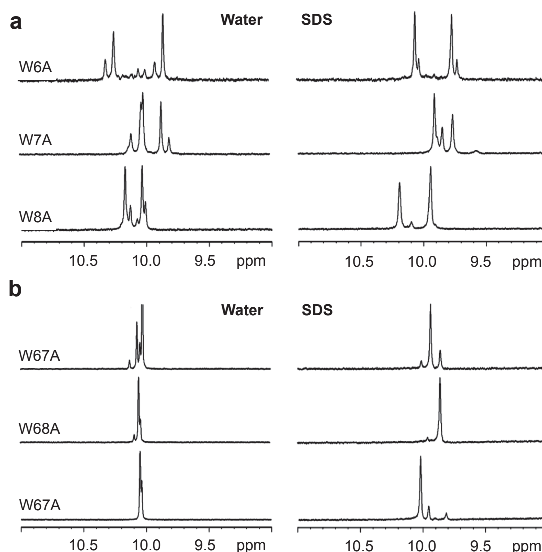

Figure 2. One-dimensional proton NMR spectra recorded for the Hε1-tryptophan region of single- (**a**) and double- (**b**) Trp-substituted Tritrp1 peptides with Ala in aqueous solution (**left**) and in the presence of d_{25}-SDS micelles (**right**) at 37 °C.

Table 2. Maximum emission wavelengths (λ_{max}) in buffer and blue shifts (nm) of the Trp-substituted Tritrp1 analogs upon binding to large unilamellar vesicles (LUVs). ePC, egg-yolk phosphatidylcholine; ePG, egg-yolk phosphatidylglycerol; and Chol, cholesterol.

Peptide	λ_{max}	**Blue Shift (nm)**	
	Buffer	**ePC:ePG**	**ePC:Chol**
Tritrp1	351	14	2
W6Y	353	18	1
W7Y	354	17	−1
W8Y	354	18	0
W67Y	354	22	1
W78Y	353	18	0
W68Y	353	23	2
W6A	353	19	0
W7A	356	16	0
W8A	355	20	1
W67A	355	17	0
W78A	354	18	0
W68A	356	16	−2

A substantial blue shift of close to 20 nm is observed for all peptides upon binding to ePC:ePG large unilamellar vesicles (LUVs). There is no significant trend in the blue shifts of the peptides in the presence of these vesicles; however, these shifts are all higher than that observed for Tritrp1 (14 nm). This can be partially explained by the lower maximum emission wavelength of Tritrp1 with ePC:ePG in buffer (351 nm) compared to 353–356 nm for the other peptides. This suggests that, already in aqueous solution, the presence of all three Trp residues simultaneously provides a slightly hydrophobic environment that influences its fluorescence emission maximum. In contrast, no considerable blue shifts were observed with ePC:Chol vesicles, suggesting that the environment of the Trp residues was not considerably changed in the presence of zwitterionic membranes.

As a complement to the blue shift experiments, the peptide-lipid interactions can also be studied by analyzing the solvent accessibility of the Trp residues using a non-ionic fluorescence quencher, such as acrylamide [44,45]. This allows us to estimate the depth of burial of the Trp residues into the membranes. The acrylamide-induced fluorescence quenching characteristics for Tritrp1 and its Trp-substituted analogs in an aqueous environment and upon interaction with LUVs are presented in Figure 3.

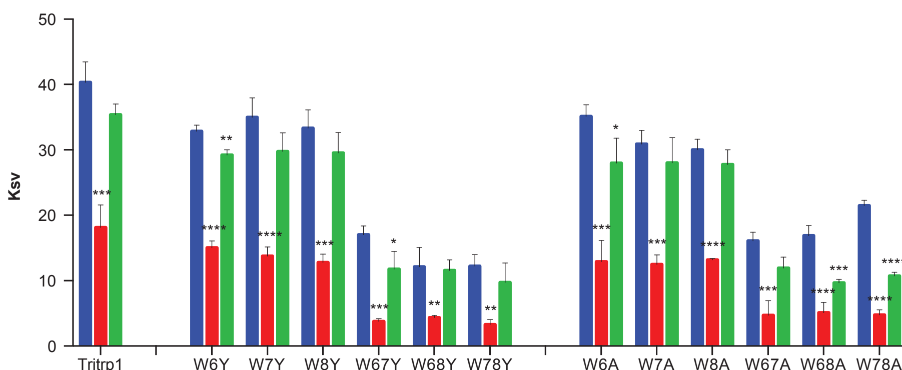

Figure 3. Stern-Volmer constants (Ksv) of the Trp-substituted Tritrp1 analogs as determined by acrylamide quenching experiments in: buffer (blue), ePC:ePG LUVs (red) and ePC:Chol LUVs (green). Results are the mean ± SD (n = 3).

A major reduction in the Stern-Volmer constants (Ksv) values is observed for all of the peptides in the presence of negatively-charged ePC:ePG vesicles, indicating that the Trp residues are not exposed to the aqueous solution. In contrast, for most peptides, the Ksv values remain mostly unchanged in the presence of ePC:Chol membranes compared to buffer alone. The Ksv results in the presence of ePC:Chol indicate that the Trp residues are not protected from the acrylamide-induced quenching. In combination with the absence of blue shifts (Figure 3), these results could be consistent with very weak binding or the absence of interactions between the peptides and these zwitterionic model membranes. However, for W68A and W78A, a statistically significant Ksv reduction was observed in the presence of ePC:Chol membranes. Interestingly, these two peptides did not exhibit a considerable blue shift (Table 2).

When comparing the results for all of the Trp-substituted peptides, no clear correlation between the specific Trp mutations and the blue shift or acrylamide quenching results was detected. However, the blue shift and acrylamide quenching results clearly illustrate a more favorable insertion of the Trp residues from the Tritrp1 analogs into the negatively-charged membranes in comparison to zwitterionic membranes.

2.5. Calcein Leakage from LUVs

Permeabilization of the bacterial membrane has been identified as one of the possible mechanisms of action for Tritrp1 [22,23,46]. Several studies have established the ability of the peptide to disturb the lipid bilayer and induce leakage from vesicle systems [22,23]. In this work, the permeabilizing ability of the Trp-substituted analogs with ePC:ePG model membrane vesicles was evaluated by measuring the leakage of calcein from these synthetic vesicles of defined composition (Figure 4). It should be noted that the leakage percentages for Tritrp1 reported here are roughly half the value compared to those reported by our group in previous studies [17,22]. This is due to a change in instrumentation to a 96-well plate reader instead of a cuvette-based spectrofluorometer, which utilizes different sample stirring conditions. However, the trends observed amongst the peptides in the two assay systems are the same.

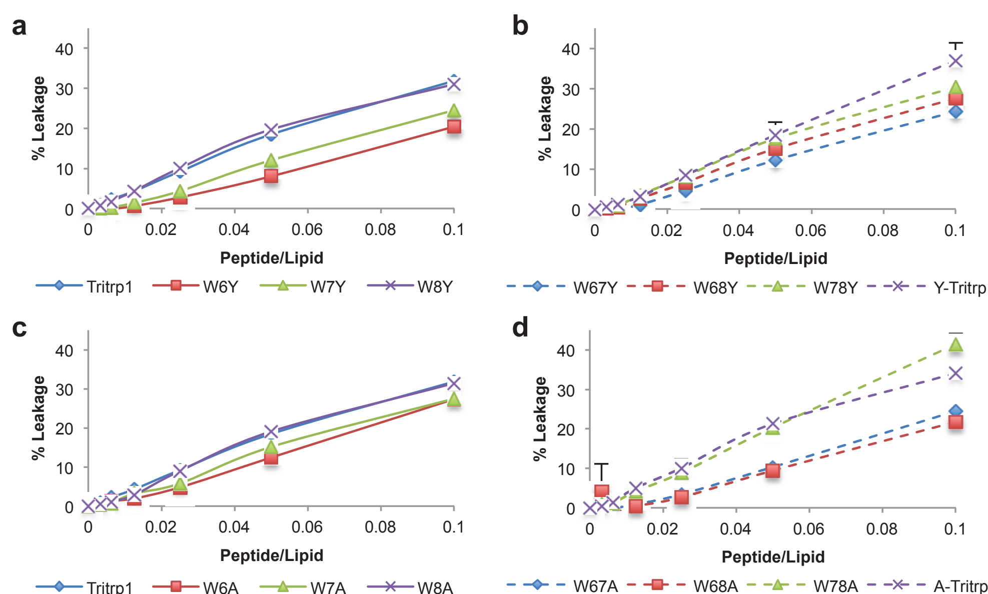

Figure 4. Calcein leakage for Tritrp1 and its analogs in the presence of ePC:ePG (1:1) vesicles. Single-substituted peptides with Tyr (**a**) and Ala (**c**). Double-substituted peptides with Tyr (**b**) and Ala (**d**). Results are the mean ± SD (n = 3).

All peptides were able to induce concentration-dependent calcein leakage in negatively-charged vesicles (ePC:ePG). The level of leakage induced by single-substituted peptides is dependent on the

position of the Trp residue being replaced (Figure 4a,c). W6Y exhibits the lowest percentage of calcein leakage, indicating that position 6 makes a higher contribution to the permeabilization ability of Tritrp1. In contrast, the peptides with substitutions at Trp8 (W8Y and W8A) retain the same leakage ability as Tritrp1, suggesting that this specific Trp is less important for membrane perturbation. The relevance of Trp7 for membrane permeabilization seems to fall between the Trp residues at positions 6 and 8, as indicated by intermediate levels of leakage for W7Y and W7A.

The results for the double-substituted peptides also support the position-dependent role in membrane permeabilization (Figure 4b,d). Peptides involving Trp6 substitutions (W67Y, W68Y, W67A and W68A) were among the peptides that showed the least amount of leakage, while W78Y and W78A give rise to higher leakage.

In order to better compare the influence of different Trp replacements on membrane permeabilization, two peptide-to-lipid ratios (P/L 0.025 and 0.1) were selected, and the results are depicted in Figure 5.

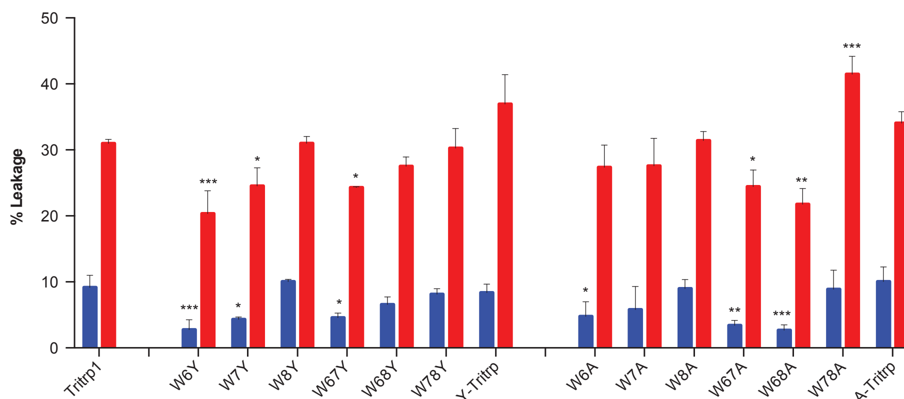

Figure 5. Calcein leakage results for Tritrp1 and its analogs in the presence of ePC:ePG (1:1) vesicles. Two different peptide/lipid (P/L) ratios are depicted as taken from Figure 4, 0.025 (blue) and 0.1 (red). Results are the mean ± SD (n = 3).

It is clear from Figure 5 that single- and double-Trp substitutions with Tyr induce a position-dependent ability to permeabilize ePC:ePG membranes. The differences among the leakage percentages of W6Y, W7Y and Tritrp1 clearly indicate the importance of these two Trp residues at both P/L ratios (Figure 5). A strong tendency to affect the membrane disturbing ability of the peptides by mutating Trp located at position 6 and 7 can be inferred, with Trp6 being more relevant than Trp7. Mutations of Trp8 did not affect the membrane perturbing ability of the peptides, as detected by the lack of statistically significant difference between the means of W8Y, W8A and Tritrp1. These results correlate very well with the antimicrobial activities described in Table 1, where Trp6 substitutions resulted in reduced antimicrobial activity, and no change in bactericidal activity was observed for W8Y. A similar trend was also observed for the single substitutions with Ala; however, differences among these peptides were within their standard deviations, and only W6A *vs.* Tritrp1 at a low P/L ratio exhibited a statistically significant difference (Figure 5, blue). Nevertheless, for the

double-substituted peptides, the results indicate that W67Y, W67A and W68A induced considerably lower leakage than Tritrp1, again illustrating the importance of Trp6 and Trp7. Interestingly, the differences in leakage levels induced by double-Ala-substituted peptides could not be correlated directly with their antimicrobial activities, with MICs of 64–128 μM for W67A, W68A and W78A. This could be related to the broad range of peptide concentrations involved, which is the result of the two-fold dilution setup of the MIC assays.

The peptides with triple substitutions in the Trp residues illustrated an interesting phenomenon, where Y-Tritrp and A-Tritrp were observed to have similar leakage levels as Tritrp1. However, the antimicrobial activities of these two peptides were reduced (Table 1), especially with A-Tritrp having an MIC outside the range of our experiment (>128 μM). For these two peptides, the membrane permeabilization of ePC:ePG vesicles does not seem to correlate with their antimicrobial potency. Previously reported data for Y-Tritrp and a peptide similar to A-Tritrp did not show considerable calcein leakage of negatively-charged liposomes [21,22]. The difference in membrane composition and experimental setup might contribute to the difference observed in leakage behaviour. Lack of direct correlation between calcein leakage and antimicrobial activity has been observed in the past [47], indicating that additional experiments are needed to provide a more accurate description of the mechanism of action of these two Tritrp1 analogs and perhaps other AMPs.

Calcein leakage experiments are normally used to illustrate the importance of the negatively-charged membranes over the zwitterionic membranes. In this work, the preference of Tritrp1 and its analogs to interact with negatively-charged membranes was initially identified by the fluorescence experiments described above (Section 2.4). In order to visualize the permeabilizing ability of the peptides in the presence of a simple eukaryotic model membrane, the calcein leakage experiments were performed using ePC:Chol (2.5:1) vesicles (Figure 6). Additionally, as described for the leakage assays with the ePC:ePG vesicles, two peptide-to-lipid ratios (P/L) were selected, and the results are depicted in Figure 7.

The leakage induced by all of the Tritrp1 analogs with zwitterionic vesicles (Figure 6) was considerably lower than the leakage induced for the negatively-charged vesicles (Figure 4). These results confirm the preference of the peptides for negatively-charged membranes, as previously described for Trp-substituted peptides with Ala and Tyr residues (Table 2 and Figure 3). Interestingly the parent peptide, Tritrp1, also exhibited a similar preference as described by the blue shift (Table 2) and acrylamide quenching experiments (Figure 3). However, large calcein leakage was observed at P/L ratios of 0.05 or higher (Figure 6a,c). This could be an indication that a threshold peptide concentration (P/L > 0.025) is required in order to trigger the permeabilization process in the zwitterionic membranes.

As depicted in Figure 7 for the ePC:Chol membranes, all of the Trp-substituted peptides exhibited a lower ability to induce calcein leakage compared to the parent peptide (Tritrp1), even at high peptide-to-lipid ratios. These results indicate that the substitution of any of the Trp residues for Ala or Tyr in Tritrp1 is sufficient to impede the permeabilization effect on these membranes. The lack of membrane perturbing ability for the Tritrp1 analog peptides is in agreement with the low levels of Trp membrane insertion observed in the blue shift (Table 2) and acrylamide quenching fluorescence studies (Figure 3). Similar results have been described for several AMPs

when interacting with cholesterol-containing membranes [48]. The lack of membrane perturbing abilities for these membranes suggests that our analogs might exhibit higher selectivity towards bacterial membranes compared to the original Tritrp1 peptide.

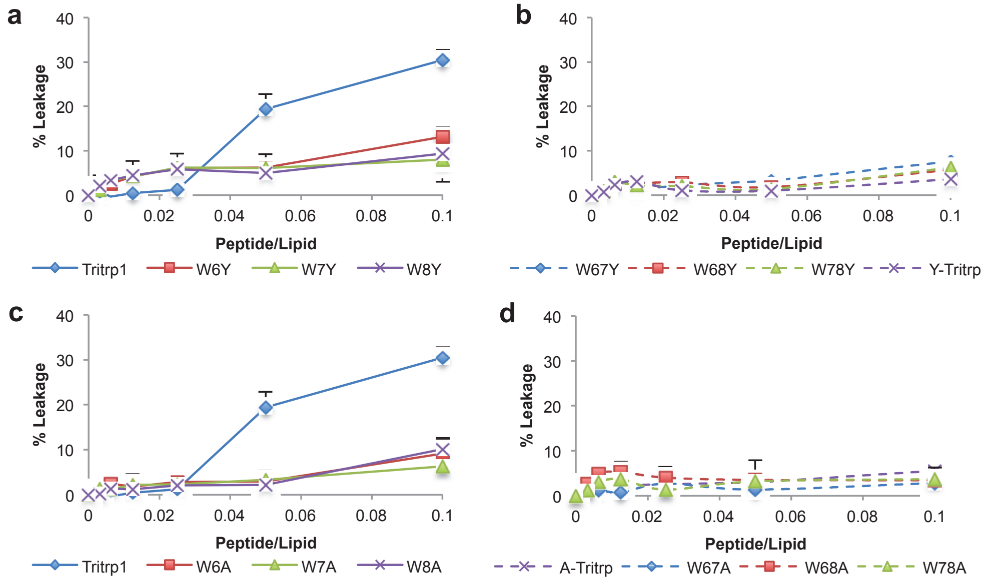

Figure 6. Calcein leakage results for Tritrp1 and its analogs in the presence of ePC:Chol (2.5:1) vesicles. Single-substituted peptides with Tyr (**a**) and Ala (**c**). Double-substituted peptides with Tyr (**b**) and Ala (**d**). Results are the mean ± SD ($n = 3$).

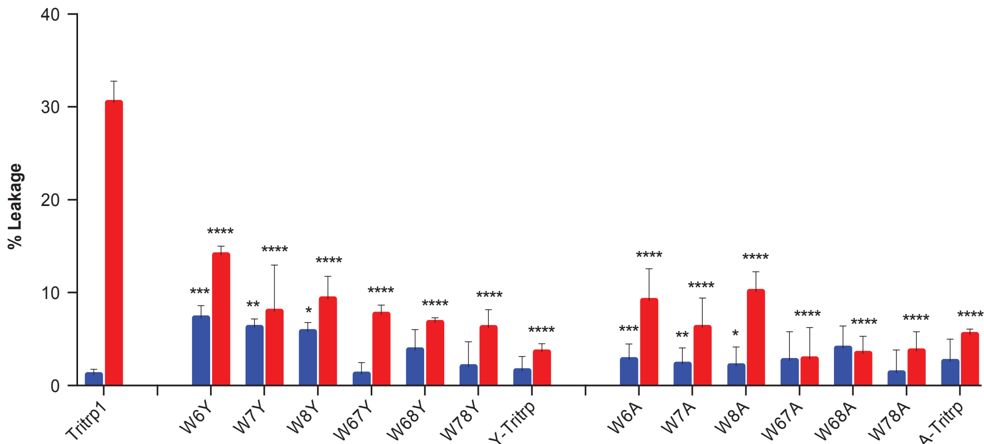

Figure 7. Calcein leakage results for Tritrp1 and its analogs in the presence of ePC:Chol (2.5:1) vesicles. Two different peptide/lipid (P/L) ratios are depicted as taken from Figure 6, 0.025 (blue) and 0.1 (red). Results are the mean ± SD ($n = 3$).

2.6. *E. coli* Inner Membrane Permeabilization

The use of membrane mimetic systems, such as LUVs, allows for the creation of well-defined lipid mixtures, but these do not represent all aspects of the highly complex and heterogeneous cytoplasmic bacterial membrane. Additionally, the peptide-to-lipid ratios used in the calcein leakage experiments might not fully represent a biological scenario. Despite the strong correlation previously described for ePC:ePG membrane permeabilization and the antimicrobial activity of Tritrp1 and its analogs, we felt that it was necessary to evaluate the ability to permeabilize an actual cytoplasmic bacterial membrane. To achieve this, we used the *E. coli ML35p* strain and the impermeable substrate ONPG (2-nitrophenyl-β-D-galactopyranose) as an indicator of membrane perturbation [49]. The effects induced by Tritrp1 and its Trp-substituted analogs on the *E. coli* inner membrane permeability are depicted in Figures 8 and 9.

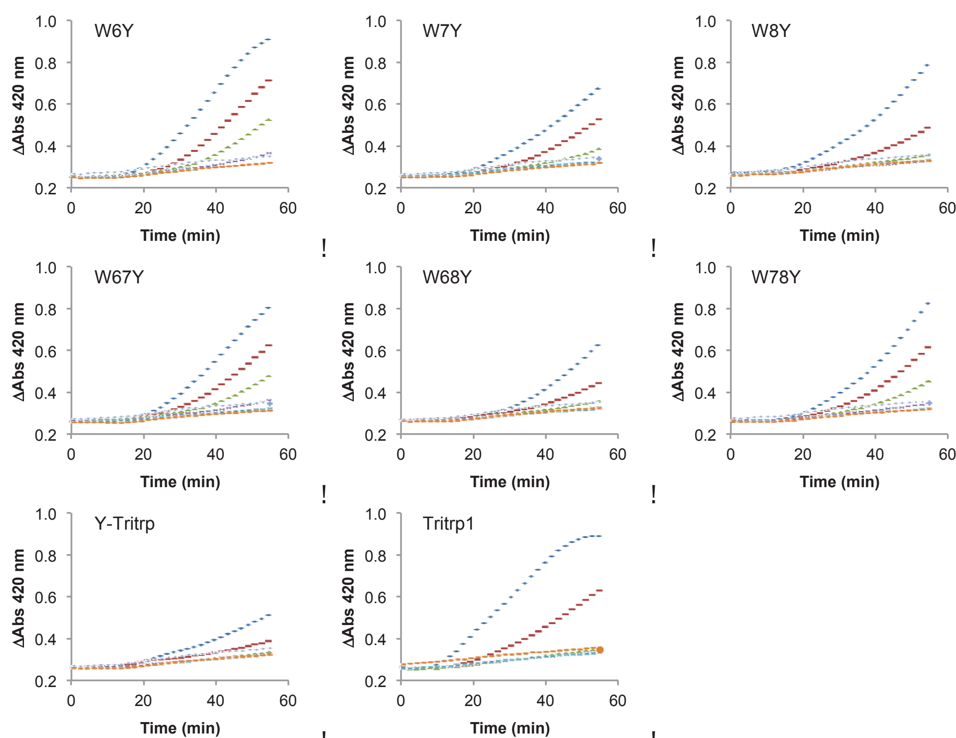

Figure 8. Inner membrane permeabilization induced by Tritrp1 and Trp-to-Tyr-substituted Tritrp1 analogs. Peptide concentrations were MIC (♦), ½ MIC (■), ¼ MIC (▲), ⅛ MIC (×), 1/16 MIC (*), 1/32 MIC (●) and 0 μM (+). The MIC values for each peptide are derived from Table 1. For peptides with a range of MICs, the higher concentration was used. The results are the average of three independent experiments, and the standard deviation (SD) for two selected peptide concentrations are depicted in Figure 10.

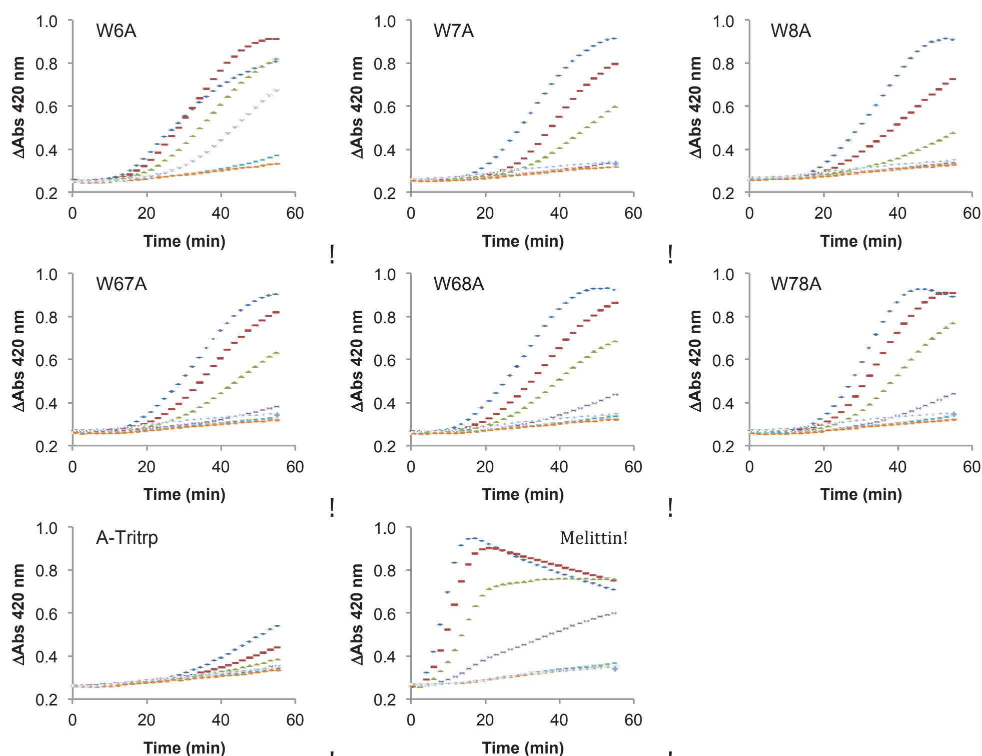

Figure 9. Inner membrane permeabilization induced by melittin and Trp-to-Ala-substituted Tritrp1 analogs. Peptide concentrations were MIC (♦), 1/2 MIC (■), 1/4 MIC (▲), 1/8 MIC (×), 1/16 MIC (*), 1/32 MIC (●) and 0 µM (+). The MIC values for each peptide are derived from Table 1. For peptides with a range of MICs, the higher concentration was used. For A-Tritrp, the highest peptide concentration selected was 128 µM. The results are the average of three independent experiments, and the standard deviation (SD) for two selected peptide concentrations are depicted in Figure 10.

In these assays, the permeabilization of the *E. coli* inner membrane results in an increase of the A_{420} due to the hydrolysis of ONPG by β-galactosidase, an enzyme that is located in the cytoplasm [49]. An increase in the absorbance was detected over 60 min for most peptides in this study, indicating that the membranes were permeabilized by the peptides. At concentrations close to their MICs, the peptides seem to trigger membrane permeabilization after 20 min of incubation. At concentrations less than a quarter of the MIC, there is generally little membrane permeabilization caused by the peptides. In comparison to Tritrp1 (Figure 8), the Ala-derived peptides exhibited a higher level of membrane permeabilization at the MIC, indicating a strong tendency of these peptides to disturb the permeability of the inner membrane (Figure 9). However, it is important to consider that the MIC for most Ala-derived peptides is considerably higher than Tritrp1. The Tyr-derived peptides

exhibited membrane permeabilization profiles similar to Tritrp1 at their MICs (Figure 8). However, compared with the well-known cytotoxic and membrane-active melittin peptide [50,51] (Figure 9), the membrane perturbing activity of Tritrp1 and its analogs is not as strong and takes a longer time to build up. This indicates that distinct mechanisms of action may be involved in the antimicrobial activity of these peptides.

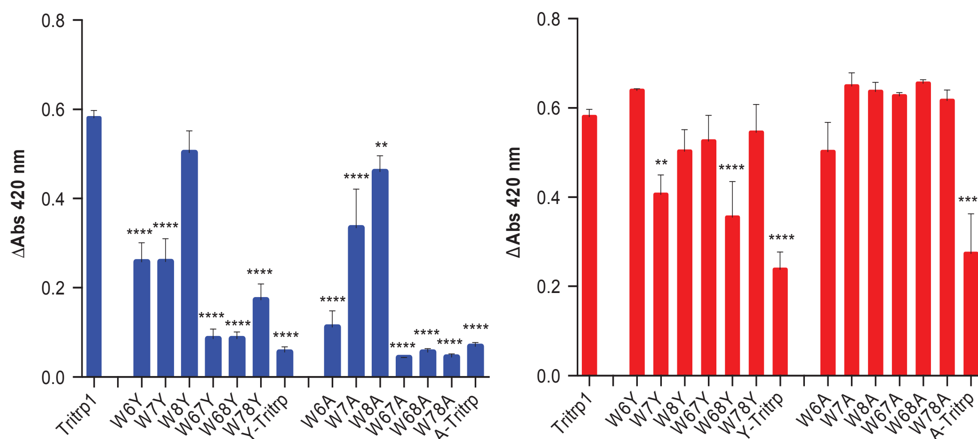

Figure 10. Change in absorbance (ΔAbs_{420}) after 55 min of incubation of *E. coli* ML35p in the presence of Tritrp1 and its analogs at 4 µM (blue) and at the respective MICs for each peptide (red). For peptides with a range of MICs, the higher concentration was considered. Results are the mean ± SD (*n* = 3).

In order to identify the effect of the individual Trp substitutions on the membrane activity among all of the peptides in this study, the levels of permeabilization, represented by the changes in A_{420} after 55 min of incubation are depicted in Figure 10. Only two concentrations were selected, the MIC of the peptide and 4 µM, the latter being used to compare across all Tritrp1 analogs.

At their MICs, most of the Tyr- and Ala-derived peptides induce a similar level of ONPG hydrolysis compared to Tritrp1, which strongly suggests that their antimicrobial activity involves a membrane perturbing mechanism of action (Figure 10, red). However, some peptides (W7Y, W68Y and Y-Tritrp) show statistically significant lower membrane activity in comparison to Tritrp1 at their respective MICs (Figure 10, red). These results indicate that other mechanism(s) of action may contribute to the bactericidal activity of these peptides. Interestingly, A-Tritrp did not have a strong permeabilization ability for the *E. coli* inner membrane at 128 µM, which correlates with its lack of antimicrobial activity, despite causing significant effects against the ePC:ePG vesicles in the calcein leakage assays (Figure 4).

Using a common concentration of 4 µM to compare across all peptides, a trend emerged depending on the position of the Trp substitution (Figure 10, blue). Modifications of Trp6 substantially reduced the ability of the peptide to permeabilize the bacterial membrane. W6A and W6Y had a statistically significant lower permeabilizing capability in comparison to Tritrp1.

Similarly W7A and W7Y also exhibited a considerably lower ability to permeabilize the inner membrane of *E. coli* (Figure 10, blue). In contrast, the permeabilizing ability of W8Y was not significantly affected (Figure 10, blue), suggesting that Trp8 is not as important for the activity of the peptide. These results indicate that the differences in the ability to permeabilize bacterial membranes are responsible for the differences in antimicrobial activity observed for the Tritrp1 analogs (Table 1). These results are in agreement with the position-dependent effects already described in the calcein leakage experiments (Figure 5).

The case of Y-Tritrp is interesting due to the statistically significant lower membrane permeabilization observed in *E. coli*. The peptide also exhibited an MIC of 16 μM (Table 1) and a Tritrp1 comparable membrane disturbing ability in ePC:ePG vesicles (Figures 4 and 5). It is feasible that Y-Tritrp exerts its antimicrobial effect though a different mechanism, which, in combination with membrane permeabilization, might lead to cell death. A similar behavior was described for a Tritrp1 peptide analog, where all Trp residues were substituted by 5-hydroxy-Trp [24]. This peptide lacked the ability to permeabilize synthetic and bacterial membranes, but preserved a strong antimicrobial activity against *E. coli*. Its mechanism of action is not completely understood yet, but was related to either outer membrane destabilization or binding intracellular targets, which would induce the inhibition of macromolecular synthesis [24].

The significantly lower ability of A-Tritrp to permeabilize the bacterial inner membrane (Figure 10) could be correlated with the lack of antimicrobial activity against *E. coli* (Table 1). Considering the high percentage of calcein leakage observed previously for A-Tritrp (Figure 5), this work suggests that permeabilization of the inner membrane of *E. coli* correlates better than calcein leakage to the antimicrobial activity.

Altogether, our experiments show that the three Trp residues located at the core of tritrpticin do not make the same contribution to the antimicrobial activity. We know from previous work that Trp position is important for the cytotoxic activity of α-helical peptides [52]. Similarly, the antimicrobial activity of lactoferricin-derived peptides was strongly dependent on its Trp residues [19]. Accordingly, in the case of Tritrp1, the Trp residues in each of the three positions contribute differently to the antimicrobial activity of the peptide, as well as to the mechanism(s) of action that may be involved in causing bacterial death. The Trp residue located at position 6 was found to be the most important Trp residue for permeabilization *in vitro* and *in vivo*. Furthermore, this Trp proved to be crucial for the antimicrobial activity of Tritrp1. On the other hand, the Trp at position 8 did not have a substantial influence on the antimicrobial activity and membrane permeabilization properties of Tritrp1.

2.7. QSAR Analysis

An evaluation of the relative importance of the three Trp residues on the antimicrobial activity of tritrpticin was also attempted *in silico*. The use of a variety of amino acid descriptors for the quantitative structure-activity relationships (QSAR) analysis allows for the correlation of amino acids in a particular antimicrobial sequence to its biological potency [53,54]. A theoretical model was developed for the 15-amino acid residue bovine lactoferricin (LFB) peptide (FKCRRWQWRMKKLGA). This multivariate model, in addition to the native sequence of LFB,

was developed on the basis of experimental results obtained for several substituted variants of the peptide, including substitution of its two Trp residues (for details, see [53–55]). The QSAR model was able to confirm that the second Trp was the most important amino acid residue for the antimicrobial activity of the LFB peptide [53]. When the same model was used to evaluate tritrpticin, Trp8 and, to a lesser degree, Trp7 were identified as the most relevant Trp residues for its antimicrobial activity. Unfortunately, this result did not coincide with our experimental data, where Trp6 was clearly shown to be more relevant for the antimicrobial activity against *E. coli*. This indicates that factors other than the three Trp residues also have a large effect on the antimicrobial activity of tritrpticin. Indeed Tritrp1 adopts a distinct three-dimensional micelle-bound structure when compared to the 15-residue LFB peptides [22,35,56]. In order to validate this theoretical approach further, it might be interesting in the future to develop a new model based on our data reported here for Tritrp1. This would allow us to determine whether the new model can be useful to predict available data for related Trp-rich peptides, such as indolicidin and puroindolines [25,57–60].

3. Experimental Section

3.1. Materials, Peptides and Bacterial Strains

Phospholipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). SDS-d_{25} was obtained from Cambridge Isotopes Laboratories (Andover, MA, USA). The peptides were purchased from GenScript Inc. (Piscataway, NJ, USA). They were synthesized using standard 9-fluorenylmethyoxycarbonyl (Fmoc) chemistry, and their identities and purities (>95%) were validated by mass spectrometry and HPLC, respectively. Melittin purified from honey bee venom and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

E. coli ATCC 25922 was purchased from American Type Culture Collection (Manassas, VA, USA). *E. coli* ML35p was kindly provided by Dr. Robert Lehrer at the UCLA David Geffen School of Medicine.

3.2. Antibacterial Activity

The minimum inhibitory concentration (MIC) of Tritrp1 and its Trp-substituted analogs against *E. coli* ATCC 25922 was measured by the standard broth microdilution method [61]. Bacteria (5×10^5 CFU/mL) were incubated overnight at 37 °C in Mueller-Hinton broth (MHB) and in the presence of peptides diluted in a two-fold concentration series ranging from 0.25–128 µM in a 96-well polypropylene plate. The reported MIC values correspond to the minimum peptide concentration where bacterial growth was not observed. After overnight incubation, 10 µL from the first three wells without bacterial growth for each peptide in the MIC plate were diluted $1:10^6$ in MHB, and 100 µL of this dilution was plated in MHB-agar. After incubation overnight at 37 °C, the minimum bactericidal concentration (MBC) was reported as the minimum peptide concentration where no colony formation was detected.

3.3. [1]H Nuclear Magnetic Resonance (NMR) Spectroscopy

The interaction of Tritrp1 and its Trp-substituted peptides with SDS micelles was studied by one-dimensional (1D) ^{1}H NMR spectroscopy. The peptides (0.2 mM) were dissolved in 9:1 H_2O:D_2O, and the NMR spectra were acquired at 310 K in a Bruker Avance 700 MHz Ultrashield NMR spectrometer equipped with a Cryo-Probe (Bruker Corporation, Milton, ON, USA), using 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as the internal chemical reference. NMR spectra were then acquired for the peptide samples dissolved in 30 mM SDS-d_{25} micelles. The pH of all of the NMR samples was between 3.5 and 4.5.

3.4. Large Unilamellar Vesicles (LUVs) Preparation

LUVs were prepared by adding the necessary volume of egg-yolk phosphatidylcholine (ePC), egg-yolk phosphatidylglycerol (ePG) and cholesterol dissolved in chloroform. Two LUV systems were prepared: ePC:ePG (1:1) and ePC:cholesterol (2.5:1). The organic solvent was initially removed by evaporation in a stream of nitrogen gas and the remaining solvent was evaporated under vacuum overnight. The lipid films were resuspended in Tris-buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) by vigorous vortexing and then freeze-thawed five times using liquid nitrogen and lukewarm water. LUVs of 100 nm in diameter were produced by extrusion through two 0.1-µm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA, USA). Calcein-containing LUVs were prepared by resuspending the dry lipids in Tris-buffer containing 70 mM calcein. After LUV extrusion, free calcein was removed by gel filtration using a Sephadex G-50 column. The concentration of lipids in the LUVs was measured by the Ames phosphate assay [62], performed in triplicate.

3.5. Tryptophan Fluorescence and Acrylamide Quenching

The tryptophan fluorescence was monitored using a Varian Cary Eclipse fluorimeter (Agilent Technologies, Santa Clara, CA, USA) equipped with a multicell sample holder and temperature control set to 37 °C. The peptides (1 µM) in Tris-buffer were excited at 280 nm (slit width: 5 nm) and the emission spectra were measured from 300 to 500 nm (slit width: 10 nm) in the absence and presence of LUVs (30 µM). The difference in the maximum emission wavelength between the peptide in buffer and in the presence of LUVs is taken as the blue shift.

For the acrylamide quenching experiments, sequential additions of 5 µL of 4 M acrylamide stock solution (up to 0.15 M) were made to the sample containing peptide (1 µM) and LUVs (30 µM). The tryptophan fluorescence was recorded after each acrylamide addition. The fluorescence intensity changes were analyzed through Stern-Volmer plots, where the quenching constants (Ksv) were calculated using the Equation:

$$\frac{Fo}{F} = 1 + Ksv[Q]$$

where Fo is the initial fluorescence of the peptide and F is the fluorescence at the acrylamide quencher concentration $[Q]$.

3.6. Calcein Leakage

The ability of Tritrp1 and its analogs to permeabilize the phospholipid bilayer was measured by following the leakage of calcein induced in calcein-loaded LUVs. The calcein-loaded LUVs (2.5 μM) were incubated in the presence of different concentration of peptides (0–0.25 μM) in a 96-well plate and incubated for 15 min at 37 °C with constant shaking. The calcein fluorescence was measured by excitation at 485 nm and emission at 520 nm in an Eppendorf PlateReader AF2200 (Eppendorf, Mississauga, ON, Canada). The fluorescence of calcein-loaded LUVs in the absence of peptide was subtracted from all values, and Triton X-100 (0.1%) was used to establish the fluorescence intensity of 100% leakage. Results were calculated as the percentage of maximum leakage.

3.7. E. coli Inner Membrane Permeabilization

The permeabilization of the bacterial inner membrane induced by Tritrp1 and its Trp-substituted analogs was measured as described by Epand *et al.* [49]. This method uses the *E. coli* strain ML35p, which constitutively expresses the cytoplasmic β-galactosidase enzyme and lacks the *lac* permease transporter. In the presence of membrane permeabilizing agents, the membrane impermeable substrate 2-nitrophenyl-β-D-galactopyranoside (ONPG) is hydrolyzed by β-galactosidase, and the formation of its product can be monitored by absorbance at 420 nm.

E. coli ML35p was grown at 37 °C in Luria-Bertani (LB) broth from a single colony until OD_{600} ~0.6. The cells were collected and washed three times with incubation buffer (10 mM Na^{+}-phosphate, pH 7.4, 100 mM NaCl and 300 μg/mL LB) and added to a final OD_{600} of 0.3 in the presence of 0.5 mM ONPG and two-fold peptide concentration series in a 96-well plate. The absorbance at 420 nm of the wells was measured every 2 min approximately for 60 min using a Perkin Elmer Victor™ X4 multi-label plate reader (Waltham, MA, USA) with shaking and temperature control at 37 °C.

3.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 [63]. Statistical significant differences between the Tritrp1 analogs and the control peptide (Tritrp1) were established by using one-way ANOVA and Dunnett's *post hoc* test. The *p*-value scale is as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

4. Conclusions

The position of the Trp residues in tritrpticin has a very important role in the membrane perturbing ability of this peptide. Not all residues were equally relevant for the antimicrobial mechanism of action. Our studies of the permeabilization of synthetic membranes established that Trp6 was the most important Trp residue for membrane disruption in contrast to Trp8. The same phenomenon was observed in a more biologically relevant scenario using assays that measure the permeabilization of the *E. coli* inner membrane in intact bacteria. These membrane effects were directly correlated with the antimicrobial potency of Tritrp1 and its Trp-to-Tyr and Trp-to-Ala

analogs. These results not only highlight the importance of the Trp residues for the antimicrobial activity of Tritrp1, but also were able to assign individual contributions to each Trp depending on its location in the peptide sequence. This work contributes to a better understanding of the role of Trp residues in the antimicrobial activity of tritrpticin.

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Author Contributions

Mauricio Arias, Leonard T. Nguyen and Hans J. Vogel conceived of and designed the experiments; Mauricio Arias, Leonard T. Nguyen and Andrea M. Kuczynski performed the experiments, while Tore Lejon performed the QSAR calculations. Mauricio Arias, Leonard T. Nguyen, Tore Lejon and Hans J. Vogel analyzed the data. Mauricio Arias, Leonard T. Nguyen and Hans J. Vogel wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Differential Susceptibility of Bacteria to Mouse Paneth Cell α-Defensins under Anaerobic Conditions

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Abstract: Small intestinal Paneth cells secrete α-defensin peptides, termed cryptdins (Crps) in mice, into the intestinal lumen, where they confer immunity to oral infections and define the composition of the ileal microbiota. In these studies, facultative bacteria maintained under aerobic or anaerobic conditions displayed differential sensitivities to mouse α-defensins under *in vitro* assay conditions. Regardless of oxygenation, Crps 2 and 3 had robust and similar bactericidal activities against *S. typhimurium* and *S. flexneri*, but Crp4 activity against *S. flexneri* was attenuated in the absence of oxygen. Anaerobic bacteria varied in their susceptibility to Crps 2–4, with Crp4 showing less activity than Crps 2 and 3 against *Enterococcus faecalis*, and *Bacteroides fragilis* in anaerobic assays, but *Fusobacterium necrophorum* was killed only by Crp4 and not by Crps 2 and 3. The influence of anaerobiosis in modulating Crp bactericidal activities *in vitro* suggests that α-defensin effects on the enteric microbiota may be subject to regulation by local oxygen tension.

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1. Introduction

Defensins are broad-spectrum microbicides with activities against diverse microbes and certain viruses [1]. The α-defensin subfamily consists of amphipathic ~4.5 kDa peptides that share a common triple-stranded β-sheet topology. α-Defensins also have nine conserved residue positions, including Arg and Glu positions that form a salt bridge, a conserved Gly residue on a beta turn, and six Cys residues that form the characteristic tridisulfide array [2]. Although these canonical features are highly conserved, the remaining 22–25 residue positions may be occupied by varied amino acids, creating diverse α-defensin molecules with often differing target cell specificities [1,3]. Members of the α-defensin subfamily kill bacteria *in vitro* by electrostatic attractions between the cationic peptides and the electronegative bacterial cell envelope and subsequent hydrophobic interactions with phospholipid acyl chains that induce membrane disruption [4–6]. In addition, certain α-defensins bind and inactivate lipid II thereby inhibiting cell wall biogenesis [7,8].

Enteric α-defensins are released apically by Paneth cells into the lumen of small intestinal crypts [9–11]. In the small bowel, secreted Paneth cell α-defensins and additional host defense molecules confer immunity against certain pathogens, and they determine the composition of the ileal microbiota [12–14]. α-Defensins constitute the majority of bactericidal peptide activity released by Paneth cells, and mice that are defective in Paneth cell homeostasis are subject to dysbiosis and blooms of select bacterial species [15–18]. In addition, Paneth cell α-defensins

persist in mouse colonic lumen, although their role in colonic innate immunity is uncertain, given the 10^{12} to 10^{14} bacteria per gram of tissue luminal contents in that environment [19,20].

The gastrointestinal tract is colonized by complex microbial consortia, which are critical in mucosal protection, immunological development, nutrition and metabolism [20–22]. It is estimated that 99% of intestinal microbiota are strict anaerobes, predominantly members of the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria [20,23], and they constitute a potential infectious challenge if homeostasis of the intestinal epithelium is disrupted. In mouse ileum, the composition of the microbiota is determined by Paneth cell α-defensins, perhaps by selection of peptide-tolerant bacterial species [14]. For example, the relative numbers of Firmicutes and Bacterioidetes in ileum of mice expressing a human DEFA5 transgene (*DEFA5* (+/+)) and congenic FVB mice are markedly different [14,20]. These findings illustrate how Paneth cell secretion of a single additional α-defensin can influence the commensal population, and they provide rationale for characterizing the effects of enteric α-defensins on anaerobic bacteria.

Although antimicrobial activities of α-defensins have been studied extensively in the presence of oxygen [1,24–26], their microbicidal effects against anaerobes of the gastrointestinal microbiota have remained mostly unknown. Against facultative periodontal bacteria, antibacterial activities of HNPs 1–3 under aerobic and anaerobic conditions varied with the microbial target [27,28]. Under both conditions, the more electropositive rabbit NP-1 α-defensin peptide proved more potent than human neutrophil α-defensins (HNPs), suggesting that mouse α-defensins, also strongly cationic, may be particularly bactericidal under anaerobic conditions. Human α-defensin HD5 and human β-defensins (hBDs) 1–3 also showed variable antimicrobial activities against anaerobes in assays that measured membrane potential as an index of bacterial viability [29], and HD5 was active against facultatives but had low activity against strict anaerobes *in vitro*. Among mouse α-defensins, Crp4 has shown selective bactericidal activities against certain, but not all, anaerobic bacterial species [30]. Here, we report on the relative bactericidal activities of mouse Paneth cell α-defensins against anaerobic and facultative bacteria under strict anaerobic conditions. Under these conditions, the activity of individual mouse Paneth cell α-defensins was highly variable against facultatives as a function of anaerobiosis. Anaerobic bacterial species, including *Bacteroides fragilis* (Bacteroidetes) and *Clostridium difficile* (Firmicutes), phyla whose numbers *in vivo* are affected by Paneth cell α-defensins [31], also displayed variable sensitivity to these α-defensins.

2. Results and Discussion

2.1. α-Defensin Activities against Facultative Bacteria under Aerobic and Anaerobic Conditions

To test whether mouse α-defensins assayed in an anaerobic environment against facultative and strict anaerobic bacterial species retain structural integrity, we assessed peptide homogeneity and molecular masses by AU-PAGE (Figure 1A) and MALDI-TOF MS and showed that peptides maintained their disulfide arrays under anaerobic conditions. Samples of proCrp4, Crp2, Crp3, and Crp4 dissolved in 0.01% acetic acid, 10 mM PIPES, and 1% (v/v) Brucella broth to replicate assay conditions were incubated aerobically or anaerobically and tested for spontaneous disulfide bond reduction in the absence of oxygen. After 2 h under anaerobic conditions, the four peptides

(Figure 1B) had atomic masses equal to native, oxidized peptides, showing that anaerobic conditions did not reduce disulfide bonds to free thiols. Also, peptide mobilities in AU-PAGE, a gel system that separates α-defensin disulfide bond variants or foldamers at high resolution [32], were those of the native peptides (Figure 1A). Thus, the tridisulfide arrays of these α-defensins were unaffected by anaerobic assay conditions.

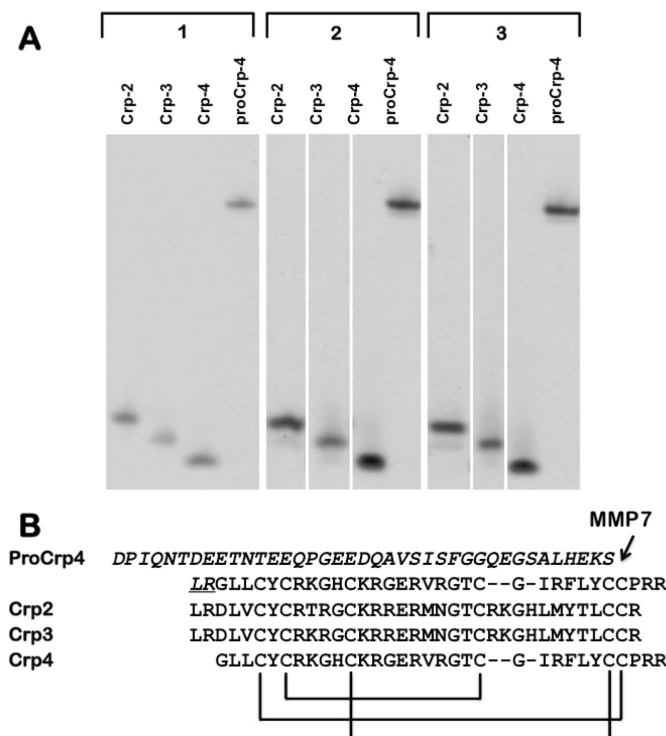

Figure 1. Acid-Urea PAGE of α-Defensins after Anaerobic Incubation. (**A**) cryptdin (Crp)2, Crp3, Crp4, and proCrp4 were incubated under anaerobic conditions (see Experimental) for 2 h and analyzed by AU-PAGE. (1) Aerobic control peptides dissolved in in 0.01% acetic acid, (2) Peptides incubated under anaerobic conditions in 0.01% acetic acid, (3) Peptides incubated in 0.01% acetic acid, 1% Brucella broth (BRU, see Experimental) broth under anaerobic conditions as described in the Experimental section; (**B**) ProCrp4, Crp2, Crp3, Crp4 primary structures are shown for reference with disulfide pairings shown below the Crp4 sequence. Arrow at right indicates the final cleavage event in proCrp processing by matrix metalloproteinase-7, the activating convertase. Dashes were introduced to maintain the cysteine spacing in proCrp4 and Crp4 for alignment with Crp2 and Crp3.

The bactericidal activities of mouse Crps 2–4 were compared against facultative bacterial species under aerobic and anaerobic conditions. *In vitro*, Crps 2–4 kill 99.9% of most bacteria at ≤3 μM peptide levels when assayed under normoxia [13,19,24]. Here, we measured their relative activities in the presence or absence of air over 1 to 15 μM peptide levels. Under either condition, Crps 2–4 were bactericidal against *S. typhimurium*, *S. flexneri*, and *E. coli* ML35 (Figures 2C–H and 3A,D,G), and Crps 2 and 3 had robust and similar bactericidal activities against *S. typhimurium* and *S. flexneri*, regardless of oxygenation. However, under anaerobiosis, Crp4 bactericidal activity was reduced markedly against *S. flexneri* and modestly so against *S. typhimurium*. Regardless of assay conditions, proCrp4 lacked activity (Figure 2A,B), consistent with its lack of activity prior to proteolytic activation by MMP-7 [33]. As expected from previous determinations of *in vitro* bactericidal activities [2,24], Crp4 potency was greater than that of Crps 2 and 3 when assays were performed in air (Table 1). However, under anaerobic conditions, the activity of 15 μM Crp4 against *S. flexneri* was significantly lower than Crps 2 and 3. Thus, direct cell killing of facultative bacteria by individual Paneth cell α-defensins varies as a function of the presence or absence of oxygen.

2.2. Sensitivity of E. coli to Mouse α-Defensins under Aerobic and Anaerobic Conditions

To examine whether pathogenic *E. coli* strains are as susceptible to mouse Paneth cell α-defensins as a laboratory-adapted strain under anaerobic conditions, three strains of *E. coli* were tested for defensin sensitivity. All *E. coli* strains were susceptible to Crps 2–4, and Crp4 had the greatest activity against all strains, regardless of assay conditions. On the other hand, pathogenic *E. coli* ci and EPEC strains survived exposure to 1–5 μM Crps 2 and 3 better under anaerobic conditions than in air (Table 1, Figure 3B,C,E,F), and *E. coli* ci was less susceptible to Crp4 under anaerobic conditions (Figure 3H).

2.3. Differential Effects of Crps 2–4 against Anaerobic Bacteria

The susceptibility of anaerobic bacteria to α-defensins were determined for Crps 2–4, showing that the four species tested responded differently to specific peptides. *C. difficile* (Firmicutes), *B. fragilis* (Bacteroidetes) and the facultative anaerobe *E. faecalis* (Firmicutes) were selected for study, because the ratio of Firmicutes to Bacteroidetes of the ileal microbiota change markedly as a function of qualitative differences in Paneth cell α-defensins [31]. Also, we chose the veterinary and human pathogen *F. necrophorum*, because Fusobacteria have been implicated in exacerbating inflammatory bowel disease and in colon cancer [34,35]. Under anaerobic conditions, Crps 2 and 3 showed greater bactericidal activity against *E. faecalis*, and *B. fragilis* than Crp4 (Figure 4B, C), greater activity against *C. difficile* at ≤5 μM but the same activity as Crp4 at 20 μM peptide levels (Figure 4A). However, Crps 2 and 3 both lacked activity against *F. necrophorum* in contrast to Crp4, which was highly bactericidal against *F. necrophorum* (Figure 4D). Although Crp4 was the most bactericidal of the known mouse α-defensins in previous *in vitro* assays performed in room air [24], the results of the current study are consistent with the low Crp4 activity reported against *B. fragilis* [30], which is sensitive to Crps 2 and 3 (Figure 4). Therefore, anaerobic bacteria vary in

susceptibility to mouse Paneth cell α-defensins, and individual α-defensins differ in their activities against anaerobic bacterial species.

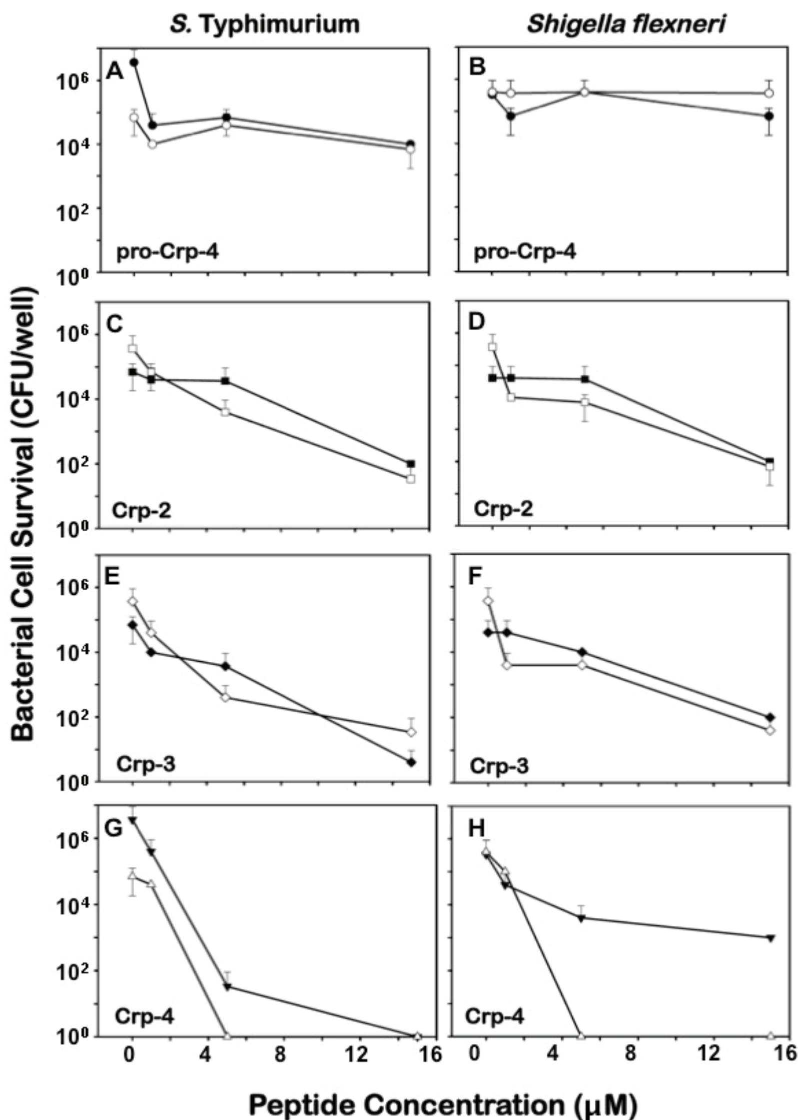

Figure 2. Bactericidal Activities of Mouse α-Defensins against Facultative Organisms under Aerobic and Anaerobic Conditions. *S. typhimurium* 14028s (**A**, **C**, **E**, **G**) and *Shigella flexneri* BS497 (**B**, **D**, **F**, **H**) were Target Organisms for 0, 1, 5, or 15 µM proCrp4 (A, B), Crp2 (C, D), Crp3 (E, F), and Crp4 (G, H) under Both Aerobic (open symbols) and Anaerobic (Closed Symbols) Conditions. Data from three independent experiments are expressed as the mean ± standard deviations.

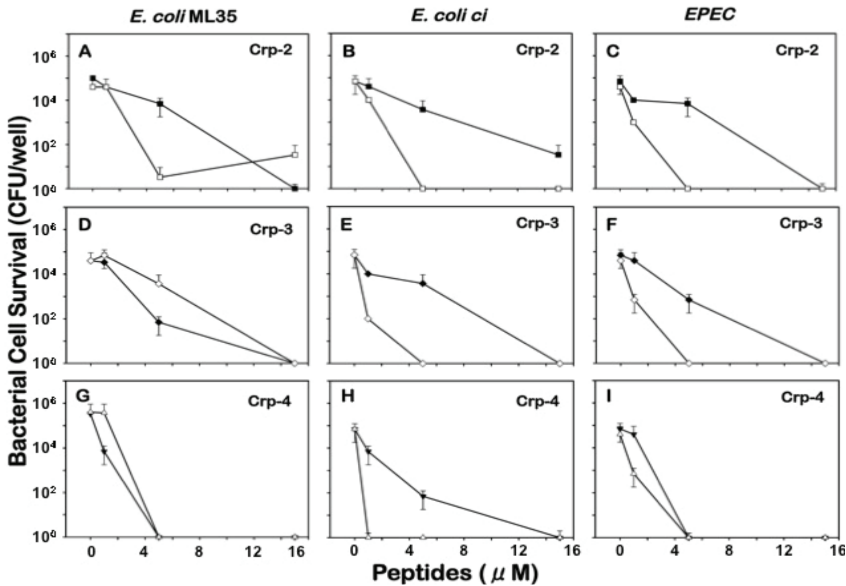

Figure 3. Bactericidal Activities of Crps 2–4 against *E. coli* Strains under Aerobic and Anaerobic Conditions. At concentrations of 1, 5, or 15 µM Crp2 (**A**–**C**), Crp3 (**D**–**F**), and Crp4 (**G**–**I**) were assayed for bactericidal activity against *E. coli* ML35 (A, D, G), *E. coli* clinical isolate (B, E, H), and an EPEC strain (C, F, I) under aerobic (open symbols) and anaerobic (closed symbols) conditions. Data shown are from three independent experiments expressed as means ± standard deviations.

Table 1. Minimum Bactericidal Concentrations (MBC) Values were Determined as the Lowest Peptide Concentrations that Reduced the Number of Viable Bacteria by 99.9%. Due to the large difference between tested concentrations, a range is given.

Bacteria	Oxygen Status	Crp2 MBC (µM)	Crp3 MBC (µM)	Crp4 MBC (µM)
Shigella flexneri BS497	aerobic	5–15	1–5	1–5
	anaerobic	90% [a]	99% [a]	99% [a]
Salmonella Typhimurium WT	aerobic	5–15	5–15	1–5
	anaerobic	99% [a]	5–15	1–5
E. coli ML35	aerobic	1–5	5–15	1–5
	anaerobic	5–15	5–15	1–5
E. coli clinical isolate	aerobic	1–5	1	1
	anaerobic	5–15	5–15	1–5
EPEC	aerobic	1–5	1–5	1–5
	anaerobic	5–15	5–15	1–5
Clostridium difficile	anaerobic	1–5	1–5	5–15
Bacteroides fragilis	anaerobic	5–15	5–15	0%[a]
Fusobacterium necrophorum	anaerobic	0% [a]	60% [a]	1–5
Enterococcus faecalis	anaerobic	5–15	5–15	90% [a]

If 99.9% killing was not reached, then the percentage of bacteria killed at the highest tested concentration, 15 µM, is shown.

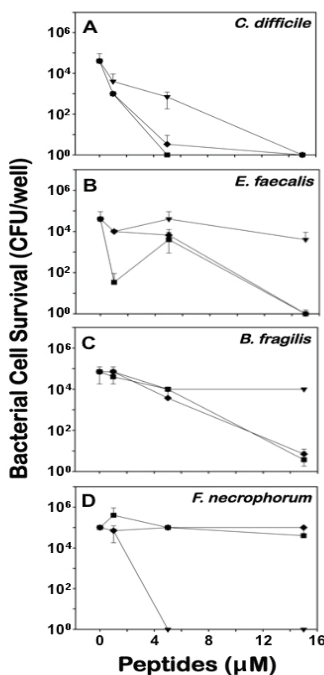

Figure 4. Bactericidal Activities of Crps 2-4 against Anaerobic Bacteria. *C. difficile* ATCC 9689 (**A**), *E. faecalis* ATCC 29214 (**B**), *B. fragilis* ATCC 25285 (**C**), and *F. necrophorum* ATCC 25286 (**D**) were incubated with 1, 5, or 15 μM Crp 2 (-■-), Crp3 (-◆-), and Crp4 (-▼-) under anaerobic conditions in three independent experiments. Data are expressed as the means ± standard deviations.

2.4. Comparative Bactericidal Activities of Mouse α-Defensin Mixtures

Inbred strains of mice, C57BL/6 mice in particular, are polymorphic with respect to Paneth cell expression of α-defensins [2]. Specifically, most inbred strains, including BALB/c, FVB, C3H/HeJ/N, and 129/SvJ strains as well as outbred Swiss mice, produce high levels of Crps 1–6 [36]. However, C57BL/6 mice lack the *Defa1*, *2*, *4* and *6* genes for Crps 1, 2, 4, and 6, expressing instead high levels of Crps 20, 21, 23, and 27 [2]. Because the peptides tested in this study are absent from C57BL/6 mice, the most frequent background for genetic modification, we tested the bactericidal activity of α-defensin mixtures from outbred Swiss and C57BL/6 small intestine [24,36].

α-Defensin mixtures were purified from Swiss and C57BL/6 mouse small intestinal protein extracts by sequential gel permeation and cation exchange chromatography [19,37]. Because they exhibited variable peptide-specific sensitivities to Crps 2–4 (Figure 4), *F. necrophorum* and *C. difficile* were selected as target organisms for comparisons of the native α-defensin mixtures. Despite qualitative differences in α-defensin content of the two mixtures [2,33], the bactericidal activities of the mixed α-defensin preparations were the same against either anaerobic species (Figure 5).

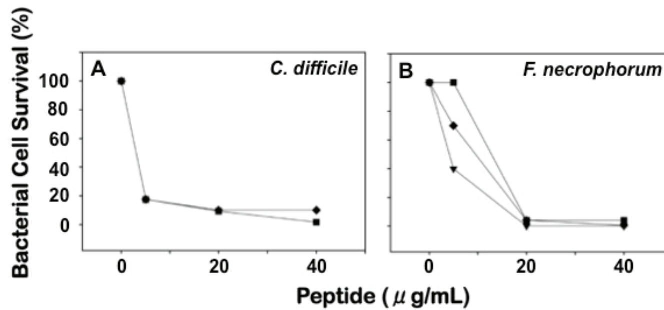

Figure 5. Bactericidal Activities of Swiss and C57BL/6 Native α-Defensin Mixtures against *C. difficile* and *F. necrophorum*. *C. difficile* ATCC 9689 (**A**) and *F. necrophorum* ATCC 25286 (**B**) were test anaerobes for bactericidal assays using preparations of α-defensins from complete ileum of mice that have qualitatively different α-defensins [2]. Bacteria were incubated with 5, 20, or 40 µg/mL of the following: Crp4 (-▼-), outbred Swiss Crps (-■-), or C57BL/6 Crps (-◆-). Surviving bacteria were counted with data expressed as the percent of bacterial survival in each sample relative to control samples lacking peptides (Experimental Section). Data from three independent experiments are expressed as means ± standard deviations.

2.5. Discussion

To simulate the anaerobic conditions under which α-defensin-microbial interactions are believed to occur in the ileum and colonic lumen, we have tested the effect of anaerobiosis on mouse α-defensin bactericidal activity *in vitro*. Unexpectedly, the activity of Crp4, a consistently potent microbicide when assays are performed in air, was attenuated against the facultative pathogen *S. flexneri* under anaerobic conditions (Figure 2). In addition, differential Crp2–4 activities were evident against *E. coli* strains, with Crps 2 and 3 exhibiting attenuated activities against the two pathogenic *E. coli* strains tested under anaerobic conditions (Figure 3). Moreover, *E. faecalis*, and *B. fragilis* were sensitive to Crps 2 and 3 at low peptide levels, with Crp4 again showing markedly lower activity at 15 µM peptide levels when assayed under anaerobiosis (Figure 4). In direct contrast to these results, only Crp4 was bactericidal against *F. necrophorum* (Figure 4), revealing extensive variability of α-defensin-mediated killing as a function of anoxia. Given the variability of individual Crp activities and the differential effects of oxygen on them, we compared native α-defensin mixtures from C57BL/6 and outbred Swiss mice against *F. necrophorum* and *C. difficile*. Against these strict anaerobes, the combined bactericidal activities of the different Crp mixtures were equivalent (Figure 5). Thus, although the anaerobic environment alters the activities of individual Crp peptides against specific bacterial species, mixtures of distinctly different Crp peptides in molar ratios reflecting those in Paneth cell secretions, are similar in their overall bactericidal effects against the anaerobes tested.

Anatomically restricted to the small bowel, Paneth cells secrete dense core granules rich in α-defensins and host defense molecules apically into the lumen [38–40]. Luminal α-defensins

confer immunity to oral infection and define the composition of the ileal microbiota [14,41], and defects in delivery of activated Paneth cell α-defensins to the lumen affect enteric innate immunity adversely [40,42]. Although Paneth cell α-defensins are secreted only in the small bowel, the peptides can be recovered from distal colonic lumen [19,37]. Because the colonic microbial burden is orders of magnitude greater than in small bowel and colonic α-defensins are less abundant than in small intestine, α-defensins may not affect the colonic microbiota by direct peptide-mediated cell killing. In mouse studies where Paneth cell α-defensins were shown to alter the composition of the ileal microbiota, the composition of the cecal and colonic microbiota were unaffected by Paneth cell α-defensins [14]. Also, peptides that are not bactericidal when assayed under in anaerobic conditions may have bacteriostatic affects under those conditions, and that possibility has not been tested in the studies presented here.

The differential susceptibilities to cryptdins of bacteria grown anaerobically may result from alterations in membrane composition and metabolism. The α-defensins studied here cause direct bactericidal action by peptide-mediated membrane disruption at the concentrations tested [43–45]. In response to environmental changes or chronic peptide exposure, bacteria may acquire resistance to antimicrobial peptides by altering membrane fluidity and by decreasing the net electronegative charge of the bacterial cell surface [46–48]. For example, *S. typhimurium* modifies its membrane via lipid A acylation [47], and bacteria regulate lipid A modification in response to Mg^{2+} concentrations [46]. As anaerobiosis influences virulence and pathogenesis by processes such as iron acquisition and sequestration [49–52], oxygen tension, much like iron concentration, may signal changes that alter susceptibility to α-defensins by inducing lipid A or lipoteichoic acid modification. Alternatively, anaerobiosis may alter membrane energetics or bacterial growth, increasing susceptibility to specific α-defensins under anaerobic conditions as was observed for *S. flexneri* with Crp4.

Variations in the microbiota such as those associated with changes to Paneth cell α-defensin composition have implications in host health and disease. Bacteria can modulate the composition of the microbiota by competing for growth-limiting resources or by production of direct microbicides such as bacteriocins and lantibiotics [53,54]. In addition, proteases secreted by *E. faecalis*, for example, convert proCrp4 to mature Crp4 *in vitro* [37], suggesting that bacteria may regulate the microbiota by activating or degrading AMPs in the lower gastrointestinal tract. In humans, changes in the microbiota and α-defensin expression levels are two of among many factors associated with ileal Crohn's disease [42,55,56], and changes to the microbiota are linked to chronic inflammatory disorders and systemic diseases, including ulcerative colitis, obesity, cancer, diabetes, allergic reactions, and cardiovascular disease [57–60]. Because of the role of anaerobic commensal organisms in overall health, understanding the interplay between host defense peptides, including α-defensins, and the mainly anaerobic intestinal flora may have consequences in innate immunity and in disease.

3. Experimental Section

3.1. Preparation of Recombinant and Synthetic Peptides

Crp4 and proCrp4 were produced by recombinant methods and purified to homogeneity as described [61,62]. Briefly, recombinant peptides were expressed in *Escherichia coli* as *N*-terminal His6-tagged fusion proteins using the pET-28a expression vector (Novagen Inc., Madison, WI) and isolated by affinity purification. Affinity purified recombinant fusion proteins were cleaved with CNBr to separate the vector coded fusion protein from the expressed α-defensin, diluted, lyophilized, and recombinant peptides were purified to homogeneity by C18 RP-HPLC as before [33,61,63]. Peptide homogeneity was assessed by analytical RP-HPLC and acid-urea (AU)-PAGE [32], and peptide masses were verified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Microflex LRF mass spectrometer in linear mode (Bruker Daltonics, Maynard, MA, USA).

Homogeneous preparations of Crp2 and Crp3 were synthesized using Boc chemistry for solid phase peptide synthesis as described for human alpha-defensins [64–66]. The molecular masses of resultant Crp2 and Crp3 in their reduced forms were verified by electrospray ionization mass spectrometry to be within experimental error of their calculated theoretical values. After HPLC purification, the synthetic polypeptides were oxidatively folded at 0.25 mg/mL in 50 mM Tris/HCl buffer containing 1 M guanidinium HCl, 3 mM reduced glutathione, and 0.3 mM oxidized glutathione, pH 8.3, followed by HPLC purification to homogeneity (Figure 1A). The formation of three intra-molecular disulfides was ascertained by the loss of 6 mass units upon folding.

3.2. Purification of Tissue-Derived Mouse Enteric α-Defensins

α-Defensins were isolated from complete mouse ileum, consisting of organ plus luminal contents, as described [19,36]. Briefly, segments of complete ileum excised from outbred Swiss or C57BL/6 mice immediately after euthanasia were homogenized in 100 mL ice-cold 60% acetonitrile, 1% trifluoroacetic acid (TFA), incubated at 4 °C overnight, clarified by centrifugation, and lyophilized. Extract proteins dissolved in 5 mL of 5% acetic acid were chromatographed on 10 × 120 cm Bio-Gel P-60 columns (Bio-Rad), and α-defensins were identified as rapidly migrating peptides in P-60 fractions by MALDI-TOF MS and AU-PAGE analysis [32,36]. α-Defensins were purified further by cation exchange chromatography of pooled α-defensin-containing fractions and peptide quantities were determined using the Pierce Protein Assay [19].

3.3. Bacterial Species and Culture Conditions

Anaerobic manipulations were performed in a Bactron II anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA). *Bacteroides fragilis* ATCC 25285, *Clostridium difficile* ATCC 9689, *Enterococcus faecalis* ATCC 29214, and *Fusobacterium necrophorum* ATCC 25286 were cultured anaerobically on pre-reduced anaerobically sterilized (PRAS) Brucella blood agar plates and in PRAS Brucella broth (Anaerobe Systems, Morgan Hill, CA, USA). Wild-type *Salmonella enterica* serovar Typhimurium 14028s, *Shigella flexneri* BS497 [67], *Escherichia coli* ML35

ATCC 43827, *E. coli* clinical isolate (*E. coli* ci) obtained from The University of California Irvine Medical Center (Orange, CA, USA), and enteropathogenic *E. coli* E2348/69 (EPEC) [68] were cultured on trypticase soy agar (TSA) and in trypticase soy broth (TSB) under both aerobic and anaerobic conditions (Table 1). We thank Drs. Mike Cox and Jeremy McDonald (Anaerobe Systems, Inc., Morgan Hill, CA, USA) for *B. fragilis* ATCC 25285, *C. difficile* ATCC 9689, *E. faecalis* ATCC 29214, and *F. necrophorum* ATCC 25286 and for advice for culturing anaerobic bacteria, Dr. Philippe Sansonetti (Pasteur Institute, Paris, France) for *Shigella flexneri* BS497, and Dr. Gail Hecht (University of Illinois, Chicago, IL, USA) for EPEC E2348/69. Media and solutions were degassed and with all plasticware were equilibrated inside the anaerobic chamber for a minimum of 18 h before use. PRAS supplies were manufactured and packed under anaerobic conditions to avoid exposure to oxygen and oxygen damage. Cultures were grown at 37 °C with (aerobes) or without (anaerobes) agitation.

3.4. Bactericidal Assay

The sensitivity of bacteria to α-defensins was assayed in 96-well polypropylene round-bottom microtiter plates (Corning). Samples of proCrp4, Crp2, Crp3, Crp4, and tissue-derived Paneth cell α-defensin peptide mixtures in 5 µL 0.01% acetic acid were mixed with 35 µL 10 mM PIPES plus 1% (v/v) of appropriate medium (PIPES-media). Purified Crps were assayed in triplicate dilution series at 1, 5, or 15 µM, and tissue-derived peptide mixtures at 5, 20, and 40 µg/mL. Exponential phase bacterial cells were deposited by centrifugation at 2000 *g* for 2 min, washed with 10 mM PIPES-media and resuspended in 10 mM PIPES-media. Bacterial cells were added to a final cell densities that ranged from 1×10^5 to 2×10^6 CFU/mL, incubated for 1 h at 37 °C with gentle, intermittent agitation, then diluted serially ten-fold from 1:10 to $1{:}10^6$ in TSB or BRU. Diluted cell suspensions were incubated 24–48 h at 37 °C, and bactericidal activity was assessed by absence of growth, correlated to CFU/well, and survivors were enumerated based on the number of dilutions required to extinguish growth. In testing native α-defensin mixtures, results were expressed as percent of input bacteria that survived peptide exposure: % bacterial killing = [(CFU per well exposed to peptide/CFU per well not exposed to peptide) × 100]. Minimum bactericidal concentrations (MBC) of individual peptides were determined as the lowest peptide concentration that reduced bacterial cell survival by 1000-fold, or that reduced cell viability by 99.9%.

4. Conclusions

To simulate the anaerobic conditions under which α-defensin-microbial interactions are thought to occur in the ileum and colonic lumen, we have tested the effect of anaerobiosis on mouse α-defensin bactericidal activity *in vitro*. Facultative bacteria maintained under aerobic or anaerobic conditions displayed differential sensitivities to mouse α-defensins, and anaerobic bacteria varied extensively in their susceptibility to Crps 2–4. Comparisons of native α-defensin mixtures from C57BL/6 and outbred Swiss mice against *F. necrophorum* and *C. difficile* showed that the combined bactericidal activities of the different α-defensin mixtures were similar. Thus, although the anaerobic environment alters the activities of individual Crp peptides against specific bacterial

species, mixtures of distinctly different Crp peptides in molar ratios reflecting Paneth cell secretions, are similar in their overall bactericidal effects against the anaerobes tested. These findings suggest that the effects of Paneth cell α-defensins on the enteric microbiota may be subject to regulation by local oxygen tension.

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Author Contributions

J.R.M and A.J.O. designed the study; J.R.M. performed experiments; W.L. and M.E.S. provided reagents; M.E.S. provided technical advice; J.R.M. drafted the manuscript; W.L., M.E.S., and A.J.O. edited the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Antimicrobial Activity of Chemokine CXCL10 for Dermal and Oral Microorganisms

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Abstract: CXCL10 (IP-10) is a small 10 kDa chemokine with antimicrobial activity. It is induced by IFN-γ, chemoattracts mononuclear cells, and promotes adhesion of T cells. Recently, we detected CXCL10 on the surface of the skin and in the oral cavity. In the current study, we used broth microdilution and radial diffusion assays to show that CXCL10 inhibits the growth of *Escherichia coli*, *Staphylococcus aureus*, *Corynebacterium jeikeium*, *Corynebacterium striatum*, and *Candida albicans* HMV4C, but not *Corynebacterium bovis*, *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus sanguinis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, *Poryphromonas gingivalis*, or *C. albicans* ATCC 64124. The reason for the selective antimicrobial activity is not yet known. However, antimicrobial activity of CXCL10 may be related to its composition and structure, as a cationic 98 amino acid residue molecule with 10 lysine residues, 7 arginine residues, a total net charge of +11, and a theoretical pI of 9.93. Modeling studies revealed that CXCL10 contains an α-helix at the N-terminal, three anti-parallel β-strands in the middle, and an α-helix at the C-terminal. Thus, CXCL10, when produced on the surface of the skin or in the oral cavity, likely has antimicrobial activity and may enhance innate antimicrobial and cellular responses to the presence of select commensal or opportunistic microorganisms.

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1. Introduction

CXCL10 (IP-10) is a small 10 kDa chemokine. Structurally, it falls into the CXC family of chemokines, which differs from the C, CC, and CX3C families based on variations in the separation of N-terminal cysteines [1,2]. CXCL10 is induced by IFN-γ and produced by monocytes, fibroblasts, and endothelial cells [3], as well as activated neutrophils and eosinophils [4]. Under inflammatory situations, CXCL10 chemoattracts monocytes, macrophages, natural killer cells, dendritic cells, and cytotoxic and helper T cells (specifically Th1) [5,6] and promotes adhesion of T cells. CXCL10 is expressed in higher levels with multiple disease states, including cancer [5], infectious diseases [7], psoriasis [8], and various autoimmune diseases [9,10]. Along with the chemoattractant activity in immune responses, CXC chemokines are active in the regulation of angiogenesis. CXCL10 is missing the ELR (Glu-Leu-Arg) motif, indicating that it acts as an inhibitor of angiogenesis [11]. Additionally, members of the CXC chemokine family function as antimicrobial peptides, serving to enhance innate antimicrobial defense [2,12]. CXCL10 has antimicrobial activity against *Escherichia coli* [2,12],

Staphylococcus aureus [2], *Listeria monocytogenes* [12], *Streptococcus pyogenes* [13], *Bacillus anthracis* [14,15], and *Candida albicans* [2].

Recently, we and others have detected CXCL10 in and on the surface of the skin [8,10] and in the oral cavity [16,17]. These findings suggest that CXCL10 may enhance the antimicrobial barriers in these areas. To test this, we assessed the antimicrobial activity of CXCL10 for microorganisms commonly found as commensals or pathogens on the skin and in the oral cavity.

2. Results

2.1. Activity of CXCL10 on Microorganisms Commonly Found on the Skin

CXCL10 had antimicrobial activity against select microorganisms that commonly inhabit the skin (Tables 1 and 2). However, the activity of CXCL10 was also sensitive to the conditions of the antimicrobial assays, particularly the saline and media composition of the diluent used for each microorganism in the assay. SMAP28 was used as a control peptide and inhibited the growth of these microorganisms.

Table 1. Broth microdilution assays showing the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of CXCL10 for microorganisms commonly found as commensals or pathogens on the skin.

Microorganism	CXCL10 µg/mL MIC (Standard Error)	CXCL10 µg/mL MBC (Standard Error)	SMAP28 µg/mL MIC (Standard Error)	SMAP28 µg/mL MBC (Standard Error)
S. aureus	>50.00	>50.00	4.17 (1.04)	6.25 (0.00)
E. coli	>50.00	>50.00	3.13 (0.00)	3.13 (0.00)
C. bovis	>50.00	>50.00	6.25 (0.00)	6.25 (0.00)
C. striatum	5.21 (1.04)	25.00 (0.00)	0.07 (0.01)	0.07 (0.01)
C. jeikeium	>50.00	>50.00	0.31 (0.00)	0.31 (0.00)

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays were performed in triplicate for each microorganism. *S. aureus* and *E. coli* were cultivated in Mueller Hinton Broth (MHB) at 37 °C for 24 h and *C. bovis*, *C. striatum*, and *C. jeikeium* were cultivated in Brain Heart Infusion Broth containing 0.1% Tween 80 at 37 °C (BHI-T80) for 48 h.

S. aureus was resistant to CXCL10 in the broth microdilution assay (MIC, MBC > 50.00 µg/mL; Table 1) and the viable plate count assay (MIC > 100.00 µg/mL) [18], but susceptible to CXCL10 in the radial diffusion assay (MIC 125.89 µg/mL; Table 2). *E. coli* was resistant to CXCL10 in the broth microdilution assay (MIC, MBC > 50.00 µg/mL; Table 1), but susceptible to CXCL10 in viable plate count assay (MIC < 100.00 µg/mL) [19] and in the radial diffusion assay (MIC 3.13 µg/mL). *C. bovis* was resistant to CXCL10 in the broth microdilution assay (MIC, MBC > 50.00 µg/mL; Table 1); the viable plate count assay (MIC > 100.00 µg/mL) [20], and the radial diffusion assay (MIC > 200.00 µg/mL; Table 2). *C. jeikeium* was resistant to CXCL10 (MIC, MBC > 50.00 µg/mL) in the broth microdilution and radial diffusion assays. In contrast, *C. striatum* was sensitive to CXCL10 in the broth microdilution (MIC, 5.21 µg/mL; MBC 25.00 µg/mL) and radial diffusion (MIC, 19.59 µg/mL) assays.

Table 2. Radial diffusion assays showing the minimal inhibitory concentration (MIC) of CXCL10 for microorganisms commonly found as commensals or pathogens on the skin.

Microorganism	CXCL10 µg/mL MIC (Standard Error)	SMAP28 µg/mL MIC (Standard Error)
S. aureus	125.89 (0.00)	10.49 (1.66)
E. coli	11.62 (4.23)	3.69 (0.75)
C. bovis	>200.00	17.24 (0.43)
C. striatum	3.42 (1.55)	8.00 (1.62)
C. jeikeium	21.52 (0.23)	4.44 (0.26)

Minimal inhibitory concentration (MIC) assays were performed in triplicate for each microorganism. *S. aureus* and *E. coli* were cultivated in Mueller Hinton Broth (MHB) at 37 °C for 24 h; and *C. bovis*, *C. striatum*, and *C. jeikeium* were cultivated in Brain Heart Infusion Broth containing 0.1% Tween 80 at 37 °C (BHI-T80) for 48 h.

2.2. Activity of CXCL10 on Microorganisms Commonly Found in the Oral Cavity

CXCL10 did not have antimicrobial activity against bacteria that commonly inhabit the oral cavity (Table 3). In radial diffusion assays, CXCL10 did not inhibit the growth *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus sanguinis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, or *Poryphromonas gingivalis*. Again, SMAP28 was used as a control peptide and inhibited the growth of these microorganisms.

Table 3. Radial diffusion assays showing the minimal inhibitory concentration (MIC) of CXCL10 for microorganisms commonly found as commensals or pathogens in the oral cavity.

Microorganism	CXCL10 µg/mL MIC (Standard Error)	SMAP28 µg/mL MIC (Standard Error)
S. mutans	>200.00	40.51 (2.70)
S. mitis	>200.00	125.89 (0.00)
S. sanguinis	>200.00	61.55 (2.80)
F. nucleatum	>1000.00	39.47 (5.62)
P. gingivalis 381	>1000.00	69.70 (22.74)
P. gingivalis ATCC 33277	>1000.00	74.42 (11.32)
A. actinomycetemcomitans	>1000.00	26.18 (3.81)

Assays were performed in triplicate for each microorganism. *S. mutans*, *S. mitis*, and *S. sanguinis* were cultivated in trypticase soy broth with yeast extract (TSBYE) at 37 °C; *F. nucleatum* was cultivated in Schaedler's broth at 37 °C in an anaerobic environment; *P. gingivalis* was cultured in Tryptic Soy Broth supplemented with vitamin K1 and hemin at 37 °C in an anaerobic environment; and *A. actinomycetemcomitans* was cultivated in TSBYE at 37 °C in 5.0% CO_2.

2.3. Activity of CXCL10 on C. albicans Commonly Found on the Skin and in the Oral Cavity

C. albicans ATCC 64124 was resistant to CXCL10 in the broth microdilution (MIC, MBC > 50.0 µg/mL) and radial diffusion (MIC > 50.0 µg/mL) assays (Table 4). In contrast,

C. albicans HMV4C was resistant to CXCL10 in the broth microdilution assay (MIC, MBC > 50.0 µg/mL), yet susceptible to CXCL10 in the radial diffusion assay (MIC 23.90 µg/mL).

Table 4. Broth microdilution and radial diffusion assays showing the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of CXCL10 for *C. albicans* commonly found as a commensal or pathogen on the skin and in the oral cavity.

Microorganism	CXCL10 µg/mL MIC (Standard Error)	CXCL10 µg/mL MBC (Standard Error)	SMAP28 µg/mL MIC (Standard Error)	SMAP28 µg/mL MBC (Standard Error)
Broth microdilution assay				
C. albicans ATCC 64124	>50.00	>50.00	12.50 (0.00)	12.50 (0.00)
C. albicans HMV4C	>50.00	>50.00	12.50 (0.00)	16.67 (4.17)
Radial diffusion assay				
C. albicans ATCC 64124	>1,000.00	n/a	39.73 (18.60)	n/a
C. albicans HMV4C	23.90 (10.38)	n/a	18.90 (2.13)	n/a

Minimal inhibitory concentration (MIC) assays were performed in triplicate for each strain. *C. albicans* ATCC 64124 and *C. albicans* HMV4C were grown on trypticase soy agar (TSA) overnight and then cultivated in RPMI 1640 at 37 °C for 3 h. n/a = not applicable.

2.4. Proposed Structure of CXCL10

The structure model of the protein CXCL10 was predicted by I-TASSER [21], and is shown in Figure 1A. The top templates used by I-TASSER include: 1o80A and 1lv9A. A confidence score (C-score) was used to estimate the accuracy of the I-TASSER model. This score is based on the clustering structural density/consensus significance of multiple threading templates. In the present study, the model was very accurate with a C-score of −0.47, estimated TM-score = 0.65 ± 0.13, and estimated RMSD = 4.8 ± 3.1 Å. Shown in Figure 1B is the local accuracy estimation. The residues between 29 and 93 have higher resolution (with predicted distance to native below 4 Å), which correspond to three anti-parallel β-strands in the middle and an α-helix at the *C*-terminal.

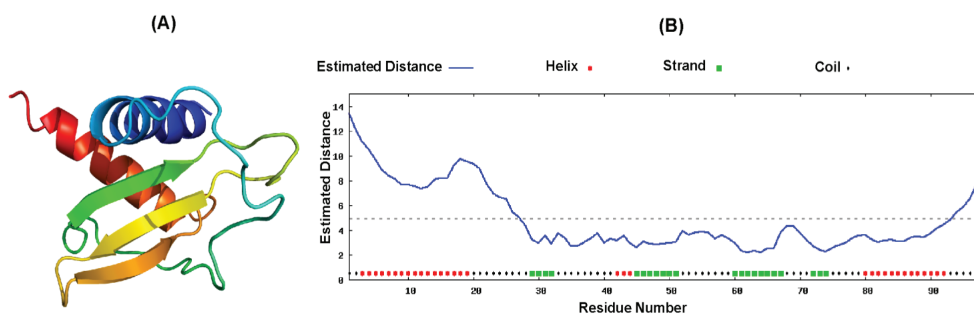

Figure 1. The structure modeling results of CXCL10. (**A**) I-TASSER model; (**B**) Estimated local accuracy.

3. Discussion

In the current study, we found that CXCL10 has selective antimicrobial activity. In broth microdilution and radial diffusion assays, CXCL10 inhibits the growth of *E. coli*, *S. aureus*, *C. jeikeium*, *C. striatum*, and *C. albicans* HMV4C, but not *C. bovis*, *S. mutans*, *S. mitis*, *S. sanguinis*, *F. nucleatum*, *A. actinomycetemcomitans*, *P. gingivalis* strain 381 and ATCC 33277, or *C. albicans* ATCC 64124 (Tables 1–4). Antimicrobial activity of CXCL10 may be related to its strong cationic composition and unique structure of three anti-parallel β-strands in the middle and an α-helix at the C-terminal. Thus CXCL10, when produced on the surface of the skin and in the oral cavity, may enhance innate antimicrobial and cellular responses to the presence of select microorganisms.

The skin and oral cavity are continually exposed to high concentrations of commensal and environmental microorganisms and both of these sites produce a vast diversity of antimicrobial substances as part of their innate immune host defense. This includes antimicrobial peptides, antimicrobial proteins, enzymes, and antimicrobial lipids. For example, the skin produces over 13 different antimicrobial substances [22]; the gingival crevicular fluid contains over 24 antimicrobial substances [23–25]; and saliva contains over 45 antimicrobial substances [23–25].

Cole and colleagues asked whether antimicrobial concentrations of CXCL10 could be achieved *in vivo* [12]. Based on the amount of CXCL10 produced from IFN-γ stimulated peripheral blood mononuclear cells and the density of mononuclear cells in lesions, they hypothesized that CXCL10 could be produced in areas of chronic inflammation in μg/mL amounts. Furthermore they propose that CXCL10 concentrations *in vivo* in plasma of individuals with meliodosis can also reach the MIC concentrations they observed in their study. We and others have recently detected CXCL10 on the surface of the skin and in the oral cavity. In a study of 23 subjects, surface skin wash fluid contained 0.0 to 59.3 pg/mL of CXCL10 (unpublished findings [26]); in a study of 20 subjects, saliva contained 634.0 ± 158.9 pg/mL CXCL10 [27]; in a study of 52 subjects, gingival crevicular fluid contained 36.0 to 3672.0 pg/mL CXCL10 [16]; and in a study of 6 subjects, gingival crevicular fluid contained 48.7 to 29,280.0 pg/mL CXCL10 [17]. These sites also have an incredible abundance and diversity of commensal and opportunistic microflora [28–32]. The presence of CXCL10 at these sites suggests that it may contribute to innate host defense.

CXCL10 has been reported to have antimicrobial activity against *E. coli* [2,12], *S. aureus* [2], *L. monocytogenes* [12], *S. pyogenes* [13], *B. anthracis* [14,15], and *C. albicans* [2]. In this study we extend these findings and report that CXCL10 has selective antimicrobial activity against microorganisms found on the skin and in the oral cavity, thus contributing to antimicrobial host defense by other antimicrobial peptides, antimicrobial proteins, enzymes, and antimicrobial lipids in these areas. Among microorganisms commonly found on the skin, *S. aureus* and *C. bovis* were more resistant to CXCL10 and *E. coli*, *C. jeikeium*, and *C. striatum* were more susceptible to CXCL10. Antimicrobial activity was likely dependent upon the saline and media composition of the diluents used in these assays. This was not an unexpected finding. Cole and colleagues nicely showed that the antimicrobial activity of CXCL10 for *E. coli* was dependent upon the saline concentration of the media [12]. *E. coli* was very susceptible to CXCL10 in 10 mM sodium phosphate buffer, pH 7.4 (MIC, 4.4 μg/mL); moderately susceptible in phosphate buffer with 50 mM NaCl (MIC, 25.0–50.0 μg/mL);

and more resistant in phosphate buffer with 100 mM NaCl (MIC > 50.0 μg/mL). Similarly, the antimicrobial activity of CXCL10 for *S. pyogenes* was diminished in the presence of 150 mM NaCl [13].

The effect of saline and media composition of the diluent impacts the outcomes of the assays and it would be reasonable to assume that *in vivo*, antimicrobial activity of CXCL10 would be greater in microenvironments with higher concentrations of CXCL10 and lower amounts of saline and protein from serous fluid or plasma.

Yang and colleagues found that *S. aureus* was susceptible to CXCL10 [2]. In the current study, we found that *S. aureus* was resistant to CXCL10 in broth microdilution assays (MIC, MBC > 50.00 μg/mL; Table 1) yet susceptible to CXCL10 in radial diffusion assays (MIC 125.89 μg/mL; Table 2). The reason for this difference is not known but again may be related to the saline and media composition of the diluents used in the broth microdilution assay or may be related to differences among strains. Differences among strains do occur. For example, Yang and colleagues found that *C. albicans* was susceptible to CXCL10 [2]. We found that *C. albicans* ATCC 64124 was resistant to CXCL10 (MIC > 50.0 μg/mL in the broth microdilution assay; Table 4); *C. albicans* HMV4C was resistant to CXCL10 (MIC > 50.0 μg/mL in the broth microdilution assay; Table 4), yet *C. albicans* HMV4C was susceptible to CXCL10 (MIC 23.90 μg/mL in the radial diffusion assay; Table 4).

Interestingly, bacteria commonly found in the oral cavity were resistant to CXCL10. *C. albicans*, an opportunistic pathogen on the skin and in the oral cavity, was resistant or susceptible to CXCL10, depending upon the strain. Differences among strains do occur and this would warrant using a number of strains of each species in subsequent work.

The exact mechanism for the antimicrobial activity of CXCL10 for bacteria is not yet known but is likely related to its unique composition and structure. CXCL10 contains 10 lysine residues, 7 arginine residues, has a net charge of +11, and has a theoretical pI of 9.93. This likely facilitates the physical attraction of CXCL10 for the negatively charged microbial surface [12] and activity is influenced by the composition of components in the surrounding environment. Antimicrobial activity of CXCL10 is known to decrease in complex microbiological media and antimicrobial activity rapidly decreases as the concentration of NaCl increases in assay diluents [12,13]. What happens next is not well known. CXCL10 may possibly interact and form pores in the microbial cytoplasmic membrane. Alternately, CXCL10 may target specific cytoplasmic membrane proteins. One proposed target is FtsX, an integral cytoplasmic inner membrane protein thought to form the substrate translocation channel of a putative ABC transporter [14]. In cells treated with CXCL10 and processed for immunoelectron microscopy, CXCL10 bound to the bacterial cell membrane and was associated with loss of cellular integrity [14].

We wanted to compare the modeled structure of CXCL10 with the structure of CXCL9. Egesten and colleagues assessed the structure of chemokine CXCL9 (MIG) with antimicrobial activity [13]. The *N*-terminus of CXCL9 contained a region composed of 3 antiparallel β-strands and the *C*-terminus contained a region composed of an α-helix. When areas of this molecule were synthesized and tested against *S. pyogenes*, the N-terminus did not have antimicrobial activity but the *C*-terminus did [13]. Yang and colleagues assessed the structure of CXCL10 [2]. CXCL10 had a secondary structure with a number of uniquely rich segments. It is rich in positively charged amino acid residues and has a uniquely positive charged C-terminus. In the current study, we found that the

global topology of CXCL10 was similar to that of CXCL9 (Figure 1A): the middle of CXCL10 contained a region composed of 3 antiparallel β-strands and the C-terminus contained a region composed of an α-helix (Figures 1A). The major difference is that our model contains one α-helix at the *N*-terminus (Figure 1A). When further modeled, it would appear that the *C*-terminus α-helix would be expected to bind to other proteins and thus may be involved in antimicrobial activity similar to that proposed for CXCL9 (Figure 2).

4. Experimental Section

4.1. Microorganisms and Culture Conditions

Representative microorganisms commonly found on the skin surface were used and cultivated as previously described [33]. *S. aureus* ATCC 29213 was cultivated in Mueller Hinton Broth (MHB) at 37 °C for 24 h; *E. coli* ATCC 12795 was cultivated in MHB at 37 °C for 24 h; and *C. bovis* ATCC 7715, *C. jeikeium* ATCC 43734, and *C. striatum* ATCC 7094 were cultivated in Brain Heart Infusion Broth containing 0.1% Tween 80 (BHIB-T80) at 37 °C for 48 h.

Representative microorganisms commonly found in the oral cavity were also used and cultivated as previously described [33,34]. *S. mutans* ATCC 25175, *S. mitis* ATCC 49456, and *S. sanguinis* ATCC 10556 were cultivated in trypticase soy broth with 0.6% yeast extract (TSBYE) at 37 °C; *F. nucleatum* ATCC 25586 was cultivated in Schaedler's broth at 37 °C in an anaerobic environment; *P. gingivalis* strain 381 and *P. gingivalis* ATCC 33277 were cultured in Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA) supplemented with vitamin K1 and hemin (Sigma Chemical Co., St Louis, MO, USA) at 37 °C in an anaerobic environment; and *A. actinomycetemcomitans* ATCC 43718 was cultivated in TSBYE at 37 °C in 5.0% CO_2.

C. albicans ATCC 64124 and *C. albicans* HMV4C were grown on trypticase soy agar (TSA) and cultivated in RPMI 1640 at 37 °C for 3 h. Their identities were confirmed with a Bruker Daltonik MALDI Biotyper (State Hygienic Laboratory, University of Iowa Research Park, Coralville, IA, USA).

Prior to the antimicrobial assays, all cultures were transferred to fresh medium and incubated for 3 h in their respective culture conditions. Cultures were then adjusted to contain ~1.0×10^8 cfu/mL (optical density 0.108; 600 nm; Spectronic 20D1, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then diluted to 10^{-2} to 10^{-3}-fold in fresh media (depending upon the specific microorganism).

4.2. Chemokine and Antimicrobial Peptide

Recombinant CXCL10 (Peprotech, Rocky Hill, NJ, USA) was used. CXCL10 was suspended in 0.01% acetic acid to a concentration of 1000.0 µg/mL, gently mixed, dispensed as 100 µL aliquots into cryotubes, and frozen until use.

Sheep myeloid antimicrobial peptide (SMAP) 28 (NeoMPS, Inc., San Diego, CA, USA) was used as a positive control peptide [35] with antimicrobial activity against many of the microorganisms used in this study [36]. SAMP28 is robust and antimicrobial activity is not affected *in vitro* [37] or *in vivo* [38] by the presence of complex microbiological media and increased concentrations of NaCl. SMAP28 was suspended in 0.01% acetic acid to a concentration of 1000.0 µg/mL, gently mixed, dispensed as 100 µL aliquots into cryotubes, and frozen until use.

4.3. Broth Microdilution Assay

A broth microdilution assay was used to determine the antimicrobial activities of CXCL10 and SMAP28 for bacteria [35] and a modified broth microdilution assay was used to determine the antimicrobial activities of CXCL10 and SMAP28 for *C. albicans* [39]. Briefly, 0.01% acetic acid was added to the wells of column 1 to 12 of microtiter plates (Immulon 1 plates, ISC Bioexpress, Kaysville, UT, USA). 100 µL of CXCL10 or SMAP28 was then added to the wells in column 1 and diluted 2-fold from column 1 to column 10 using a multi-channel pipetter. 100 µL of bacterial culture was added to each well from column 1 to column 11. Column 11 served as the positive growth control (in diluent). 100 µL of sterile culture media was added to the wells in column 12 and used as the sterility controls and plate blanks. Alternately, 100 µL of a 3 h *C. albicans* culture in RPMI with resazurin (Alamar Blue, Invitrogen Corp., Carlsbad, CA, USA) was added to each well from column 1 to column 11. Column 11 served as the positive growth control (in diluent). 100 µL of sterile RPMI with resazurin was added to the wells in column 12 and used as the sterility controls and plate blanks. After set up, the plates were incubated overnight at 37 °C in the appropriate culture conditions. The microbial growth in the presence of CXCL10 or SMAP28 was assessed at 600 nm in the spectrophotometer (PowerWaveX, Bio-Tek instruments, Inc., Winooski, VT, USA). *C. albicans* growth in the presence of CXCL10 or SMAP28 was assessed by measuring the metabolic reduction of resazurin to resorufin using an excitation wavelength of 544 nm and an emission wavelength of 590 nm (SpectraMax M2e Multi-Mode Microplate Reader, Molecular Devices, LLC, Sunnyvale, CA, USA).

The minimal inhibitory concentration (MIC) was determined to be the lowest concentration of peptides that inhibited microbial growth by more than 50% of the positive growth control in each row. The wells were cultured onto the respective agars and the minimal bactericidal concentration (MBC) was determined to be the lowest concentration of peptides that inhibited microbial growth on agar. Broth microdilution assays were performed in triplicate for each microorganism.

4.4. Radial Diffusion Assay

A radial diffusion assay was used to determine the antimicrobial activities of CXCL10 and SMAP28 for *S. aureus*, *E. coli*, *C. bovis*, *C. jeikeium*, and *C. striatum* [12] and a modified radial diffusion assay was used to determine the antimicrobial activities of CXCL10 and SMAP28 for *S. mutans*, *S. mitis*, *S. sanguinis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *P. gingivalis* [36]. Briefly, 4.0×10^6 microorganisms were suspended in 25.0 mL of 1% agarose in 0.01 sodium phosphate buffer, pH 7.4 at 50 °C and pipetted into square Petri dishes. When solidified, 2.0 mm diameter wells were punched into the agar. Five microliters of CXCL10 or SMAP28 at 1000.0, 500.0, 250.0, 125.0, 62.5, and 0.0 µg/mL were added per well. The plates were incubated at 37 °C. After 3 h, 10.0 mL of 1.0% agarose containing 6.0% of the respective microbial media was added and allowed to solidify. The plates were incubated at 37 °C in their respective culture conditions. At 18 h, clear zones representing microbial growth inhibition were measured. Radial diffusion units were calculated as the diameter of the clear zone less the 2.0 mm diameter of the well ×10. The radial diffusion units were plotted against the $\log_{10}$ of the peptide concentration and the MIC was

determined to be anti-$\log_{10}$ of the x-intercept. The radial diffusion assays were performed in triplicate for each microorganism.

4.5. Viable Plate Count Assay

A viable plate count assay was used to assess the direct effects of CXCL10 on the viability of dermal and oral microorganisms. For this, microorganisms were cultivated as described above, adjusted to contain ~1.0×10^8 cfu/mL (optical density 0.108; 600 nm; Spectronic 20D1, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and then diluted 10^{-4}-fold in sterile water. 50 µL containing ~500 cfu, was added to 50 µL of CXCL10 in 0.01% acetic acid, SMAP28 in 0.01% acetic acid, or 0.01% acetic acid. After incubation for one hour at 37 °C, 25 µL was removed and spotted in triplicate on the respective agar for each microorganism. After incubation for 24 h, colonies were counted.

4.6. Modeling the Structure of CXCL10

The structure of CXCL10 was predicted using the iterative threading assembly refinement (I-TASSER) server [21,40]. Briefly, the 98 amino acid residue FASTA format sequence of CXCL10 was threaded against the PDB library using the meta-threading algorithm LOMETS to identify homologues templates. Fragments that were excised from the threading templates were then reassembled into full-length models by replica exchange Monte Carlo simulations with the threading unaligned regions (mainly loops) built by ab initio folding. The lowest free-energy conformation was selected by clustering the Monte Carlo simulation structures using SPICKER39. Next, fragment assembly simulation was performed again starting from the SPICKER cluster centroids, where the spatial restraints collected from both the LOMETS templates and the analogy PDB structures by TM-align were used to guide the reassembly simulations. Finally, the models were refined in the atomic-level by the fragment guided molecular dynamics (FG-MD) simulations.

To examine the biological function of the CXCL10 sequence, the COACH algorithm [41] was used to match the I-TASSER model to the protein function library BioLiP [42]. As shown in Figure 2, the CXCL10 was predicted to interact with a peptide (18 residues from the extracellular portion of the receptor CXCR-1) using the template interleukin-8 (PDB ID: 1ilpA).

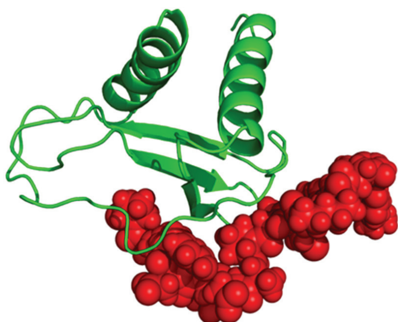

Figure 2. The CXCL10 (in green cartoon) is predicted to interact with a peptide (in red spheres).

5. Conclusions

CXCL10, when produced on the surface of the skin and in the oral cavity, may have a broader role than previously thought in enhancing innate antimicrobial and cellular responses to the presence of microorganisms. In this study we show that CXCL10 has antimicrobial activity against select microorganisms commonly found on the skin or in the oral cavity. Antimicrobial activity may be due to the unique characteristics of CXCL10.

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Author Contributions

In this study, we assessed the ability of CXCL10 to have antimicrobial activity against microorganisms commonly found on the skin (Grant O. Holdren and Amber M. Bates) and in the oral cavity (David J. Rosenthal, Amber M. Bates and Carol L. Fischer) using broth microdilution assays (Grant O. Holdren, David J. Rosenthal and Amber M. Bates) and radial diffusion assays (Grant O. Holdren, David J. Rosenthal, Amber M. Bates and Carol L. Fischer). The proposed structure of CXCL10 was determined at the Department of Computational Medicine & Bioinformatics, The University of Michigan (Jianyi Yang and Yang Zhang). Nicole K. Brogden and Kim A. Brogden conceived the study and Nicole K. Brogden, Carol L. Fischer and Kim A. Brogden wrote the main manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Structure-Dependent Immune Modulatory Activity of Protegrin-1 Analogs

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Abstract: Protegrins are porcine antimicrobial peptides (AMPs) that belong to the cathelicidin family of host defense peptides. Protegrin-1 (PG-1), the most investigated member of the protegrin family, is an arginine-rich peptide consisting of 18 amino acid residues, its main chain adopting a β-hairpin structure that is linked by two disulfide bridges. We report on the immune modulatory activity of PG-1 and its analogs in neutralizing bacterial endotoxin and capsular polysaccharides, consequently inhibiting inflammatory mediators' release from macrophages. We demonstrate that the β-hairpin structure motif stabilized with at least one disulfide bridge is a prerequisite for the immune modulatory activity of this type of AMP.

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1. Introduction

The innate immune system protects the host by rapid detection and elimination of invading pathogens. Phagocytic cells are equipped with pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) [1], scavenger receptors, and others that facilitate rapid detection of invading pathogens [2,3]. Phagocytes are also equipped with bactericidal compounds like lysozymes and host defense cationic peptides that facilitate rapid killing of pathogens [4–6]. Antimicrobial peptides (AMPs) are ubiquitous in many host cells and found as preformed structures stored in granules inside the immune cells that can be instantly released and activated [7–10]. AMPs are also induced and synthesized during infection, which helps increase their level to augment host defense [7–11].

Upon bacterial infection, one of the proposed mechanisms of antibacterial activity of AMPs is insertion into bacterial membranes, causing the rupture and death of bacteria. The ruptured bacterial membrane fragments and leaked cytosol contents contain potent pathogen-associated molecular patterns (PAMPs) that activate Toll-like receptors (TLRs), leading to the release of inflammatory mediators [12,13]. However, the excessive release of inflammatory mediators also causes uncontrolled immune activation and sepsis-like symptoms [14–16]. Therefore, AMPs play an important role in dampening the acute release of proinflammatory mediators by binding to pathogen-related TLR ligands and inhibiting their bioactivity and/or, for some AMPs, by directly intervening in the TLR signaling cascade [17–20]. Endotoxin, also known as lipopolysaccharide (LPS) or lipooligosaccharide (LOS), is a major component of the outer membrane in Gram-negative bacteria. The direct interaction of AMPs with LPS is well documented and underlies the neutralizing activity or the immune modulatory effects *in vitro* and *in vivo* [17,21]. We observed that while AMPs dampen proinflammatory cytokine release induced by LPS, they also amplify respiratory burst in macrophages, possibly to ensure the killing of invading pathogens [22]. The important role of AMPs

in host defense is due to their ability to exert both antibacterial activity and immune modulatory activity on host cells [23–25]. This dual role of AMPs is important for clearing invading pathogens and resolving subsequent inflammation [26].

Protegrins are the main porcine AMPs that belong to the cathelicidin family of host defense peptides and consist of five members, PG-1, 2, 3, 4, and 5 [27,28]. PG-1 is the most abundant and most characterized member of the protegrins. PG-1 is an arginine-rich AMP that consists of 18 amino acids, including four cysteines; its main chain adopts a β-hairpin structure that is linked with two disulfide bridges (Figure 1) [29–31]. PG-1 is assumed to exert its antibacterial activity by forming β-barrel pores across the phospholipid membranes, leading to cell death [32]. PG-1 forms dimers that when inserted into the bacterial membranes build octameric transmembrane pores, causing major leak of potassium ions and subsequent cell rupture, as shown by the molecular dynamic simulation studied by Kaznessis [33]. Due to its cationic character, PG-1 acts rapidly by binding electrostatically to anionic bacterial lipid membranes [34–36]. PG-1, like other AMPs, possesses potent antibiotic-like activity and avoids antibiotic resistance systems due to its rapid, nonspecific effect on bacterial cell membranes [37,38]. It was estimated that between 10 and 100 pores per bacterial cell are required to exert a bactericidal effect on *E. coli* [39].

While several studies have extensively investigated the structural determinants of PG-1 antibacterial activity [20,39–45], the structural determinants of PG-1 required for immune modulatory activity are not known. In the search for PG-1 analogs that exhibit enhanced bactericidal activity with reduced toxicity, several synthetic PG-1 analogs have been reported [27,28,46–52]. The major structural characteristics of PG-1 peptide required for antibacterial activity include: (a) β-hairpin fold stabilized with two disulfide bridges connecting Cys-6 and Cys-15 and Cys-8 and Cys-13, respectively, and intra-chain hydrogen bonding; (b) cationic nature; and (c) amphipathicity of the peptide [38,39]. Synthetic analogs containing the two disulfide bridges are more active than analogs containing a single or no disulfide bridge [38,53,54]. The increased cationic character of the synthetic peptide due to an increased number of arginine residues resulted in enhanced antibacterial activity [55]. PG-1, as a cationic peptide, has the ability to bind to anionic components of bacterial membranes; this includes LPS and capsular polysaccharide (CPS) polymers which act as Toll-like receptor (TLR) ligands and thus function as potent inducers of inflammatory responses in macrophages [56,57].

In this study we investigated the immune modulatory activity of PG-1 and several of its analogs and evaluated the importance of disulfide bridges as well as hydrogen bonding potential of its main chain on the ability to neutralize TLR ligand bioactivity in macrophages, consequently dampening inflammatory mediators' release. We report that synthetic PG-1 analogs adopting (and maintaining) the β-hairpin fold bearing at least one disulfide bridge exert potent immune modulatory activity against meningococcal LOS as TLR4 ligand and CPS polymers as TLR2 and TLR4 ligand.

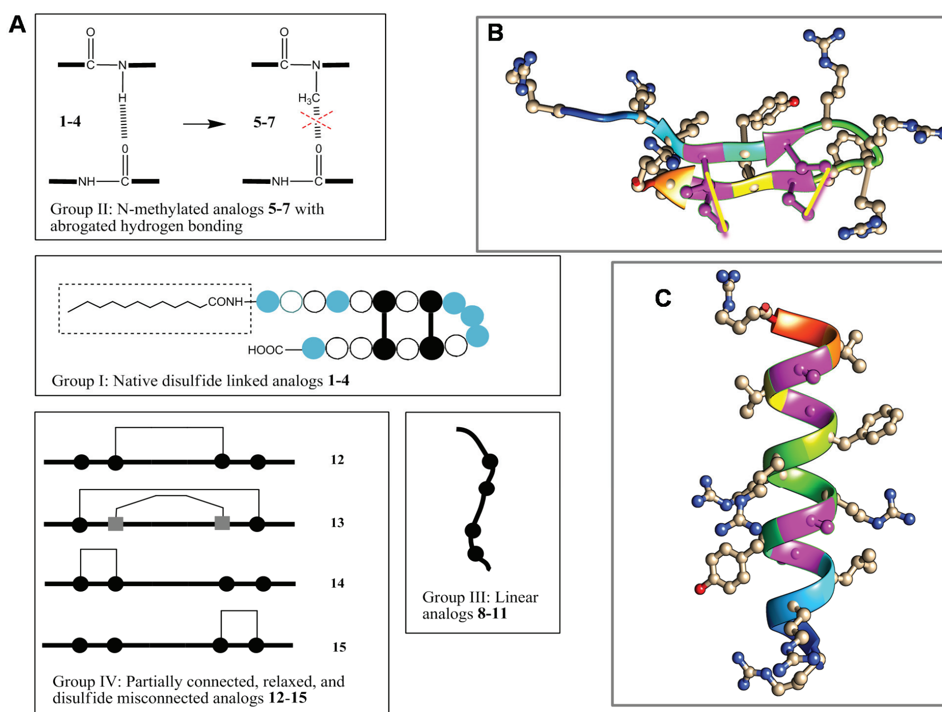

Figure 1. PG-1 and its analogs used in this study. (**A**) Schematic overview of analogs. [= arginine or homoarginine (**4**); = cysteine or homocysteine (**13**); absence of H-bonding in **5**–**7** is indicated ()]; the dotted box indicates the acyl chain in analog **3**. Three-dimensional structure model of PG-1 (**B**) and of its linearized non-cysteine containing analog **8** (**C**) with alanine replacements. The 3D structure of the PG-1 β-hairpin fold is predicted by I-TASSER based on the published crystal structures (PDB # 1PG1 and 1ZY6) and visualized using Chimera software. Cysteine residues that form disulfide bridges are magenta-colored. The 3D structure of a linearized analog **8** adopting a coil fold is achieved when four alanine residues are replaced by cysteines. The coil fold is predicted by I-TASSER and visualized by Chimera. Alanine residues are magenta-colored.

2. Results and Discussion

As a first line of innate host defense against invading pathogens, AMPs can exert significant immune modulatory activity on mammalian cells [58]. In this respect, human alpha-helical LL-37 cathelicidin has been extensively studied [17,21,57,59,60]. We previously reported that porcine cathelicidin, PG-1, inhibits meningococcal LOS immune stimulatory activity and reduces TNFα and nitric oxide release from human and murine macrophages, respectively [22], indicating that a similar mechanism applies to this beta-forming peptide. Herein, we extended our investigations to several analogs of PG-1 (Figure 1 and Table 1) and tested their immune modulatory activity against TLR

ligand endotoxin and capsular polysaccharides CPS prepared from *Neisseria meningitidis*. To investigate the immune modulatory activity of the PG-1 analogs, we employed human and murine macrophage cell lines stimulated with TLR ligands that have been preincubated with these peptides (2 µg/mL, a physiologically relevant dose). The immune modulatory activity was assessed as the inhibition of proinflammatory cytokines TNFα and IL-1β released from stimulated human THP-1 monocyte-like macrophages [57,61]. Inhibition of nitric oxide release from murine RAW264 macrophages was also used to assess the immune modulatory activity of the derivatives. All analogs were non-toxic when used at this low dose (2 µg/mL per 10^6 macrophages) and did not affect the viability of macrophages as assessed by the trypan blue exclusion method [62] (data not shown). None of the PG-1 analogs tested in this study induced the release of cytokines TNFα and IL-1β or nitric oxide when added to macrophages without TLR ligands.

Table 1. PG-1 and its analogs used in this study and their activities.

Compound	Sequence										LOS Activity Inhibition (%)	CPS Activity Inhibition (%)
	1 2 3 4 5	6	7	8	9 10 11	12	13	14	15	16 17 18		
1 (I)	NH_2-R G G R L	C	Y	C	R R R	F	C	V	C	V G R-$CONH_2$	99.9	99
2 (I)	NH_2-*R G G R L*	*C*	*Y*	*C*	*R R R*	F	*C*	*V*	*C*	*V G R*-$CONH_2$	98.3	94.7
3 (I)	C12-R G G R L	C	Y	C	R R R	F	C	V	C	V G R-$CONH_2$	74.2	22.5
4 (I)	NH_2-R G G R L	C	Y	C	R R R	F	C	V	C	V G R-$CONH_2$	100	100
5 (II)	NH_2-R G G R L	C	Y	C	R R R	F	C	V	C	V G R-$CONH_2$	60	52
6 (II)	NH_2-R G G R L	C	Y	C	R R R	F	C	V	C	V G R-$CONH_2$	16	21
7 (II)	NH_2-R G G R L	C	Y	C	R R R	F	C	V	C	V G R-$CONH_2$	0	15
8 (III)	NH_2-R G G R L	A	Y	A	R R R	F	A	V	A	V G R-$CONH_2$	33	23
9 (III)	NH_2-R G G R L	C(Me)	Y	C(Me)	R R R	F	C(Me)	V	C(Me)	V G R-$CONH_2$	0	5.7
10 (III)	NH_2-R G G R L	M	Y	M	R R R	F	M	V	M	V G R-$CONH_2$	4.6	24
11 (III)	NH_2-R G G R L	M(O)	Y	M(O)	R R R	F	M(O)	V	M(O)	V G R-$CONH_2$	20.5	4.6
12 (IV)	NH_2-R G G R L	C(Me)	Y	C	R R R	F	C	V	C(Me)	V G R-$CONH_2$	84.2	84.7
13 (IV)	NH_2-R G G R L	C	Y	C	R R R	F	C	V	C	V G R-$CONH_2$	99.9	88.4
14 (IV)	NH_2-R G G R L	C	Y	C	R R R	F	C(Me)	V	C(Me)	V G R-$CONH_2$	99.9	99.9
15 (IV)	NH_2-R G G R L	C(Me)	Y	C(Me)	R R R	F	C	V	C	V G R-$CONH_2$	88.4	91.6

D-Amino acid residues are in italics; C: homocysteine; C(Me): *S*-methyl-cysteine; C12: dodecanoyl; F: *N*-methyl-phenylalanine; M(O): methionine oxide; R: homoarginine; and Y: *N*-methyl-tyrosine.

Specifically, we investigated the immune modulatory response towards completely or partially linearized analogs, testing the importance of disulfide bridges of PG-1 (Compound **1**). We also tested the importance of intra-/inter-chain hydrogen bonding potential, as H-bonding is presumed to be a major driving force behind oligomerization of PG-1 at bacterial membranes [63]. The analogs used in this work are categorized into four groups based on their common structural characteristics (Figure 1 and Table 1). Group I derivatives **1**–**4** include the native PG-1 (**1**) and all maintain disulfide bridge connectivity. As expected, the D-amino acid analog **2** has very similar activity to the parent peptide **1** and effectively neutralized meningococcal LOS and CPS bioactivity, leading to inhibition of TNFα (Figures 2 and 3), IL-1β (Figures 4 and 5), and nitric oxide (Figures 6 and 7) release even when used

at low dose of 2 µg/mL [52]. Peptide **3** contains a dodecanoyl (C12) moiety at the *N*-terminus of **1**, making it more hydrophobic. Compared to PG-1, acylation did not improve immune modulatory activity against meningococcal LOS (Figures 2 and 4) and was even less effective against neutralizing CPS polymers (Figures 3 and 5). This observation is contrary to LL-37, for which N^{α}-terminus acylation markedly enhanced its immune modulatory activity against LOS and CPS polymers [57]. Furthermore, acylation of the cathepsin G (CG) peptide, which has an α-helical structure, led to enhancement of its activity [64,65]. The length of fatty acyl chain matters and C12 chain length were found to be optimal for enhancing CG peptide activity when compared to shorter or longer chain lengths ranging from C4–C18, as previously shown [66]. Therefore, acylation of peptides with a β-hairpin structure like PG-1 may potentially interfere with peptide oligomerization, which is a very refined process prerequisite to its activity [32]. Analog **4** contains in place of its six arginine residues homoarginine residues, thus making it more apolar as compared to the parent peptide **1** while retaining the positively-charged guanidinium groups. Side-chain guanidinium groups of Arg residues were shown to be critical for PG-1 interaction with bacterial surface phosphate moieties [67,68]. It can be seen that the replacement of arginines with bulkier homoarginines resulted in immune modulatory derivative **4**, the most potent that we have tested so far against meningococcal LOS and CPS (Figures 2, 3, 6, and 7). We note that the number of arginines was found to be critical for protegrin antimicrobial activity [69]. Tang *et al*. showed that reducing the number of arginines dramatically reduced antibacterial activity due to a reduction in membrane insertion and the inability of arginine to electrostatically bind to the phosphate groups on lipid A [55,69,70]. The presence of phosphate groups is critical for interaction with cationic peptides, and elimination of these negative charges by phosphoethanolamine or carbohydrate residues replacement confers resistance to AMPs like polymyxin B and LL-37 [71]. PG-1 is also shown to bind more efficiently to LPS from *Pseudomonas aeruginosa* as compared to LPS from *Burkholderia cepacia* [72]. The reduced binding due to substitution of phosphate head groups with 4-aminoarabinose in *B. cepacia* lipid A is thought to be the major determinant of resistance [72].

2.1. β-hairpin Analogs with Impaired H-Bonding

Group II derivatives **5**–**7** maintain the native disulfide bridge connectivity but differ from **1**–**4** in having N^{α}-methylated residues, Tyr-7 and/or Phe-12 in sequence positions 7 and 12. Replacement of the native N^{α}-amino group in a peptide bond (-CONH-) with its N^{α}-methylated surrogate, -CON(CH3)- [73], was done in order to eliminate hydrogen bonding of the main chain peptide H-bond donor (=NH) in positions 7 and/or 12 as depicted in Figure 1A. Such derivatives should exhibit weaker inter-/intra-chain H-bonding at these sites to the main chain carbonyl group acceptors that were shown to be involved in PG-1 oligomerization [74,75]. Indeed, as can be seen in Figures 2–5, the ability of analogs **5**–**7** to neutralize meningococcal LOS and CPS was dramatically reduced in both assays. We find that the effect of N-methylation is site-specific, and more pronounced in position 7 as compared to position 12. This quantitative difference in effect can be rationalized by position 7 being in the center of the putative dimerization β-strand domain of PG-1, as opposed to position 12 at the edge of PG-1's dimerization domain. We inferred that dimerization is important for PG-1 to have its TLR-mediated effect. This demonstrates that main chain H-bonding plays a critical

role in a peptide’s binding to LOS and to CPS. In support of our findings, Giacometti *et al*. reported that synthetic PG-1 analog IB-367 neutralized LPS and led to significant reduction in TNFα levels, consequently preventing endotoxin-induced mortality in an *in vivo* rat model [20].

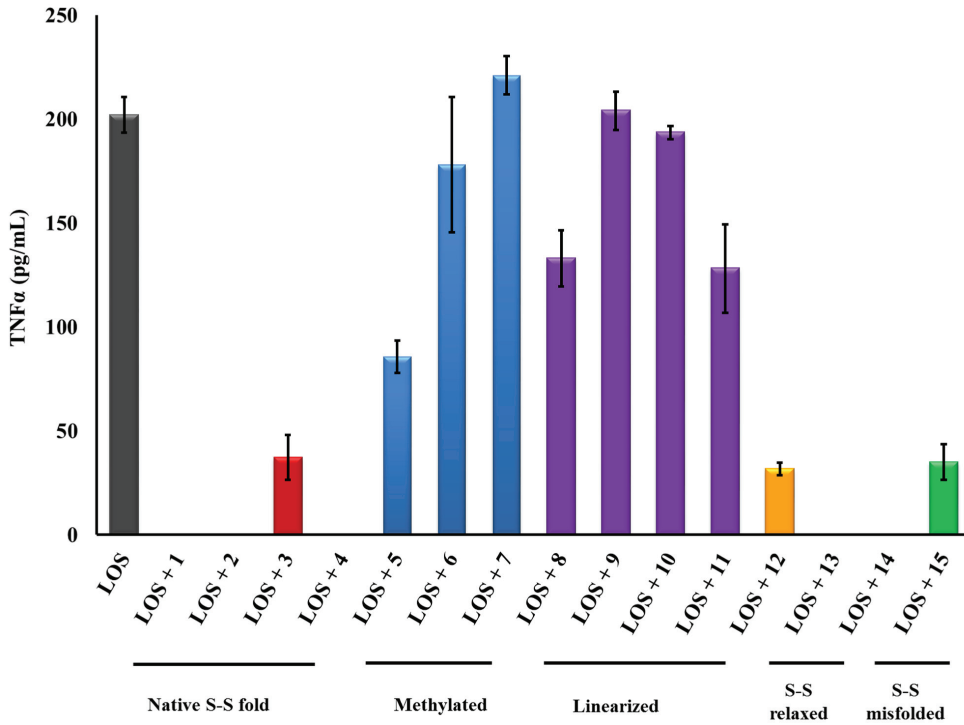

Figure 2. PG-1 analogs neutralized meningococcal LOS activity and inhibited TNFα release. TNFα was released from human macrophage-like THP-1 cells induced overnight with meningococcal LOS (5 ng/mL ~ 2.5 pmole/mL) preincubated with or without 2 μg/mL of PG-1 or its derivatives for 30 min at 37 °C. TNFα release was measured by ELISA. Error bars represent ±SD from the mean of duplicate measurements. This experiment is representative of two independent experiments. Methylated: *N*-methylated tyrosine or phenylalanine derivatives; S-S: disulfide bridges.

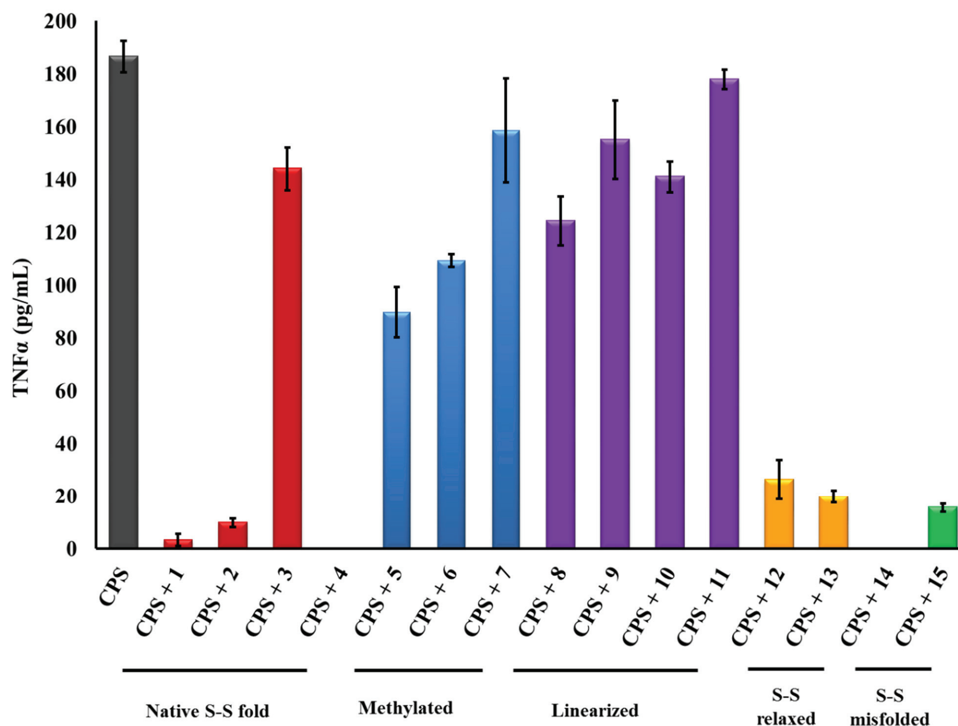

Figure 3. PG-1 analogs neutralized meningococcal capsular polysaccharide (CPS) polymer activity and inhibited TNFα release. CPS polymers were purified from the endotoxin-deficient serogroup B meningococcal NMB-*lpxA* mutant designated CPS. TNFα was released from human macrophage-like THP-1 cells induced overnight with meningococcal CPS polymers (25 μg/mL) pre-incubated with or without 2 μg/mL of PG-1 or its derivatives for 30 min at 37 °C. TNFα release was measured by ELISA. Error bars represent ±SD from the mean of duplicate measurements. This experiment is representative of two independent experiments. Methylated: N-methylated tyrosine or phenylalanine derivatives; S–S: disulfide bridges.

2.2. Linear PG-1 Analogs Lacking β-Hairpin

It is well established that the PG-1 peptide β-hairpin structure fold is essential for its antibacterial activity. However, it is not known whether this fold is also required for the peptide's immune modulatory activity. To this end, the Group III analogs **(8–11)** represent "linearized" versions of PG-1 that do not contain native disulfide bridges of 6–15 and 8–13. We substituted the four Cys residues in analogs **8–11** with residues of increasing bulkiness and apolar character (Ala, Cys(S-me), Met(O), and Met, in that order). As can be seen in Table 1, "linearization" of PG-1 dramatically reduced its immune modulatory activity against meningococcal LOS and CPS (Table 1), as it failed to inhibit the release of TNFα (Figures 2 and 3) or IL-1β (Figures 4 and 5) from stimulated THP-1

cells. Analogs **8** and **9** also failed to inhibit nitric oxide release from murine RAW264 macrophages stimulated with LOS or CPS doses (Figures 6 and 7). We also note that linearized peptides lost between 50-fold and 4000-fold of their antibacterial activity (data not shown), which is consistent with previously published reports [30,40,76]. As predicted by computational modeling of analog **8** (Figure 1C), the peptide adopts a linear coil structure rather than a β-hairpin fold native peptide structure. A previous study by Lai *et al*. designed cysteine-free linearized PG-1 analogs that adopted the β-hairpin fold by using D-proline instead of arginine at position 10, which allowed peptides to form a β-hairpin fold [77]. These linearized peptides containing D-proline maintained the β-hairpin fold and exerted good antibacterial activity [77]. It remains to be determined whether these D-proline-containing linearized peptides would also possess immune modulatory activity. The binding of PG-1 to LPS inhibits the biological activity of LPS and prevents it from activating TLR4, thus inhibiting the consequent release of cytokines TNFα and IL-1β from macrophages. Likely, the β-hairpin fold affords a peptide conformation that facilitates binding to LPS via the negative charges of the lipid A phosphate head groups and via hydrophobic interactions with lipid A fatty acyl chains, similar to what has been proposed for other AMPs. In particular, a parallel emerges with the fish defense peptides, pardaxins, where adaptive changes in the overall peptide shape enable binding to lipid A head groups as well as to hydrophobic fatty acyl chains [78]. A similar pattern of interaction was also observed between LPS and the horseshoe crab major AMP, tachyplesin 1 [79].

2.3. PG-1 Analogs with Altered Disulfide Connectivity

Finally, analogs **12**–**15** of Group IV address the potential importance of a native or alternative protegrin disulfide fold in complex formation with LOS and CPS. Here we report a limited number of such derivatives: analog **13** bears a "relaxed" disulfide formed by two homocysteine residues in place of cysteines; analog **12** bears a single native disulfide, Cys-8–Cys-13, in which the non-bridged cysteines were S-protected by a methyl group. Both analogs **12** and **13** reduced TNFα release from THP-1 cells (Figures 2 and 3) and nitric oxide release from RAW264 macrophages in a manner comparable to that of the parent PG-1 (Figures 6 and 7). Therefore, the replacement of only two cysteines with homocysteine (double native S–S connectivity) or S-methycysteine (single S–S connectivity), which potentially leads to a more relaxed β-hairpin fold structure with one or two native folded disulfides, did not substantially reduce the peptide's immune modulatory activity. We also report on the two analogs that bear a single disulfide that is *non*native, or "misconnected": analog **14** with a bridge connecting Cys-6 and Cys-8, and analog **15** with a bridge connecting Cys-13 and Cys-15 (Figure 1A). Surprisingly, we found that the misconnected disulfide bridges do not exert a deleterious effect because both analogs retain potent immune modulatory activity comparable to parent peptide **1**. Both analogs **14** and **15** neutralized meningococcal LOS and CPS activity and inhibited TNFα (Figures 2 and 3) release from human THP-1 cells and nitric oxide release from murine RAW264 macrophages stimulated with doses of meningococcal LOS (Figure 6) or CPS (Figure 7). Analog **15** also inhibited IL-1β release from THP-1 cells (Figures 4 and 5). Taken together, the data suggest that disulfide bridge alteration and/or misconnection does not impair immune

modulatory activity. We therefore assume that the active β-hairpin fold can still be maintained by these modified peptides.

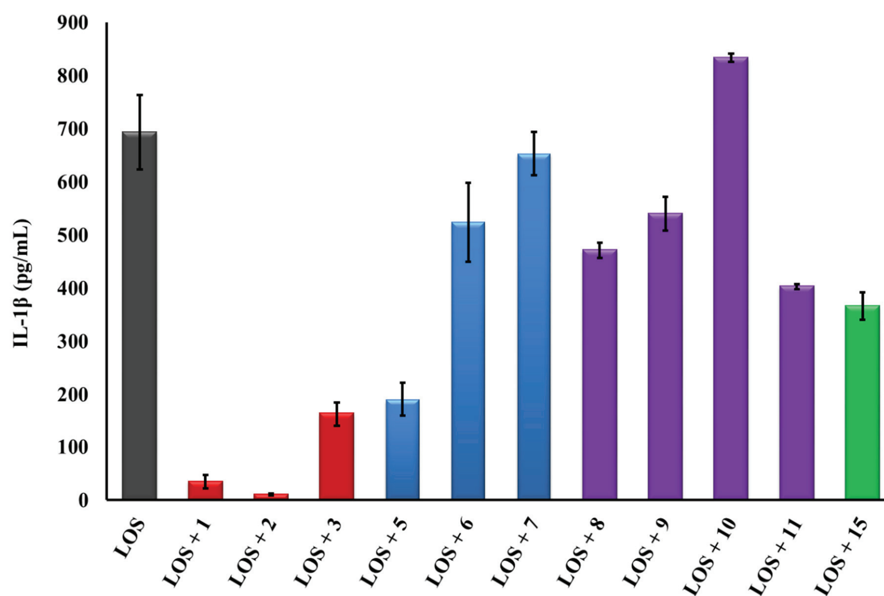

Figure 4. PG-1 analogs neutralized meningococcal LOS activity and inhibited IL-1β release. IL-1β was released from human macrophage-like THP-1 cells induced overnight with meningococcal LOS (5 ng/mL ~ 2.5 pmole/mL) preincubated with or without 2 µg/mL of PG-1 or its derivatives for 30 min at 37 °C. IL-1β release was measured by ELISA. Error bars represent ±SD from the mean of duplicate measurements. This experiment is representative of two independent experiments. Methylated: *N*-methylated tyrosine or phenylalanine derivatives; S-S: disulfide bridges.

The ability of AMPs to exert both antibacterial and immune modulatory effects points to their therapeutic potential. LPS released from gut microbiota circulating in blood and tissues due to increased permeability of the gut mucosa can cause inflammation [80–82]. This microbial translocation is associated with immune activation and inflammation in chronic diseases like HIV infection [83] and chronic kidney disease [84]. Host-derived cationic peptides bind to PAMPs and neutralize their immune stimulatory activity, thereby reducing immune activation and inflammatory state.

In summary, porcine PG-1 is a very potent immune-modulatory AMP capable of complex formation with a wide range of bacterial lipopolysaccharides as well as negatively charged capsular polysaccharides. We demonstrated its ability to effectively dampen major inflammatory signaling pathways such as those of the host during the course of infection. This is the first attempt to characterize the structure-immunomodulatory relations of PG-1, a short disulfide-linked AMP. Our results suggest that the immunomodulatory activity of PG-1 is more tolerant to major structural alterations as compared to its AMP activity. This includes retaining activity while one of the native

disulfides is missing and/or is misconnected. Retaining one disulfide is, however, essential as linear analogs proved inactive. Importantly, the elimination of two hydrogen (H)-bonding sites, believed to be important in the oligomerization of PG-1 on bacterial surfaces via main chain modification (methylation), proved to abrogate immunomodulatory activity of the peptide, suggesting that its active LPS-complexed form is an oligomer similar to PG-1's AMP action. With this in mind, studies are underway to characterize the solution structures of some of these analogs in order to shed more light on this interesting phenomenon. Although statistical analysis was not performed on our peptide screening data, the number of technical replicates does suggest trends. We recognize that further work is required to test the conclusions drawn from this proof-of-concept study. Thus, future experiments are planned to further investigate the most promising peptides that exhibit potent immune modulatory activity.

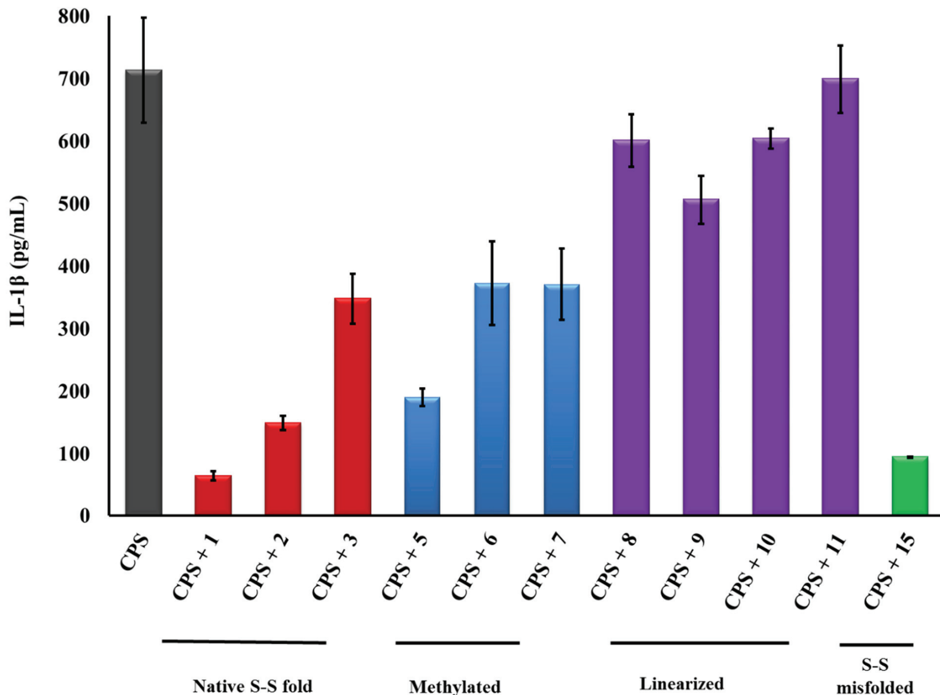

Figure 5. PG-1 analogs neutralized meningococcal capsular polysaccharide (CPS) polymers activity and inhibited IL-1β release. CPS polymers were purified from the endotoxin-deficient serogroup B meningococcal NMB-*lpxA* mutant designated CPS. IL-1β was released from human macrophage-like THP-1 cells induced overnight with meningococcal CPS polymers (25 μg/mL) pre-incubated with or without 2 μg/mL of PG-1 or its derivatives for 30 min at 37 °C. IL-1β release was measured by ELISA. Error bars represent ±SD from the mean of duplicate measurements. This experiment is representative of two independent experiments. Methylated: N-methylated tyrosine or phenylalanine derivatives; S–S: disulfide bridges.

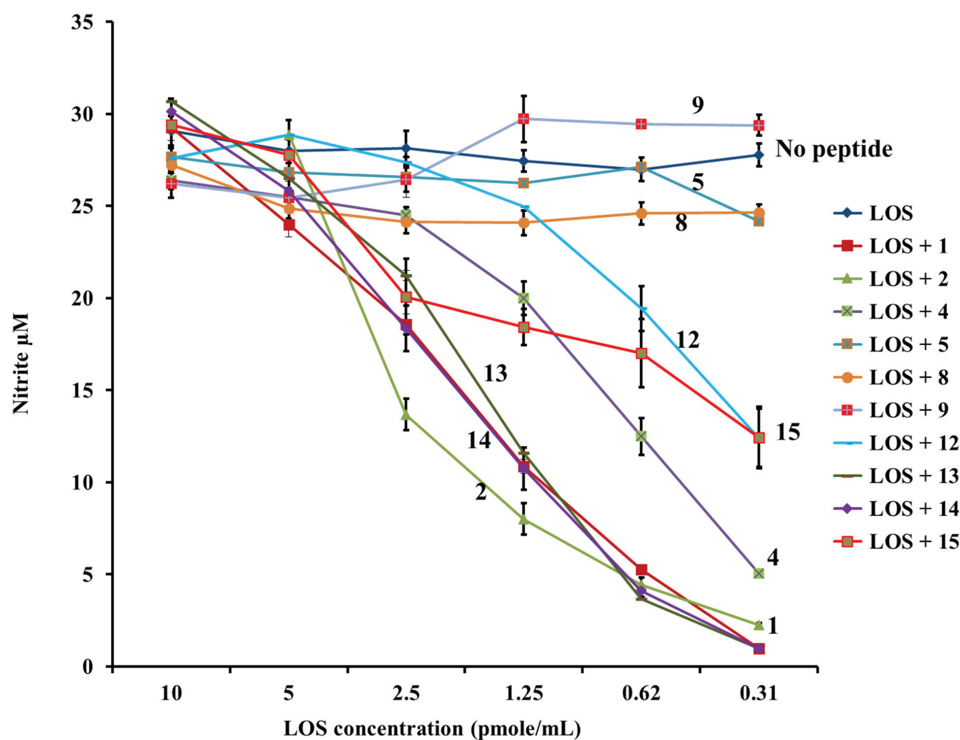

Figure 6. PG-1 analogs neutralized meningococcal LOS bioactivity and inhibited nitric oxide release. Nitric oxide was released from murine RAW264 macrophages induced overnight with meningococcal LOS doses pre-incubated with or without 2 µg/mL of PG-1 or its analogs for 30 min at 37 °C. Nitric oxide release was measured by the Greiss method. Error bars represent ± SD from the mean of duplicate measurements. This experiment is representative of two independent experiments.

3. Experimental Section

3.1. Reagents

RPMI 1640 medium, Dulbecco's Eagle medium, fetal bovine serum (FBS), penicillin/streptomycin, sodium pyruvate, and nonessential amino acids were obtained from Cellgro Mediatech (Herndon, VA, USA). Human and mouse TNFα and IL-1β ELISA kits were from R&D Systems (Minneapolis, MN, USA). THP-1 and RAW264 cell lines were purchased from ATCC (Manassas, VA, USA). Meningococcal lipooligosaccharides (LOS/LPS) that activate TLR4 and meningococcal capsular polysaccharides (CPS) polymers that induce TLR2 and TLR4 signaling were prepared as previously described [56]. CPS polymers were purified from the LPS-deficient serogroup B *Neisseria meningitidis lpxA* mutant [56].

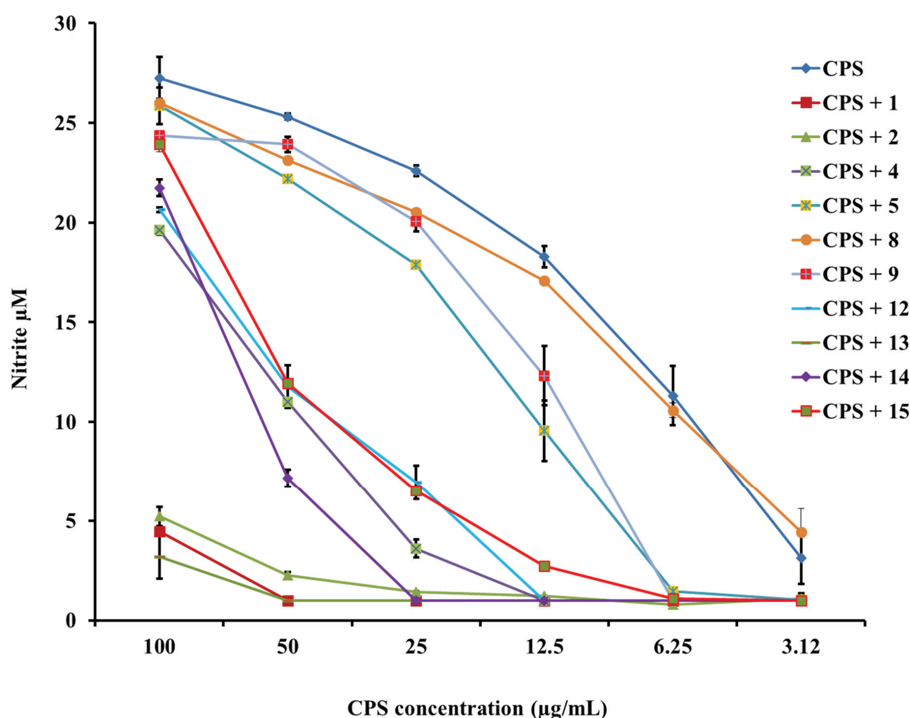

Figure 7. PG-1 analogs neutralized meningococcal capsular polysaccharide (CPS) polymer activity and inhibited nitric oxide release. CPS polymers were purified from the endotoxin-deficient serogroup B meningococcal NMB-*lpxA* mutant designated CPS. Nitric oxide was released from murine RAW264 macrophages induced overnight with doses of meningococcal CPS polymers pre-incubated with or without 2 µg/mL of PG-1 or its analogs for 30 min at 37 °C. Nitric oxide release was measured by the Greiss method. Error bars represent ±SD from the mean of duplicate measurements. This experiment is representative of two independent experiments.

3.2. PG-1 Analog Synthesis

The PG-1 and its analogs (Table 1) used in this study were prepared by Fmoc/tBu solid-phase peptide synthesis, as previously described [85]. Fmoc-Cys(Trt)-OH and Fmoc-Hcy(Trt)-OH were used for incorporation of Cys and Hcy. Following deprotection/cleavage in TFA, the peptides were purified as all-reduced species by preparative reversed-phase- (RP) HPLC using gradients of acetonitrile in 0.1% aqueous TFA [85]. For PG-1 analogs bearing one or two disulfides, connecting Cys or Hcy residues in PG-1 sequence positions 6, 8, 13, and 15, disulfide formation was affected by air oxidation in water in the presence of charcoal using purified, all-reduced peptides [86]. The completion of oxidation was monitored by analytical RP-HPLC and mass spectrometry. The oxidized peptides were purified by RP-HPLC and lyophilized. The final peptide purity (>95%) was

confirmed by RP-HPLC and peptide masses were confirmed by mass spectrometry. All peptides were used in the form of their TFA salts. The stock solutions were prepared in 0.1% aqueous acetic acid and were ultrafiltered prior to their use. When tested for their antimicrobial activity against *Neisseria gonorrhoeae* strain FA19 [58], PG-1 (**1**) and its linearized analog (**8**) demonstrated similar potencies to those published under similar conditions [87].

3.3. Cell Cultures

THP-1 human monocyte-like cells were grown in RPMI 1640 with L-glutamate supplemented with 10% FBS, 50 IU/mL of penicillin, 50 µg/mL of streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids. Culture flasks were incubated at 37 °C with humidity under 5% CO_2. Murine macrophages RAW264 were grown in Dulbecco's Eagle medium, supplemented and incubated as noted above.

3.4. Cellular Activation

Human THP-1 (monocyte-like cells) and murine RAW264 macrophages were stimulated with TLR ligands with or without preincubation with PG-1 and its analogs (Table 1). Purified meningococcal CPS samples were freshly dissolved in pyrogen-free sterile H_2O at 1 mg/mL stock concentration and vortexed for 2 min. Working CPS concentrations (ranging from 100 µg/mL to 1 µg/mL) were made in duplicate wells using sterile PBS by serial fold dilutions in the 96-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 50 µL final volumes. PG-1 analogs (2 µg/mL) or PBS equivalent volumes were added to designated wells and preincubated for 30 min at 37 °C. Freshly grown THP-1 cells and murine macrophages, each adjusted to 10^6 cell/mL and 250 µL aliquots, were dispensed into each well at a final cell density of 250×10^3 in the designated 96-well plates. The plates were incubated overnight at 37 °C with 5% CO_2 and humidity. Supernatants from stimulated cells were harvested and stored at −20 °C until use.

3.5. Cytokine Profiles

The cytokines TNFα and IL-1β, released from THP-1 cells, were quantified by DuoSet ELISA (R&D Systems), as previously described [61]. All experiments were performed twice ($n = 2$) with technical duplication in each experiment.

3.6. Nitric Oxide Induction by Murine Macrophages

Freshly grown adherent RAW264 macrophages were harvested, washed and re-suspended in Dulbecco's complete media, counted and adjusted to 10^6 cell/mL. Two hundred fifty microliter aliquots were then dispensed into each well of a 96-well plate at a final cell density of 250×10^3 prior to stimulation with TLR ligands with or without PG-1 analogs, as mentioned above. The induced RAW264 macrophages were incubated overnight at 37 °C with 5% CO_2 and supernatants were harvested and saved. Nitric oxide release was quantified using the Greiss chemical method, as previously described [61].

3.7. Cellular Viability Assessment

Trypan blue exclusion method was used to assess the viability of macrophages (1×10^6/mL) incubated with 2 µg/mL of PG-1 or its analogs overnight at 37 °C with 5% CO_2, as described above [22,62].

3.8. Computational Modeling of PG-1's 3-D Structure and Its Linearized Analogs

Three-dimensional structures of the parent PG-1 peptide and its linearized analog **8** were predicted using I-TASSER [88], and the generated PDBs were visualized by Chimera software [89]. The prediction of PG-1 was based on the published crystal structures PDB # 1PG1 and 1ZY6. The following amino acid sequences were used to generate 3D structure prediction: PG-1: NH_2-RGGRL CYCRRRFCVCVGR-$CONH_2$; Compound **8**: NH_2-RGGRLAYARRRFAVAVGR-$CONH_2$.

4. Conclusions

Protegrin, as a major porcine leukocyte AMP, exerts potent immune modulatory activity. The data presented here suggest that adoption of the β-hairpin structure, stabilized with at least a single disulfide bridge, is a prerequisite for immune modulatory potential. Active PG-1 analogs neutralized LOS and CPS bioactivity and markedly reduced inflammatory mediators' release from macrophages.

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Author Contributions

Susu M. Zughaier and Jan Pohl conceived and designed the experiments. Susu M. Zughaier and Pavel Svoboda performed the experiments, analyzed the data, and contributed reagents, materials, and analysis tools. Susu M. Zughaier and Jan Pohl wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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Mechanisms of Antimicrobial Peptide Resistance in Gram-Negative Bacteria

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Abstract: Cationic antimicrobial peptides (CAMPs) are important innate immune defenses that inhibit colonization by pathogens and contribute to clearance of infections. Gram-negative bacterial pathogens are a major target, yet many of them have evolved mechanisms to resist these antimicrobials. These resistance mechanisms can be critical contributors to bacterial virulence and are often crucial for survival within the host. Here, we summarize methods used by Gram-negative bacteria to resist CAMPs. Understanding these mechanisms may lead to new therapeutic strategies against pathogens with extensive CAMP resistance.

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1. Introduction

Cationic antimicrobial peptides (CAMPs) have microbicidal properties towards a variety of pathogens including bacteria, viruses, fungi and parasites. They are a large and varied group of peptides produced by many organisms ranging from prokaryotes to vertebrates (over 1200 have been identified thus far) [1]. These peptides contain little consensus in their amino acid sequences, though they largely maintain certain key features: they are cationic, amphipathic and relatively hydrophobic [2]. These attributes are thought to allow CAMPs to interact with bacterial membranes which contain anionic head groups and hydrophobic fatty acid chains. The CAMPs then destabilize bacterial membranes, which can involve pore formation, leading to cell lysis [3]. Some CAMPs may also have intracellular targets whose inhibition can lead to disruption of cell wall, protein and nucleic acid synthesis, as well as the direct induction of cell death [4].

In addition to their roles in host defense, at least one class of CAMPs has been harnessed and used clinically to treat bacterial infections. The polymyxins, derived from the Gram-positive bacterium *Bacillus polymyxa*, are a class of antibiotics that have seldom been used due to nephrotoxic and neurotoxic side effects [5]. However, due to the catastrophic increase in antibiotic resistance, these drugs are increasingly used as last-line antibiotics to treat infections with multi-drug resistant Gram-negative pathogens [5]. Currently, two polymyxins are in use clinically, polymyxin B and colistin (polymyxin E).

While CAMPs can kill a variety of pathogens, Gram-negative bacteria represent a major target. Due to the intense pressure that CAMP-mediated killing exerts on bacteria, some species have evolved ways to resist the action of these antimicrobials. Resistance to CAMPs is thought to have a significant negative effect on the ability of the host to prevent and fight bacterial infections, and also threatens the utility of polymyxins in the clinic. Unfortunately, due to their recent increased use, resistance to polymyxins is already on the rise [6]. Taken together, the development of CAMP resistance likely allows Gram-negative bacteria to avoid killing by both the host immune

system and polymyxin antibiotics. In this review, we will summarize the variety of methods used by Gram-negative pathogens to survive in the face of CAMPs, as well as the clinical implications of resistance to these peptides.

2. Surface Remodeling

The outer surface of Gram-negative bacteria represents the first barrier to CAMPs and is therefore often modified to enhance resistance (Figure 1). One of the main ways in which killing by CAMPs can be avoided is through an increase in bacterial surface charge. Host CAMPs contain a region of highly positive charge and are attracted to negatively charged molecules, such as the surface of many bacteria. Thus, increasing surface charge prevents access of CAMPs to the vulnerable bacterial outer membrane [4].

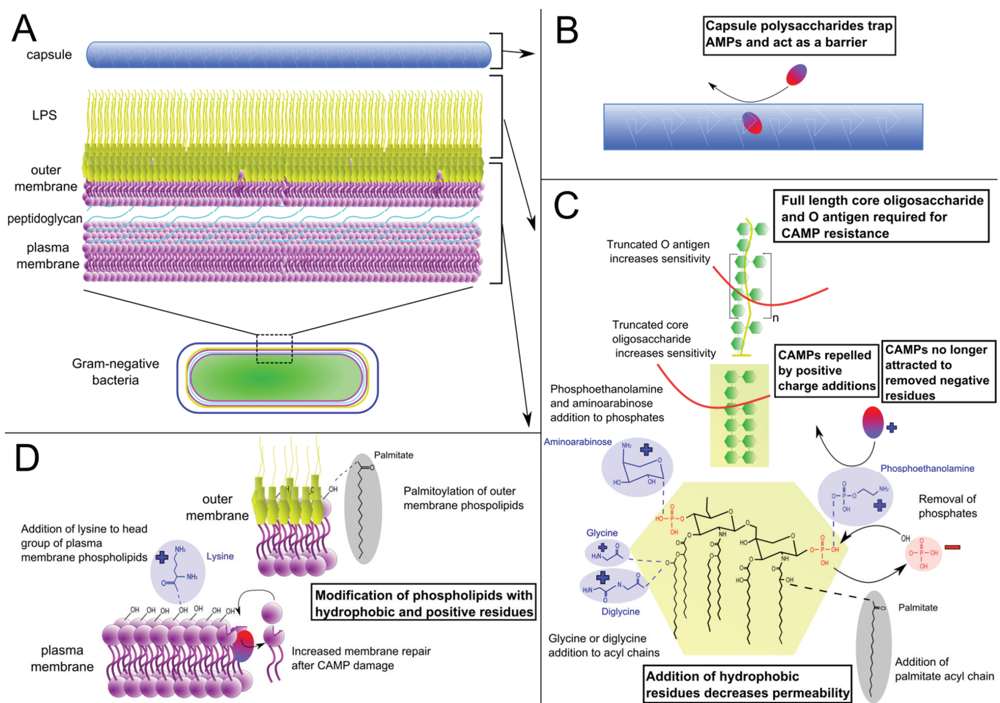

Figure 1. Bacterial surface modifications that enhance cationic antimicrobial peptide (CAMP) resistance. Gram-negative bacterial cell wall structure (**A**), with magnification of (**B**) capsule, (**C**) lipopolysaccharide, and (**D**) outer and plasma membranes. Lipopolysaccharide (LPS) structure varies greatly across species; depicted is a representative *E. coli* LPS structure, with modifications from various other species.

The surface of Gram-negative bacteria is largely composed of the glycolipid lipopolysaccharide (LPS), serving as one of the initial barriers against extracellular stresses. Specifically, LPS is a major constituent of the outer leaflet of the outer membrane phospholipid bilayer, which envelops

the peptidoglycan containing periplasm and the inner membrane (Figure 1A). It is comprised of the hydrophobic lipid A whose acyl chains insert into the membrane, the diverse core oligosaccharide, and the O-antigen comprised of repeating subunits (Figure 1C). In particular, the lipid A and core oligosaccharide often contain multiple negatively charged residues, such as phosphate groups.

2.1. Lipopolysaccharide Modifications

To mitigate the negative charge of LPS, numerous species of bacteria add positive residues to this structure, often to the lipid A. Common positively charged additions to LPS are cationic sugars. For example, the amine containing sugar aminoarabinose is added to a lipid A phosphate group in *P. aeruginosa* as well as *Salmonella typhimurium*, resulting in increased survival of both bacteria in the presence of polymyxin B [7,8]. Aminoarabinose is also present on the LPS of *Burkholderia* species [9]. However, this addition seems to be required for *Burkholderia* survival and so has not been directly linked to CAMP resistance. *Francisella novicida* adds another amine containing sugar, galactosamine, to its single lipid A phosphate group, similarly promoting polymyxin B resistance. Demonstrating the contribution of this modification to pathogenesis, deletion mutants lacking the galactosamine modification are highly attenuated, with a 5 log decrease in virulence in murine infections [10,11]. Increased CAMP resistance is also linked to cationic sugar addition in *Bordetella pertussis* and *Bordetella bronchiseptica*, in which glucosamine groups are added to both lipid A phosphates [10,12].

Other amine containing moieties, such as amino acids, are also added to the lipid A component of LPS to counteract its negative charge. For example, specific strains of *Vibrio cholerae*, the causative agent of the human disease cholera, add glycines to their lipid A [13]. While most strains of *Vibrio cholerae* are sensitive to CAMPs, the O1 El Tor strain, responsible for the current cholera pandemic [14], has a much higher level of resistance. Hankins *et al*. have shown that this strain of *V. cholerae* adds glycine and diglycine amino acid residues to lipid A acyl chains, increasing the net positive charge of LPS and the bacterial cell surface [13].

Phosphoethanolamine is another amine containing group that can be added to lipid A, as is the case in *Neisseria gonorrhoeae*, the causative agent of gonorrhea. This phosphoethanolamine residue is added to one of the phosphate groups of lipid A, under the control of the *lptA* gene that is required for its ability to resist CAMP-mediated killing [15]. Importantly, this increased resistance to CAMPs facilitates the establishment of a more severe disseminated form of gonorrheal infection [16]. Phosphoethanolamine addition to LPS also occurs in *Salmonella typhimurium* and in colistin-resistant strains of *A. baumannii* [17], where it increases resistance to polymyxin B [18].

In addition to adding positive charge to counteract the negative residues on LPS, some bacteria remove negative residues as an alternative mechanism of mitigating overall negative charge. The anionic phosphate groups of lipid A are major negative residues on LPS and are thus targets for removal. In *F. tularensis*, the 4' lipid A phosphate is removed by the phosphatase LpxF, leaving only one phosphate group on lipid A [19]. In *lpxF* deletion mutants that cannot remove the 4' phosphate, there is greatly increased susceptibility to polymyxin B, as well as the loss of lethality in a mouse intradermal infection [19,20]. Interestingly, the *lpxF* gene was exogenously expressed in *E. coli* whose lipid A normally has two phosphate groups. These modified *E. coli* lack a 4' lipid A phosphate and

consequently display a >15-fold increase in polymyxin MIC [21]. The negatively charged phosphate groups on lipid A are a target for removal in many other pathogenic bacterial species, including *Porphyromonas gingivalis* [22], *Bacteroides fragilis* [23] and *Helicobacter pylori* [24]. Together, these data clearly demonstrate that CAMP resistance can be induced by removal of negatively charged lipid A residues.

Distinct from the alteration of charge, another strategy for generating CAMP resistance is to increase the hydrophobicity of LPS. Hydrophobic lipid chains, added to lipid A phosphates, the glucosamine backbone or existing acyl chains, serve to increase LPS saturation and decrease overall permeability, preventing CAMPs from inserting into the membrane [25]. In *Salmonella*, acyl chains are added to the glucosamine backbone and phosphates of lipid A by PagP [26]. *pagP* deletion mutants exhibited increased membrane permeability [26] and were nearly 4 times more susceptible to the antimicrobial peptide C18G (a synthetic CAMP derived from human platelet factor IV) [27]. Enhanced acylation of lipid A also occurs in *E. coli* and *Yersinia enterocolitica* [26].

Many of the LPS modifications described above are tightly regulated and induced upon exposure to CAMPs. The well characterized PhoPQ two component regulatory system of *Salmonella typhimurium* controls several modifications that lead to CAMP resistance [28]. It plays a major role in pathogenesis, since deletion mutants lacking this system had over a 4 log virulence defect during murine infection [29]. The fact that this regulatory system contributes to CAMP resistance suggests that avoiding killing by these antimicrobials is critical for virulence (Table 1). The sensor kinase PhoQ senses environmental stresses, such as low Mg^{2+} and Ca^{2+}, as well as those encountered by bacteria within macrophage phagosomes, even directly detecting the presence of the CAMPs LL-37 and C18G [30], leading to activation of the response regulator PhoP [31]. PhoP subsequently activates the PmrAB two component system [31]. PmrAB signaling leads to modification of lipid A phosphates with aminoarabinose, increasing charge, and 2-hydroxy myristate, increasing hydrophobicity and decreasing permeability [32]. The PhoPQ and PmrAB systems play similar roles in *Pseudomonas aeruginosa* [33] and *Serratia marcescens* [34], while PmrAB functions in lipid A modification in *Acinetobacter baumannii* in the absence of PhoPQ [35]. These data highlight distinct ways that bacteria inducibly modify lipid A to resist CAMPs. However, lipid A is not the only portion of LPS that is a target for modification.

In addition to lipid A modifications, the O-antigen and core sugars have also been implicated in CAMP resistance. In *Brucella abortus*, transposon mutants that lack O-antigen showed significantly decreased survival in polymyxin B and were attenuated in a mouse model [36]. Mutants in *Burkholderia cenocepacia* with a truncated core were unable to grow in high concentrations of polymyxin B as did the wild type strain, and they were additionally outcompeted in a lung infection model [37]. Full length core and O-antigen thus can significantly contribute to CAMP resistance and have an important impact on virulence.

It is important to note that many CAMP resistant bacteria use several of the strategies listed above to mitigate the negative charge of their LPS. For example, *Helicobacter pylori* not only decreases negative charge by removing a phosphate group, it also adds in its place a positively charged phosphoethanolamine [24], further increasing the charge of its lipid A. This results in extensive resistance to polymyxin B, with an MIC 25× higher than that of a deletion strain lacking these

modifications. Many other Gram-negative bacteria use multiple strategies to mitigate the negative charge of LPS, and also modify other membrane components as well to further enhance CAMP resistance [52].

Table 1. Links between CAMP resistance and virulence of Gram-negative pathogens. Examples of CAMP resistance mechanisms that additionally have an impact on virulence. * Regulatory proteins shown to be responsible for CAMP resistance regulation, but regulate other processes as well.

Species	Modification	CAMP Resistance	Impact on Virulence	Ref.
Brucella abortus	LPS O antigen	Transposon mutants lacking O antigen show decreased survival to polymyxin B at 5–40 µg/mL	Transposon mutant unable to persist six weeks after mouse intraperitoneal infection	[36]
Burkholderia cenocepacia	LPS inner core oligosaccharide	*B. cenocepacia* require full length core oligosaccharide to grow in 100 µg/mL polymyxin B	Mutants with truncated core oligosaccharide were completely outcompeted by parent strain in rat lung infection model	[37]
Legionella pneumoniae	*rcp*, homolog of *pagP*, responsible for palmitoyl addition to lipid A	Mutants in *rcp* show 50% decrease in MIC to polymyxin B and synthetic CAMP C18G	Deletion mutants showed decreased survival in macrophages and were outcompeted by the parental strain in mouse lung infection	[38]
Neisseria gonnorhoeae	Mtr efflux pump	MICs are 8× higher for PG-1 and 30× higher for LL-37 in WT compared to *mtr* mutant	Deletion mutant completely outcompeted by WT after 3 day mouse genital tract infection	[39]
Proteus mirabilis	ZapA secreted metalloprotease	Purified ZapA readily degrades LL-37 and human beta-defensin-1 *in vitro*	4 log decrease in virulence in mouse urinary tract infection with ZapA mutant	[40]
Pseudomonas aeruginosa	AcrAB efflux pump	Mutant in a*crB* 10× more susceptible to CAMP-containing BALF, as well as diminished survival in 0.1 µg/mL polymyxin B, 30 µg/mL HNP-1, and 0.1 µg/mL HBD-1 + 2	1–3 log decrease in virulence of deletion mutant over 72 h mouse infection using a pneumonia model	[41]
	LasA cleavage and release of syndecan-1 from host immune cells	Shed syndecan-1 can bind Pro/Arg rich CAMPs	3 log decrease in virulence when syndecan-1 is absent in KO mouse lung infection, with 1/3 reduction in mortality	[42–44]

Table 1. *Cont.*

Species	Modification	CAMP Resistance	Impact on Virulence	Ref.
Salmonella typhimurium	Various	Transposon mutagenesis yielded 12 mutants that were susceptible to CAMP protamine at 1mg/mL	11 of 12 mutants with high protamine susceptibility had decreased virulence in mouse intragastric infection	[45]
	Aminoarabinose addition to lipid A through *pmrF*	*pmrF* deletion mutant unable to add aminoarabinose to lipid A, and is more sensitive to CAMPs	Mice orally infected with mutants had double the survival time as WT-infected mice. Competition infections with WT and deletion mutants show that CAMPs CRAMP and matrilysin alone not responsible for attenuation	[46]
	* PmrAB mediated addition of aminoarabinose to lipid A	Inactivation of *pmrA* results in 19× reduction in polymyxin B MIC, while overexpression results in 3× increase	*pmrA* deletion mutants show decreased lethality in mice by oral but not intraperitoneal infection	[8,47]
	* SlyA regulatory protein	*slyA* mutant is susceptible to 1 µg/mL polymyxin B, and SlyA protein binds to promoter of *ugtL* resistance gene	Deletion mutants have LD50 >4 log higher for oral infection and >5 log higher for peritoneal infection in mice	[48,49]
	* PhoP regulatory protein	Mutants increase sensitivity to human and rabbit neutrophil granules, as well as rabbit CAMP NP-1	Deletion mutants in *phoP* show 4 log reduction in virulence in mouse peritoneal model of infection, and *phoP*/*phoQ* deletion of *S. typhi* was a safe vaccine candidate in humans	[28,29, 50]
Yersinia enterocolitica	Unspecified LPS modifications, possibly RosAB	Pathogenic *Y. enterocolitica* strains were more resistant to polymyxin B than non-pathogenic environmental strains when grown at 37 °C	Environmental strains not known to cause disease like the polymyxin resistant pathogenic strains	[51]

* Regulatory proteins shown to be responsible for regulation of antimicrobial peptide resistance, but regulate other processes as well.

2.2. Phospholipid Modifications

In addition to LPS, phospholipids are the other major component of the Gram-negative outer membrane. Similar to LPS, phospholipids in the outer membrane can also be modified to increase CAMP resistance (Figure 1D). *S. typhimurium* uses its PhoPQ system to not only modify LPS, but also to modify phospholipids that reside in the outer membrane. PhoPQ-activated PagP adds palmitoyl groups to phospholipids, similar to its modification of lipid A described above. This leads to an increase in the levels of palmitoylated phosphatidylglycerols within the outer leaflet of the outer membrane, which are less polar and more hydrophobic than many other phospholipids in the outer membrane. Increased hydrophobicity in the outer membrane may decrease permeability,

similar to the effect in lipid A palmityolation [53]. Therefore, localizing these modified phospholipids to the outer leaflet of the membrane results in increased CAMP resistance.

In addition, the inner membrane may be modified to increase CAMP resistance. Addition of lysine to phospholipids (lysylation) within the plasma membrane increases the charge of anionic phosphatidylglycerol to a cationic form, and thus is able to help repel cationic CAMPs and reduce their binding to the membrane. Though best studied in Staphylococcus aureus, these lysylated phospholipids are also present in Gram-negative species [54] including *Rhizobium tropici* [55] and *Caulobacter crescentus* [56].

It has also been suggested that the PagP protein mentioned above may act as part of an acute membrane repair response, facilitating rapid membrane repair after damage caused by CAMPs [53]. In addition, it has been hypothesized that one of the reasons that CAMPs do not efficiently damage eukaryotic host membranes is that eukaryotic cells have a much more robust form of membrane repair than bacteria [57]. Thus it is possible that bacteria with increased membrane repair capacity could survive higher concentrations of CAMPs, simply repairing the membrane as it is damaged. Dorschner *et al.* suggest that proteins involved in membrane repair are prime candidates for investigation of microbial resistance [58]. There is still a lack of concrete evidence demonstrating that this occurs, but membrane repair may be an important facet of CAMP resistance and warrants further investigation.

2.3. Capsule Production

Beyond alterations to the bacterial membranes, the outer surface of bacteria can be further modified to protect against CAMPs. The bacterial capsule is a protective layer external to the outer membrane that acts as an additional barrier and is comprised primarily of long chained repeating polysaccharides [59]. *Klebsiella pneumoniae* capsule provides increased resistance against cationic defensins, lactoferrins and polymyxins. Furthermore, there is a direct correlation between higher amounts of capsular polysaccharide, decreased levels of CAMPs binding to the outer membrane, and increased resistance to polymyxins [60]. Capsule-mediated resistance to CAMPs is likely critical for bacterial virulence during *in vivo* infection as an acapsular mutant was unable to cause pneumonia in a mouse model [61]. It should be noted, however, that the capsule can provide resistance to other immune pressures in addition to CAMPs, such as complement and phagocytosis, and thus the attenuation of the acapsular mutant is not necessarily due to a decrease in CAMP resistance.

In *Neisseria meningitidis*, capsule production was shown to increase resistance to the human CAMP LL-37 [62]. Survival in the presence of LL-37 was 100-fold lower in a deletion strain lacking capsule compared to wild-type. Furthermore, upon exposure to sublethal levels of LL-37, the capsule biosynthetic genes *siaC* and *siaD* were upregulated and contributed to increased capsule production [62].

In addition to those mentioned above, numerous Gram-negative species express a polysaccharide capsule. Further, *P. aeruginosa* has also been shown to use its capsule to resist CAMPs [63]. Taken together, the data described here illustrate how Gram-negative pathogens can use numerous modifications to LPS, phospholipids, and the production of a polysaccharide capsule to resist CAMPs and protect their membranes.

3. Biofilms

Bacteria can further resist CAMPs through their organization into specialized structures known as biofilms. In addition to free floating, planktonic bacterial populations, bacteria can form biofilms on diverse surfaces. These structures consist of sessile bacteria adhering to a surface in a highly organized manner that allows for circulation of nutrients [64]. Bacteria in a biofilm often secrete a slimy extracellular matrix that both aids in adherence to surfaces and acts as a barrier to outside stressors. This extracellular matrix can be composed of various compounds including cellulose, teichoic acids, proteins, lipids and nucleic acids [65]. Biofilms can form on environmental surfaces such as hospital equipment, allowing these populations to persist, and likely contributing to the growing problem of hospital-acquired infections. They can even form on ventilators and catheters, giving them access to mucosal sites in patients and further promoting their infectivity. Biofilms are also able to form on biological surfaces such as teeth or the respiratory tract, often facilitating the establishment of chronic infections [64].

The general organization of the bacteria and extracellular components contributes to the protection offered by the biofilm structure. As a biofilm matures, it progresses from a thin homogeneous structure to a thicker, more heterogeneous form that contains many substructures. These can include stacks of bacteria forming "mushroom" shaped structures [66]. This is observed in *Pseudomonas aeruginosa*, which forms biofilms that display exceptional resistance to CAMPs and antibiotics, in some cases over 1000 times as great as their planktonic form [67]. *Pseudomonas* biofilms contain a high level of the polysaccharide alginate, which is known to cause significant alterations to biofilm structure. A strain that overproduces alginate formed biofilms that were much thicker and more structurally heterogeneous, an architecture that acts as a more effective diffusion barrier to CAMPs [68]. Additionally, expression of *Pseudomonas* biofilm genes in *E. coli*, whose biofilms are normally flat and unstructured, resulted in the formation of biofilms with more complex architecture, correlating with increased resistance to the polymyxin antibiotic colistin. This increased resistance was not observed against other antibiotics such as ciprofloxacin, indicating that this protection may be specific to CAMPs [69]. Biofilm structure can vary greatly across different species and strains, which may account for some of the differences in CAMP susceptibility in various biofilms.

Specific components of the extracellular matrix have been shown to be critical for resistance to CAMPs. Anionic alginate in *P. aeruginosa* not only contributes to biofilm structure but can also bind to and induce conformational changes in invading CAMPs [70]. The CAMP-alginate complexes then oligomerize, hindering their ability to enter the biofilm [71]. Further, polysaccharides from biofilms of *K. pneumoniae* and *Burkholderia pyrrocinia* are able to bind and sequester CAMPs [72]. Adding these polysaccharides to *E. coli* increased its MIC to CAMPs LL-37, human beta defensin 3, and Bac7(1-35). Extracellular DNA also forms an integral component of *P. aeruginosa* [73] and *S. typhimurium* [74] biofilms, and can also contribute to CAMP resistance. The negative charge of DNA allows it to bind and sequester cations from the surrounding environment. This results in an environment with a low concentration of cations, which is an activating signal for the previously

mentioned PhoPQ system. This therefore results in the activation of CAMP resistance genes via PhoPQ that lead to LPS and other modifications [73].

In addition to signaling by PhoPQ, biofilms have several other inducible defenses against CAMPs. *P. aeruginosa* encodes the inducible biofilm gene *psrA*, which has been linked with greatly increased levels of CAMP resistance [75]. This gene was upregulated 3-fold in the presence of the CAMP indolicidin. Deletion mutants lacking *psrA* were less able to form biofilms, and showed significantly increased killing when challenged with indolicidin or polymyxin B. Pamp *et al*. have shown that tolerance to colistin in *Pseudomonas* biofilms is due to metabolically active cells within the biofilm. While the less metabolically active cells in the biofilm were killed by colistin, a spatially distinct subset of more active cells were able to resist killing. These cells were able to upregulate PmrAB-regulated resistance genes responsible for lipid A modification [76]. Overall, biofilms confer bacteria with the ability to form a hardy structure that can withstand and resist destruction by high concentrations of CAMPs, as well as many other types of antimicrobials.

4. Efflux Pumps

Efflux pumps are complexes of mostly membrane bound proteins that move toxic compounds out of cells. Bacterial efflux pumps are active transporters, either directly requiring ATP or using an existing electrochemical potential gradient. These complexes play important roles in antibiotic resistance, as many bacteria use them to resist major classes of antibiotics, including fluoroquinolones, macrolides, tetracyclines, glycylcyclines, beta lactams and aminoglycosides [77]. In addition, bacterial efflux pumps contribute to colonization and persistence, likely in part by defending against host antimicrobials such as CAMPs [78]. Indeed, there are many examples of Gram-negative bacteria that use efflux pumps to increase survival and virulence *in vivo* even in the absence of antibiotics including *Salmonella typhimurium* [79,80], *Salmonella enteritidis* [81], *Enterobacter cloacae* [82], *Borrelia burgdorferi* [83], *P. aeruginosa* [84], *K. pneumoniae* [41], *V. cholerae* [85] and *N. gonnorhoeae* [39].

In addition to other resistance mechanisms described above, *K. pneumoniae* uses the AcrAB-TolC efflux pump system, known to mediate resistance against fluoroquinolones, to resist CAMPs. When the AcrB component of the efflux pump system was knocked out, mutant bacteria exhibited increased sensitivity to fluoroquinolones as well as polymyxin B [41]. The *acrB* mutant also exhibited 10-fold lower survival in bronchoalveolar lavage fluid, which contains many CAMPs, and specifically displayed increased sensitivity to the human alpha defensin HNP-1 as well as human beta defensins HBD-1 and HBD-2. Importantly, this increased susceptibility correlated with a 1–3 log attenuation of the mutant in a mouse pneumonia model [41].

Another pathogen that expresses efflux pumps to increase CAMP resistance is *Yersinia enterocolitica*. A human gut pathogen, *Y. enterocolitica* has a high level of resistance to human CAMPs, at least in part due to the action of the RosAB efflux pump system. A *rosAB* deletion mutant was more sensitive than wild-type to the CAMPs polymyxin B, cecropin P1 (produced in pig bladders) and melittin (found in bee venom) [86]. This pump acts as a potassium antiporter, using a potassium gradient that pumps K^+ ions into the cell as it pumps out harmful CAMPs. Interestingly, the RosAB pump is activated at 37 °C and in the presence of CAMPs, similar to

conditions encountered within the host during infection [86]. Under these conditions, pathogenic *Y. enterocolitica* strains are more resistant to CAMPs than non-pathogenic strains or a control *E. coli* strain [51]. While this was not explicitly shown to be due to the RosAB pump and could be due to another temperature regulated system, the data suggest that RosAB-mediated CAMP resistance is likely important for maintaining pathogenicity in *Y. enterocolitica*.

N. gonorrhoeae possess the Mtr (multiple transferrable resistance) efflux pump which facilitates resistance to numerous antimicrobials. This three protein system has been shown to pump out various hydrophobic compounds, such as bile salts and fatty acids, which can cause membrane damage. This pump also confers resistance to CAMPs as well. *mtr* deletion mutants had significant growth defects in the presence of PG-1, a protegrin produced by porcine macrophages [87], and the MIC of the human CAMP LL-37 and horseshoe crab-derived tachyplesin-1 were also reduced in the *mtr* deletion mutant. Thus, the Mtr efflux pump is able to recognize a variety of CAMP structures and remove them from the bacterial cell [87]. This efflux pump is highly relevant for *in vivo* survival; gonococci lacking *mtr* were completely outcompeted by the wild type strain in a competitive infection of the mouse genital tract [39] and this was correlated with the levels of CAMP resistance *in vitro* [88]. The closely related *Neisseria meningitidis*, which can cause meningitis in humans, also expresses the *mtr* efflux pump and it was similarly shown to contribute to CAMP resistance [89].

The RND family of efflux pumps in *Vibrio* species has a similar activity in mediating resistance to polymyxins and bile acids. *V. cholerae* has at least six loci that encode RND family proteins, including the VexB protein which can mediate CAMP resistance. When this protein is deleted from a virulent strain, the mutant bacteria exhibit increased susceptibility to polymyxin B as well as bile acids, which are found in the GI tract that *V. cholerae* infects. Further, this deletion mutant was unable to effectively colonize the gut of mice when compared to the wild-type strain [90]. The closely related *Vibrio vulnificus*, which can cause wound infections and sepsis, encodes a different efflux pump, TykA, which is responsible for resistance to the CAMPs protamine and polymyxin B [91].

Efflux pumps have been shown to be important for resistance to a wide range of antibiotics and there has been much interest in using efflux pump inhibitors to enhance antibiotic treatment [92]. However, the extensive evidence that these pumps can enhance CAMP resistance and play a role in virulence suggests that efflux pump inhibitors may also be used therapeutically to sensitize bacteria to innate immune defenses. Inactivating bacterial efflux pumps responsible for CAMP resistance could enhance the ability of the host CAMPs to clear infections, while at the same time increasing sensitivity to antibiotics.

5. Binding and Sequestering CAMPs

When confronted with a large concentration of CAMPs, some bacteria are able to bind and sequester these peptides so they cannot reach the bacterial membrane. One method for binding external CAMPs is through the release of negatively charged molecules that will attract these amphipathic antimicrobials. Negatively charged proteoglycans are found in abundance on the surface of fibroblasts and epithelial cells, and can be cleaved and released by bacterial enzymes at rates that exceed that of baseline release. For example, the connective tissue proteoglycan decorin is one of the major secreted products of human fibroblasts [93], and when incubated with *P. aeruginosa* or

P. mirabilis, it is cleaved to release several products, including dermatan sulphate. This degradation occurs in the presence of bacteria conditioned media, purified *P. aeruginosa* elastase, or alkaline proteinase, even in the absence of fibroblast enzymes. This released dermatan sulphate was able to efficiently bind neutrophil derived α-defensin unlike the full length uncleaved decorin molecule. This free and soluble dermatan sulphate was able to nearly completely inhibit killing by defensins at concentrations 10 times above the MIC for *P. aeruginosa* [94].

Similarly, *P. aeruginosa* takes advantage of the release of the cell surface heparin sulfate proteoglycan syndecan-1. This proteoglycan is found on the surface of epithelial cells, and is shed during tissue injury as a soluble ectodomain. Incubating epithelial cells with cell culture supernatants from *P. aeruginosa* led to cleavage of syndecan-1 and release of its soluble ectodomain [42]. This activity was found to be dependent on the *P. aeruginosa* protein LasA, which is a known virulence factor and has been previously shown to modify other proteins. Shedded ectodomains of syndecan-1 are able to bind and interfere with the antimicrobial activity of CAMPs, specifically those that are Pro/Arg-rich like cathelicidins [42], likely due to charge based interactions. This was also demonstrated *in vivo*, with increased syndecan-1 shedding from epithelial cells during *P. aeruginosa* lung infection in a mouse model [43]. The virulence of the pathogen was dependent on syndecan-1 shedding, as there was a 3 log decrease in virulence if syndecan-1 was absent or rendered resistant to shedding [43]. Syndecan-1 ectodomains not only bind to CAMPs but can also bind and interfere with a range of other immune signaling molecules [42] such as cytokines and matrix metalloproteases [95]. It is not yet known the downstream effect that this binding would have on the greater immune response, but immune modulation in addition to direct interference with CAMPs may together account for the observed virulence decrease [43].

The fact that proteoglycans are able to interfere with host CAMP activity suggests that the bacterial capsule, which is rich in polysaccharides, may also be able to capture and sequester CAMPs [4]. Acapsular mutants often have decreased virulence *in vivo*, and *K. pneumoniae* acapsular mutants are more susceptible to α- and β-defensins [60]. This idea was further strengthened by evidence from Llobet *et al.*, showing that the anionic polysaccharide component (CPS) of the bacterial capsule is able to impart CAMP resistance in *K. pneumoniae* and *P. aeruginosa* [63]. Purified CPS was able to increase the resistance of acapsular mutants, and was shown to bind to soluble CAMPs in a charge-dependent manner. This resulted in fewer peptides reaching the surface of the bacteria. After exposure to CAMPs, these anionic polysaccharides are released by the bacteria to bind and sequester the antimicrobials [63]. It is possible that other encapsulated bacteria can use this mechanism to enhance CAMP resistance as well.

Another component that can be released to trap CAMPs is part of the bacterial cell membrane itself, in the form of enclosed vesicles budding off from the surface known as outer membrane vesicles (OMVs). OMV release is a normal part of bacterial cell growth [96] and may be used for a variety of processes such as toxin delivery [97]. In *E. coli*, membrane stress, especially from accumulation of proteins in the outer membrane, induces an increase in OMV formation. As the targets of CAMPs are bacterial membranes, CAMPs can be bound and sequestered in these vesicles, diverting them from the membranes of living bacteria. This notion is supported by the fact that mutants that over produce OMVs are 6-fold more resistant to killing by polymyxin B, while a

mutant lacking vesicle release was 10-fold more susceptible [98]. *Vibrio cholerae* has adapted its OMV response to aid in CAMP resistance as well. In the presence of sublethal concentrations of polymyxin B, it was noted that OMVs released from the bacteria were larger and had altered protein content [99]. These OMVs were better able to protect against CAMPs, as co-incubating bacteria with them doubled the level of protection against LL-37 when compared to OMVs produced by bacteria in the absence of polymyxin B. The polymyxin B induced OMVs contained elevated levels of the protein Bap1, which was shown to mediate the increased CAMP protection by binding to but not degrading LL-37. Thus, OMV release can act as an inducible defense against CAMPs that can significantly increase levels of resistance.

6. Proteolytic Degradation

In addition to mechanisms to block access of CAMPs to bacteria, or pump them out of the cell, direct inactivation of these antimicrobials offers another means by which bacteria can combat them. As summarized below, diverse bacteria produce proteases that degrade CAMPs, an activity that is highly reliant upon the structural motifs of the target peptide. The human CAMP LL-37 is a linear, alpha helical peptide and is thus more susceptible to degradation by proteases than CAMPs with non-linear structures containing disulfide bonds such as defensins [100]. *P. aeruginosa* produces an elastase that is capable of rapidly degrading LL-37 *in vitro*, with its bactericidal activity completely inactivated within 1 hour [101]. Structural analysis showed that cleavage occurred at 4 peptide bonds all located within the regions of LL-37 that have bactericidal activity. Further, a *Proteus mirabilis* proteinase and *E. faecalis* gelatinase degrade and inactivate LL-37 *in vitro*, allowing for survival of bacteria in the presence of otherwise lethal doses of this antimicrobial [101]. In *S. typhimurium*, the omptin family protease PgtE degrades various alpha-helical CAMPs, including human LL-37 and its murine ortholog CRAMP [102]. Strains with deletions of this gene had 2-fold lower MICs to both CAMPs, while overexpression of *pgtE* increased the MIC by 8-fold. Interestingly, *pgtE* expression is regulated by PhoPQ, highlighting another example of the many CAMP resistance mechanisms controlled by this two-component system.

Even though linear CAMPs are quite sensitive to degradation by proteases, they can be shielded and protected by binding to proteins such as extracellular actin. *In vivo*, LL-37 can bind to released actin molecules, preventing the access of degradative proteases while still maintaining its antimicrobial activity [103]. High levels of extracellular actin were found in areas of cell necrosis, which often occurs at sites of infection [103]. Thus, linear CAMPs like LL-37 can be protected and rendered much less vulnerable to proteolytic degradation *in vivo* due to their complexing with other proteins.

Many non-linear CAMPs are more resistant to degradation than linear CAMPs. This is due at least in part to intramolecular disulfide bonds, such as those found in the defensins, which contain a canonical array of 6 disulfide linked cysteines [104]. These linkages create non-linear tertiary structure that is much more stable in the environment and resistant to protease degradation [105,106]. However, some bacteria have evolved proteases to degrade even these CAMPs with increased stability. The protein OmpT is another omptin family protease and contributes to CAMP resistance in *E. coli*. Stumpe *et al*. have shown that this outer membrane protein degrades the CAMP protamine [107] which is thought to conform to a nonlinear structure involving three disulfide

bonds [108]. Both the OmpT and PgtE omptin proteins are present in *Shigella flexneri* and *Yersinia pestis*, suggesting that these pathogens may also use omptins to degrade CAMPs [109].

B. cenocepacia has two zinc dependent metalloproteases that have been shown to degrade CAMPs, ZmpA and ZmpB. Each of these proteins can degrade a variety of peptides, including a wide range of CAMPs. These proteins have distinct substrates, as only ZmpA can degrade linear LL-37, while only ZmpB degrades non-linear human beta defensin-1 [110]. Other CAMPs like protamine, elafin and SLPI (all of which are non-linear with disulfide bonds) were degraded by both, but they seemed to be digested into different fragments by each protease. These proteases are additionally important for the virulence of *B. cenocepacia*, since deletion mutants of each protease individually results in decreased lung pathology in a mouse infection model [111,112].

Along with its previously mentioned proteinase, *P. mirabilis* encodes the virulence factor ZapA that is involved in CAMP degradation. The ZapA protein is a secreted extracellular metalloprotease that is able to degrade a wide variety of targets, including host defense proteins such as immunoglobulins and complement components [113]. It is also able to target host CAMPs, including not only LL-37, but also disulfide bond containing defensin HBD-1. Proteolysis of LL-37 and HBD-1 by ZapA resulted in 6 and 9 fragments respectively, completely inactivating both proteins [113]. Importantly, absence of ZapA in *P. mirabilis* results in a 4 log decrease in bacteria in a mouse urinary tract infection model, suggesting that the degradation of host antimicrobials is vital to the virulence of this pathogen [40]. Many additional examples of bacteria directly degrading (or causing the degradation of) CAMPs likely exist, and presumably make important contributions to *in vivo* virulence.

Bacteria can also take advantage of host enzymes with CAMP-degrading activity. Reduced killing of *P. aeruginosa* by beta defensins in the broncho-alveolar fluid of cystic fibrosis patients has been shown to be due to the release of proteolytic cathepsins from macrophages, which are able to degrade host beta defensins. The release of cathepsins is due at least in part to the release of inflammatory mediators such as IL-13 and IFN-γ, which has been suggested to result from immune activation by LPS from *P. aeruginosa* and other commensal Gram negative bacteria [114]. Thus, *P. aeruginosa* is able to take advantage of the host immune response, facilitating the release of CAMP degrading enzymes.

7. Modulation of CAMP Expression

Cationic antimicrobial peptides are present in steady state at all mucosal sites of the body to prevent infection by invading microorganisms. However, in the context of an infection, CAMPs can also be upregulated to help fight the invading microbes. Stimulation of increased CAMP production occurs mainly through innate immune recognition of microbes [115] and subsequent signaling by Pattern Recognition Receptors (PRRs). For example, the host PRR Toll-like Receptor 4 (TLR4) recognizes and signals for a proinflammatory response, including CAMP induction, in response to LPS.

This also means that modulation of microbial components like LPS, such that they cannot be detected by host PRRs, can lead to decreased PRR signaling and thus lower levels of CAMPs. Modification of lipid A structure, which can greatly affect CAMP resistance (Section 2.1), can also have a significant effect on TLR4 signaling. In *P. gingivalis*, removal of a single phosphate group

from lipid A results in both increased resistance to polymyxin B [116] and reduced activation of TLR4 [117]. In addition, incorporation of a seventh acyl chain in *Salmonella* lipid A similarly results in increased CAMP resistance and decreased TLR4 signaling [118]. These examples highlight that modification of LPS structure can both directly (repel or prevent binding of CAMPs) and indirectly (reduced induction of CAMPs) lead to CAMP resistance.

Interestingly, LPS modifications that increase CAMP resistance in some bacteria actually increase detection by TLR4 [119]. For example, palmitoylation of lipid A in *P. aeruginosa* results in increased resistance to CAMPs but a more inflammatory LPS [120], indicating that CAMP resistance and evasion of TLR4 signaling do not always occur in tandem. It is possible that those modifications that are able to provide both increased CAMP resistance and evasion of TLR4 signaling are more beneficial to bacteria than modifications that provide only one of these attributes. Alternatively, increased inflammation may promote pathogenesis by some bacteria, and in these cases increased CAMP resistance as well as increased TLR4 signaling may be beneficial to the pathogen.

The link between tandem alterations of CAMP resistance and inflammatory signaling extends to other bacterial components and host receptors as well. Similar to LPS and TLR4, bacterial lipoproteins (BLP) are recognized by the host PRR Toll-like Receptor 2 (TLR2), leading to initiation of inflammatory signaling. In *Francisella novicida*, the CRISPR-Cas protein Cas9 plays a regulatory role in repressing the expression of a BLP [121]. This BLP repression leads to enhanced resistance to polymyxin B, as well as direct suppression of this TLR2 ligand and thus evasion of signaling (including CAMP induction) by this host receptor [122]. Many diverse PRRs detect bacteria, and avoiding recognition by these receptors could represent a broad and critical strategy to subverting the induction of CAMPs.

Another mechanism of limiting host inflammatory signaling, and thus CAMP induction, may be provided by the bacterial capsule. By facilitating resistance to CAMPs, the capsule prevents damage to bacterial membranes that contain activators of host signaling such as LPS and BLPs. As such, the capsule serves as a barrier to prevent the release of bacterial components that can be recognized by PRRs. This in turn prevents induction of higher levels of CAMPs. For example, in *P. aeruginosa*, the absence of capsule leads to an increased sensitivity to CAMPs as well as increased induction of beta defensins during murine infection [123]. Even further, the capsule polysaccharides of *P. aeruginosa* also activate the anti-inflammatory immune receptors CYLD and MKP-1, which results in the release of anti-inflammatory molecules that downregulate beta defensin production [123], illustrating yet another indirect mechanism by which the capsule facilitates resistance to CAMPs.

Shigella, which cause varying degrees of bacterial dysentery in children and adults, are able to downregulate the CAMP response of their human hosts through an as yet undetermined mechanism. Biopsies from *Shigella* infected colons show that there is a significant downregulation of transcripts encoding LL-37 and HBD-1 in epithelial cells, corresponding with decreased LL-37 and HBD-1 protein in the majority of the infected biopsies [124]. This downregulation was detected up to day 30, after which LL-37 levels began to increase above healthy control levels. Inhibition of LL-37 production was observed during *in vitro* infections of macrophage and epithelial cell lines, and was shown to be dependent upon *Shigella* plasmids within the host cells acting by an unknown

mechanism [124]. It is known, however, that the MxiE protein controls the injection of plasmid encoded effectors into the host cell leading to this CAMP inhibition. Downregulation of CAMPs early in the infection likely enhances the ability of *Shigella* to adhere to mucosal surfaces and infect host epithelial cells [125].

In addition to the examples cited above, bacteria have a wide range of mechanisms of altering or avoiding the host immune response (for a review of these see Hornef *et al.* [126]). Many of these could result in decreased levels of CAMPs, which could aid in colonization and infection by bacteria. As CAMP levels are dynamic and intertwined with the overall immune response, it is likely that diverse strategies to limit immune signaling indirectly play important roles in CAMP resistance.

8. Relevance

We have highlighted in this review that resistance to CAMPs in Gram-negative bacteria is often a crucial virulence strategy. While there are abundant correlations between bacterial traits that increase CAMP resistance and affect virulence (Table 1), it is in most cases yet to be proven during *in vivo* infection that resistance to CAMPs facilitates pathogenesis. This is likely due in part to the possible redundancy of numerous host CAMPs, and therefore the difficulty in deleting a sufficient number of CAMPs from a host in many cases to observe robust phenotypes.

CAMP resistance also affects susceptibility to cationic antibiotics used in the clinic, the polymyxins. Resistance to host cationic antimicrobials may even be facilitated by resistance to polymyxins. Clinical strains of *A. baumannii* that are polymyxin resistant are significantly more resistant to both LL-37 and lysozyme [127]. This has also been demonstrated in *Enterobacter cloacae*, where colistin heteroresistant strains are resistant to lysozyme after initial treatment with colistin [128]. This cross resistance between polymyxins and host antimicrobials may interfere with the use of CAMPs as therapeutic tools, including not only polymyxins but many other CAMPs still in development [129]. This is a great concern in an era in which new antibacterials are not keeping pace with the emergence of resistance. This brings up the possibility of instead targeting CAMP resistance mechanisms therapeutically, which would theoretically increase their susceptibility to polymyxins in addition to reducing the virulence of pathogens.

9. Conclusions

Gram-negative pathogens use many diverse mechanisms to resist killing by cationic antimicrobial peptides. Bacteria can alter surface structures to repel CAMPs, establish biofilms to increase resistance, use efflux pumps to pump them out, sequester them, produce proteases to degrade them, or alter immune responses to prevent their induction. It is no surprise that these mechanisms (with the exception of immune modulation) are also used by bacteria to gain resistance to antibiotics and that many of the pathogens mentioned in this review are noted for their ability to resist antibiotics. In many instances, CAMP resistance increases the virulence of bacterial pathogens. The combination of increased antibiotic resistance and virulence due to CAMP resistance makes pathogens very dangerous in the clinical environment. Thus it is imperative to devise new ways to combat or reverse CAMP resistance in Gram-negative bacteria. Further understanding the mechanisms of

CAMP resistance may be fruitful in deciphering new ways to combat highly virulent and antibiotic-resistant Gram-negative pathogens.

Acknowledgments

We have attempted to summarize a vast literature and have cited specific examples of mechanisms used by Gram-negative bacteria to resist CAMPs. We sincerely apologize to the authors of relevant studies that we did not cite.

Author Contributions

Victor I. Band wrote the manuscript and conducted the literature review. David S. Weiss provided broad ideas and structure, as well as revisions.

Conflicts of Interest

The authors declare no conflict of interest.

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Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria

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Abstract: Antimicrobial peptides, or AMPs, play a significant role in many environments as a tool to remove competing organisms. In response, many bacteria have evolved mechanisms to resist these peptides and prevent AMP-mediated killing. The development of AMP resistance mechanisms is driven by direct competition between bacterial species, as well as host and pathogen interactions. Akin to the number of different AMPs found in nature, resistance mechanisms that have evolved are just as varied and may confer broad-range resistance or specific resistance to AMPs. Specific mechanisms of AMP resistance prevent AMP-mediated killing against a single type of AMP, while broad resistance mechanisms often lead to a global change in the bacterial cell surface and protect the bacterium from a large group of AMPs that have similar characteristics. AMP resistance mechanisms can be found in many species of bacteria and can provide a competitive edge against other bacterial species or a host immune response. Gram-positive bacteria are one of the largest AMP producing groups, but characterization of Gram-positive AMP resistance mechanisms lags behind that of Gram-negative species. In this review we present a summary of the AMP resistance mechanisms that have been identified and characterized in Gram-positive bacteria. Understanding the mechanisms of AMP resistance in Gram-positive species can provide guidelines in developing and applying AMPs as therapeutics, and offer insight into the role of resistance in bacterial pathogenesis.

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1. Introduction

Antimicrobial peptides (AMPs) and the bacterial resistance mechanisms against them have been co-evolving for eons. A diverse array of life forms can produce AMPs, which can be used to promote immune defenses, nutrient acquisition or elimination of rival organisms from the environment. As a result, AMPs are found in a multitude of environments, ranging from mammalian tissues to soil and aquatic environments. This ubiquitous presence of AMPs in the environment provides strong selective pressure to drive the development of bacterial resistance against these peptides.

AMPs are typically small, charged, amphipathic molecules that can be produced in a variety of structures. Though structurally diverse, most AMPs work by interacting with the bacterial cell surface, followed by disruption of cellular integrity. Accordingly, the majority of bacterial resistance mechanisms function by limiting the interaction of AMPs with the bacterial cell surface. Mechanisms of AMP resistance include trapping or sequestering of peptides, outright destruction of AMPs by proteolysis, removal of AMPs from the cell via active transport, and structural modification of the cell surface to avoid interaction with AMPs. Many of these resistance mechanisms are upregulated in response to AMPs, allowing the bacteria to adaptively counter the effects of AMPs. Loss of

these resistance mechanisms can impair the ability of bacteria to colonize plant or animal hosts and can attenuate virulence for many pathogens. Mechanisms of resistance may evolve specifically within a bacterial lineage or be genetically transferred from other AMP-resistant organisms.

In this review, we evaluate the available literature on Gram-positive bacterial resistance mechanisms to antimicrobial peptides. This review highlights methods of AMP resistance based on mode of action and location within the Gram-positive bacterial cell. We begin with an overview of resistance mechanisms that act on AMPs extracellularly, and then discuss bacterial cell surface alterations. Finally, we consider removal of AMPs from the bacterial cell via transport.

2. Extracellular Mechanisms of Resistance: Enzymatic Degradation and AMP Blocking

The initial site of AMP interaction is at the bacterial cell surface. As a result, extracellular mechanisms of AMP inactivation have evolved as a first line of defense to minimize damage to the bacterial cell. Extracellular AMP resistance mechanisms have arisen in two main forms: enzymatic inactivation and sequestration (see Table 1 and Figure 1). The majority of these direct targeting mechanisms have evolved to recognize cationic AMPs. Cationic AMPs are positively charged peptides that may differentially target negatively charged moieties on the outer cell envelope, including teichoic acids, lipid II, and phosphatidylglycerol [1–3].

2.1. Extracellular Proteases

The degradation of AMPs by proteases is a mechanism of resistance found in many Gram-positive species, including *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* [4–6]. AMP-degrading proteases generally have broad substrate specificity, are typically found in mammalian pathogens, and include both metallopeptidases and cysteine proteases [7,8]. This section will present several examples of AMP-degrading proteases produced by Gram-positive bacteria and detail their effects on resistance.

AMP-degrading proteases are often secreted by bacteria into their surrounding extracellular environments. Gelatinase, an extracellular metallopeptidase produced by some strains of the opportunistic pathogen *E. faecalis*, cleaves the human cathelicidin, LL-37, resulting in the loss of antimicrobial activity *in vitro* [4]. The production of gelatinase by *E. faecalis* is associated with bacterial dissemination in animal models of disease and with increased incidence of dental caries in humans [9,10]. One example of a secreted protease made by *S. aureus* that confers AMP resistance is the aureolysin enzyme [5]. Aureolysin can hydrolyze the C-terminal bactericidal domain of LL-37, rendering the AMP inactive [11]. An infection model using human macrophages revealed that aureolysin contributes to Staphylococcal persistence within the phagosomal compartment [12], an environment that contains high levels of the antimicrobial peptide, LL-37 [13]. Additionally, some species of Staphylococci possess proteases that combat anionic AMPs such as dermcidin, a negatively charged peptide secreted by human sweat glands [14]. SepA (or SepP1) made by *S. epidermidis*, is a secreted metalloprotease that can cleave and inactivate dermcidin [6,15]. The SepA protease appears to specifically target dermcidin *in vitro* [6,16].

Table 1. Summary of Gram-positive Antimicrobial Peptides (AMP) Resistance Mechanisms.

Name	Mechanism of Action	Antimicrobial Resistance	Organisms	Reference
AMP Degradation				
Aureolysin	Protease	LL-37	*S. aureus*	[5,11]
Gelatinase	Protease	LL-37	*E. faecalis*	[4,10]
SepA	Protease	dermcidin	*S. epidermidis*	[6,16]
SpeB	Protease	LL-37	*S. pyogenes*	[4,21,22]
Sequestration/Competition for AMP target				
M Protein	Binding at surface	LL-37	*S. pyogenes*	[24]
PilB	Binding at surface	cathelicidins	*S. agalactiae*	[25]
SIC	Extracellular binding	α-defensins, LL-37, lysozyme	*S. pyogenes*	[26,27]
Staphylokinase	Extracellular binding	Cathelicidin, defensins	*S. aureus*	[28,29]
LciA	Binding at surface	Lactococcin A	*L. lactis*	[30,31]
Capsule	Binding/shielding	Polymyxin B, HNP-1	*S. pneumoniae*	[32]
Exopolysaccharide	Shielding/ Sequestration	LL-37, hBD-3, dermcidin	*S. epidermidis*	[33–35]
LanI lipoproteins	Binding or competition	lantibiotics	*L. lactis*, *B. subtilis*, other lantibiotic producers	[36–38]
Cell Surface Modifications				
DltABCD	D-alanylation of teichoic acids	daptomycin, vancomycin, nisin, defensins, protegrins	*S. aureus*, *L. monocytogenes*, *B. cereus*, *C. difficile*, *S. pyogenes*, *S. agalactiae*, *B. anthracis*, *S. suis*	[2,39–45]
MprF	Lysylation of phoshatidylglycerol	defensins, thrombin-induced platelet microbicidal protein	*S. aureus*, *L. monocytogenes*, *B. anthracis*, *M. tuberculosis*	[46–50]
OatA	Peptidoglycan O-acetylase	lysozyme	*S. aureus*, *S. epidermidis,* *S. lugdunensis*, *E. faecalis*, *L. monocytogenes*	[51–54]
PdgA	Peptidoglycan *N*-acetylglucosamine deacetylase A	lysozyme	*S. pneumoniae*, *E. faecalis*, *S. suis*, *L. monocytogenes*, *B. anthracis*	[55–58]
NamH	*N*-acetylmuramic acid hydroxylase	lysozyme	*M. smegmatis*	[59]

Table 1. *Cont.*

Name	Mechanism of Action	Antimicrobial Resistance	Organisms	Reference
AMP Efflux				
One-component transporter				
LmrB	ABC transporter	LsbA/LsbB	*L. lactis*	[60]
QacA	ABC transporter/alteration of membrane structure	thrombin-induced platelet microbicidal protein (tPMP)	*S. aureus*	[61]
BceAB type				
AnrAB	ABC transporter	nisin, gallidermin, bacitracin, β-lactams	*L. monocytogenes*	[62,63]
BceAB	ABC transporter	Bacitracin [a], actagardine, mersacidin, plectasin	*B. subtilis* [a], *S. mutans*	[64–68]
BraAB	ABC transporter	nisin, nukacin ISK-1, bacitracin	*S. aureus*	[69]
PsdAB	ABC transporter	nisin, enduracidin, gallidermin, subtilin	*B. subtilis*	[66]
MbrAB	ABC transporter	bacitracin	*S. mutans*	[35]
SP0812-SP0813	ABC transporter	bacitracin, vancoresmycin	*S. pneumoniae*	[70]
SP0912-SP0913	ABC transporter	bacitracin, lincomycin, nisin	*S. pneumoniae*	[71]
VraDE	ABC transporter	bacitracin, nisin, nukacin ISK-1	*S. aureus*	[69,72–76]
VraFG	ABC transporter	nisin, colistin, bacitracin, vancomycin, indolicidin, LL-37, hBD3	*S. aureus*, *S. epidermidis*	[69,72,75, 77–79]
YsaCB	ABC transporter	nisin	*L. lactis*	[80]
BcrAB type				
BcrAB(C)	ABC transporter	bacitracin	*B. licheniformis*	[81]
BcrAB(D)	ABC transporter	bacitracin	*E. faecalis*	[82,83]
LanFEG type				
As-48EFG(H)	ABC transporter	AS-48	*E. faecalis*	[84]
CprABC	ABC transporter	nisin, galidermin, other lantibiotics	*C. difficile*	[85,86]
EpiFEG(H)	ABC transporter	epidermin, gallidermin	*S. epidermidis*	[87]
LtnFE(I)	ABC transporter	lacticin 3147	*L. lactis*	[88,89]
McdFEG	ABC transporter	macedocin	*S. macedonicus*	[90]
MrsFGE	ABC transporter	mersacidin	*Bacillus sp. HIL Y-84, 54728*	[91,92]
MutFEG	ABC transporter	mutacin II	*S. mutans*	[93]
NisFEG(I)	ABC transporter	nisin	*L. lactis*	[37,94]
NukFEG(H)	ABC transporter	nukacin	*S. warneri*	[95,96]
SboFEG	ABC transporter	salivaricin B	*S. salivarius*	[97]
ScnFEG	ABC transporter	streptococcin A-FF22	*S. pyogenes*	[98]
SmbFT	ABC transporter	Smb, haloduracin	*S. mutans*	[99]
SpaFEG	ABC transporter	subtilin	*B. subtilis*	[36,100]

[a] Confers only bacitracin resistance in *B. subtilis*.

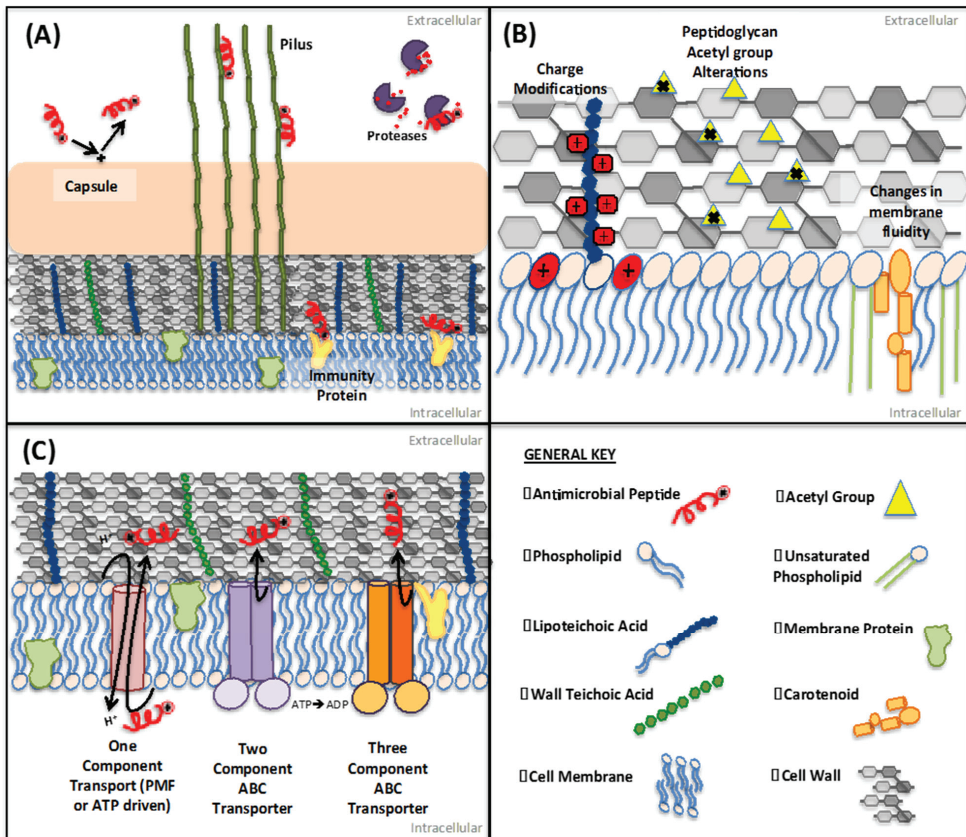

Figure 1. Overview of Antimicrobial Peptide Resistance Mechanisms in Gram-Positive Bacteria. (**A**) Extracellular mechanisms of AMP resistance include peptide degradation by secreted proteases, AMP sequestration by secreted or membrane associated protein (e.g., pili, immunity proteins, M proteins), or blocking by capsule polysaccharides; (**B**) Cell wall and membrane modifications include: Alteration of charge by lysination of the phospholipid head groups or D-alanylation of the lipoteichoic backbone, modification of the cell wall by deacetylation of *N*-acetylglucosamine or *O*-acetylation of *N*-acetylmuramyl residues, and alterations in membrane fluidity by phospholipid tail saturation or carotenoid additions; (**C**) Transport mechanisms of antimicrobial efflux from the cell include: ATP-driven ABC transporters composed of a single, double, or triple protein pump and involve a supplementary immunity protein, or single protein transporters driven by proton motive force.

Gram-positive proteases are also capable of targeting AMPs at the bacterial surface. SpeB is a cysteine proteinase secreted by the pathogenic bacterium *Streptococcus pyogenes* [17]. SpeB has broad substrate specificity and cleaves AMPs, such as LL-37, and other host proteins such as fibrin, immunoglobulins, and other immune modulators [4,18–21]. In an example of adaptive

resistance, SpeB was found to complex with the host α_2-macroglobulin (α_2M) proteinase inhibitor during infection [22]. The catalytically active SpeB-α_2M complexes are retained on the bacterial cell surface by association with the *S. pyogenes* G-related α_2M-binding protein (GRAB) [22,23]. The SpeB-α_2M complex has higher proteinase activity against LL-37, relative to free SpeB, and reduces killing of *S. pyogenes in vitro* [22].

2.2. Protein-Mediated Sequestration

Sequestration is another extracellular mechanism of AMP resistance [24–29,101]. Some Gram-positive bacteria produce extracellular or surface-linked proteins that directly bind to AMPs and block access to the cell membrane. Mechanisms of protein-mediated AMP sequestration vary between species and strains. We have highlighted specific examples of AMP sequestration mechanisms identified amongst strains of *S. pyogenes*, *S. aureus*, *Streptococcus agalactiae*, and *Lactococcus lactis*.

Proteins that inhibit AMP activity through binding can be secreted into the extracellular environment to inhibit contact of bactericidal peptides with the cellular surface. For example, the Streptococcal inhibitor of complement (SIC) produced by *S. pyogenes* is a hydrophilic, secreted protein that sequesters many AMPs, thereby preventing them from reaching cell-surface targets [102]. SIC binds to α-defensins, LL-37, and lysozyme, neutralizing the AMPs and inhibiting their bactericidal activity against *S. pyogenes* [27,102,103]. SIC production promotes bacterial survival *in vitro* and increases the virulence of *S. pyogenes* in animal models of disease [26,104]. Staphylokinase secretion by *S. aureus* is another example of an extracellular AMP resistance mechanism. Production of the staphylokinase protein by *S. aureus* occurs through the lysogenic conversion of the hlb β-hemolysin toxin gene by a bacteriophage harboring the *sak* gene [105–107]. Staphylokinase binds the murine cathelicidin mCRAMP *in vivo* and also complexes with human defensins HNP-1 and HNP-2 to reduce their bactericidal effects [28,29]. Studies of staphylokinase binding suggest that the staphylokinase-cathelicidin complex promotes host tissue invasion by activating the conversion of plasminogen to the host extracellular matrix-degrading enzyme, plasmin, although the role this conversion plays in Staphylococcal virulence remains unclear [29,101,108].

Proteins attached to the cellular surface can also bind AMPs to prevent contact with cell-associated targets. Examples of such proteins include the M1 protein of *S. pyogenes* and the pilus subunit, PilB of *S. agalactiae*. M1 of *S. pyogenes* can be found on the surface of most clinical isolates and has been linked to both host tissue adherence and invasive disease [109]. A hyper-variable extracellular portion of the M1 protein was shown to bind LL-37 and prevent the AMP from reaching the cell membrane [24]. The sequestration of LL-37 by M1 also promotes Streptococcal survival in neutrophil extracellular traps (NETs) by reducing LL-37 activity [24]. Like the M proteins of *S. pyogenes*, pili are also associated with invasive disease and promotion of host cell adherence by *S. agalactiae* [110,111]. Pili are large, filamentous, multimeric protein complexes expressed on the cell surface of *S. agalactiae* and other bacteria. Expression of the Streptococcal pilin subunit, PilB, promotes association of LL-37 with the bacterial cell surface and correlates with increased resistance to the murine cathelicidin mCRAMP *in vitro* [25]. In addition, pilB mutants of *S. agalactiae* (GBS) exhibit reduced fitness relative to wild-type strains in murine

infection models [25]. These data suggest that in addition to the adhesin properties of pili, pilus-mediated binding of AMPs also contributes to *S. agalactiae* virulence within the host.

Another family of membrane-associated AMP resistance proteins encompasses the LanI immunity proteins of some bacteriocin producer strains. LanI proteins are typically encoded near a bacteriocin biosynthetic operon and provide protection against the bacteriocin made by the producer bacterium [112,113]. LanI-type immunity proteins are lipoproteins that anchor to the bacterial cell surface and confer resistance by either binding directly to AMPs or outcompeting AMPs by binding directly to the cellular target [114–117]. The LanI lipoproteins often work in concert with LanFEG transporters, possibly acting as substrate-binding partners for specific lantibiotics. The best characterized of the transporter-associated LanI proteins are the NisI and SpaI lipoproteins found in strains of *L. lactis* and *Bacillus subtilis*, respectively [36,37,118] (described in transporter section). But, several lantibiotic producers encode only a LanI immunity protein and do not encode an apparent LanFEG transporter (e.g., PepI of *S. epidermidis* [119], lactocin S [120] of *L. sakei* and epicidin 280 of *S. epidermidis* [121]). In these systems, the LanI lipoprotein confers full immunity to the associated lantibiotic. Though some LanI structures have been characterized, LanI lipoproteins generally have low, if any, homology to one another [116,122]. Thus, it is unclear if mechanism of action for LanI-mediated immunity is conserved between different LanI lipoproteins.

2.3. Inhibition of AMP Activity by Surface-Associated Polysaccharides

Extracellular polysaccharide production has long been recognized as a factor that promotes both virulence and host colonization by many bacteria [123–125]. Extracellular polysaccharides are composed of structurally diverse polymers that are enzymatically produced by some Gram-positive species [126,127]. Extracellular polysaccharides that are attached to the cellular surface through covalent linkages with the cell wall are known as capsules (capsular polysaccharide, or CPS), while loosely attached polymers are referred to as exopolysaccharides, or EPS [128–130]. Polysaccharide-mediated AMP resistance is thought to occur by shielding the bacterial membrane via binding or electrostatic repulsion of AMPs [34,131].

The production of capsular polysaccharides provides resistance to a variety of AMPs and other antimicrobials and can allow some bacteria to evade host detection. Capsule-AMP binding can be mediated by the electrostatic interaction of positively charged AMPs with the negatively charged polysaccharide capsule [32]. For example, free capsular extracts from *Streptococcus pneumoniae* bind both polymyxin B and the α-defensin HNP-1, preventing these AMPs from reaching the cell membrane and promoting bacterial survival *in vitro*. Additionally, both polymyxin B and HNP-1 promote release of the capsule from *S. pneumoniae* without loss of cell viability, suggesting that capsule release may be a mechanism of AMP resistance by sequestering AMPs away from the bacterial cell surface [32]. In another example, production of the exopolysaccharide intercellular adhesion, PIA, by *S. epidermidis* reduces killing by human defensin hBD-3, cathelicidin (LL-37), and the anionic AMP dermcidin. PIA is hypothesized to shield the bacterial membrane from the effects of AMPs [33,34]. Predictably, PIA production is associated with *S. epidermidis* virulence in multiple animal infection models [132,133]. However, while many exopolysaccharide capsules can

provide resistance to AMPs, this protection is not universal to all capsule-producing Gram-positive bacteria [134–136].

3. Membrane and Cell Wall Modifications

The bacterial cell wall and membrane comprise a major target for the bactericidal activity of AMPs [137–139]. Bacteria frequently modify cell surface components to counter the effects of AMPs by reducing the net negative charge of the cell, altering membrane fluidity, or directly modifying AMP targets [140–142].

3.1. Repulsion of AMPs

Many AMPs target bacterial cells through electrostatic interactions with the cell surface [137–139]. The net charge of the bacterial cell surface is generated by anionic components of the cell membrane and cell wall, such as phospholipids and teichoic acids [143–145]. In turn, positively charged AMPs are attracted to the negatively charged bacterial cell surface [144,145]. Hence, a broad strategy of resistance to positively charged AMPs is to alter the components on the cell surface to decrease the net negative charge of the cell, thereby limiting the electrostatic interaction of AMPs with the bacterial cell surface.

One component of the bacterial cell membrane that carries a negative charge is phosphatidylglycerol [144,145]. But in many Gram-positive bacteria, the negative charge on phosphatidylglycerol can be masked via the addition of a positively charged amino acid by the multipeptide resistance factor protein, MprF [146,147]. MprF is an intergral lysyl-phosphatidylglycerol synthetase that synthesizes and translocates aminoacylated-phosphatidylglycerol to the external membrane layer of the bacterial cell. MprF synthases were initially found to incorporate a positively charged lysine into phosphatidylglycerol (Lys-PG), decreasing the net negative charge on the bacterial membrane. In *S. aureus*, *Listeria monocytogenes*, *E. faecalis*, *Enterococcus faecium*, *B. subtilis*, and *Bacillus anthracis*, the aminoacylation of phosphatidylglycerol by MprF confers resistance to positively charged AMPs [47–49,148–150]. Additionally, an MprF homolog is present in *Mycobacterium tuberculosis*, which also confers resistance to positively charged AMPs. This MprF homolog, LysX, carries out the same functions as MprF, with the addition of a lysyl-tRNA synthetase activity [46,151]. Lysinylation of phosphatidylglycerol confers resistance to a broad spectrum of AMPs, including human defensins, gallidermin, nisin, lysozyme, daptomycin, polymyxin B, and vancomycin (Table 1) [46,150–159]. In addition to lysine modifications, some MprF orthologs can modify membrane phosphatidylglycerol with multiple amino acids, including alanine and arginine [149,160]. The enhanced antimicrobial resistance provided by aminoacylation of phosphatidylglycerol is also associated with increased virulence for several Gram-positive pathogens [46,48,49,161,162].

The Dlt pathway is another enzymatic mediator of AMP resistance that has been identified and studied in many Gram-positive genera including *Staphylococcus*, *Listeria*, *Enterococcus*, *Bacillus*, *Clostridium*, *Streptococcus*, and *Lactobacillus* [2,40–45,163–168]. The enzymatic functions of the DltABCD proteins lead to the D-alanylation of teichoic acids and lipoteichoic acids of the cell

wall [169]. The addition of D-alanine to the backbone of teichoic acids can mask the negative charge present along these glycopolymers, thereby leading to increased surface charge and lower attraction of positively charged antimicrobials [169]. Similar to MprF, D-alanylation of teichoic acids by the Dlt pathway leads to a global change in charge distribution across the cell surface, allowing resistance to a broad range of cationic AMPs including vancomycin, nisin, gallidermin daptomycin, polymyxin B, lysozyme, and cathelicidins [2,39,141,163,166,170–172].

In addition to charge modification of teichoic acids, high-resolution microscopy of Group B *Streptococcus* revealed that D-alanylation could increase cell wall density, leading to increased surface rigidity [173]. Accordingly, D-alanylation may confer resistance to AMPs both by reducing the electrostatic interactions between AMPs and the cell surface and by decreasing the permeability of the cell wall [173]. As AMPs are ubiquitous within animals, D-alanylation of the cell wall can affect host colonization for pathogens and non-pathogenic species [41,152,164,174,175].

3.2. Target Modification

The cell wall is a common antimicrobial target for Gram-positive organisms. As a result, bacteria have evolved multiple modifications that limit antimicrobial targeting of the cell wall. Lysozyme, or *N*-acetylmuramide glycanhydrolase, an antimicrobial enzyme, is an important component of the host innate immune defense. Lysozyme is cationic at physiological pH, which facilitates its interaction with negatively charged bacterial surfaces. The cationic and muramidase activities of lysozyme directly target the bacterial peptidoglycan, the primary constituent of the cell wall [176]. The muramidase domain of lysozyme hydrolyzes the β-1,4 linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid of peptidoglycan, leading to the breakdown of the peptidoglycan macromolecular structure and eventual lysis of the cell [177–179]. As a result, bacterial resistance mechanisms have evolved to counter both the muramidase and cationic activities of lysozyme. In this section, we detail the mechanisms by which peptidoglycan is modified to limit lysozyme activity.

Two peptidoglycan modifiers that contribute to AMP resistance in some Gram-positive bacteria are the enzymes PgdA and OatA. It is proposed that the modifications made by both of these enzymes lead to steric hindrance between AMPs and the cell surface, thereby limiting the contact between lysozyme and its target [180]. PgdA deacetylates *N*-acetylglucosamine residues of peptidoglycan, generating a less favorable substrate for lysozyme [181–184]. PgdA was first implicated as a peptidoglycan deacetylase in the respiratory pathogen *S. pneumoniae*. PdgA and other peptidoglycan deacetylase orthologs have been shown to contribute to AMP resistance in many bacteria, including *Enterococcus*, *Streptococcus*, *Listeria* and *Bacillus* species [55–58,180,183,185]. Moreover, deacetylation of peptidoglycan enhances colonization and virulence in several pathogens, including *E. faecalis*, *L. monocytogenes* and *S. pneumoniae* [185–187]. As *N*-acetylglucosamine deacetylases are encoded within the genomes of most Gram-positive bacteria, these enzymes likely contribute to lysozyme and host colonization in many more species.

OatA (also known as Adr in *S. pneumoniae*) is another type of peptidoglycan modifying enzyme found in Gram-positive bacteria that confers resistance to lysozyme [188–190]. OatA performs *O*-acetylation at the C6-OH group of *N*-acetylmuramyl residues in peptidoglycan [188–190].

O-acetylation of *N*-acetylmuramyl residues is thought to prevent lysozyme from interacting with the β-1,4 linkages of peptidoglycan by steric hindrance [180]. OatA and orthologous proteins have been characterized in *Staphylococcus*, *Enterococcus*, *Lactococcus*, *Bacillus*, *Streptococcus* and *Listeria* species [51,52,54,58,180,187,191]. Like deacetylation mechanisms, *O*-acetylation of peptidoglycan is likely to be widespread among Firmicutes and has been noted to contribute to virulence in animal models of infection [52,54,187,190,192].

A peptidoglycan modifier unique to *Mycobacterium* is the enzyme NamH (N-acetylmuramic acid hydroxylase). NamH hydroxylates *N*-acetylmuramic acid residues leading to the production *N*-glycolylmuramic acid. The modification of peptidoglycan by NamH was determined to confer lysozyme resistance in *Mycobacterium smegmatis* [59]. It is likely that NamH confers lysozyme resistance to Mycobacterial species through the generation of *N*-glycolylmuramic acid, as NamH is well conserved in Mycobacterial genomes. It is hypothesized that *N*-glycolylmuramic acid residues may stabilize the cell wall; however, the mechanism of resistance is not fully understood [193]. However, recent work suggests that the presence of an *N*-glycolyl group blocks lysozyme from accessing the β-1,4 peptidoglycan bonds, preventing the muramidase activity of lysozyme and leaving the cell wall intact [59].

3.3. Alterations to Membrane Order

Apart from AMP repulsion and AMP target modifications as mechanisms of resistance, other changes in membrane composition can also reduce the susceptibility of bacteria to AMP-mediated killing. Alterations in Gram-positive membrane composition appear to contribute to AMP resistance by affecting the peptide interactions with the cell membrane. In particular, the degree of membrane fluidity appears to be an important determinant of AMP susceptibility.

One example of a membrane alteration that confers AMP resistance is the saturation of membrane fatty acids. Investigations into the cell membrane components of nisin-resistant *L. monocytogenes* showed that some resistant strains contained a higher proportion of saturated (straight chain) fatty acids versus unsaturated (branched chain) fatty acids [194,195]. Additionally, a nisin resistant strain of *L. monocytogenes* produced lower concentrations of the lipid head group phosphatidylglycerol and less diphosphatidylglycerol than a nisin-susceptible strain [194–196]. This nisin-resistant strain also contained higher concentrations of the lipid head group, phosphatidylethanolamine, while the anionic membrane component, cardiolipin, was decreased [197]. These studies suggest that higher concentrations of saturated fatty acids, a decrease in phophatidylglycerol and an increase in phophatidylethanolamine head groups in the *Listeria* membrane lead to a decrease in cell membrane fluidity [194–197]. It is proposed that the decrease in membrane fluidity increases nisin resistance by hindering nisin insertion into the membrane [197].

The addition of other membrane components can also increase rigidity and lead to resistance to host AMPs and daptomycin in *S. aureus* [198]. Increased membrane rigidity in some Gram-positive organisms can result from carotenoid overproduction [199,200]. Carotenoids are organic pigments made of repeating isoprene units that are produced by plants, bacteria, and fungi [201]. Carotenoids, such as staphyloxanthin made by *S. aureus*, can stabilize the leaflets of the cell membrane by increasing order in the fatty acid tails of membrane lipids and lead to decreased susceptibility to

AMPs [199,202,203]. This stabilization of fatty acid tails leads to an increase in cell membrane rigidity, which is suggested to limit insertion of AMPs into the membrane [204,205].

Though a higher concentration of saturated fatty acids in the membrane confers AMP resistance in some bacteria, other bacteria increase unsaturated fatty acid concentrations to increase resistance. In *S. aureus*, increased levels of unsaturated membrane lipids increase the resistance to the host AMP, tPMP (thrombin-induced platelet microbicidal proteins) [206]. Unsaturated fatty acids contain double bonds along the length of their carbon chain, which causes lipid disorder, thereby increasing membrane fluidity and impacting resistance to antimicrobials [206,207]. Other studies in AMP resistance found that methicillin-resistant *S.aureus* isolates that developed resistance to daptomycin also had increased resistance to host tPMPs and the human neutrophil peptide, hNP-1. These co-resistant strains have a phenotype defined by increased cell wall thickness and increased membrane fluidity [198]. It is hypothesized that these altered membrane arrangements may prevent efficient AMP insertion into the membrane [198,206,207].

At present, there is no clear explanation as to how alterations in membrane fluidity or rigidity lead to AMPs resistance. From the examples discussed above, it could be argued that the degree of fluidity required for resistance to a particular AMP may be as varied as the structures of the AMPs themselves, or perhaps is constrained to groups with similar mechanisms of action.

4. AMP Efflux Mechanisms

Transport, or efflux, is a common mechanism used by Gram-positive bacteria for the removal of toxic compounds and antimicrobials from cells. The majority of antimicrobial peptide efflux mechanisms consist of multi-protein ABC (ATP-binding cassette) transporter systems, which use ATP to drive the transport of substrates across or out of the cell membrane [208]. There are three primary types of ABC transporter systems implicated in Gram-positive AMP resistance: three-component ABC-transporters, two-component ABC-transporters, and single protein multi-drug resistance transporters, or MDR pumps [209]. All ABC-transporters are composed of two distinct domains: the transmembrane domain (permease) and the nucleotide-binding domain (NBD), which facilitates ATP-binding [209]. A less common efflux mechanism that has been identified is the Major Facilitator (MFS) Transporter module, which facilitates small solute transport via a chemiosmotic ion gradient [210]. This section will present the key types of AMP transporters found in Gram-positive bacteria and highlight the AMP resistance characteristics of these systems.

4.1. Three-Component (LanFEG) Transporter Systems

Three-component ABC transporters, or LanFEG systems, are best characterized in AMP-producing bacteria. LanFEG systems are members of the ABC-type 2 sub-family of transporters, and consist of one protein with a nucleotide-binding domain (LanF) and two distinct transmembrane permeases (LanE and LanG) [211]. The majority of the characterized LanFEG systems are self-immunity mechanisms that provide protection against bacteriocins (typically lantibiotics) made by bacteriocin producer strains [38,112] (Table 1). The LanFEG transporters are

often found in conjunction with LanI membrane-associated lipoproteins that can function in tandem with the transporter to provide greater resistance to AMPs [112,212,213].

The best-characterized LanFEG transporters are the NisFEG and SpaFEG systems found in strains of *L. lactis* and *B. subtilis* that produce the lantibiotic AMPs nisin and subtilin, respectively. Both NisFEG and SpaFEG provide resistance to their cognate substrates, but full resistance is achieved in concert with their associated substrate-binding lipoproteins, NisI and SpaI [100,213–215]. Immunity to the lantibiotic nukacin ISK from *Streptococcus warneri* does not involve a LanI protein, but instead contains a distinct membrane-associated protein termed NukH [96,216]. In contrast to the LanI proteins, NukH is not a lipoprotein; however, NukH does appear to function as a substrate-binding partner to the NukFEG transporter. Similar to LanI, NukH confers partial immunity to nukacin ISK, but full immunity requires the complete NukFEGH system [216,217].

Most characterized LanFEG systems confer resistance only to the AMP made by a producer strain, although examples have been identified that provide resistance to multiple AMP substrates in non-producer bacteria. In *Clostridium difficile*, the CprABC transporter (a LanFEG ortholog) confers resistance to nisin, gallidermin, and likely other structurally dissimilar lantibiotic peptides [85,86]. The regulation of immunity and AMP biosynthetic genes are typically coupled in bacteriocin producer strains [112]. The ability of the CprABC system to confer resistance to multiple unrelated peptides may result from the uncoupling of the immunity mechanism from bacteriocin synthesis. But non-producers that have immunity genes in the absence of AMP biosynthetic operons can have relaxed substrate specificity that allows for recognition of multiple bacteriocins. Thus, Lan transporter cross-immunity to multiple AMPs could provide a significant competitive advantage to non-producer bacteria. Indeed, a homology search for LanFEG proteins reveals that the genomes of many other Firmicutes encode predicted bacteriocin transporters that are not coupled with apparent bacteriocin synthesis genes. Hence, like other antibacterial resistance mechanisms, the LanFEG systems have found their way into non-producing species [85,86].

4.2. Two-Component ABC-Transporter Systems

Two-component ABC-transporters make up the majority of transporter-mediated AMP resistance characterized in non-AMP producing bacteria. The canonical two-component ABC-transporter consists of one nucleotide-binding protein and a separate membrane-spanning permease [218,219]. Unlike most LanFEG systems, two-component transporters often provide resistance to multiple AMPs and are common among Gram-positive bacteria. As outlined in Table 1, numerous examples of these transporters have been identified that can provide resistance to AMPs produced by humans and bacteria, including cyclic peptides and some non-peptide antibiotics [218,220].

There are two main types of two-component ABC-transporter systems that confer resistance to AMPs among Gram-positive bacteria. The first and most common type is often referred to as the BceAB group [218,221]. BceAB transporter systems contain an archetypal ATP-binding protein of about 225–300 amino acids and a larger permease component that ranges in size from 620–670 amino acids. The prototype of this transporter group, BceAB, was first identified as a bacitracin resistance mechanism in *B. subtilis* [67,68]. Since the identification of BceAB, dozens of similar transporters have been discovered in pathogenic and non-pathogenic Gram-positive species, including *S. aureus*,

L. monocytogenes, *S. pneumoniae*, and *L. lactis* (see Table 1 for examples) [62,71,77,80]. Members of the BceAB group have demonstrated resistance to a wide-range of bacteriocins, mammalian and fungal defensins, peptide antibiotics, and other antimicrobial compounds (Table 1). Although many of the BceAB transporters confer resistance to AMPs *in vitro*, the roles of these transporters in the virulence of pathogenic species are not known.

Another common type of a Gram-positive ABC-transporter that confers AMP resistance is the BcrAB(C) system. The BcrAB(C) transporter confers resistance to bacitracin and was originally identified in a bacitracin producer strain of *Bacillus licheniformis* [81]. BcrAB transporters can be distinguished from the BceAB systems by size and topology: BcrA is an ATP-binding cassette that ranges from about 280–320 amino acids, while the BcrB permease modules are smaller, at approximately 200–250 amino acids. BcrAB is often encoded with a third protein, BcrC (or BcrD), which allows for higher resistance to bacitracin than the BcrAB transporter alone [81,222,223]. Initially it was hypothesized that BcrC functioned as part of the BcrAB ABC-transporter, however it was later demonstrated that BcrC acts as an undecaprenyl pyrophosphate (UPP) phosphatase that competes with bacitracin for UPP [222]. The BcrAB transporters are predicted to be structurally similar to the LanFEG transporters, though the Lan systems function through two dissimilar permease components, while Bcr systems operate with only one permease subunit (BcrB) [38,82,218]. Aside from the bacitracin producer strains, BcrAB and orthologous transporters have been shown to confer resistance to bacitracin in many strains of *E. faecalis*, as well as some *Streptococcus* and *Clostridium* species [35,82,83,224].

4.3. Single Membrane Protein Antimicrobial Transporters

Multi-drug resistance (MDR) ABC-transporters are common bacterial mechanisms of resistance to peptide and non-peptide antibiotics [225]. Though these transporters are most common among characterized mechanisms for non-peptide antimicrobial resistance in Gram-positive bacteria, there are examples of MDR transporters that confer resistance to AMPs. One notable MDR AMP resistance mechanism consists of the LmrA/B proteins encoded by some *L. lactis* strains [60,226]. A LmrA MDR efflux pump was first described in a non-producer strain of *L. lactis* [226]. LmrB is an ortholog of LmrA found in *L. lactis* strains that produce the bacteriocins LsbA and LsbB [60]. LmrA/LmrB are membrane proteins with six predicted transmembrane segments and a *C*-terminal, nucleotide-binding domain [60]. LmrA provides broad resistance against a long list of peptide antibiotics and cytotoxic compounds, while LmrB confers resistance to the two bacteriocins LsbA and LsbB [60,226]. A BLASTp homology search revealed the presence of additional orthologs of LmrA/B encoded within the genomes of hundreds of Gram-positive Firmicutes, though the function and significance of these remains unknown.

A less common type of single-protein transporter involved in antimicrobial peptide resistance is exemplified by the QacA transporter of *S. aureus* [61]. QacA is a member of the major facilitator superfamily (MFS) of membrane transport proteins, which use proton motive force, rather than ATP, to drive the efflux of substrates [227]. QacA confers resistance to a variety of toxic dyes, antiseptics and disinfectants [228,229]. In addition to cationic toxins, QacA provides resistance to thrombin-induced platelet microbicidal protein (tPMP), a host-derived antimicrobial peptide [61].

QacA-dependent tPMP resistance was found to confer a survival advantage in an animal model of infection, and increased resistance to tPMP in *S. aureus* also correlates with endocarditis in humans [61,230]. QacA orthologs have also been identified in other staphylococci, as well as in *Enterococcus* and *Bacillus* species, though the ability of these orthologs to transport AMPs is not understood [231,232].

5. Conclusions

Antimicrobial peptides are diverse in both structure and function and are produced by all forms of life. As such, AMPs are an ancient defense mechanism, and resistance mechanisms to AMPs have been selected for as long as AMPs have existed. Gram-positive bacteria are ancient producers of AMPs and as a consequence, these organisms likely developed some of the first AMP resistance mechanisms.

Herein we have detailed a wide variety of AMP resistance mechanisms found in Gram-positive bacteria (summarized in Figure 1). AMPs resistance mechanisms can be broad spectrum, such as MprF and the Dlt pathway which function by decreasing the net negative charge of the bacterial cell surface, thereby reducing the attraction for positively charged AMPs from the cell. Conversely, AMP resistance mechanisms can be highly specific and only confer resistance to a single peptide. AMP resistance mechanisms can be confined to a particular species or genus, such as NamH in *Mycobacterium*, or can be distributed among multiple species, such as the LanFEG systems. AMPs resistance mechanisms are dynamic; they can be passed from species to species via bacteriophages or horizontal gene transfer, and can change specificity and function over time through evolution [85,86,105,233]. Under selective pressure, AMP resistance mechanisms can evolve to suit the needs of a particular species in its own niche [234].

At present, many AMPs are being investigated as potential antimicrobial therapies [235–240]. AMP drug development should be carefully vetted because like any naturally-produced antimicrobial, cognate resistance mechanisms for AMPs are already present in the producer bacterium. While these resistance mechanisms may be found more frequently in producer strains, each has the propensity to be passed on to other genera or species within a shared environmental niche. Because the presence of AMPs provides high selective pressure for the acquisition of resistance, it is important to consider the potential for resistance mechanism transfer between bacteria when developing AMPs for clinical use [241,242]. Additionally, depending on the AMP resistance mechanism that is selected for, a multitude of issues may arise if the mechanism of resistance is broad-spectrum. A broad-spectrum AMP resistance mechanism could restrict the already limited clinical treatment options for use against some Gram-positive pathogens and may undermine our own immune response by conferring resistance to our own innate immune system peptides [243].

Antimicrobial peptide resistance is not as well characterized for Gram-positive bacteria as it is for Gram-negative bacteria. Thus, it is likely that many more mechanisms of antimicrobial resistance remain to be discovered in Gram-positive species. As more AMPs are found, new Gram-positive AMP resistance mechanisms will undoubtedly be revealed.

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Author Contributions

K.L.N., E.K.C. and S.M.M. wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Resistance to Antimicrobial Peptides in Vibrios

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Abstract: Vibrios are associated with a broad diversity of hosts that produce antimicrobial peptides (AMPs) as part of their defense against microbial infections. In particular, vibrios colonize epithelia, which function as protective barriers and express AMPs as a first line of chemical defense against pathogens. Recent studies have shown they can also colonize phagocytes, key components of the animal immune system. Phagocytes infiltrate infected tissues and use AMPs to kill the phagocytosed microorganisms intracellularly, or deliver their antimicrobial content extracellularly to circumvent tissue infection. We review here the mechanisms by which vibrios have evolved the capacity to evade or resist the potent antimicrobial defenses of the immune cells or tissues they colonize. Among their strategies to resist killing by AMPs, primarily vibrios use membrane remodeling mechanisms. In particular, some highly resistant strains substitute hexaacylated Lipid A with a diglycine residue to reduce their negative surface charge, thereby lowering their electrostatic interactions with cationic AMPs. As a response to envelope stress, which can be induced by membrane-active agents including AMPs, vibrios also release outer membrane vesicles to create a protective membranous shield that traps extracellular AMPs and prevents interaction of the peptides with their own membranes. Finally, once AMPs have breached the bacterial membrane barriers, vibrios use RND efflux pumps, similar to those of other species, to transport AMPs out of their cytoplasmic space.

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1. Introduction

Vibrios are γ-proteo-bacteria ubiquitous in aquatic environments. They have evolved the capacity to colonize a broad series of hosts from protozoans to metazoans. Vibrios are normally present in the tissues of healthy animals. Sometimes they become pathogenic in wild marine animals such as corals, in particular as a result of environmental changes including shifts in seawater temperature and salinity, or, for aquacultured animals, upon exposure to high animal densities or stressful farming practices [1]. Currently, vibrioses are recognized as a major factor limiting the development of aquaculture. In addition, vibrios can cause severe disease outbreaks in human populations, the best known example being cholera. Again, environmental drivers—such as temperature changes, severe rainfalls that lower water salinity, and insufficient sanitation—govern the occurrence of the disease in human populations [2].

Vibrios have developed tropism for epithelial tissues that line both the outside and inside of cavities and lumen of their diverse hosts. They can colonize the keratinized epithelium of skin as well as the gastrointestinal tract. By lining the cavities and surfaces of structures throughout the

body, epithelia act as a first line of defense against pathogens. Epithelia also produce antimicrobial peptides (AMPs) [3,4], conferring to the host an immune arsenal broadly conserved among metazoans. When the host's epithelial barriers are breached, vibrios encounter phagocytes, key components of the animal immune system. These phagocytes infiltrate infected tissues and use reactive oxygen and nitrogen species (ROS and RNS) as well as AMPs to kill phagocytosed microorganisms intracellularly or deliver their antimicrobial content extracellularly to circumvent infection. Interestingly, recent works have shown that vibrios are able to colonize and survive inside phagocytes [5,6].

AMPs from metazoans are often cationic peptides that initially interact electrostatically with the membranes of bacteria, which carry negatively-charged lipopolysaccharide (Gram-negative) or teichoic acids (Gram-positive). Many AMPs then insert into the membranes and form deleterious pores or channels [7]. Alternatively, AMPs can bind to essential components of bacterial membranes or translocate across to reach the cytoplasm, where they interfere with essential cellular processes such as nucleic acid, protein, enzyme, and cell wall syntheses [8–13]. In addition, AMPs produced by a given host can be synergistic, combining their mechanisms of action to fight bacterial pathogens [14]. However, it has become clear that the activity of AMPs goes far beyond their antimicrobial properties; these peptides are also involved in many immunomodulatory functions including inflammation, wound healing, chemotaxis, cell differentiation, angiogenesis, regulation of oxidative stress, regulation of adaptive immunity, and epithelia homeostasis [4,15]. Accordingly, AMPs are also called Host Defence Peptides.

Importantly, the tissues of healthy metazoans host an abundant microbiota, which itself has the capacity to produce AMPs, contributing to protection against pathogenic microbes. Prokaryotic AMPs are frequently referred to as bacteriocins, a generic name that covers classes of compounds with diverse structures and mechanisms of action. Bacteriocins may be peptides created by complex biosynthetic pathways that enable the inclusion of unconventional amino acids, as well as nucleotides and siderophores [16,17]. Many of these prokaryotic AMPs are cationic, although this is not a general rule. Like metazoan AMPs, some target the bacterial membranes while others target specific receptors and behave as inhibitors of key metabolic pathways. Still others combine different mechanisms of action (for review see [17]).

When confronted with such a complex immune arsenal, how do vibrios avoid the chemical defenses of their hosts and associated microbiota? What can we learn from their ability to colonize immune cells/tissues that produce high local concentrations of AMPs? In the context of the extensive antibiotic use that has led to emergence of broad-spectrum antibiotic-resistant bacteria [18], AMPs are often seen as an alternative to conventional antibiotics. However, an increasing number of studies have shown the diversity of mechanisms by which bacteria can also avoid the action of AMPs. Thus, the emergence of "superbugs" resistant to both antibiotics and AMPs is a potential risk of using AMPs as an antibiotic alternative. However, understanding the mechanism by which bacteria have evolved the capacity to live in AMP-producing tissues should allow us to develop strategies to prevent AMP-resistance.

2. Antimicrobial Peptides in Host-Vibrio Interactions

2.1. Vibrios Colonizing Epithelial Surfaces

Many species of vibrios pathogenic for human and animal species have evolved the capacity to colonize epithelia (Table 1). Among these, the species of vibrios pathogenic for humans, *Vibrio cholerae* and *Vibrio parahaemolyticus,* cause major enteric disorders. While *V. parahaemolyticus* disrupts the intestinal epithelium [19], *V. cholerae* induces inflammatory responses and innate immune cell infiltration in the small intestine without affecting the integrity of the mucosal tissue [20]. Diarrhea caused by such enteric infections leads to intense dehydration and is recognized as a major factor in morbidity and mortality worldwide. Virulence factors of the diarrheagenic vibrios are expressed upon intimate association with host epithelial cells and, in many instances, include the secretion of toxins. Vibrios causing gastrointestinal infection need to penetrate the mucous layer before attaching to intestinal epithelial cells, a process usually mediated by fimbriae or pilus structures (e.g., toxin-co-regulated pilus (TCP)). Subsequently, the bacteria secrete important virulence factors such as cholera toxin (CT) and hemagglutinin/protease (HA/protease) (for review see [21]). In *V. parahaemolyticus*, colonization of the intestine is dependent on the type III secretion system (T3SS2) [22] and further secretion of a T3SS2-secreted effector, VopZ, which also inhibits host mucosal defenses [23].

As in humans, many vibrios colonize the epithelial surfaces of animals, both vertebrates and invertebrates. Again, this often requires a first step of binding to the mucus covering the epithelium. In some cases, epithelium colonization is part of a mutualistic process. For instance, in the squid, the luminescent *Vibrio fischeri* colonizes the crypts of the squid light organ, which consists of a series of deep invaginated epithelium-lined crypt spaces [24]. In other cases, invasion of the epithelium is part of the pathogenic process. For instance, in the rainbow trout, *Vibrio anguillarum* colonizes both the skin and the intestinal epithelia, causing a fatal hemorrhagic septicaemia [25]. Similarly, in the coral *Oculina Patagonica*, the pathogenic *Vibrio shiloi* penetrates into the epithelial cells of the coral, multiplies, and produces a toxin that inhibits photosynthesis of the coral symbiotic algae (for review see [26]).

Table 1. Vibrios colonizing epithelia.

Species or Strain	Host	Tissues	References
V. cholerae	human	intestine	[20]
V. vulnificus	human	skin, wounds	[27]
V. parahemolyticus	human	intestine	[19]
V. anguillarum	fish	skin, intestine	[25]
V. shiloi	coral	oral ectoderm	[26]
V. coralliilyticus	coral	oral ectoderm	[28]
V. fisheri	squid	light organ	[24]

2.2. AMPs and Epithelial Defenses

Mammalian epithelial tissues such as the epidermis but also the respiratory, gastrointestinal and genitourinary tracts are in direct contact with the environment, thus, constant interaction between microorganisms and the immune system occurs at these sites. In vertebrates, epithelial tissues provide the first line of protection as they trigger the immune response. Mammalian epithelial cells function as both a physical barrier and as immune active cells, producing a number of immune-related molecules [29]. Therefore, colonizing vibrios face a diversity of chemical weapons expressed in epithelial tissues. Indeed, in animals, virtually all epithelia have been found to express AMPs either constitutively or in response to damage and/or infection (Table 2).

In humans, AMPs are expressed in a broad range of epithelial cell types, either constitutively or in response to infection. The major AMPs and proteins of human epithelia include the small cationic α- and β-defensins, the human cathelicidin LL-37 (hCAP-18) and the bactericidal/ permeability-increasing protein (BPI). The average concentration of defensins in these epithelial cells reaches the 10–100 µg/mL range with higher local concentrations due to the uneven distribution of defensins [3]. BPI is expressed in mucosal epithelia including the esophagus and the colon [30]. LL-37 is expressed in the squamous epithelia of the airways, mouth, tongue, esophagus and large intestine [31–33] as well as in inflamed skin [34]. Human β-defensins are expressed by kidney, skin, pancreas, gingiva, tongue, esophagus, salivary gland, cornea, and airway epithelium [35]. In the small intestine, the antimicrobial C-type lectins HIP/PAP are expressed [4,36] together with enteric α-defensins, which are major AMPs exclusively expressed by Paneth cells located at the bottom of the intestinal crypts [37]. Importantly, the epithelial lining of the small intestine is the site at which *V. cholerae* adheres after passing through the gastric acid barrier and penetrating the mucin layer of the small intestine [38].

The human enteric α-defensins HD-5 and HD-6 are components of the secretory granules of Paneth cells. They are released in the lumen of the small intestinal crypts upon exposure to bacteria and bacterial antigens. Their contribution to enteric mucosal immunity has been clearly evidenced in transgenic mice expressing the human Paneth cell α-defensin, HD-5 [39]. While HD-5 has direct antimicrobial activity against bacteria, HD-6 acts by creating nanonets that entrap bacteria and prevent further dissemination [40]. Paneth cells of mice also secrete their own α-defensins into the lumen of small intestinal crypts, and local concentrations have been estimated to be 25–100 mg/mL at the point of release [37]. Paneth cells were also shown to express LPLUNC1 which co-localizes with HD-5 in the secretory granules. LPLUNC1 is a protein similar to BPI which does not display antimicrobial activity *in vitro* but binds lipopolysaccharide (LPS) and inhibits the TLR4-signaling pathway in response to *V. cholerae* O1 LPS. LPLUNC1 mRNA is also the most highly up-regulated transcript in the small intestine during acute phase cholera [41].

In fish, epithelial defenses include a series of AMPs whose expression varies according to peptide families, fish species and tissues (for recent reviews see [42,43]). Indeed, fish AMPs are abundant in mucosal linings such as the skin, gills, and intestine, suggesting an important role in immunity [44]. These include AMPs similar to those found in mammals, namely β-defensins, cathelicidins, hepcidins and histone-derived AMPs together with α-helical peptides AMPs (pleurocidin,

piscidins, and moronecidin, among others). Fish β-defensin genes have the highest basal expression in skin epithelium, which is induced by a variety of bacterial challenges such as *Aeromonas hydrophila* and *Vibrio anguillarum*. Interestingly, tissue-specific production of β-defensins has been described in salmonids where variants of this family can be differentially up-regulated in the intestine or gill tissues following bacterial challenge [45]. Hepcidin, which is both an AMP and a hormone expressed in liver, is also expressed by the skin epithelium and intestine. Fish hepcidin genes can be induced by exposure to both Gram-positive and Gram-negative bacteria. Cathelicidin is expressed in diverse epithelia including skin, gill and intestine. In the Atlantic cod, its expression in the gills was induced by *Aeromonas salmonicida* but not by *V. anguillarum* [46]. Like β-defensins, salmonid cathelicidins are produced in several mucosal tissues where variants display differential expression [47]. Moreover, transcripts of a homologue of the human bactericidal/permeability-increasing protein (BPI) have been found in the skin, intestine and gills of various fish species [48,49]. Finally, histone-derived AMPs are released in the epithelial mucosal layer of wounded fish skin [50]; they are expressed by mucus-producing globlet cells, the cells in which all the AMPs from fish skin accumulate [43].

In marine invertebrates, AMPs are also expressed by a broad range of epithelial cell types. Homologues of human BPI are produced by diverse invertebrate species. In the squid *Euprymna scolopes*, *Es*-LBP1 was found in the light organ of juvenile squids colonized by *V. fischeri*, but not in aposymbiotic squids. Expression was localized within the deep crypt spaces where the symbiotic vibrios reside and along the surface of the epithelia [51]. In the oyster *Crassostrea gigas*, a homologue of human BPI, *Cg*-BPI, is produced by various epithelial cell types including the intestine, gills, and mantle [52]. In addition, the *Cg*-Defm defensin is expressed by the oyster mantle, the shell-forming secretory epithelium [53]. Expression of *Cg*-BPI and *Cg*-Defm was constitutive in the epithelia of oysters infected with vibrios [14]. Recently, a novel AMP rich in lysine residues was extracted from oyster gills. This AMP called *Cg*-Molluscidin is predicted to form a α-helix [54]; its regulation in response to infection is still unknown. Moreover, as in fish, oyster epithelia accumulate histones displaying antimicrobial activity against vibrios [55]. These antimicrobial histones are released in response to infection or injury by infiltrating hemocytes, the circulating immune cells of the oyster, by a mechanism reminiscent of neutrophil extracellular traps in vertebrates [56].

2.3. Vibrios Adapted to Intracellular Life in Phagocytes

Vibrios have traditionally been considered extracellular organisms. In recent years, however, vibrios (*V. cholerae* and *V. mimicus*) have been shown to also adopt intracellular stages in phagocytes from the environment, the amoebae [75–77] (Table 3). Similarly, live vibrios have been found inside professional phagocytes within the hosts they colonize. In vertebrates, *V. cholerae* can survive inside human macrophages; this intracellular stage is required for the T6SS-mediated secretion of factors causing actin cross-linking in host cells [5]. In invertebrates, a *V. splendidus*-related strain referred to as *V. tasmaniensis* LGP32 can survive in hemocytes, the circulating immune cells of the oyster (Table 3). Hemocyte invasion was accompanied by reduced production of reactive oxygen species and altered phagosome mutation [6]. While vibrios

pathogenic for fish can adopt intracellular stages in epithelial cells [78,79], to our knowledge they have not been reported to invade professional phagocytes.

Table 2. Antimicrobial peptides (AMPs) expressed in epithelial tissues.

Species	AMP Family	Examples	Epithelial Tissues	References
Human	α-defensins	HD-5, HD-6	Small intestine, female genital tract	[37,57]
	β-defensins	hBD-1/-2/-3	Respiratory tract, large intestine, urogenital epithelium, oral cavity, skin	[58–62]
	Cathelicidins	LL-37(hCAP-18)	Skin, gastrointestinal tract, epididymis, lungs, oral cavity, ocular surface	[31,63,64], for review see [65]
	Bactericidal-permeability increasing proteins	BPI	Esophagus, respiratory tract, large intestine	For review see [66]
	C-type lectins	HIP/PAP	Small intestine	[36]
Fish	β-defensins	omDB-1/-2/-3/-4	Skin, gills, intestine	[45,67]
	Cathelicidins	rtCATH_1/-2A-2B, asCATH-1/-2	Skin, gills, intestine	[47,68]
	Liver-expressed antimicrobial peptides (LEAPs)	HFIAP-1/-2/-3 Hepcidin (LEAP-1), LEAP-2 Sal-1 Sal-2	Skin, intestine	[69], for review see [44]
	α-helical peptides	Pleurocidin, Piscidins Chrysophsins Moronecidin	Skin, gills	[70–72]
	Bactericidal-permeability increasing proteins	BPI	Intestine, gills	[48,49]
	Histone-derived AMPs	Parasin-1 Hipposin Oncorhyncin	Skin mucus	[73,74] [44,50]
Squid	LPS-binding/ Bactericidal-permeability increasing proteins	*Es*-LBP1	Light organ	[51]
Oyster	CS-αβ defensins	*Cg*-Defm	Mantle tissue	[53]
	Bactericidal-permeability increasing proteins	*Cg*-BPI	Gills, mantle, labial palps, gastrointestinal tract	[52]
	Histone-derived AMPs	*cv*H2B-1/-2/-3/-4	Gills	[55]
Coral	Cysteine Rich peptides	Damicornin Mytimacin-like	Oral ectoderm	[28]
	LPS-binding/Bactericidal-permeability increasing proteins	LBP–BPI	Oral ectoderm	[28]

Table 3. Vibrios colonizing phagocytes.

Species or Strain	Host Cells	References
V. cholerae O1, O139	amoebae	[75,77]
V. cholerae	human macrophages	[5]
V. mimicus	amoebae	[76]
V. tasmaniensis LGP32	oyster hemocytes	[6]

2.4. AMPs of Phagocytes

Intracellular vibrios must face the potent chemical defenses of phagocytes, professional immune cells that circulate in the animal bloodstream and infiltrate infected tissues. Phagocyte defences include reactive oxygen species, which are particularly active during phagocytosis; hydrolytic enzymes including lysozyme; as well as AMPs, which are produced and stored by phagocytic cells (Table 4).

Table 4. AMPs expressed in phagocytes.

Species	AMP	Examples	Phagocytes	References
Human	α-defensins	HNP-1/-2/-3/-4	Neutrophils	[80]
	β-defensins	hBD-1/-2	Macrophages, Dendritic cells	[81,82]
	Cathelicidins	LL-37	Neutrophils	[89,90]
	Liver-expressed antimicrobial peptides (LEAPs)	Hepcidin	Granulocytes Macrophages	[91,92]
	Bactericidal-permeability increasing proteins	BPI	Neutrophils, (Eosinophils/to a lesser extent)	[30,93,94]
Fish	α-helical peptides	Piscidins	Granulocytes	[85]
	LPS-Binding/Bactericidal-permeability increasing proteins	LBP/BPI	Head–kidney leukocytes	[49]
Oyster	CS-αβ defensins	*Cg*-Defh-1/h2	Hemocytes	[1]
	Big-defensins	*Cg*-big-defensin-1/-2/-3	Hemocytes	[87]
	Proline-rich peptides	*Cg*-Prp	Hemocytes	[14]
	Bactericidal-permeability increasing protein	*Cg*-BPI	Hemocytes	[14,52]
	Histone-derived AMPs	H1- and H5-like histones	Hemocytes	[56]

Human phagocytes (neutrophils and macrophages) are indeed known to express a broad diversity of AMPs. Neutrophils express α-defensins, stored in azurophil granules that fuse with the phagolysosome to kill internalized bacteria, and the LL-37 cathelicidin, stored in secretory granules which release their content extracellularly. α-defensin expression is constitutive and their release is regulated by diverse microbial signals. In neutrophil phagolysosomes, the concentration of defensins has been estimated at ~10 mg/mL [80]. In addition, human neutrophils express the BPI antimicrobial protein [30]. In human macrophages, where *V. cholerae* is able to survive, AMPs such as LL-37,

hepcidin and human β-defensin 1 and 2 can control intracellular pathogens [81,82]. Indeed, the crucial role of LL-37 in intracellular killing of mycobacteria has been extensively documented [83,84].

In fish, less information is available on AMPs expressed by phagocytes. AMPs of granulocytes include the α-helical peptide piscidin [85] and hepcidin in the seabream [86]. The BPI/LBP protein is constitutively expressed in head kidney leukocytes from Atlantic cod [49]. However, attention must be paid to the potential infiltration of phagocytes in tissues when AMPs expression is analyzed. Therefore, further studies are needed to determine whether the AMP expression in fish is restricted to a specific cell type or tissue. It is also not known whether fish phagocytes serve as a niche for any given *Vibrio* species.

Hemocytes of invertebrates also produce a large array of AMPs. Upon infection, oyster hemocytes massively migrate to infected tissues, bringing their antimicrobial content to the site of infection, and actively phagocytose bacteria. In oyster hemocytes, where the *V. tasmaniensis* strain LGP32 was found to survive, AMPs include defensins, big-defensins, proline-rich peptides, as well as a BPI antimicrobial protein (for review see [1]). BPI is stored in large cytoplasmic granules while the intracellular localization of the other AMPs is not yet known. Expression of BPI and big-defensin 1 and 2 is induced in hemocytes of infected oysters, whereas defensin expression is not regulated by the infection [14,87].

Similar to phagocytes from metazoans, amoebae, which can host diverse *Vibrio* species, produce pore-forming polypeptides such as the well-known amoebapores. These peptides are stored in cytoplasmic granules and can rapidly perforate human and bacterial cells. Amoebapores combat the growth of phagocytosed bacteria by permeabilizing their membranes inside the digestive vacuoles [88].

3. Known Mechanisms of Resistance/Evasion to AMPs in Vibrios

3.1. Outer Membrane Remodeling

As electrostatic interactions often play a crucial role in the initial interaction of cationic AMPs with bacterial membranes, both Gram-negative and Gram-positive bacteria have evolved strategies to neutralize the net negative charge of cell surface molecules with amine-containing substitutions. Thus, D-alanylation of teichoic acids, which are major components of the Gram-positive cell wall, confers AMP-resistance in a variety of Gram-positive bacteria including *Staphylococcus aureus* and *Bacillus cereus* [95,96]. More generally, aminoacylation of bacterial cell surface phosphatidylglycerols with L-lysine, L-alanine, or D-alanine confers resistance to cationic AMPs in both Gram-positive and Gram-negative bacteria [97].

LPS, the major constituent in the Gram-negative outer membrane, is composed of three regions: the anionic Lipid A membrane anchor, the core oligosaccharide and the *O*-antigen polysaccharide [98]. Hankins *et al.* have shown that *V. cholerae* O1 and O139 share identical asymmetrical hexa-acylated Lipid A structures [99] composed of a β 1'-6 linked glucosamine disaccharide with unmodified 1- and 4'-phosphate groups, which is acylated at the 2-, 3-, 2'- and 3'-positions. Myristate (C14:0) and 3-hydroxylaurate (3-OH C12:0) are ester-linked to the hydroxyl groups on

the 2'- and 3'-linked fatty acyl chains (Figure 1). As in *V. cholerae*, the presence of a hydroxylated secondary acyl chain has been reported in the Lipid A structure of *V. fischeri* [100].

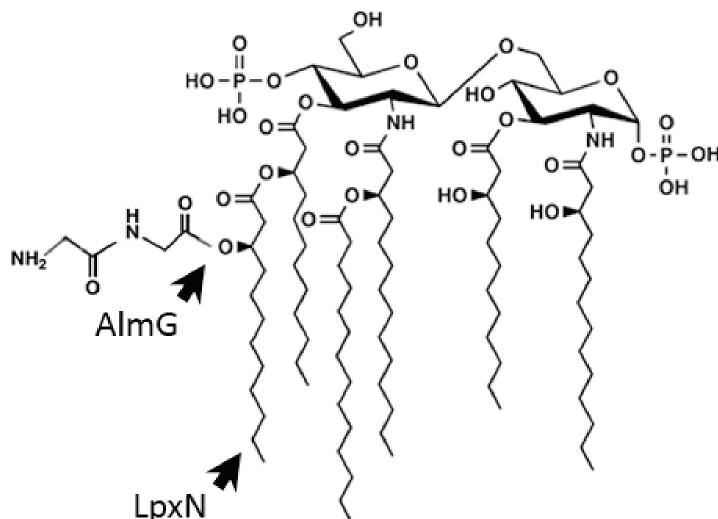

Figure 1. Structure of modified lipid A from *V. cholerae* O1 El Tor. The structure of *V. cholerae* lipid A was established by Hankins *et al.* (2011) [99]. It is composed of a β 1′-6 linked glucosamine disaccharide with unmodified 1- and 4′-phosphate groups, which is acylated at the 2-, 3-, 2′- and 3′-positions. Myristate (C14:0) and 3-hydroxylaurate (3-OH C12:0) are ester-linked to the hydroxyl groups on the 2′- and 3′-linked fatty acyl chains. The 3-hydroxylaurate secondary acyl chain transferred by the LpxN acyltransferase is required for AMP resistance. Similarly, the di-Glycine residues transferred by the AlmG to the hexa-acylated lipid A of *V. cholerae* O1 El Tor strains are crucial for AMP-resistance [101].

Polymyxin B (PmB) has been extensively used to study the molecular basis of bacterial resistance to cationic AMPs in Gram-negative bacteria. Indeed, this peptide produced by the Gram-positive *Paenibacillus polymyxa* disrupts the cell envelope of Gram-negative bacteria by associating with the anionic LPS as well as with acidic glycerophospholipids [102]. To resist to AMPs, Gram-negative bacteria can neutralize their cell membrane by transferring phosphoethanolamine or aminoarabinose to phosphate groups on the lipid A domain of LPS [103].

In *V. cholerae*, the secondary acyltransferase VC0212 (LpxN or MsbB), which transfers a 3-hydroxylaurate group to penta-acylated Lipid A, contributes to the resistance of an El Tor strain to AMPs including PmB and LL-37 [99,104]. Thus, the higher susceptibility of the *vc0212* mutant displaying incomplete Lipid A might be due to the greater permeability of its bacterial membrane. However, recent data by Hankins *et al*. demonstrated that the presence of a 3-hydroxyl group on the secondary acyl chain provides a site for esterification of glycine residues in a unique strategy necessary for resistance to PmB in *V. cholerae* [101] (Figure 1).

Three *V. cholerae* proteins, VC1577 (AlmG), VC1578 (AlmF), and VC1579 (AlmE) sharing sequence homology with the machinery involved in D-alanylation of teichoic acids in Gram-positive bacteria are essential for Lipid A modification with glycine and diglycine residues through aminoacyl esterification (Figure 1). Interestingly, sequence alignments comparing the classical (susceptible to PmB) and the El Tor (resistant to PmB) biotypes of *V. cholerae* revealed that the classical strain O395 has a nonsense mutation, resulting in a truncated AlmF carrier protein lacking the conserved serine [101]. The authors discovered that classical strains lack glycine-modified Lipid A. Upon *alm* mutation, the minimum inhibitory concentration (MIC) of PmB against El Tor strains dropped dramatically (~100 times) from 96–128 μg/mL to 0.5–1.0 μg/mL, showing that glycine modification of Lipid A is an essential mechanism of AMP resistance in *V. cholerae*. This study provides a well-defined mechanism for the different PmB-resistant phenotypes observed in *V. cholerae* classical and El Tor biotypes. Why classical strains appear to have lost carrier protein functionality and thus AMP resistance is a puzzling evolutionary question.

To date, it is unknown whether modifications of *Vibrio* LPS are induced upon exposure to sublethal concentrations of cationic AMPs, as shown in other bacterial species like Salmonella Typhimurium, which regulate their LPS structure, contributing to resistance to cationic AMP [105]. Changes in *Salmonella* LPS structure, regulated by the two-component system PhoPQ, include reducing average O-antigen chain-length, acylating, deacylating, and hydroxylating lipid A, derivatizing lipid A and LPS core phosphates with cationic groups (for recent review see [106]). Homologues of PhoPQ are found in *Vibrio* species, however, the potential role of PhoPQ in resistance to AMPs has not been described to date.

3.2. Induction of the Envelope Stress Response

As discussed above, many AMPs create damage to bacterial membranes as part of their mechanism of action. Sensing external stress is therefore crucial to combating membrane injury before the damage becomes irreversible. One of the strategies by which bacteria respond to outer membrane stress and modulate gene expression is via the alternate σE factor, encoded by the *rpoE* gene. Under non-stress conditions, σE is inactivated by its cognate anti-sigma factor localized to the inner membrane. When activated by envelope stress, *i.e.*, misfolding of outer membrane proteins, σE promotes the expression of factors that help preserve and/or restore cell envelope integrity. Certain outer membrane proteins can serve as upstream signal sensors to modulate the activity of σE [107]. In *V. cholerae,* the major outer membrane OmpU is a key determinant of σE production [108]. Such dependence on a single factor contrasts with the regulation of σE in *E. coli*, in which numerous factors contribute to its activation and none is dominant.

In *V. cholerae*, σE plays a role in outer membrane stress response and resistance to AMPs. Thus, deficiency of σE confers to *V. cholerae* greater sensitivity to the antimicrobial peptide P2, a synthetic derivative of human BPI. Consistent with the *ompU*-dependent activation of σE, lack of OmpU in *V. cholerae* also conferred a greater sensitivity to AMPs [109,110]. Similar results were obtained for the oyster pathogen *V. tasmaniensis* LGP32 in which OmpU contributed to resistance to the oyster antimicrobials *Cg*-Defm and *Cg*-BPI [111]. However, in both *V. cholerae* and *V. tasmaniensis*, OmpU-mediated resistance was much lower than that conferred by Lipid A

remodeling [101]. Moreover, in *V. tasmaniensis* LGP32, the major negative effect of the *ompU* deletion on pathogenicity was attributed to impaired capacity to invade the oyster immune cells rather than lower resistance to oyster AMPs [6].

3.3. AMP Titration by Outer Membrane Vesicles

One σE-dependent mechanism whose role in AMP resistance has been less studied is outer membrane vesicle release. OMVs form the insoluble fraction of Gram-negative bacteria extracellular products; they are extruded from the bacterial cell surface and entrap some of the underlying periplasmic contents [112,113]. OMVs are key players in the interaction between Gram-negative bacteria and both the prokaryotic and eukaryotic cells from their environment [114]. Whereas it is now well established that *Vibrio* spp. constitutively release OMVs during cell growth [115–117], only recent studies in *E. coli* [118] and *Vibrio* spp. [119,120] have shown that the release of OMVs protects bacteria against membrane-active AMPs.

In *V. cholerae*, earlier work demonstrated that under envelope stress conditions, the small regulatory RNA VrrA is expressed in a σE-dependent manner to down-regulate OmpA, which in turn reduces envelope stress by promoting OMV release [121]. More recently, we found that physiologically relevant amounts of OMVs produced in the presence of a sub-lethal concentration of PmB provide protection against human cathelicidin LL-37, increasing the MIC of LL-37 by four-fold. This cross-protection has been attributed to the presence of the biofilm-associated extracellular matrix protein Bap1, which is associated with OMVs in larger amounts when bacteria are grown in the presence of PmB. The Bap1 protein can therefore trap LL-37, leading to increased resistance of *V. cholerae* towards LL-37 [119].

In *V. tasmaniensis* LGP32, OMVs provide significant and dose-dependent protection against AMPs [120]. Indeed, OMVs increased the MIC of PmB from 2–16-fold at OMV concentrations ranging from 6.25–50 μg/mL. This protective effect was attributed to the binding of PmB to OMVs; no proteolytic degradation of the peptide was observed. Interestingly, the addition of oyster plasma to the culture medium strongly stimulated the release of OMVs by *V. tasmaniensis* LGP32. This indicates that as in *E. coli,* in which sub-lethal concentrations of AMPs promote OMV release [118], OMV release in vibrios is likely up-regulated by membrane-active agents in oyster plasma. Consistent with this hypothesis, LGP32 lacking the major outer membrane protein OmpU, which controls envelope stress signaling in vibrios [108], showed a hypervesiculation phenotype (Figure 2A).

Altogether, these recent studies indicate that OMVs are a potent strategy used by vibrios to trap membrane-active AMPs such as PmB or LL-37, forming a protective shield that prevents interaction with the membranes of the bacterial cell (Figure 2B). Although OMVs released by vibrios can contain specific proteases like the recently identified vesicular serine protease Vsp (VS_II0815) of *V. tasmaniensis* LGP32, there is to date no evidence of AMP degradation by OMV-encapsulated content [119,120].

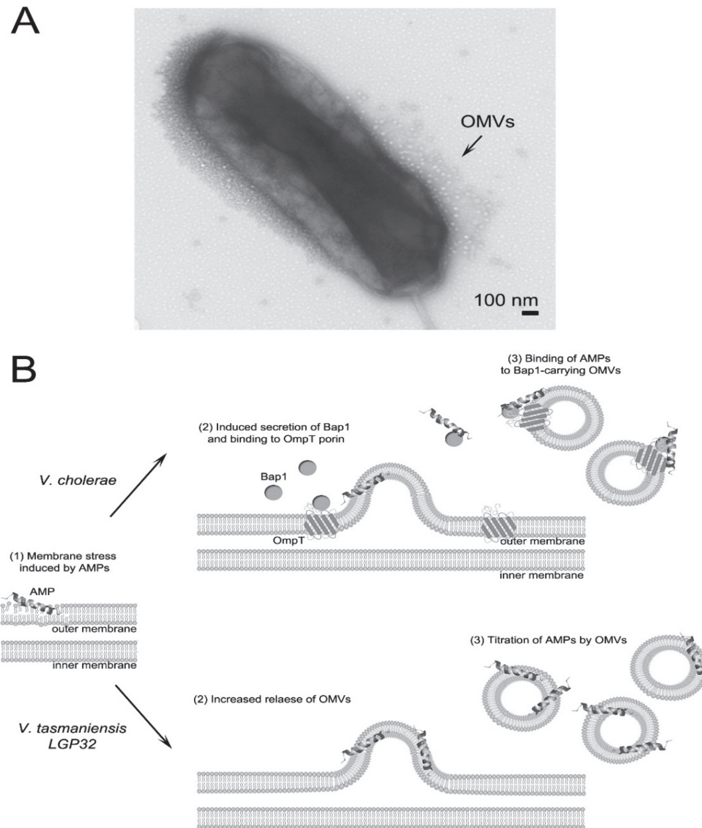

Figure 2. Model for AMP-titration by outer membrane vesicles (OMVs) in *V. cholerae* and *V. tasmaniensis*. (**A**) OMVs released in the extracellular medium by the hypervesiculating Δ*ompU* mutant of *V. tasmaniensis* strain LGP32. Logarithmic phase cultures were negatively stained and observed by transmission electron microscopy as described in [120]; (**B)** The role of OMVs in the protection against AMPs has been recently described in two species of vibrios. In *V. cholerae*, OMVs cross-protect against the human cathelicidin LL-37 when bacteria are exposed to sublethal concentrations of PmB. Those OMVs are associated with Bap1 protein which serves as a ligand for LL-37. The association of Bap1 to OMVs is mediated by the outer membrane protein, OmpT [119]. In *V. tasmaniensis*, OMVs produced in the absence of AMPs are sufficient to titrate PmB and confer a potent dose-dependent protection against PmB. Although the molecular basis of PmB binding to *V. tasmaniensis* OMVs remain unknown, it is speculated that titration may occur by PmB insertion in the OMV membranes. The release of OMVs was shown to be strongly enhanced by the contact of *V. tasmaniensis* with oyster plasma [120]. In both species, OMV release is thought to create a protective membranous shield that prevents the interaction of membrane-active AMPs with the bacterial membranes.

3.4. Efflux of AMPs

The involvement of efflux pumps in antimicrobial resistance, especially in antibiotic resistance, is well established in Gram-negative bacteria [122,123]. There are five different active efflux systems described in bacteria: the ATP-binding cassette superfamily (ABC), the small multidrug resistance family (SMR), the multi antimicrobial extrusion protein family (MATE), the major facilitator superfamily (MFS), and the resistance-nodulation-cell division superfamily (RND) [124]. In terms of antimicrobial resistance, the RND family efflux pumps are particularly important in Gram-negative bacteria. RND efflux systems are composed of an outer membrane protein homologous to the transmembrane β-barrel TolC protein of *E. coli*, a periplasmic membrane fusion protein (MFP), and an integral cytoplasmic membrane pump protein belonging to the RND superfamily of transporters (for review see [125]). These three components function to form a channel to extrude substrates from the cell envelope into the environment. The *V. cholerae* VexAB-TolC [126,127], the *E. coli* and *Salmonella enterica* AcrAB-TolC [128,129], and the *Pseudomonas aeruginosa* MexAB-OprM systems [130] function as RND efflux systems.

In *V. cholerae*, six RND efflux pumps have been described: VexAB, VexCD, VexEF, VexGH, VexIJK, and VexLM [131]. Among them, four are required for antimicrobial resistance *in vitro*. VexAB is the main efflux pump involved in the resistance to antimicrobials including bile acids, detergents, antibiotics, and PmB. The MIC of PmB dropped by four-fold (from 110–27 μg/mL) after *vexB* mutation in *V. cholerae* [127,132]. Moreover, the MIC of PmB against the *vexB* mutant was comparable with the MIC against the RND-null strain, indicating that only VexAB is involved in resistance to PmB [127]. Besides VexAB, VexGH also contributes to antibiotic (novobiocin and ampicillin) and detergent resistance but to a lesser extent than VexAB. Indeed, a decrease in the MIC can be observed only for a *vexBH* double mutant but not for the *vexH* single mutant, compared to the wild-type and *vexB* single mutant strains [133]. Finally, VexCD and VexIJK appeared to efflux bile acids and detergents, respectively [127,132]. VexEF and VexLM do not participate in antimicrobial resistance, but are required for the full virulence of *V. cholerae* by influencing the production of the major effectors of virulence, *i.e.*, cholera toxin and the toxin co-regulated pilus [133]. In *V. parahaemolyticus*, proteomic identification of membrane proteins up-regulated in strains that artificially evolved resistant to AMPs, (including the fish AMP pleurocidin) led to the identification of TolC [134]. Unfortunately, its role in AMP resistance in *V. parahaemolyticus* has not been investigated further.

In addition to efflux pumps, a K^+ pump encoded by the *trkA* gene has been described in *V. vulnificus*, and its role in AMP and serum resistance investigated [135]. The *trkA* gene product, TrkA, is a cytoplasmic protein bound to the inner side of the cytoplasmic membrane [136]. In *V. vulnificus*, the *trkA* mutant exhibited attenuated growth at intermediate potassium concentrations and was more sensitive to human serum protamine and PmB than was the wild type. Indeed, in contrast to the wild-type strain, the *trkA* mutant lysed in the presence of 10–20 μg/mL of protamine, and 5–15 μg/mL of PmB [135]. Moreover, TrkA was found to be important for *V. vulnificus* virulence in mice [135].

3.5. Suppression of AMP Expression

Pathogenic bacteria have developed multiple modalities to combat the antimicrobial response of their hosts. In addition to the structural modifications reviewed above, which increase their resistance to AMPs, they also use transcriptional repression as a strategy to evade the host immune system. Thus, the down-regulation of AMPs can be considered a general mechanism to facilitate invasion of pathogenic bacteria, including vibrios.

In humans, where the interaction of *V. cholerae* with intestinal epithelial cells is a critical step in the disease process, down-regulation of the cathelicidin LL-37, but not of the defensin HBD-1 has been reported in the presence of enteric pathogens including *V. cholerae* O139 [137]. The authors showed that cholera toxin (CT) was the predominant molecule associated with the regulation of AMPs by *V. cholerae* spp. *in vitro* and *in vivo* using intestinal epithelial cells and ileal loop experiments, respectively [137]. Moreover, multiple signaling pathways activated downstream of intracellular accumulation of cAMP contribute to the CT-mediated suppression of LL-37 in intestinal epithelial cells [137]. However, a more recent study on small intestine biopsies of patients with *V. cholerae* O1 infections did not show transcriptional repression of AMP genes in the small intestine [138], a discrepancy that might be explained by differences in transcriptional regulation *in vivo* and *in vitro*. *In vivo*, the expression of hBD-1, -3 and -4 did not vary with the infection, whereas hBD-2 mRNA levels were significantly higher at the acute stage of cholera than at the convalescent stage and in healthy controls. Paneth cell-derived HD-5 and HD-6, which were all expressed at high levels in controls, were not affected by the infections. While no transcriptional repression could be observed, the authors reported that hBD-2, HD-5 and LL-37 peptides are normally present in the small intestine epithelium and amounts decrease at the acute stage of watery diarrhea. Lower amounts of HD-5 could result from degranulation of the Paneth cells in response to infection. The processes regulating hBD-2 and LL-7 levels remain to be characterized.

In invertebrate hosts, similar downregulation of antimicrobial peptides and proteins has been observed during vibrioses. For instance, the coral pathogen, *V. coralliilyticus*, represses the expression of the damicornin, an AMP expressed by the scleractinian coral *Pocillopora damicornis* [28]. Indeed, damicornin transcripts increased during the first 6 days after infection with *V. coralliilyticus*, directly followed by a dramatic decrease from days 9–18. Conversely, no transcriptional change was observed when *P. damicornis* was exposed to a nonvirulent state of *V. coralliilyticus* [28]. Since *V. coralliilyticus* enters into the ectodermal coral tissue within 6 days, the authors suggested that a first phase of infection, involving bacterial recognition by host cells, triggers a nonspecific inflammatory response that activates damicornin gene transcription. In a second phase, following bacterial invasion, the pathogen suppresses damicornin transcription. This study represents the first characterization of the immunosuppression of AMP expression in an invertebrate-vibrio model of pathogenesis. More recently, using a global RNAseq approach, the same authors showed that not only damicornin, but also a mytimacin-like and a LBP-BPI gene displayed decreased expression during a successful *V. coralliilyticus* infection [139].

In mollusks, repression of AMP transcription has not been demonstrated *per se*. Indeed, upon infection of oysters with the pathogen *V. tasmaniensis* LGP32, major hemocyte movements occur

which, by bringing AMP-producing hemocytes to infected tissues, create an apparent depletion in *Cg*-Defm and *Cg*-BPI transcripts in the circulating hemocytes. However, those transcripts accumulate at the same time in the hemocyte-infiltrated tissues [14]. A similar apparent repression of defensin expression was observed in the circulating hemocytes of a heterologous host, the mussel, infected with *V. tasmaniensis* LGP32 [140]. However, to date, the only AMP whose transcription is likely down-regulated by LGP32 is a proline-rich peptide from the oyster which acts by synergism with the other AMPs [14].

4. Conclusions

While vibrios have evolved the capacity to colonize immune tissues such as epithelia and phagocytes, only recent studies have started to investigate the mechanism by which they can survive the high AMP concentrations they encounter. Among their potent mechanisms of resistance to AMPs, vibrios use novel mechanisms of membrane remodeling. In particular, some highly resistant strains substitute their hexaacylated Lipid A with a diglycine residue to reduce the negative charge of their surface thereby lowering the electrostatic interaction with cationic AMPs. As a response to envelope stress, which can be induced by membrane-active agents including AMPs, vibrios release outer membrane vesicles to create a protective membranous shield that traps AMPs and prevents interaction of the peptides with their own membranes. Finally, once AMPs have breached the bacterial membrane barriers, vibrios can use RND pumps similar to those of other species to transport AMPs out of their cytoplasmic space. Although suppression of AMP transcription has been described in some host–pathogen interactions, this mechanism of immune evasion appears to be more specific to given strains/species than universal among vibrios.

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Author Contributions

All authors have contributed to the research presented and the writing of the present review.

Conflicts of Interest

The authors declare no conflict of interest.

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Augmentation of Cationic Antimicrobial Peptide Production with Histone Deacetylase Inhibitors as a Novel Epigenetic Therapy for Bacterial Infections

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Abstract: The emergence of antibiotic resistance seriously threatens our ability to treat many common and medically important bacterial infections. Novel therapeutics are needed that can be used alone or in conjunction with antibiotics. Cationic antimicrobial peptides (CAMPs) are important effectors of the host innate defense that exhibit broad-spectrum activity against a wide range of microorganisms. CAMPs are carried within phagocytic granules and are constitutively or inducibly expressed by multiple cell types, including epithelial cells. The role of histone modification enzymes, specifically the histone deacetylases (HDAC), in down-regulating the transcription of CAMP-encoding genes is increasingly appreciated as is the capacity of HDAC inhibitors (HDACi) to block the action of HDACs to increase CAMP expression. The use of synthetic and natural HDACi molecules to increase CAMPs on mucosal surfaces, therefore, has potential therapeutic applications. Here, we review host and pathogen regulation of CAMP expression through the induction of HDACs and assess the therapeutic potential of natural and synthetic HDACi based on evidence from tissue culture systems, animal models, and clinical trials.

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1. Antimicrobial Peptides—An Innate Defense Against Microbial Pathogens

Confidence in currently licensed antibiotics to effectively treat and control bacterial infections has seriously waned in recent years with the emergence of multidrug resistance in several medically important bacterial species [1]. While new compounds are under development, novel strategies are needed to out-pace the selection for resistance mutations [2]. CAMPs are relatively small (<10 kDa), cationic and amphipathic peptides that form an important component of the host innate defense against invading pathogens [3]. CAMPs have been isolated from a wide variety of animals, both vertebrates and invertebrates, plants, fungi, and bacteria, and these innate effectors exhibit broad-spectrum activity against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, protozoa, yeast, fungi and viruses [4]. CAMPs are broadly classified into five major groups based on three dimensional structural studies and amino acid composition, namely (a) peptides that form α-helical structures; (b) peptides rich in cysteine residues; (c) peptides that form β-sheets (d) peptides rich in specific amino acids e.g., histatin (rich in histidine), cathelicidins (rich in proline) and indolicidins (rich in tryptophan); and (e) peptides composed of rare and modified amino acids.

The mechanism by which CAMPs exert their antimicrobial activity involves disruption of the plasma membrane leading to the lysis of the target cell [5]. Hence, CAMPs are excellent candidate antimicrobial agents that can act against a broad range of pathogens alone or potentially, as adjunctive therapies for existing antibiotics. A few peptides have already entered clinical trials for the treatment of impetigo, diabetic foot ulcers and gastric helicobacter infections [6]. The potential therapeutic effect of CAMPs against sexually transmitted infections, including human immunodeficiency virus (HIV) and herpes simplex virus (HSV) infections [7], has also been investigated.

2. Pathogens Can Regulate HDAC-Mediated Expression of CAMPs

An alternative approach to directly challenging infectious agents with CAMPs is to induce CAMP expression therapeutically. Indeed, pathogens have evolved the opposite strategy of down-regulating CAMP expression to better establish themselves in the host. For example, several bacterial pathogens down-regulate the cathelicidin LL-37, secretory leukocyte protease inhibitor (SLPI), and, or human beta defensins (e.g., HBD-1, HBD-2, HBD-3) in tissue culture cells [8,9], and animal infection models [10]. These results are consistent with the detection of significantly lower concentrations of CAMPs in infected individuals. For example, significantly lower vaginal concentrations of SLPI were detected in non-pregnant women with gonorrhea or *Chlamydia* infections compared to uninfected healthy controls [11]. Similarly, down-regulation of LL-37 and HBD-1 transcription was detected in gut biopsies from individuals with *Shigella dysenteriae* [12], genetic evidence suggests pathogen-mediated suppression of gene transcription is responsible.

The mechanism(s) by which bacteria down-regulate CAMP-encoding genes has not been resolved, but some bacterial pathogens can alter host gene expression at the level of chromatin remodeling. It is now well understood that regulation of gene expression can occur at several checkpoints: transcriptional, post-transcriptional, translational and post-translational stages. At the level of transcription, chromatin modifications play a very important regulatory role as chromatin remodeling is controlled by chromatin modifying enzymes [13], of which the histone deacetylases (HDAC) are an important family. HDAC control the availability of DNA binding sites to transcription factors by removing the acetyl groups from the surface of specific amino acids located in the N-terminal of histone proteins [14]. The balance between the histone acetylases (HA) and HDAC has been suggested to regulate transcription of several genes in multiple locations and collectively can cause global genomic and proteomic changes (Figure 1A).

The discovery that bacterial pathogens can alter host gene expression by altering the balance between HA and HDAC enzymes is a fascinating insight into the intimate evolution of microbes within a host. An early seminal study in 2009 by Garcia and colleagues [15] demonstrated that infection of THP-1 cells with *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis, led to suppression of a broad range of antimicrobial peptides and proteins namely cathelicidin, defensins (DEFB1, DEFB4, DEFA1, DEFA4 and DEFA6), azurocidin-1, lysozyme and cystatin A. A chromatin immunoprecipitation (CHIP) assay revealed a significant fold decrease in the acetylation and a proportional increase in the methylation of histone H3 at the promoters of the genes described above, suggesting the observed transcriptional changes were due to pathogen's effects on chromatin remodeling enzymes, specifically histone deacetylases and methylases.

A handful of other reports have analyzed bacterial-induced regulation of HDAC expression. Yin and Chung reported that the oral pathogens *Poryphyromonas gingivalis* and *Fusobacterium nucleatum* modulated HDAC1 and, or HDAC2 expression in a human immortalized human keratinocyte cells and primary gingival epithelial cells [16]. *P gingivalis* caused the most significant suppression of HDAC gene transcription and lower numbers of bacteria able to significantly reduce HDAC-1 mRNA compared to HDAC-2 mRNA, which required a higher multiplicity of infection. It is not yet clear which HDACs influence the expression of specific CAMP-encoding genes. However, using small inhibitory RNAs, Kallsen and colleagues showed that knockdown of HDAC1, but not HDAC2 or HDAC3 expression in human lung adenocarcinoma A549 cells, increases HBD-1 gene expression, from which it is hypothesized that HDAC1 may play a regulatory role for HBD-1 expression in A549 cells [17]. The events by which other pathogens can suppress CAMP expression *in vitro* and *in vivo* are described in Figure 1B.

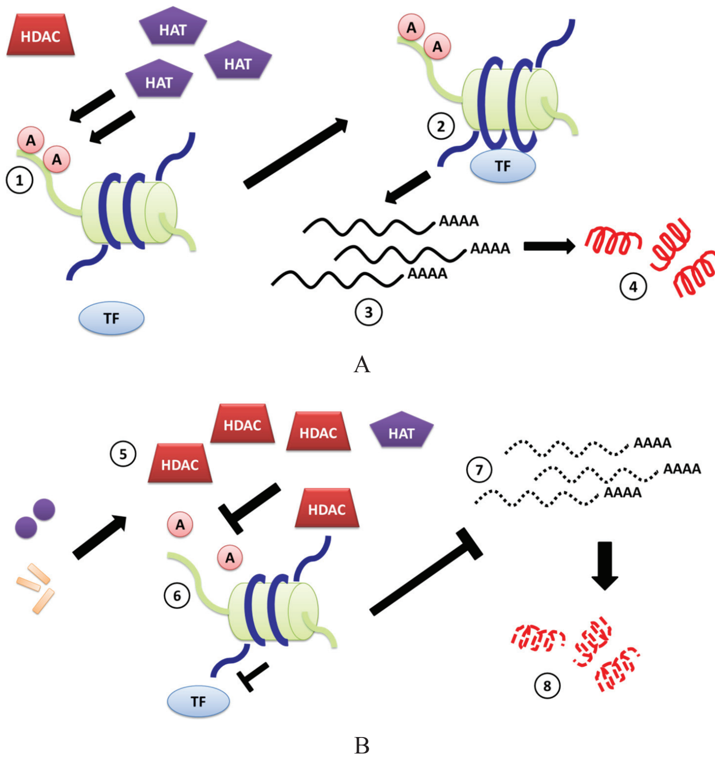

Figure 1. *Cont.*

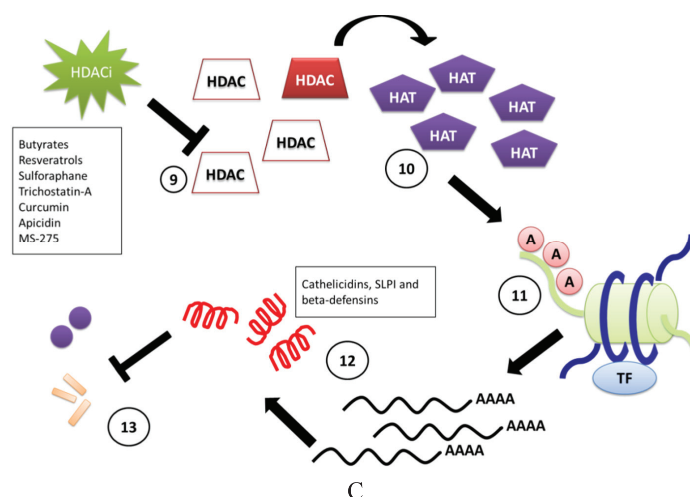

Figure 1. Pictorial description of chromatin remodeling resulting from the interplay between chromatin modifying enzymes and pathogens. (**A**) There is scientific evidence to assume that the enzyme histone acetylase adds acetyl groups to specific amino acids on the free *N*-terminal of histone proteins (not embedded in the octamer) (1), as a result of which the chromatin coiled around the histone octamers undergoes changes in spatial configuration exposing parts of DNA to which transcription factors bind (2). This change in DNA topography allows for a particular region of DNA to be transcribed and translated (3,4), thus regulating some important cellular functions including immune responses to pathogen invasion; (**B**) Several pathogens have evolved mechanisms to induce HDAC expression, which causes removal of acetyl groups attached to *N*-terminal histones, affecting the transcription and translation of many genes, including those involved in pathogen recognition, immunity and CAMP production (6–8). Examples include *Shigella dysenteriae*, *Vibrio cholerae* and *Anaplasma phagocytophilum* and *Porphyromonas gingivalis*. The mechanism(s) by which bacteria induce HDAC expression has not been well studied, although bacterial toxins or other cellular components (*i.e.*, lipopolysaccharide, LPS) have been shown to play a role in some cases (5); (**C**) Chromatin remodeling can be regulated by HDACi, which directly interact with the HDAC enzyme and cause a state of hyperacetylation (9,10). This hyperacetylation can lead to a global change in gene expression. HDACi-induced over-expression of CAMP-encoding genes (11,12) has led to the idea of developing the HDACi as novel therapeutics for controlling bacterial infections in conjunction with antibiotic treatment (13).

3. Induction of CAMPs by HDACi

HDAC inhibitors (HDACi) inhibit the activity of HDAC enzymes and several HDACi have been isolated from natural sources while others have been chemically synthesized [18]. HDACi can regulate transcription of a gene by inhibiting the HDAC enzyme from removing an acetyl group from histone

tails. This inhibition leads to remodeling of the chromatin that is bound to histone octamers to increase the available binding sites on DNA for transcription factors and other regulatory proteins [19]. Recent studies using cDNA arrays have suggested that treatment of multiple cancer cell lines with HDACi affect gene expression in as many as 7%–10% of genes [20]. The number of genes affected by HDACi-treatment depended of several factors including time of culture, concentration, and the particular HDACi used [21].

HDAC inhibitors (HDACi) can prevent HDAC-mediated down-regulation of gene expression, and HDACi have been extensively evaluated for treating several cancers. A few HDACi have been approved for use by FDA or are in clinical trials [22]. Apart from their anti-cancer activities, HDACi have also been evaluated for other immunomodulatory properties and have attracted intellectual property interests from the pharmaceutical companies [23,24]. Interestingly, several reports in recent years have suggested that some HDACi induce the expression of CAMPs (Table 1). A review of the recent literature demonstrating CAMP-inducing activities of synthetic or natural HDACi follows (see Figure 1C).

Table 1. HDACi that have been evaluated for the capacity to induce CAMPs *in vitro* and *in vivo*.

HDAC Inhibitor	System Tested	Effect on CAMP mRNA Expression	Reference
Butyrate	Human lung epithelial cell line EBC-1	Cathelicidin ↑	[25]
	Human bronchial epithelial cell line VA10	Cathelicidin ↑ HBD-1 ↑	[26]
	Human airway epithelial cells NCI-H292	Cathelicidin ↑	[27]
	Human lung epithelial cell line A549	HBD-1 ↑	[17]
	Human primary gingival epithelial cells infected with *P. gingivalis* and *F. nucleatum*	HBD-2 ↑	[16]
	Human monocyte cell line U937	HBD-1 ↓	[26]
	Adult patients with shigellosis	Cathelicidin ↑	[28]
Resveratrol	Human keratinocyte cell line HaCaT	Cathelicidin ↑	[29]
	Topical administration in female hairless mice	Cathelicidin ↑	[30]
	Human monocyte cell line U937	Cathelicidin ↑	[29]
	Pseudomonas aeruginosa-infected A549 cells	HBD-2 ↓	[31]
Pterostilbene	Human monocyte cell line U937	Cathelicidin ↑	[29]
Polydatin	Human keratinocyte cell line HaCaT	HBD-2 ↑	[32]
Sulforaphane	Liver tissue from SFN-treated C57BL/6 mice	MBD-10 ↑	[33]
	Human intestinal epithelial cell lines Caco-2, HT-29 and SW480	HBD-2 ↑	[34]
	Mouse monocyte macrophage cell line RAW 264.7	SLPI ↑	[35]
	Nasal lavage from healthy human adults who ingested SFN-containing broccoli shake homogenate	SLPI ↑	[36]
Trichostatin-A	Human primary gingival epithelial cells infected with *P. gingivalis* and *F. nucleatum*	HBD-2 ↑	[16]
	Human lung epithelial cell lines A549 and NCI-H727	HBD-1 ↑	[17]
	Human airway epithelial cells NCI-H292	Cathelicidin ↑	[27]
Curcumin	Human cell lines: U937, HT-29 and HaCaT	Cathelicidin ↑	[37]
Apicidin	Human lung epithelial cell line A549	HBD-1 ↑	[17]
MS-275	Human lung epithelial cell line A549	HBD-1 ↑	[17]

4. Butyrates

Butyrate is a short chain fatty acid derived from the microbial fermentation of dietary fibers in the colon and are proven to have beneficial effects on health including the prevention of certain cancers, including colon cancer [38]. The beneficial effects of butyrate are attributed to its capacity to regulate gene expression through its action as a HDACi [39]. Ingram and colleagues were first to report that butyrate increased the level of acetylated histones in cultured HeLa and Friend erythroleukemic cells [40]. Butyrate inhibits most HDAC members except HDAC-6 and -10 (class III and class II HDACs, respectively). During inhibition of HDAC activity, HAT activity continues, which results in histone hyperacetylation [41]. Butyrate can exist in different forms and many forms have similar biological properties. These compounds have also have been studied for their ability to induce CAMPs. Butyrate was initially approved for treatment via an oral route for managing ulcerative colitis and urea cycle disorders [42,43]. However, due its foul smell, butyrate makes it unsuitable for oral therapy in humans. In recent literature, butyrate has been delivered to *in vivo* systems in the form of enema or creams to treat shigellosis and chronic dermatitis, respectively [28,44].

4.1. Butyrate-Induced Expression of Cathelicidins

Kida and colleagues were among the first to demonstrate the ability of sodium butyrate (SB) to induce cathelicidins in human lung epithelial cell line EBC-1 [25]. They observed a dose-dependent increase in the levels of cathelicidin mRNA expression in EBC-1 cells in response to SB-treatment, and results were confirmed at the protein level by immunoblot. Similar observations were made by Liu *et al.*, who reported reduced LL-37 expression in NCI-H292 human airway and nasal epithelial cells in response to SB-treatment [27]. Based on a luciferase reporter assay, the mechanism by which SB appears to increase LL-37 is through increased binding of transcription factor AP-1 to a specific region of the cathelicidin gene promoter sequence. CHIP assays suggested that SB-treatment of EBC-1 cells also augmented the acetylation of histone H3 and H4 at the cathelicidin gene promoter. This hypothesis was supported by immunoblot analysis, indicating that the augmentation of histone acetylation of the cathelicidin promoter participates in the SB-stimulated induction of cathelicidin gene expression in EBC-1 cells [25].

The therapeutic potential of SB as an anti-infective is further supported by a randomized, double-blind, placebo-controlled, clinical trial in adults with shigellosis. Delivery of SB (80 mM) as an enema to test subjects twice daily for 3 days resulted in higher LL-37 expression in the rectal epithelia compared to levels in subjects given saline in parallel. Stool concentrations of LL-37 remained significantly higher in the test group and an early reduction of macrophages, pus cells, IL-8 and IL-1β in the stool was observed as well as improvement in rectal histopathology [28].

Following the discovery of SB HDACi activity, Steinmann *et al.* [26] reported the ability of phenyl butyrate (PB) to induce cathelicidin expression in the immortalized human bronchial epithelial cell line, VA10. Incubation of VA10 with increasing doses of PB (0.25–4 mM) for 24 h resulted in a strong induction of cathelicidin mRNA [26]. Similar observations were also made with a human colonic adenocarcinoma HT-29 cells, human renal carcinoma A498 cells, and human leukemic monocyte lymphoma U937 cells, although the changes in LL-37 expression varied between cell

types. Steinmann and colleagues also observed that co-stimulation of VA10 cells with 4 mM PB in the presence of vitamin D3 ($1,25(OH)_2D_3$) resulted in more than an 80-fold increase of cathelicidin mRNA levels over those treated with Vitamin D3 or PBA alone, and a ~100-fold increase compared to controls, indicating a synergistic induction [26]. These data were confirmed at the protein level with LL-37-specific antibodies. No significant change in histone acetylation on the proximal promoter of the cathelicidin gene was detected using a CHIP assay to assess the acetylation status of histones H3 and H4 in PB-treated VA10 cells, however, and thus in contrast to SB, the effect of PB on chromatin remodeling remains unknown.

4.2. Butyrate-Induced Expression of Beta-Defensins

Kallsen and colleagues observed that butyrate significantly upregulated the expression of HBD-1 in A549 cells in a temporal manner, with highest expression observed between 36 and 48 h post-treatment [17]. Yin and Chung subsequently reported that infection of gingival epithelial cells (GEC) with *P. gingivalis* caused a three-fold increase in HBD-2 expression and this effect was further enhanced when GECs were pretreated with 2 mM SB. A more pronounced increase in HBD-2 transcript levels was observed in *P. gingivalis*-infected GECs when pretreated with a combination of SB and Trichostatin-A (TSA) [16]. In contrast, *F. nucleatum* infection of GECs induced a strong response of HBD-2 gene expression, and while no additive effects were observed in cells pre-treated with SB, a combination of SB and TSA induced a very strong upregulation of HBD-2 mRNA [16]. Interestingly, the response to PB appears to be cell-specific. While PB treatment of VA10 cells significantly upregulated the transcription of the HBD-1 gene [26], PB treatment of U927 cells down-regulated DEFB1 gene expression. Based on this finding, the potential for cell-specific differences in responses to HDACi should be carefully considered when developing other HDACi for potential therapeutic use.

5. Resveratrol and Structurally-Related Molecules

Resveratrol (RESV; 3,5,4-trihydrostilbene) is a natural polyphenolic alcohol found in high quantities in plants and is produced in response to external stress, like UV irradiation, fungal infection or injury [45]. The molecule has attracted attention due to its beneficial properties in reducing the incidence of heart diseases. However, RESV also exhibits anti-oxidant, anti-inflammatory and anti-proliferative effects [46]. The compound has also been tested for its anticancer properties in tumors from different sites of the body [47].

RESV has been reported to demonstrate HDACi activity against specific members of class I and II HDAC enzymes. Molecular docking studies with HDAC-2, -4, -7 and -8 revealed that RESV fits into the binding pocket of all four HDACs and more specifically interacts with the binding pocket of the enzymes, which contains the active site with a zinc ion [48]. Further studies by Venturelli and colleagues employing specific fluorometric profiling assays revealed that treatment of HeLa cells with 50–100 µM RESV only moderately inhibited most members of HDAC family (HDAC 1–11). Significant inhibition of HDAC-1 and HDAC-4 was observed with a 100 µM dose of RESV.

In a recent study by Park and colleagues, RESV-treatment of HaCaT keratinocyte cells significantly induced the production of LL-37 mRNA [30]. Western blotting and ELISA assays with whole cell lysates and culture supernatants from with resveratrol-treated HaCaT cells showed a similar increase in LL-37 peptide levels. This RESV-mediated induction was dependent on the ceramide signaling pathway. Hence, co-incubation of HaCaT cells with RESV and *N*-oleoylethanolamine (NOE), an inhibitor of ceramidase, the enzyme that converts ceramide to sphingosine, significantly inhibited RESV-induced LL-37 expression at both the mRNA and protein level [30]. Similar observations were made with dimethylsphingosine and SKI, which are inhibitors that block the conversion of sphingosine to sphingosine-1-phosphate, suggesting that RESV-induced expression of LL-37 was regulated by the ceramide metabolic pathway. This hypothesis is further supported by the demonstration that topical administration of RESV on murine epidermis led to increased expression of the murine homolog of LL-37, cathelicidin-related antimicrobial peptide (CRAMP), and that this result was dependent on the sphingosine-1-phosphate-induced signaling [30].

In a separate study, Guo *et al.* observed that exposure of U937 cells to RESV for 18 h induced significant expression of cathelicidin. However, a stronger induction was observed when RESV was combined with 1,25$(OH)_2D_3$ [29]. Analysis of RESV-treated U937 cells by flow cytometry using anti LL-37 antibodies confirmed the inductive effects of RESV alone and in combination with 1,25$(OH)_2D_3$. Interestingly HaCaT cells treated with RESV at 10 μM for 18 h did not induce cathelicidin expression. However, when combined with 1,25$(OH)_2D_3$, RESV caused a three-fold increase in LL-37 mRNA level.

The efficacy of RESV to induce host effectors during a bacterial infection was brought into question by Cerqueira and colleagues, who found a significant down-regulation of HBD-2 transcripts in *Pseudomonas aeruginosa*-infected A549 cells when the cells were pre-treated with RESV (100 μmol) [31]. Further studies with different cell lines and pathogens are needed to better define the therapeutic potential of RESV against bacterial infection.

5.1. Pterostilbene

Pterostilbene is a stilbenoid found in rich quantities in blueberries and grapes, and is chemically related to RESV. It is a type of phytoalexin, which are agents produced by plants to fight infections. Like RESV, pterostilbene exhibits anti-oxidant, anti-inflammatory and anticancer activities [49]. Chen and colleagues reported that treatment of RPMI8226 multiple myeloma cells and HEK 293 cells with 10 μM of pterostilbene strongly induced histone acetylation and specifically prevented HDAC1 digestion by thermolysin [50]. Currently only one report suggests that pterostilbene induces CAMPs *in vitro*. Guo and group reported that pterostilbene treatment of U937 cells induced significant expression of cathelicidin. This increase was enhanced when pterostilbene was combined with 1,25$(OH)_2D_3$, leading to a three-fold increase in expression of cathelicidin compared with 1,25$(OH)_2D_3$ alone. Results from a flow cytometry assay designed to detect LL-37 peptide confirm these results [29]. In the same study, co-treatment of U937 cells with pterostilbene with calcipitriene and paracalcitol (pharmaceutical analogs of 1,25$(OH)_2D_3$) led to a 3–20 -fold increase in expression of cathelicidin at mRNA level.

5.2. Polydatin

Polydatin, also known as piceid (resveratrol-3-O-β-mono-D-glucoside, polydatin), is the glycoside form of RESV and is found in very high concentration in the grape *Polygonum cuspidatum*. Polydatin has the glucoside group bonded in position C-3 and substitutes a hydroxyl group giving rise to conformational changes of the molecule leading to increase in its biological properties. Like RESV and pterostilbene, polydatin is known to regulate oxidative and inflammatory pathways [51]. Currently, no reports exist to suggest that polydatin might be able to inhibit HDAC enzymes; however, it is highly anticipated that polydatin would also exhibit HDACi activities due to its structural similarity with RESV. A study by Ravagnan and colleagues revealed that pretreatment of HaCat cells with polydatin, alone or in combination with RESV for 24 h, induced HBD-2 expression at the mRNA level. This observation was confirmed by an ELISA for HBD-2, where a combination of polydatin and RESV induced as high as 191 ng/mL HBD-2 peptide in the culture supernatants compared to 9 ng/mL in untreated control HaCaT cells [32].

6. Sulforaphane

Sulforaphane (SFN) is a natural isothiocyanate, first isolated from broccoli, and a potent inducer of phase 2 detoxification enzymes and an inhibitor of phase 1 enzymes that activate chemical carcinogens. SFN has been shown to induce apoptosis and prevent tumors in mouse models [18]. SFN is also known to inhibit the HDAC family of enzymes. An early investigation of the HDACi activity of SFN by Myzak and colleagues showed that cytoplasmic and nuclear extracts from human embryonic kidney 293 cells treated with SFN had diminished HDAC activity along with a concomitant increase in histone acetylation compared to untreated cells [52]. A subsequent study from the same group demonstrated that the treatment of human colon cancer cells HCT116 with 35 μM SFN caused a significant decrease in HDAC-2 and HDAC-3 protein levels [53].

SFN has also been assessed for its ability to induce CAMPs both *in vitro* and *in vivo*. Treatment of intestinal epithelial cell lines (Caco-2, HT-29 and SW480) with SFN-induced HBD-2 mRNA expression in a time- and dose-dependent manner and increased levels of HBD-2 peptide as measured by an ELISA [34]. Similarly, SFN treatment of RAW 274.7 cells, a mouse leukaemic monocyte macrophage cell line, resulted in increased SLPI transcription [35]. In a recent study, Meyer and colleagues reported that healthy nonsmoking adults who ingested SFN-containing broccoli shake homogenate for three consecutive days demonstrated a significant increase in SLPI levels in nasal lavages. These investigators also showed that SFN-induced SLPI expression appears to be regulated by the Nrf2 transcription factor in that SLPI secretion was significantly decreased in cells transduced with Nrf2-specific shRNA [36]. This finding is consistent with an earlier study in which administration of SFN to C57BL/6J wild type and Nrf2 knockout mice resulted in increased expression of the beta defensin-10 gene [33].

Investigation of the use of SFN to treat *Helicobacter pylori* infections revealed other possible mechanisms by which SFN may have a therapeutic effect. SFN directly inhibited extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevented benzo[a]pyrene-induced stomach tumors in a mouse model when given orally [54]. The urease

enzyme produced by *H. pylori* is critical for establishment of gastric colonization, and a recent follow-up study by the same group demonstrated that SFN inactivates *H. pylori* urease by forming a dithiocarbamate complex between the isothiocyanate group of SFN and cysteine thiols of urease [55]. Yanaka and colleagues also showed that *H. pylori*-infected mice given SFN-rich broccoli sprouts and a high-salt (7.5% NaCl) diet had reduced bacterial colonization, attenuated mucosal expression of TNF-α- and IL-1β, less corpus inflammation, and that high salt-induced gastric corpus atrophy was prevented [56]. The contribution of gastric cathelicidins or defensins were not evaluated in these study, but cannot be ruled out as a mechanism by which *H. pylori* colonization and inflammation were reduced.

The possibility that SFN may be effective against sexually transmitted infections was recently investigated by our group using *Neisseria gonorrhoeae* as the model pathogen, which is a pyogenic pathogen of the urogenital tract. We found that SFN-treatment of human endocervical carcinoma ME-180 cells led to an upregulation of HBD-2, HD-5 and SLPI gene expression [57] and that oral administration of SFN significantly reduced experimental colonization of female mice and suppressed the pro-inflammatory cytokine and chemokine response to infection (Yedery *et al.*, in preparation). These data support the potential use of HDACi alone or in conjunction with antibiotics to treat *N. gonorrhoeae*, which recently reached super-bug status due to the emergence of resistance to the extended cephalosporins, the last remaining monotherapy for empirical treatment of gonorrhea [58].

7. Trichostatin A

Trichostatin A (TSA, 7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-(2*E*,4*E*, 6*R*)-2,4-heptadienamide), is a natural HDACi produced by two species of *Streptomyces*, *S. platensis* and *S. sioyaensis* [59]. The R-isomer of TSA was one of the first HDACi shown to increase the levels of histone acetylation in various mammalian cell lines [60]. TSA has also been well studied for its anti-inflammatory and anti-tumor activities.

TSA also induces CAMP expression in epithelial cells. TSA treatment of the lung epithelial cell lines A549 and NCI-H727 increased HBD-1 transcription, and a CHIP analysis with an anti-acetyl-histone H3 antibody revealed that TSA induced a 2.8-fold increase in histone H3 acetylation at the DEFB1 promoter [17]. Recently, Liu and colleagues reported that treatment of NCI-H292 human airway and human nasal epithelial cells with TSA increased the level of LL-37 transcription. Co-treatment with poly(I:C) did not affect the expression of the LL-37 gene, although poly(I:C) by itself weakly induced LL-37 expression [27]. A similar observation was made at protein level using an immunoblot assay. Importantly, studies Yin and Chung reported increased levels of HBD-2 mRNA when TSA-pretreated GECs were infected with *P. gingivalis* and *F. nucleatum* compared to uninfected controls [16]. This promising result suggests TSA may be effective against bacterial pathogens that are known to induce host HDACs and down-regulate CAMP expression.

8. Curcumin

Curcumin (CMN), or diferuloylmethane (1,7-bis-4(hydroxy-3-methoxyphenyl)-1,6-heptadiene-3, 5-dione), is present within the rhizome of the plant *Curcuma longa*. CMN has generated significant

interest due to its anti-cancer functions, which are attributed to its ability to inhibit specific molecular signaling pathways involved in carcinogenesis [61]. CMN also possesses anti-oxidant, anti-inflammatory, anti-proliferative and anti-angiogenic properties against several cancer cell types [62]. Studies have also demonstrated that CMN inhibits class I HDAC enzymes (HDAC 1–3 and HDAC-8), and Chen *et al.* reported that CMN is able to regulate cell proliferation and apoptosis in Raji cells by downregulating the expression levels of HDAC-1,HDAC-3 and HDAC-8 proteins and by upregulating acetylated histone H4 protein expression [63].

CMN treatment of tissue culture cells also results in increased CAMP expression. In a recent report, Guo and group demonstrated that CMN treatment of U937 and HT-29 cells significantly induced expression of LL-37, but not in HaCaT cells. Elevated intracellular LL-37 levels were also detected in CMN-treated U937 cells as measured by intracellular staining and FACS [37]. Using a luciferase reporter assay it was observed that induction of cathelicidin by CMN does not require Vitamin D response elements (VDRE). Further, a CHIP assay with CMN-treated U937 cells demonstrated that CMN did not increase VDR binding to cathelicidin promoter suggesting that CMN-induced human LL-37 expression occurs through a VDR- independent mechanism [37].

9. Apicidin

Apicidin (APD) [cyclo(NO-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-xodecanoyl)], is a fungal metabolite that was originally known for its broad spectrum antiprotozoal activity against *Apicomplexan* parasites and *Plasmodium berghei in vitro* [64]. Later studies demonstrated the ability of APD to inhibit mammalian HDAC. The ability of APD to interact with HDAC depends on its unique structure, which contains an ethyl ketone as a potential zinc-binding group, a long alkyl chain as a linker, and a cyclic tetrapeptide [65]. A structural derivative of API, API-D, exhibits selective inhibition of class I subtypes HDAC-1, HDAC-2 and HDAC-3 [66]. Recent studies showed that APD induced expression of HBD-1 in A549 cells in a time-dependent fashion with mRNA levels achieving a seven-fold increase compared to untreated controls at 36 h post treatment.

10. MS-275

MS-275, also known as entinostat, is an HDACi that belongs to the 2-aminophenyl benzamides. MS-275 inhibits class I HDAC enzymes, with a high affinity for HDAC-1, HDAC-2 and HDAC-3, but has a relatively weak affinity for HDAC-8 [67]. Recently Kallsen *et al.*, reported that MS-275-treatment of A549 lung epithelial cells exhibited a temporally significant increase in the expression of HBD-1 mRNA. Results from CHIP assays revealed that acetylation of histone H3 and the trimethylation of lysine 4 at histone 3 (H3K4), which are histone modifications associated with transcriptionally active chromatin, were increased at the DEFB-1 promoter after treatment with MS-275 for 36 h [17].

11. Anti-Inflammatory Properties of HDACi

HDACi have also been extensively studied for their ability to control and regulate inflammation triggered by microbial ligands. Early studies by Segain and colleagues demonstrated that butyrate suppressed lipopolysaccharide (LPS)-induced secretion of cytokines by peripheral blood mononuclear cells. The suggested mechanism was that butyrate prevented the transmigration of NF-kB from the cytoplasm to the nucleus [68]. Tedelind *et al.* demonstrated that butyrate and propionate reduce inflammation-mediated tissue insult in a mouse colitis model [69]. In another report, Zhing and group reported the anti-inflammatory activity of RESV in LPS-exposed microglial cells. Their studies revealed a RESV-mediated down-regulation in phosphorylation levels of the transcription factors: NF-κB, CREB and MAPKs family in an mTOR-dependent manner [70]. Meng and colleagues observed that curcumin inhibited LPS-induced inflammation in rat vascular smooth muscle cells. The mechanism was dependent on the inhibition of the TLR4-MAPK/NF-κB pathways via blockage of NADPH-mediated intracellular ROS production [71]. Recently Brandenburg *et al.* demonstrated the ability of SFN to attenuate LPS-induced IL-1β, IL-6 and TNF-α expression in microglia; these investigators also reported that SFN significantly decreases the LPS-induced nitric oxide in a concentration-dependent manner [72]. In summary, these studies suggest that HDACi may have the therapeutic benefit of effectively regulating microbial infections due to the dual capacity of both inducing CAMPs and inhibiting the harmful effects of pathogen-mediated inflammation and tissue damage.

12. Conclusions and Future Directions

Due to increasing antimicrobial resistance and non-availability of vaccines for many pathogens, there is an urgent need to identify novel therapeutic strategies to combat the spread of these infections. The studies and observations reviewed in this article suggest that HDACi could serve as novel candidates, either as new antibiotics or adjunctive therapies in combination with existing control measures. Further investigation is needed, however; in particular, the testing of HDACi in infection models is needed to determine whether HDACi can out-compete the capacity of some bacterial pathogens to down-regulate CAMP expression. It is also important to determine whether HDACi can increase CAMP concentrations at mucosal surfaces to a level that overcomes other bacterial defenses against CAMPs, such as surface modifications that reduce CAMP binding and active efflux of internalized peptides. More experimentation is also needed to better understand the mode of action of the different HDACi identified to date. HDACi targeting specific HDAC enzymes will have an advantage over pan-inhibitors which inhibit multiple HDAC members. Also, profiling HDACi for their ability to induce individual CAMPs may generate more candidates for further evaluation with animal models and clinical trials. Subject to available technology, progress in this area is attainable, and should be supported due to the pressing need for novel anti-infectives.

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Author Contributions

R.D.Y. performed the literature survey and prepared the manuscript. A.E.J. and R.D.Y. together created the outline for this manuscript and A.E.J. evaluated the text for language and grammar. The figures in the manuscript were designed by R.D.Y.

Conflicts of Interest

The authors declare no conflict of interest.

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Ciprofloxacin Affects Host Cells by Suppressing Expression of the Endogenous Antimicrobial Peptides Cathelicidins and Beta-Defensin-3 in Colon Epithelia

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Abstract: Antibiotics exert several effects on host cells including regulation of immune components. Antimicrobial peptides (AMPs), e.g., cathelicidins and defensins display multiple functions in innate immunity. In colonic mucosa, cathelicidins are induced by butyrate, a bacterial fermentation product. Here, we investigated the effect of antibiotics on butyrate-induced expression of cathelicidins and beta-defensins in colon epithelial cells. Real-time PCR analysis revealed that ciprofloxacin and clindamycin reduce butyrate-induced transcription of the human cathelicidin LL-37 in the colonic epithelial cell line HT-29. Suppression of LL-37 peptide/protein by ciprofloxacin was confirmed by Western blot analysis. Immunohistochemical analysis demonstrated that ciprofloxacin suppresses the rabbit cathelicidin CAP-18 in rectal epithelia of healthy and butyrate-treated *Shigella*-infected rabbits. Ciprofloxacin also down-regulated butyrate-induced transcription of the human beta-defensin-3 in HT-29 cells. Microarray analysis of HT-29 cells revealed upregulation by butyrate with subsequent down-regulation by ciprofloxacin of additional genes encoding immune factors. Dephosphorylation of histone H3, an epigenetic event provided a possible mechanism of the suppressive effect of ciprofloxacin. Furthermore, LL-37 peptide inhibited *Clostridium difficile* growth *in vitro*. In conclusion, ciprofloxacin and clindamycin exert immunomodulatory function by down-regulating AMPs and other immune components in colonic epithelial cells. Suppression of AMPs may contribute to the overgrowth of *C. difficile*, causing antibiotic-associated diarrhea.

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1. Introduction

Antimicrobial peptides/proteins (AMPs) are important effectors of the immediate host defense, exerting antimicrobial activity and immunomodulation [1–4]. Defensins and cathelicidins are the two major classes of AMPs in mammals. LL-37 is the sole cathelicidin peptide in human and its orthologs in rabbit, mouse, and rat are CAP-18, mCRAMP and rCRAMP, respectively [5]. LL-37 and its orthologs are cationic, amphipathic, α-helical peptides. Defensins are cationic peptides, having a characteristic anti-parallel β-sheet fold and consist of six conserved cysteine residues forming three disulphide bonds [6,7]. Based on the size and disulfide linkage, defensins are classified into α-, β-, and θ-defensins. In human, six α-defensins, e.g., human neutrophil peptides (HNP)-1 to -4, human defensins (HD)-5 and -6, and four β-defensins, e.g., human β-defensin (HBD)-1 to -4

have, thus far, been characterized [6,8]. LL-37 and/or HBDs have been implicated in several functions including killing of microorganisms, neutralization of lipopolysaccharide, immune regulation, regulation of normal flora, wound healing, angiogenesis, and anticancer activities [3,5,6,8–14].

LL-37 is expressed in neutophils, monocytes, lymphocytes, mast cells eosinophils, dendritic cells, and epithelial cells of different organs [5,14]. HBDs are predominantly expressed in epithelial cells [8]. The expression of LL-37 and HBDs can be modulated by different stimuli in a cell and tissue specific manner [15]. Butyrate, a bacterial fermentation product in colon, upregulates cathelicidins in colonic epithelial cells of human and rabbit [16,17], and, thus, playing an important role in host-microbes interaction in the colonic mucosa.

Antibiotics, apart from exerting bactericidal/bacteriostatic effects on pathogens, can render pathogens susceptible to the host immune system, such as killing of bacteria by polymorphonuclear neutrophils (PMNs) [18]. On the other hand, by inducing production and release of microbial components, antibiotics may provoke proinflammatory responses in host cells [19]. Numerous antibiotics also have direct modulatory effects on immune functions [20]. Moreover, antibiotic treatment disrupts the normal colonic flora that may allow colonization and secondary infections by enteropathogens such as *Clostridium difficile*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Salmonella* spp. [21]. Alteration of the microbiota also affects immune homeostasis including expression of AMPs, leading to infections such as increasing susceptibility to *Listeria monocytogenes* [22]. *C. difficile* is the major cause of antibiotic associated diarrhoea (AAD), accounting for 10%–20% of all AAD cases [21]. Clindamycin, extended-spectrum penicillin, cephalosporin and fluoroquinolones including ciprofloxacin are the major antibiotics implicated in *C. difficile* associated diarrhoea (CDAD) [23].

expression of cathelicidins In this study, we determined the effect of several antibiotics on the constitutive and butyrate-induced in colon epithelial cells *in vivo* and/or *in vitro*. Since ciprofloxacin suppressed the butyrate-mediated induction of cathelicidins, we also investigated the influence of ciprofloxacin on the induction of human β-defensins (HBDs) *in vitro*. A genome wide microarray analysis was performed in order to profile the expression of co-regulated genes. Histone modifications and phosphorylation of MAP kinases were assessed for potential regulatory mechanisms. Lastly, to evaluate cathelicidin suppression as a causal link to CDAD, the inhibitory effect of human cathelicidin LL-37 on *C. difficile* was investigated.

2. Results

2.1. Effect of Antibiotics on Expression of the CAMP Gene Encoding LL-37 in HT-29, a Colonic Epithelial Cell Line

A selection of antibiotics, *i.e.*, ciprofloxacin, clindamycin, ofloxacin, levofloxacin, pivmecillinam, azithromycin, ceftriaxone, ampicillin, and isoniazid were screened by real-time PCR for their effect on the *CAMP* gene expression in HT-29 cells in the presence or absence of sodium butyrate (NaB). Stimulation of cells with NaB for 24 h resulted in a significant increase in *CAMP* gene expression compared to unstimulated cells (30–40 fold, $p < 0.001$). Ciprofloxacin suppressed this induction significantly ($p < 0.05$ with 100 μg/mL ciprofloxacin; $p < 0.001$ with 125 and

150 μg/mL ciprofloxacin) in a concentration dependent manner (Figure 1A). Clindamycin also exhibited significant suppression (p = 0.069, 0.016 and 0.028 with 125, 150 and 200 μg/mL clindamycin, respectively), although the degree of suppression was much lower than for ciprofloxacin (Figure 1B). Azithromycin, ofloxacin, and levofloxacin reduced the NaB-induced *CAMP* gene, but the effect was not significant (Figure 1C–E). Pivmecillinam, ampicillin, ceftriaxone, and isoniazid did not show any effect on the *CAMP* gene induction (Figure 1F–I). In the absence of NaB, no antibiotic had any effect on *CAMP* gene expression (data not shown). Thus, ciprofloxacin and to a lesser extent clindamycin significantly down-regulated NaB-induced *CAMP* expression, while other antibiotics exhibited no significant effect on the induction. Similar results were obtained, when the cells were stimulated for 48 h (data not shown). Notably, by trypan blue assay, no effect on cell viability was observed after the stimulation of the cells.

2.2. Effect of Ciprofloxacin and Pivmecillinam on the Expression of LL-37 Peptide and Its Proform hCAP18 in HT-29 Cells

Since a prominent effect of ciprofloxacin was observed on transcriptional level of LL-37 expression, we further investigated the effect on the peptide/protein level by Western blot analysis. The effect of pivmecillinam, a non-responder on *CAMP* gene expression was also evaluated in parallel. Mature LL-37 peptide (4.5 kD) was either present in low level or not detected in unstimulated culture supernatant of HT-29 cells. However, after stimulation with NaB, the expression was increased and the peptide was clearly detected (Figure 2A,C). Up-regulation of the pro-form hCAP-18 (18 kD) was also observed in the culture supernatants of NaB-stimulated cells compared to unstimulated cells (Figure 2A,C). A dose-dependent down-regulation of NaB-induced expression of hCAP-18 and LL-37 was observed with ciprofloxacin (Figure 2A). Ciprofloxacin alone had no obvious effect on constitutive expression of hCAP-18 or LL-37 (Figure 2B). Pivmecillinam had no detectable effect on constitutive or NaB-induced expression of hCAP-18 or LL-37 (Figure 2C,D). These results clearly demonstrate that ciprofloxacin dose-dependently suppresses LL-37 induction by butyrate at both transcriptional and peptide/protein levels in colon epithelial cells.

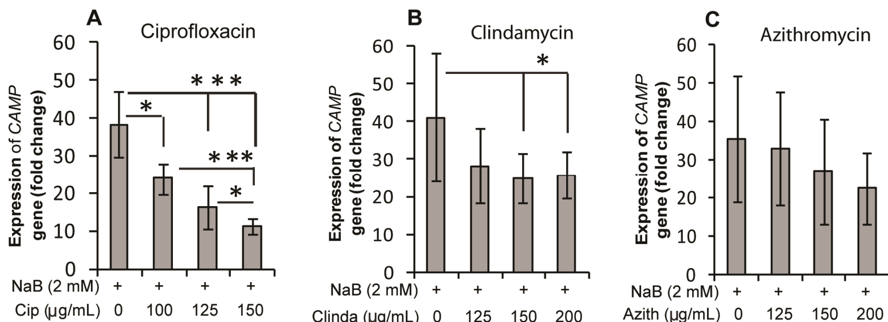

Figure 1. *Cont.*

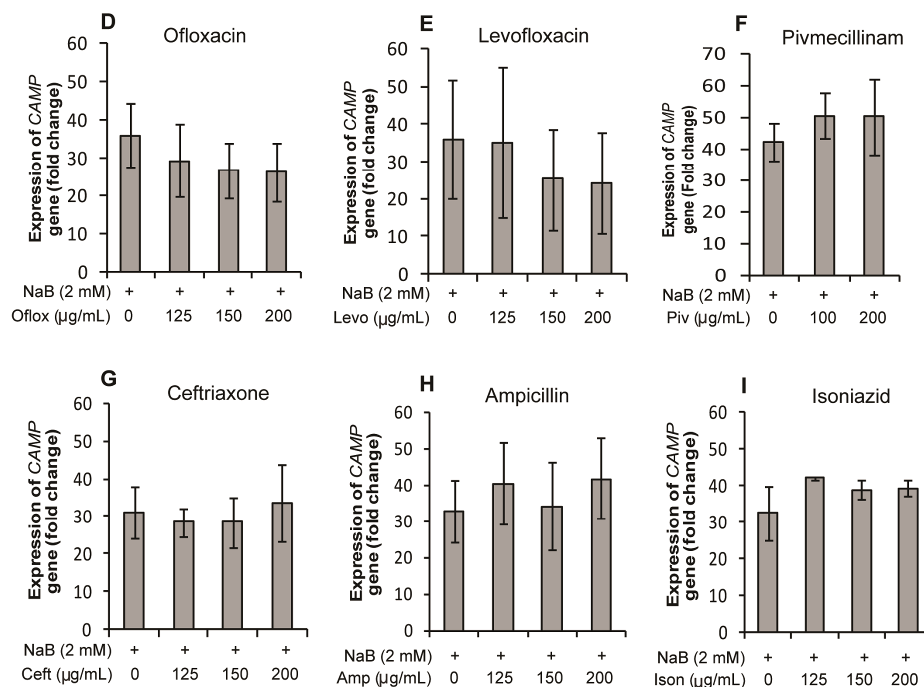

Figure 1. Effect of antibiotics on NaB-induced expression of the *CAMP* gene encoding LL-37 in HT-29 cells. HT-29 cells were stimulated for 24 h with 2 mM NaB alone or in combination with different concentrations of (**A**) ciprofloxacin; (**B**) clindamycin; (**C**) azithromycin; (**D**) ofloxacin; (**E**) levofloxacin; (**F**) pivmecillinam; (**G**) ceftriaxone; (**H**) ampicillin; and (**I**) isoniazid. RNA was extracted from cells and cDNA prepared, which was used to quantify *CAMP* gene (LL-37 transcript) expression by real time qPCR. *CAMP* expression is presented as fold change to control (untreated) cells. Data are given as mean ± SD of seven replicates. One way ANOVA of original data in case of clindamycin or log-transformed data for other antibiotics was utilized in comparing between different groups. Pair-wise effects between groups were compared by the Holm-Sidak *post hoc* comparison procedure. * $p < 0.05$, *** $p < 0.001$. NaB: sodium butyrate.

2.3. In Vivo Effect of Ciprofloxacin and Pivmecillinam on Cathelicidin Expression in Colonic Epithelia

The physiological relevance of the *in vitro* effect of ciprofloxacin and pivmecillinam on cathelicidin expression was investigated in healthy rabbits and a rabbit model of shigellosis, representing infectious diarrhoea. Immunohistochemical analysis revealed significant suppression of the rabbit cathelicidin CAP-18 expression in the rectal epithelium of healthy rabbits treated with ciprofloxacin compared to healthy untreated rabbits ($p < 0.001$) (Figure 3A,B). Notable, butyrate is present in the colon and rectum of healthy rabbits. We confirmed results from our previous study [16], demonstrating

that CAP-18 expression was significantly down-regulated in rectal epithelium of rabbits infected with *Shigella flexneri* compared to healthy rabbits ($p < 0.001$) and treatment with NaB counteracted this down-regulation. Interestingly, when ciprofloxacin was given as adjunct therapy, the induction of CAP-18 was significantly suppressed ($p < 0.001$); the level was even significantly lower than that of infected rabbits ($p < 0.001$) (Figure 3A,B). These data showed that ciprofloxacin has suppressive effect on cathelicidin expression in rectal epithelia of healthy and *Shigella*-infected rabbits. The NaB-induced reappearance of CAP-18 in rectal epithelium of *Shigella*-infected rabbits was not affected by pivmecillinam and there was a significant difference between ciprofloxacin treated and pivmecillinam treated rabbits ($p < 0.001$) (Figure 3A,B). Notably, pivmecillinam treatment reduced the expression of CAP-18 to a lesser extent than ciprofloxacin in healthy rabbits ($p < 0.01$ between ciprofloxacin treated and pivmecillinam treated healthy rabbits) (Figure 3A,B). Most likely this differential reduction of CAP-18 expression reflects effects of ciprofloxacin on both the normal flora with less butyrate production and direct on epithelial cells, while pivmecillinam only affect the butyrate production mediated by the normal flora.

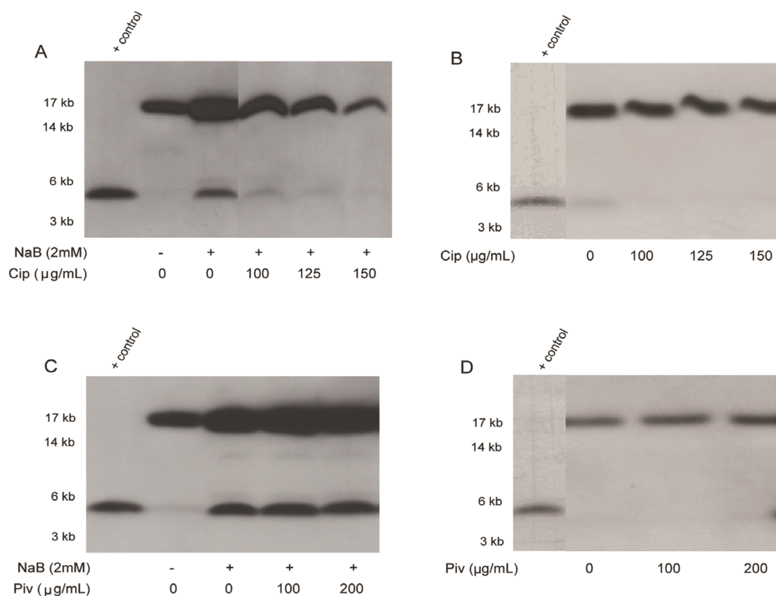

Figure 2. Effect of ciprofloxacin and pivmecillinam on LL-37 peptide and hCAP-18 in HT-29 cell supernatants. HT-29 cells were stimulated for 24 h with NaB and/or ciprofloxacin (**A**,**B**), with NaB and/or pivmecillinam; (**C**,**D**). Release of LL-37 peptide (lower band) and its proform hCAP-18 (upper band) in culture supernatants was detected by Western blot analysis. Representative pictures are given. Synthetic LL-37 peptide was included as positive control. The blot data in panel 2A are composites of two portions of a larger data set, while data of panels 2B and 2D are composites of two experiments. NaB: sodium butyrate; Cip: ciprofloxacin; Piv: pivmecilinam.

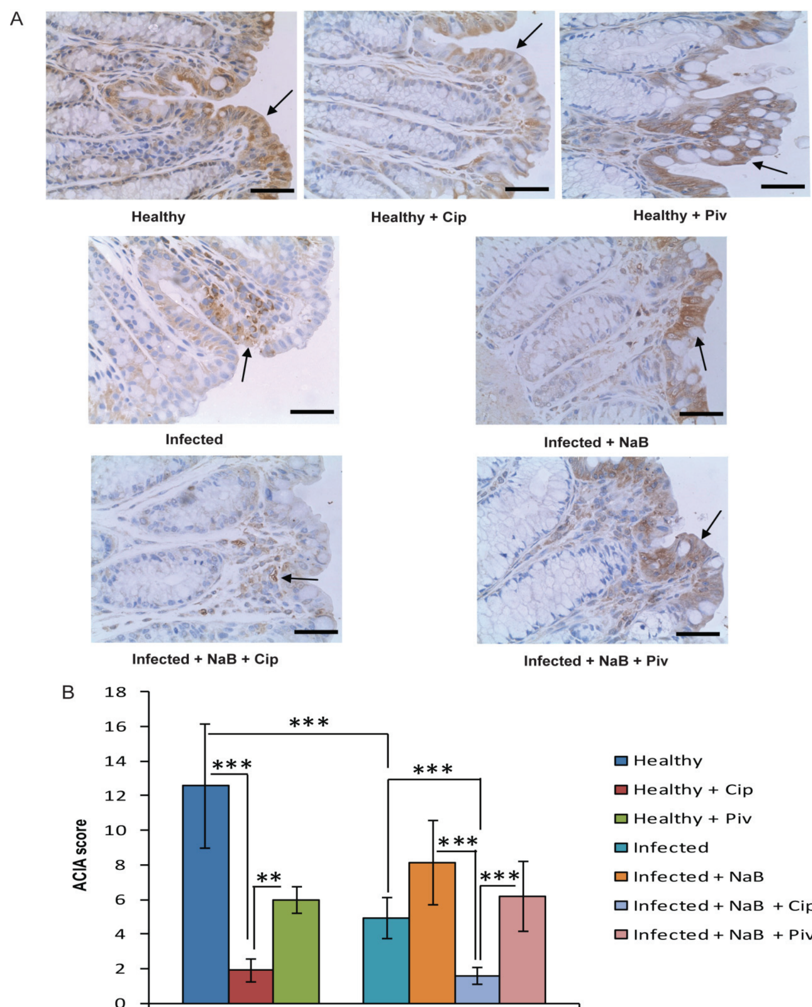

Figure 3. Effect of ciprofloxacin and pivmecillinam on CAP-18 expression in rectal epithelia of rabbit. Healthy rabbits were either treated with ciprofloxacin (n = 3) or pivmecillinam (n = 3) or left untreated (n = 3). *Shigella* infected rabbits treated with 2 mM NaB alone (n = 3) or together with ciprofloxacin (n = 3) or pivmecillinam (n = 3) or left untreated (n = 3). Mucosal sections of rectum were stained with the rabbit cathelicidin CAP-18 specific antibody. (**A**) Representative photomicrographs of CAP-18 immunostaining (arrows). Bars equal to 50 µm; (**B**) Semi-quantification of CAP-18 immunostaining are expressed as ACIA score (See materials and methods). Data are given as mean ± standard deviation. One way ANOVA of log-transformed data was utilized in comparing between different groups. Pair-wise effects between different groups were compared by the Holm-Sidak *post hoc* comparison procedure. ** $p < 0.01$, *** $p < 0.001$. NaB: sodium butyrate; Cip: ciprofloxacin; Piv: pivmecilinam.

2.4. Effect of Ciprofloxacin on the Butyrate-Induced Expression of Human β-Defensins (HBD) Transcripts in HT-29 Cells

We also examined the effect of ciprofloxacin on NaB-induced expression of β-defensins. Stimulation of HT-29 cells with NaB resulted in about 10 and 15 fold induction ($p < 0.001$) of the genes encoding HBD-1 and HBD-3, respectively. With the addition of 150 µg/mL ciprofloxacin, the induced expression of HBD-3 was reduced significantly ($p = 0.019$), whereas HBD-1 induction remained unaffected (Figure 4). NaB did not have any effect on the expression of the gene encoding HBD-2 (data not shown). These results suggest that ciprofloxacin also blocks butyrate-mediated induction of the gene encoding HBD-3 in colonic epithelial cells.

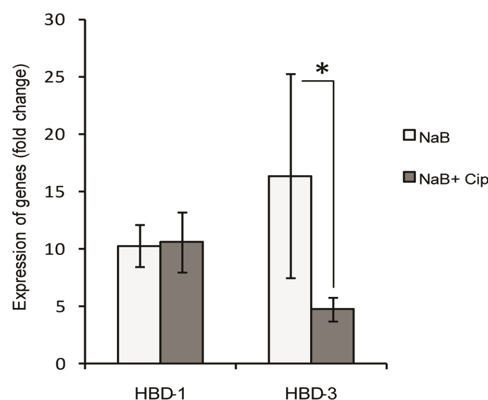

Figure 4. Effect of ciprofloxacin on butyrate-induced expression of HBD-1 and HBD-3 transcripts in HT-29 cells. HT-29 cells were stimulated for 24 h with 2 mM NaB alone or in combination with 150 µg/mL ciprofloxacin. RNA was extracted from cells and cDNA prepared, which was used to quantify expression of HBD-1 and HBD-3 transcripts by real time qPCR. Gene expression is presented as fold change to control (untreated) cells. Data are given as mean ± SD of four replicates. One way ANOVA of log-transformed data was utilized in comparing between different groups. Pair-wise effects between groups were compared by the Holm-Sidak *post hoc* comparison procedure. * $p < 0.05$. NaB: sodium butyrate; Cip: ciprofloxacin; HBD: human β-defensin.

2.5. Suppressive Effect of Ciprofloxacin on Genome-Wide Expression Profile of NaB-Induced Genes in HT-29 Cells

To further investigate the suppressive effect of ciprofloxacin on NaB-induced genes, we performed a microarray analysis on RNA, extracted from HT-29 cells that were stimulated with NaB alone or in combination with ciprofloxacin. The microarray data are deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series Accession Number GSE45220 [24]. Similar to the *CAMP* gene, the expression of several immune genes was enhanced in HT-29 cells by NaB and was subsequently suppressed by ciprofloxacin. Table 1 depicts the most interesting genes

from this set that are associated with mucosal immunity including mucins, S100 calcium binding proteins and RNase A. Genes encoding the processing enzyme kallikrein, G protein coupled receptors (GPCR), interleukin receptors, interleukin 18 and nitric oxide synthase were also co-regulated with the *CAMP* gene (Table 1). The entire list of genes that were co-regulated with the *CAMP* gene is shown in Supplementary Table S1.

Table 1. Selected Genes, up-regulated with NaB treatment and subsequently suppressed with co-administration of ciprofloxacin.

Entrez Gene ID	Gene Symbol	Description	Upregulation (NaB *vs.* Unstimulated)		Downregulation (NaB + Cip *vs.* NaB)	
			Fold Change	*p*-value	Fold Change	*p*-value
2840	GPR17	G protein-coupled receptor 17	4.37	0.0002	−2.93	0.0013
84,873	GPR128	G protein-coupled receptor 128	2.7	0.0098	−3.4	0.006
3816	KLK1	kallikrein 1	2.6	0.0012	−1.99	0.014
6035	RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	8.57	0.0011	−1.91	0.042
6274	S100A3	S100 calcium binding protein A3	10.54	0.00005	−3.02	0.016
6271	S100A1	S100 calcium binding protein A1	7.72	0.0073	−2.47	0.027
57,402	S100A14	S100 calcium binding protein A14	2.59	0.0056	−1.95	0.015
4846	NOS3	nitric oxide synthase 3 (endothelial cell)	4.05	0.0002	−3.88	0.0008
143,662	MUC15	mucin 15, cell surface associated	17.2	0.03	−4.7	0.05
394,263	MUC21	mucin 21, cell surface associated	3.27	0.0024	−2.2	0.01
3606	IL18	interleukin 18 (interferon-gamma-inducing factor)	2.93	0.0013	−14.3	0.0003
400,935	IL17REL	interleukin 17 receptor E-like	2.57	0.0052	−1.96	0.011
3554	IL1R1	interleukin 1 receptor, type I	6.32	0.0071	−3.26	0.013
3557	IL1RN	interleukin 1 receptor antagonist	3.4	0.02	−3.2	0.04

NaB: sodium butyrate (2 mM); Cip: ciprofloxacin (150 μg/mL).

2.6. Epigenetic Modifications are Involved in the Suppressive Effect of Ciprofloxacin in HT-29 Cells

Butyrate and phenylbutyrate are histone deacetylase inhibitors (HDACi) and have been demonstrated to induce *CAMP* gene expression [25–27]. To investigate potential epigenetic effects of NaB and/or ciprofloxacin in HT-29 cells, we evaluated acetylation of histone H3 and H4 by Western blot analysis of histone extracts. Phosphorylation of histone H3 has also been implicated in the induced expression of several genes such as c-fos, c-jun, additional activator protein-1 (AP-1) family genes and c-myc [28]. Hence, phosphorylation of H3 was also assessed by Western blot analysis of histone extracts. NaB augmented acetylation of histone H3 at Lys14, histone H4 at Lys16 and phosphorylation of histone H3 at Ser10 within 2 h of stimulation that lasted up to 24 h (Figure 5A–D). Ciprofloxacin exhibited no effect on the NaB-induced acetylation of H3 and H4 (Figure 5A,B). Interestingly, ciprofloxacin dose-dependently reduced the induction of phosphorylation of histone

H3 at Ser10 (Figure 5C). Changes of H3 phosphorylation in HT-29 after treatment with NaB alone or in combination with ciprofloxacin was confirmed by the immunofluorescence staining of cells (Figure 5D). Ciprofloxacin alone had no effect on histone phosphorylation and acetylation (data not shown). These findings indicate the involvement of both acetylation and phosphorylation of histones in butyrate-induced genes. However, only phosphorylation of histone H3 was correlated with the ciprofloxacin-mediated down-regulation of butyrate-induced genes.

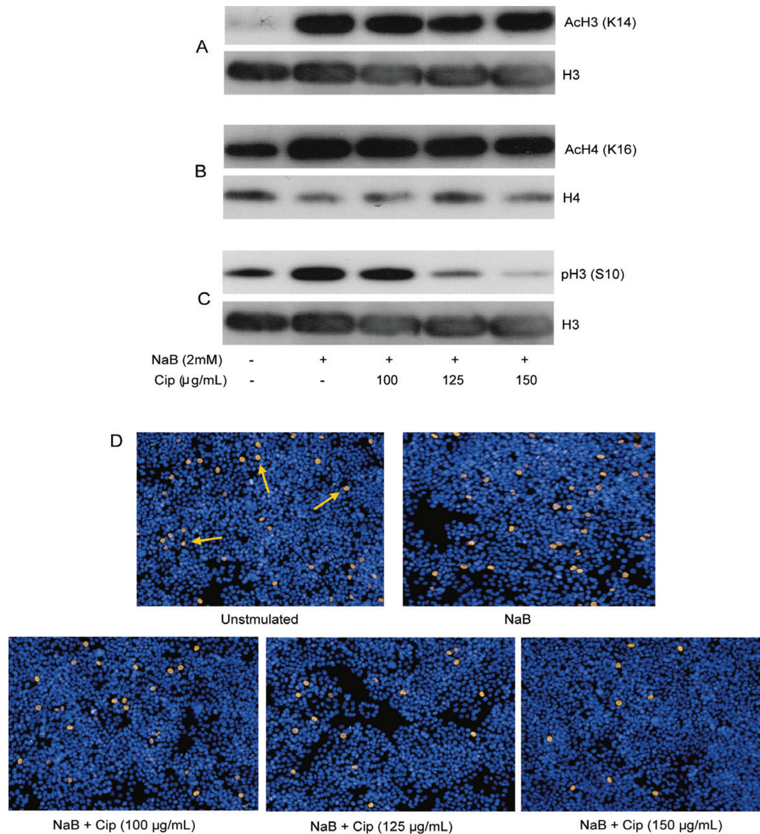

Figure 5. Histone modifications in HT-29 cells after treatment with NaB alone or in combination with ciprofloxacin. HT-29 cells were stimulated for 24 h with 2 mM NaB alone or in combination with different concentrations of ciprofloxacin. Histone was extracted from cells and utilized for Western blot analysis to detect (**A**) acetylation of histone H3 Lys14; (**B**) acetylation of histone H4 Lys16; (**C**) phosphorylation of histone H3 Ser10; (**D**) Phosphorylation of histone H3 at Ser10 was also detected by immunofluorescence staining of the cells. Arrows indicate examples of positively stained cells. NaB: sodium butyrate; Cip: ciprofloxacin; AcH3(K14): Acetylation of histone H3 at Lys14; AcH4(K16): Acetylation of histone H4 at Lys16; pH3(S10): Phosphorylation of histone H3 at Ser10.

To approach the involvement of MAP kinase signaling pathway, the phosphorylation of ERK and p38 in HT-29 cells was investigated after treatment of the cells with NaB and/or ciprofloxacin. By Western blot analysis of cell lysates, no differences between treatment groups were observed at any time point starting from 5 min to 24 h (data not shown).

2.7. *In Vitro Effect of Synthetic LL-37 Peptide on C. Difficile*

To investigate if the clinical observation that ciprofloxacin causing overgrowth of *C. difficile* might be associated with inhibitory effect on cathelicidin expression in colonic epithelia, the antibacterial activity of LL-37 against *C. difficile* was evaluated. Incubation of two clinical isolates of ciprofloxacin resistant *C. difficile* with 5 µM LL-37 led to four log reduction of colony forming unit (CFU) compared to that obtained by incubating without LL-37 ($p < 0.05$) (Figure 6), showing that LL-37 is able to kill *C. difficile in vitro.*

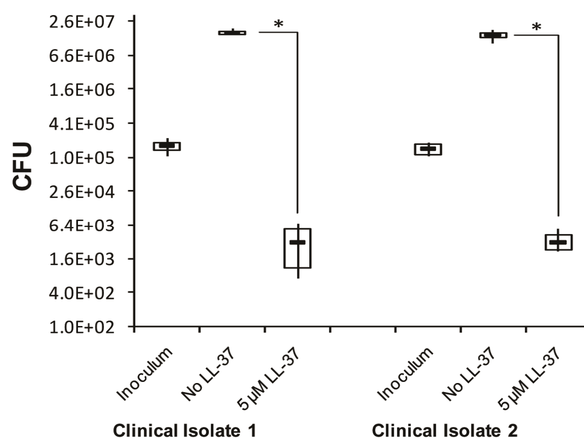

Figure 6. Inhibitory effect of synthetic LL-37 peptide on clinical isolates of *Clostridium difficile*. Two clinical isolates of *C. difficile* that were metronidazole and vancomycin sensitive but resistant to ciprofloxacin were treated with or without LL-37 peptide in MHB in wells of microtiter plate. Bacterial suspensions from individual wells were plated on blood-agar for counting of CFU. Lower and upper boundaries of the boxes and the horizontal bars in between indicate 25th percentile, 75th percentile and group median, respectively. Vertical lines through the boxes join the minimum and maximum values. Kruskal-Wallis ANOVA on Ranks of original data was utilized in comparing between different groups. Tukey test was used to compare the pair wise effects between treatment groups. * $p < 0.05$. CFU: colony forming unit; MHB: Mueller-Hinton broth.

3. Discussion

In this study, we demonstrate that ciprofloxacin and clindamycin significantly suppress butyrate-mediated induction of cathelicidin in the colon epithelial cell line HT-29. Suppressed expression of cathelicidin by ciprofloxacin was also observed *in vivo* in the rectal epithelium of

healthy rabbits, where butyrate is present, and butyrate-treated *Shigella*-infected rabbits. Moreover, ciprofloxacin suppressed butyrate-induced expression of HBD-3 in HT-29 cells. The direct effect of ciprofloxacin *in vivo* is demonstrated in healthy rabbits by higher suppression of CAP-18 by ciprofloxacin as compared to pivmecillinam. Both antibiotics disrupt the normal flora, leading to reduced butyrate level and lower CAP-18 expression. However, ciprofloxacin exerts additional suppression by directly affecting epithelial cells. The induction of the two peptides by butyrate and subsequent reduction by ciprofloxacin is correlated to the phosphorylation status of histone H3 at Ser10. Furthermore, the peptide LL-37 exhibited inhibitory effect on the growth of two ciprofloxacin resistant strains of *C. difficile in vitro*.

Ciprofloxacin, a second generation fluoroquinolone antibiotic, has a broad spectrum bactericidal activity and acts by inhibiting bacterial DNA gyrase/topoisomerase. Several antibiotics including ciprofloxacin and other fluoroquinolones are known to exert modulatory effects on immunity such as down-regulation of proinflammatory cytokines [22,29]. In addition, ciprofloxacin modulates phagocytic and killing capacity of neutrophils and macrophages [30,31], and affects the expression of toll-like receptors, CD14, CD40 and intracellular adhesion molecules in monocytes [32,33]. Furthermore, ciprofloxacin inhibits cytokine-induced nitric oxide production in human colon epithelial cells [34]. Here, we have shown that ciprofloxacin dose-dependently inhibits butyrate-induced expression of the human cathelicidin LL-37 and β-defensin (HBD)-3 in HT-29 cells. Our study indicates that the general mechanism on gene expression by ciprofloxacin is of epigenetic character through reduced phosphorylation of histone H3 that may explain the broad effects described for this antibiotic.

We did not detect any effect on LL-37 expression by ciprofloxacin in HT-29 cells in the absence of butyrate. This could be in agreement with previous observations that quinolones only exert immunomodulatory effects in the presence of co-stimulant(s) such as endotoxin, cytokines or stress [32–35]. Butyrate is constantly produced in the large intestine by the fermentation of dietary fibers, which coincides with high expression of CAP-18 and LL-37 in the rectal epithelia of healthy rabbits and humans, respectively (Figure 3 and references [16,36]). This *in vivo* induced expression of CAP-18 by butyrate in the rectal epithelium of healthy rabbits was also suppressed by ciprofloxacin. In addition, ciprofloxacin inhibited the counteracting effect of butyrate on CAP-18 expression in the rabbit model of shigellosis.

In vitro suppression of LL-37 clearly demonstrates the direct effect of ciprofloxacin on host epithelial cells. It is conceivable that the *in vivo* reduction of CAP-18 by ciprofloxacin is also a direct effect on rectal epithelial cells, not only an effect caused by killing of the microbiota. On the other hand, pivmecillinam had no suppressive effect on CAP-18 expression in epithelial cells *in vitro*, but reduced the cathelicidin levels in healthy rabbits, indicating a secondary effect due to less butyrate production by the normal flora.

Ofloxacin and levofloxacin, second and third generation of flouroquinolones, respectively, did not significantly suppress the butyrate-induced LL-37 expression in HT-29 cells. The difference in the degree of suppression might be attributed to structural differences of these fluoroquinolones. Ciprofloxacin possesses a cyclopropyl ring at the quinolone ring, but ofloxacin and levofloxacin lack

this ring. Quinolones having cyclopropyl ring were shown to have enhanced anti-leukaemic and haematopoietic effects, as opposed to the quinolones lacking this ring [37,38].

Clindamycin, a lincosamide antibiotic, at higher concentrations than ciprofloxacin significantly suppressed the induction of LL-37 by butyrate in HT-29 cells. In contrast, azithromycin, pivmecillinam, ampicillin, ceftriaxone, and isoniazid exhibited no significant suppression. These results show that the suppressive effect observed is specific for ciprofloxacin and clindamycin, indicating also a direct effect of these antibiotics on host cells and not only mediated by the disturbance of the microbiota composition.

Numerous additional genes were up-regulated in HT-29 cells after stimulation with NaB as revealed by microarray analysis, emphasizing LL-37 as a marker for mucosal immunity. Interestingly, many of these butyrate-induced genes were down-regulated by ciprofloxacin and are linked to innate immunity, including S100 calcium binding proteins, RNase A, mucins, a cathelicidin processing enzyme (kallikrein) and G protein coupled receptors (GPCR). Calgranulins (S100A8, S100A9 and S100A12) and calprotectin (heterodimer of S100A8 and S100A9) belonging to the family of S100 calcium binding proteins, which exhibit antimicrobial and immunomodulatory properties [39]. Some members of the RNase A superfamily are also involved in host defense [40]. Members of the kallikrein family were shown to process hCAP18 into LL-37 followed by subsequent cleavage to modify the activity of LL-37 in skin [41]. Interestingly, it was recently reported that doxycycline inhibits kallikrein-related peptidases and thus inhibits the generation of active LL-37 [42]. Members of G protein coupled receptors (GPCR) have been shown to mediate the function of short chain fatty acids including butyrate [43,44]. GPCR might have interesting implications on butyrate- and/or antibiotics-mediated AMP expression in colonic epithelium. Thus, our results demonstrate a broad effect of ciprofloxacin on the expression of innate immune genes that may promote secondary infections.

Western blot analysis of histone extracts of HT-29 cells revealed a rapid and persistent increase of phosphorylation and acetylation of histone H3 at Ser10 and Lys14, respectively, after stimulation of cells with NaB. Hyperacetylation was also found at Lys16 of histone H4. Notably, ciprofloxacin, at all time points (2 h, 4 h, 6 h and 24 h), dose-dependently suppressed the induced phosphorylation of histone H3, which was confirmed by immunofluorescence staining of the cells. However, ciprofloxacin did not affect the induced acetylation of histones. These findings imply epigenetic modifications as part of the ciprofloxacin mediated suppression of butyrate-induced gene expression. We have earlier shown that cyclohexamide, an inhibitor of protein synthesis, blocked the *CAMP* gene induction by NaB [27]. Therefore we propose that general histone modification(s) allow expression of genes encoding regulatory proteins for the *CAMP* gene.

Impaired immune responses have been reported as important factors for CDAD, apart from the disruption of the normal gut flora [45]. Here, we have demonstrated that LL-37 exhibits antibacterial activity against two clinical isolates of *C. difficile in vitro*, which suggests that suppression of AMPs after antibiotic treatment might favor the overgrowth of *C. difficile*. In a recent study, intra-colonic administration of mCRAMP to *C. difficile*-infected mice improved toxin A-mediated colitis outcome [46]. α-defensins were also shown to inhibit the cytotoxic effect of *C. difficile* toxin B [47]. Moreover, in a previous study, down-regulation of the antimicrobial protein RegIIIγ in the small intestinal mucosa of antibiotic-treated mice was shown to increase the colonization of gut by

vancomycin-resistant Enterococcus [48]. These findings indicate that suppression of AMPs may facilitate infection of the gut mucosa by enteropathogens, such as *C. difficile* and resultant disease manifestation. However, *C. difficile* infection in wild type and CRAMP$^{-/-}$ mice demonstrated similar colonic inflammation in both group of mice [46]. Since, mice were pretreated with antibiotic cocktail to induce *C. difficile* infection, it is possible that mCRAMP was down-regulated by the antibiotics in the colonic epithelium of wild type mice, favoring *C. difficile* infection. Moreover, Salzman *et al.* has shown that enteric defensins regulate the composition of the intestinal flora [12], suggesting a critical role of AMPs in intestinal homeostasis. Hence, by suppressing AMPs, antibiotic treatment of ciprofloxacin or clindamycin may further contribute to alteration of the microbial niche, promoting CDAD.

4. Experimental

4.1. Ethics Statement

Experiment in animal model (Research protocol # 2007-065) was approved by the Animal Experimentation Ethics Committee (AEEC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) on May 07, 2008. All experiments conformed to the rules and guidelines of icddr,b, which was developed based on the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). Animal experimentation in this study complied with the "3Rs". REPLACEMENT: Live animals had to be used to evaluate the physiological relevance of the *in vitro* effect of ciprofloxacin and pivmecillinam on cathelicidin expression. REDUCTION: Minimum numbers of animals were used. REFINEMENT: Rabbits were kept in individual cages and provided with food and water ad libitum. Rabbits were sacrificed within a very short period with an overdose of intravenous sodium pentobarbital (66 mg per kg body weight). Appropriate veterinary care was taken for assessing and preventing pain and distress by expert veterians of the animal facility.

4.2. Peptides, Antibodies, Antibiotics and Sodium Butyrate

Synthetic bioactive peptides LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and CAP-18 (GLRKRLRKFRNKIKEKLKKIGQKIQGLLPKLAPRTDY), and affinity-purified chicken anti-CAP-18 antibody were purchased from Innovagen (Lund, Sweden). Monoclonal antiserum against LL-37 was developed in mouse hybridoma cells [49]. Sodium butyrate (NaB), ciprofloxacin, pivmecillinam, ofloxacin, levofloxacin, azithromycin, clindamycin, ampicillin, ceftriaxone and isoniazid were purchased from Sigma-Aldrich (St Louis, MO, USA). Rabbit polyclonal antibodies to phospho-histone H3 (S10) or acetyl-histone H3 (K14) and mouse monoclonal antibodies to histone H3 or H4 were from Abcam (Cambridge, UK). The source of polyclonal antibody to acetyl-histone H4 (K16) was Active Motif (La Hulpe, Belgium). Rabbit polyclonal antibodies to phospho-ERK and phospho-p38 were purchased from Cell signaling technology Inc. (Danvers, MA, USA).

4.3. Cell Line and Growth Conditions

HT-29 (ATCC, HTB-38), a human colonic epithelial cell line, was maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 25 mM HEPES, 2 mM L-glutamine and penicillin-streptomycin (PEST) (Life Technologies, New York, NY, USA) at 37 °C in 5% CO_2.

4.4. Primers

The sequences of the primers for real time qPCR were: CAMP (LL-37 transcript), forward 5'-TCACCAGAGGATTGTGACTTCAAC-3' and reverse 5'-TGAGGGTCACTGTCCCCATAC-3'; human β-defensin (HBD) -1 transcript, forward 5'-ATGGCCTCAGGTGGTAACTTTC-3' and reverse 5'-CACTTGGCCTTCCCTCTGTAAC-3'; HBD-2 transcript, forward 5'-GCCTCTTCCAG GTGTTTTTG-3' and reverse 5'-GAGACCACAGGTGCCAATTT-3'; HBD-3 transcript, forward 5'-GCTGCCTTCCAAAGGAGGA-3' and reverse 5'-TTCTTCGGCAGCATTTTCG-3'.

4.5. Bacterial Strains

A clinical isolate of *Shigella flexneri* 2a, isolated from patient's stool at Dhaka Hospital of icddr,b was used to infect rabbits [16]. For the *in vitro* killing assay, two strains of *Clostridium difficile*, isolated from patients stool at Karolinska University Hospital, Huddinge, Stockholm, Sweden were used. These two clinical isolates of *C. difficile* were metronidazole and vancomycin sensitive but resistant to ciprofloxacin. All these bacterial strains were isolated from routine clinical stool samples and these clinical isolates are anonymous.

4.6. Stimulation of Cells

HT-29 Cells, grown up to 80%–90% confluence in tissue culture plates (Corning, Steuben County, NY, USA), were stimulated with 2 mM NaB and/or with different concentrations of several antibiotics for 24 or 48 h. Stimulations were performed in culture medium in the absence of FCS and PEST. Cells incubated with only culture medium served as negative control. The viability of cells was checked by trypan blue assay. To enrich peptides/proteins in culture supernatants, trifluoroacetic acid (TFA) was added to supernatants and were applied to acetonitrile (AcN)-activated OASIS cartridges (Waters, Milford, MA, USA) equilibrated in aqueous 0.1% TFA. Bound peptides/proteins were eluted with 80% aqueous AcN in 0.1% TFA and lyophilized. RNA was extracted from cells utilizing RNeasy RNA purification kit according to the manufacturer's instruction (Qiagen GmbH, Hilden, Germany). Corresponding cDNA was synthesized using a reverse transcriptase kit (Biorad Laboratories Inc., Berkeley, CA, USA).

4.7. Real-Time RT-PCR

cDNA samples from HT-29 cells were used to measure the level of the *CAMP* gene and genes encoding HBD-1, HBD-2, or HBD-3 relative to the housekeeping gene 18S rRNA. Measurements were performed by SYBRGreen based real-time quantitative RT-PCR, using a CFX-96 real time

system instrument (Biorad). Results were expressed as fold changes in the treated cells compared to control cells.

4.8. Microarray Analysis

Quality and integrity of RNA from HT-29 cells were assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Individual samples were hybridized to Affymetrix Human Gene 1.1 ST arrays. The arrays were scanned using the GeneTitan scanner. Probe cell intensity (CEL) files were preprocessed in Affymetrix Expression Console (EC, version 1.1; Santa Clara, CA, USA) using the following methods; (i) summarization: Probe Logarithmic Intensity Error (PLIER); (ii) background correction: Perfect Match probes-Guanine, Cytosine composition-based background correction (PM-GCBG); (iii) normalization: Global Median. Expression levels between control and treatment groups and between treatment groups were compared using two-tailed, Student's *t*-test.

4.9. Cell Lysis and Histone Extraction

Stimulated/unstimulated HT-29 cells were lysed with RIPA buffer (Sigma-Aldrich). After centrifugation at 13,000 rpm for 10 min at 4 °C, cell lysates were collected and the remaining cell pellets were used for histone extraction following the method of Lee *et al.* with some modifications [50]. Briefly, cell pellets were washed twice with PBS and resuspended in ice-cold 0.4 M H_2SO_4. Pellets were broken down with brief sonication and incubated overnight on a shaker at 4 °C. After centrifugation at 13,000 rpm for 1 h at 4 °C, supernatants were collected and histones were precipitated with ice-cold acetone overnight at −20 °C. The resulting translucent pellets of histones after centrifugation at 13,000 rpm for 10 min at 4 °C were air dried, resuspended in ice-cold water and used for Western blot analysis.

4.10. Western Blot Analysis

Discontinuous sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), employing 4%–12% NuPAGE Ready Gels (Life Technologies) followed by Western blot analysis were utilized for detection of LL-37 in cell culture supernatants, phosphorylation of ERK and p38 in cell lysates, and phosphorylation/acetylation of histones in histone extracts. After electrophoretic separation, materials in the gel were blotted onto polyvinyldifluoride (PVDF) membrane (Life Technologies) by electrophoretic transfer. Immunoreactivity was detected by subsequent incubation of the membrane with specific primary antibodies and corresponding secondary antibodies conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). The enhanced chemiluminescence (ECL) Western blotting detection system (GE Healthcare UK Ltd., Buckinghamshire, UK) was utilized to visualize protein/peptide bands.

4.11. Immunofluorescence Staining of Phosphorylated Histone H3 at Ser10

HT-29 cells were stimulated with NaB and/or ciprofloxacin in 96 well tissue culture imaging plate (BD Biosciences, Woburn, MA, USA). Cells were fixed with 2% paraformaldehyde followed by permeabilization with 0.1% triton-X-100 (ICN Biomedicals, Solon, OH, USA). Cells were then subsequently incubated with rabbit polyclonal antibody for phospho-histone H3 (S10) and anti-rabbit antibody conjugated with AlexaFluor 594 (Life Technologies). DAPI, a nuclear die, was added to the cells and immunofluorescence staining was detected in an Operetta image analyzer (Perkin Elmer Inc., Alameda, CA, USA).

4.12. Rabbit Model

Inbred New Zealand white rabbits (Charles River Laboratories, Wilmington, MA, USA) of either sex, aged between 2.5–3 months and weighing 1.8–1.9 kg were maintained in the animal resource facilities of icddr,b. Healthy rabbits, free of enteric pathogens (e.g., *Salmonella*, *Shigella*, and *Vibrio cholera*) and coccidia were studied. Twelve rabbits were infected with *Shigella flexneri* 2a preceded by 36 h of starvation. Bacterial suspension [10^9 CFU in 7 ml of normal saline (0.9% wt/vol, pH 7.2)] was given via sterile orogastric feeding tube to each rabbit. Rabbits developed dysentery within 24 h of bacterial inoculation. The infected rabbits were orally treated with NaB (0.14 mmol/kg body weight/dose) ($n = 3$), or NaB in combination with ciprofloxacin/pivmecillinam (20 mg/kg body weight/dose) ($n = 3$ per antibiotic group) in 20 mM sodium chloride (pH 7.2) by utilizing sterile feeding tubes. The treatments were given twice daily (at around 10 am and 4 pm) for three consecutive days. The remaining three infected rabbits were kept untreated. Healthy rabbits were either treated with ciprofloxacin ($n = 3$) or pivmecillinam ($n = 3$) or left untreated ($n = 3$). When the treatment regime was over, all rabbits were sacrificed with an overdose of intravenous sodium pentobarbital (66 mg/kg body weight) (Sigma-Aldrich). The abdomen of each sacrificed rabbit was opened and sections of rectum were collected in 10% buffered formalin and utilized for immunohistochemical evaluation.

4.13. In Situ Immunohistochemical Staining and Quantification of CAP-18 Peptide/Protein Expression in Rectal Mucosa of Rabbits

Formalin fixed tissue pieces of rectum were embedded in paraffin and cut into three micron thick sections. Sections were deparaffinized, and stained with CAP-18 antibody (6.8 μg/mL) and corresponding secondary antibody. Immunohistochemical staining of CAP-18 was analyzed by using a microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the image analysis system Quantimate Q550 (Leica). CAP-18 staining were quantified in the epithelial areas in each tissue section and the results were given as ACIA (Acquired Computerized Image Analysis) score, *i.e.*, total positively stained area x total mean intensity (1–256 levels per pixel) of the positive area divided by total cell area [51].

4.14. *In Vitro* Bacterial Killing

LL-37 peptide (5 µM) was added to the isolates (see under bacterial strains) of *C. difficile* (10^5 CFU) in Mueller-Hinton broth (MHB; Becton Dickinson, NJ, USA) in wells of microtiter plate (Nunc, Thermo Fisher Scientific, NY, USA) in a final volume of 200 µL. Control wells contained MHB alone and bacteria in MHB without LL-37. The plate was incubated for 48 h in an anaerobic jar at 37 °C. Bacterial suspension/media from individual wells were plated on blood-agar for counting of CFU.

4.15. Statistical Analyses

Statistical analyses were performed by using Sigma STAT for Windows version 3.1 and SPSS. Data were expressed as mean ± standard deviation or median with 25th and 75th percentiles. One way ANOVA was utilized in comparing effects between treatment groups. Data that were not normally distributed and/or failed the equal variance test were log transformed before ANOVA analysis. When significant effect was found between treatment groups, the pair-wise effects between treatment groups were compared by the Holm-Sidak *post hoc* comparison procedure. If the normality and/or equal variance test failed even after log-transformation, original data were analyzed by Kruskal-Wallis ANOVA on Ranks. After getting significant effect between treatment groups, the Tukey test was used to compare the pair-wise effects between treatment groups. Probabilities were regarded as significant when $p < 0.05$.

5. Conclusions

Our study shows that ciprofloxacin and clindamycin significantly downregulate butyrate-mediated induction of innate immune components in the large intestinal epithelia. The prevailing explanation for CDAD pathogenesis is that antibiotic treatment disturbs the normal flora. Here, we demonstrate that the antibiotic-mediated suppression of innate immune effectors might also contribute to CDAD.

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Author Contributions

P.S., S.J., P.B., R.R., G.H.G. and B.A. conceived and designed the experiments. P.S., A.M. and A.A.M. performed the laboratory experiments. P.S. carried out the statistical analyses. R.R. and B.A. supplied reagents/materials/analysis tools. P.S., G.H.G. and B.A. drafted the manuscript. S.J., P.B. and R.R. revised the manuscript. All authors approved the final version of the manuscript before the submission.

Conflicts of Interest

The authors declare no conflict of interest.

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