REVIEW

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Antiviral potential of cathelicidins

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ABSTRACT: The global burden of morbidity and mortality arising from viral infections is high; however, the development of effective therapeutics has been slow. As our understanding of innate immunity has expanded over recent years, knowledge of natural host defenses against viral infections has started to offer potential for novel therapeutic strategies. An area of current research interest is in understanding the roles played by naturally occurring cationic host defense peptides, such as the cathelicidins, in these innate antiviral host defenses across different species. This research also has the potential to inform the design of novel synthetic antiviral peptide analogs and/or provide rationale for therapies aimed at boosting the natural production of these peptides. In this review, we will discuss our knowledge of the antiviral activities of cathelicidins, an important family of cationic host defense peptides, and consider the implications for novel antiviral therapeutic approaches.

The global burden of morbidity and mortality arising from viral infections is high and there is an unmet need for the development of effective therapeutics. Current therapies are generally expensive, highly virus specific (requiring early viral identification) and can have a narrow window in disease progression during which application is required for clinical efficacy. These factors, and the pandemic threat posed by the possible emergence of new strains (e.g., influenza and novel coronaviruses), highlight the urgent need for novel broader-spectrum antiviral intervention strategies. Our advancing knowledge of innate host defenses against viral infection may hold the key to developing novel therapeutic approaches. The antiviral potential of naturally occurring cationic host defense peptides (CHDPs; also known as antimicrobial peptides) has recently received increasing attention, with interest in both the direct microbicidal and immunomodulatory properties of these peptides. In addition to enhancing our understanding of the roles CHDPs play in defense against viral infection, this research has the potential to inform the design of novel synthetic antiviral peptide analogs and/or provide the rationale for therapies aimed at boosting the natural production of these peptides.

Cationic host defense peptides

CHDPs constitute a critical component of the innate immune system [1] with both microbicidal properties and the potential to modify inflammation and immunity [2]. These small, positively charged peptides are conserved throughout evolution across vertebrates and invertebrates, with the two major families in mammals being cathelicidins and defensins. The broad-spectrum bactericidal potential of certain CHDPs has provided the primary focus for the field, with interest in their

KEYWORDS

• adenovirus • antimicrobial peptide • cationic host defense peptide • herpes simplex virus • HIV • influenza • innate immunity • respiratory syncytial virus • vaccinia virus • virus

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development as therapeutics for bacterial infections, either as an alternative or complementation to antibiotics. However, there is a relative paucity of research on the nature and scope of the antiviral properties of these peptides, regarding both the direct virucidal potential and the capacity to modify the outcome of viral infection through modulation of inflammation and immunity. A number of studies now suggest that CHDPs, including cathelicidins, may play important roles in host defense against viral infection and be significant in the development of novel therapeutic strategies.

In addition to their direct microbicidal activities, cathelicidins and defensins have demonstrated pleiotropic immunomodulatory and inflammomodulatory potential [3,4]. The relative significance of any direct microbicidal properties versus these modulatory roles in host defense against infection remains unclear, but is of increasing interest. These peptides may consequently offer the potential to inform the development of novel therapeutics that target both the microbe and the nature and magnitude of the innate and adaptive immune responses. Indeed, the latter approach may be of particular importance with regard to avoiding the rapid development of resistant strains. However, despite this undoubted potential of CHDPs, mechanistic studies are still in their infancy, particularly with regard to antiviral properties. This review seeks to examine the potential of cathelicidins as antiviral agents and the extent to which their capacity to modulate inflammation and immunity may be relevant in this regard.

Cathelicidins

Cathelicidins are defined by the presence of an N-terminal signal sequence, a conserved cathelin-like domain and a variable C-terminal domain, which becomes the mature functional peptide upon proteolytic cleavage. The cathelin-like domains have high sequence homology to cathelin, a porcine leukocyte protein belonging to the cystatin family of cysteine protease inhibitors. However, the cathelin-like domain of the sole human cathelicidin hCAP-18 has recently been shown to have no cysteine protease inhibitory function [5]. The mature cathelicidin peptides range from 12 to 88 amino acids and, in relative contrast to the defensins, show great diversity across species; from proline-rich structures and disulfide bond-stabilized β -hairpins, to linear peptides, which form amphipathic α -helices

upon interaction with lipid membranes [6]. Thus, although classified as the 'cathelicidin family', this diversity raises questions regarding the functional conservation between the mature cathelicidin peptides of different species. Furthermore, whereas humans, mice, rats and rabbits express only a single cathelicidin, other species, such as pigs and cows, express multiple, more divergent cathelicidins. This review will focus primarily on human CAP-18 (hCAP-18), murine CRAMP (mCRAMP) and porcine cathelicidins.

Human cathelicidin

In humans, the cathelicidin hCAP-18 is encoded by the *CAMP* gene on chromosome 3p21.3. hCAP-18 is primarily found in the specific granules of neutrophils and is cleaved extracellularly by proteinase-3 [7] to produce LL-37, a linear, 37-amino acid peptide with two leucine residues at the N-terminus, as the dominant cleavage product. In addition, hCAP-18/LL-37 can be synthesized and released by epithelial cells in an inducible manner and can be detected in a broad range of body fluids, including airway surface liquid (via bronchoalveolar lavage), sweat, saliva, semen, milk and vernix caseosa (the sebum-rich substance that covers the skin of a newborn human baby [2]). Leukocytes such as macrophages, eosinophils, lymphocytes and, to a lesser extent, mast, NK, B and T cells, can also express this peptide [8]. The LL-37 peptide was initially characterized to be α -helical in structure [9], and full mapping by 3D nuclear magnetic resonance has revealed a curved amphipathic helix with a disordered C-terminus [10]. Although LL-37 peptide is the major mature cleavage form, alternative fragments can also be generated by serine proteases (e.g., kallikreins) in keratinocytes and sweat [11,12]. In addition, an ALL-38 form can be generated by gastricin cleavage in semen [13]. Studies have also demonstrated that smaller, naturally occurring and synthetic LL-37 fragments can act as immunomodulatory molecules [14]. LL-23, a 23-mer fragment representing the N-terminus amino acid sequence of LL-37, has been shown to induce production of MCP-1 in human monocytic cells [15], and a partial 22-mer fragment of the C-terminus of LL-37 has been shown to induce secondary necrosis of apoptotic human neutrophils [16]. The capacity to modify the balance of microbicidal and immunomodulatory properties [14] illustrates the potential to manipulate peptide function in the development of potential therapeutic peptides.

The inducible expression of hCAP-18 is subject to complex transcriptional and post-transcriptional control and is upregulated in response to infectious and inflammatory signals and wounding [17–20]. Recent studies have also demonstrated the importance of 1,25-dihydroxyvitamin D3 activity on the *CAMP* gene promoter [21–24] and implicated a role for parathyroid hormone in the regulation of this cathelicidin [25]. In addition, research has identified compounds such as butyrate as having therapeutic potential as inducers of cathelicidin expression [26].

hCAP-18 is found at approximately 1.2 µg/ml in plasma, at least partly complexed with lipoproteins, under normal physiological concentrations [27]. This is estimated to be approximately 20% of the amount present in circulating neutrophils, with approximately 600 ng per 10⁶ cells. However, under inflammatory conditions, neutrophil degranulation and induction of hCAP-18 expression by epithelial and other cells leads to an increase in the concentration in inflamed organs, with an approximate threefold increase in lung lavage levels of hCAP-18 reported in infants with pulmonary or systemic infections [28]. Although challenging to extrapolate, this suggests airway surface liquid levels in the region of 20 µg/ml. Levels are also found to be raised in cystic fibrosis lung disease [29], and the expression of hCAP-18 is higher in eosinophils from asthmatics than controls [30]. hCAP-18/LL-37 has been found at very high levels (a median 1.7 mg/ml) in the skin of patients with psoriasis [31], where LL-37 has, in fact, been proposed to contribute to disease pathogenesis [32]. It is important to note that most estimates of *in vivo* cathelicidin concentration do not distinguish between the uncleaved hCAP-18 precursor and the cleaved active LL-37, which may be critical in determining functional sequelae.

The importance of hCAP-18/LL-37 to host defense is indicated by the increased susceptibility to infection (particularly periodontal) in individuals with the rare condition morbus Kostmann [33]. In this severe congenital neutropenia, neutrophils can be restored by treatment with recombinant granulocyte-colony stimulating factor; however, these cells (but not epithelial cells) are deficient in hCAP-18. In addition, hCAP-18/LL-37 levels are clearly associated with altered susceptibility to infection in human dermatological pathologies [34]. Furthermore, overexpression

of the human cathelicidin LL-37 in murine lungs enhanced the clearance of the opportunistic respiratory pathogen *Pseudomonas aeruginosa* [35], demonstrating the potential for therapeutic applicability *in vivo*. However, despite fairly broad-spectrum bactericidal activity *in vitro*, LL-37 has limited activity at the peptide concentrations characterized *in vivo* (particularly at epithelial surfaces) when studied under physiologically relevant cation conditions (review in [4]). In addition, the bactericidal properties of LL-37 can be inhibited by serum apolipoprotein [36], DNA and F-actin [37]. It is unclear as to what extent these caveats also extend to antiviral functions. However, these issues raise the question of whether cathelicidins are primarily microbicidal agents *in vivo*, or have antimicrobial effects via more indirect mechanisms.

In addition to their antimicrobial potential, cathelicidins have been demonstrated to have multiple roles in the modulation of inflammation and immunity, most of which are unaffected by cation concentrations. These are extensive and beyond the scope of this review, but are described in detail elsewhere [4]. These properties include, but are not limited to, modulation of cytokine release, induction of angiogenesis and wound healing, endotoxin neutralization, modulation of dendritic cell differentiation and function, properties as chemokines and adjuvants, and the capacity to modulate cell death (**Box 1**). LL-37 has this very broad array of properties by virtue of its capacity to directly interact with key innate immune effector cells, including monocytes and macrophages, dendritic cells, lymphocytes, epithelial cells and neutrophils. Although many of these functions are not fully mechanistically defined, a range of different receptors are implicated. These include FPR2 (also known as FPRL-1) [38–40], CXCR2 [41], MrgX2 [42], P2X7R [43] and GAPDH [44]. Furthermore, some immunomodulatory properties of cathelicidins have been demonstrated to be retained by the D-enantiomers, suggesting that they are not mediated by standard receptor-dependent mechanisms [14,45,46]. Where these modulatory functions have the capacity to significantly skew the host response to infection, such properties would be expected to have considerable potential, both in the context of bacterially and virally mediated diseases.

Murine cathelicidin

In mice, the cathelicidin mCRAMP is encoded by the *Camp* gene on chromosome 9. The

Box 1. Immunomodulatory and inflammomodulatory properties of LL-37.

- Modulation of cellular responses to RNA/DNA [32,39,150–152,155,158,167]
- Modulation of cellular responses to other inflammatory stimuli [14,43,147,148,168–171]
- Modulation of dendritic cell differentiation and function [32,161,172,173]
- Chemotaxis of neutrophils, eosinophils, monocytes, memory T cells and mast cells [38,40,41,57,174–176]
- Modulation of neutrophil function [55,159]
- Induction of mast cell degranulation [177]
- Modulation of cell death [16,160,178–185]
- Angiogenesis [186,187]
- Wound healing and cell proliferation [45,188–192]

murine protein maintains 52% amino acid sequence identity with hCAP-18 and porcine cathelicidin PR-39 in the cathelin-like domain, and 80% identity with each individually [47]. Full-length mCRAMP is cleaved to produce the active 34 amino acid, 5-kDa C-terminal peptide [48] with a tertiary structure, which was determined by NMR to be two amphipathic α -helices connected by a flexible region [49]. mCRAMP demonstrates similar expression patterns and functions to its human ortholog [47], is stored in neutrophil granules and is expressed in an inducible manner in epithelial cells and leukocytes [50–55]. However, unlike hCAP-18, expression of the *Camp* gene is not regulated by vitamin D [22].

Mice deficient in mCRAMP (*Camp*^{-/-}) have normal fetal development and fertility, and demonstrated no obvious phenotype when housed under aseptic barrier-controlled conditions [50]. However, these *Camp*^{-/-} mice have increased susceptibility to bacterial infections in multiple organ systems. These phenotypes include diminished protection against necrotic skin infection caused by Group A *Streptococcus* [50], delayed clearance of *P. aeruginosa* infection in the cornea [56] and lung [53], increased susceptibility to intestinal infection with the murine enteric pathogen *Citrobacter rodentium* [51], increased susceptibility to urinary tract infection with *Escherichia coli* [52] and increased susceptibility to pulmonary infection with *Klebsiella pneumoniae* [53]. These phenotypes clearly demonstrate the critical, nonredundant role for murine cathelicidin in host defense against bacterial infection. However, despite evidence of microbicidal properties against relevant pathogens *in vitro* [47,50,51,53], the extent to which physiological concentrations of mCRAMP are directly microbicidal *in vivo* remains unknown. In addition, mCRAMP has also been demonstrated to have modulatory properties [40,57] that could have key roles *in vivo*. Finally, recent studies [46,58], discussed

later in this review, also indicate key roles for cathelicidins in murine antiviral defenses.

Porcine cathelicidins

Pigs express a diverse group of cathelicidins with varying structural motifs and activities. These include five different protegrins (PGs), three α -helical peptides (PMAP-23, -36 and -37), two prophenins (PF-1 and -2) and the PR-39 peptide [59]. The mature peptides derived from the porcine cathelicidins are structurally very different; however, the PMAP peptides are α -helical (and more similar to LL-37 and mCRAMP), PR-39 and the prophenins are proline-rich helical peptides, and the PGs are arginine- and cysteine-rich β -sheet peptides.

The PGs were first identified in 1993 [60] and are between 16 and 18 amino acids in length. Five distinct isoforms (PG-1–5) are presently known, identified through purification (PG-1, PG-2 and PG-3) and cDNA cloning (PG-4 and -5). As with cathelicidins in other species, PGs are synthesized and stored as inactive propeptides in neutrophil granules, but are proteolytically cleaved into active products in the extracellular environment, which, in the case of PGs, is by neutrophil elastase [61].

PGs possess a unique and well-defined two-stranded β -sheet structure in solution, joined by a β -hairpin loop, and with four conserved cysteine residues forming disulfide linkages, stabilizing the peptide and facilitating interaction with biological membranes and enabling pore-forming activity. Investigations into the PG mechanism of antimicrobial action has identified that disruption of bacterial plasma membranes is one of their important properties [62,63]. However the folding of PGs, controlled by structural disulfide bridging, was not found to be essential for antimicrobial activity, but was required for permeabilization of biological membranes [64], raising interesting questions regarding the critical events in antimicrobial activity. PGs also have significant lipopolysaccharide

(LPS) binding and neutralization activity [65] and have been shown to reduce the LPS-mediated induction of TNF release from monocytic cell lines [66]. In addition, unlike other cathelicidins and many defensins, PGs are particularly resistant to physiologically relevant salt-sensitive inhibition of their antimicrobial activity [67], making them attractive templates for the development of synthetic antimicrobial compounds.

The therapeutic potential of induced or exogenous synthetic PG peptides has been investigated *in vitro* in a number of studies. While each of the PGs have been demonstrated to have microbicidal activity to some extent, the 18 amino acid PG-1 peptide has demonstrated the broadest antimicrobial activity over a wide range of Gram-positive and -negative organisms, fungi and yeasts. PG-1 has been shown to have activity against the elementary bodies of *Chlamydia trachomatis* through membrane permeabilization [68,69] and significant activity against *Mycobacterium tuberculosis* (up to 99% reduction in CFUs) has been confirmed *in vitro* [70]. Other bacteria such as *Neisseria gonorrhoeae* and *Pseudomonas* also have demonstrable and significant susceptibility to membrane damage and killing as a result of PG exposure [71,72]. In addition, the yeast phase of *Candida albicans* is also susceptible to PG-1, -2, -3 and -5, but not to PG-4 [73].

Despite these promising *in vitro* studies, evidence of protective effects in infectious disease models *in vivo* remains minimal. In one study, ectopic expression of PG-1 in transgenic mice was found to confer enhanced resistance to infection with *Actinobacillus suis*, a pathogen associated with porcine pneumonia, septicemia and abortion [74]. Exogenous PG-1 was also shown to prevent *P. aeruginosa* colonization of inoculated porcine skin wounds, and decreased bacterial counts in established skin infections [75]. Interestingly, PGs have also been proposed as potential antitumor agents through their cytotoxic activity on mammalian tumor cells [76,77], although the selectivity of the lytic activity towards malignant cells remains unknown. The extent to which these *in vivo* effects result primarily from microbicidal activity or may be due to immunomodulatory properties remains unclear, and the antiviral potential of PGs is largely undeveloped.

The cathelicidins PMAP-23, PMAP-36 and PMAP-37, named for their varying lengths [78,79], are expressed by porcine myeloid cells. The amphipathic α -helical conformation of these peptides has been hypothesized to be the

key mechanism by which PMAP molecules can integrate into biological membranes [79]. This subgroup of peptides has been demonstrated to have potent antibacterial, antifungal and antinematodal activity against organisms that include *Caenorhabditis elegans* [80] and *C. albicans* [81]. However, little is known regarding their activity towards viral pathogens.

PR-39 is a 39-amino acid porcine cathelicidin that is rich in proline and arginine residues and has a significant multifunctional repertoire of antibacterial and immunomodulatory activities that include modulation of cell death pathways [82], wound healing properties [83] and chemotactic activity [6,84,85]. The peptide has a poly-L-prolinehelical structure, enabling it to integrate into and cross biological membranes [86], and it is relatively resistant to proteolytic degradation in biological fluids, a property attributed to its high proline content [87]. Similar to other cathelicidins, PR-39 is primarily neutrophil-derived and is upregulated at sites of infection and inflammation. Little is known about the antiviral activity of this peptide, although a number of studies have demonstrated significant antibacterial activity against a range of organisms. However, rather than direct lytic activity, PR-39 is thought to act through stereospecific interactions with intracellular targets, as indicated by differences in the activity of the D-enantiomer of the PR-39 peptide [88].

With a similar poly-L-proline helical structure to PR-39, PF-1 and PF-2 are two variants, with 79- and 80-amino acid residues respectively, which can be distinguished either by an extra pyroglutamic acid or with a glutamine residue. Prophenins and prophenin derivatives have demonstrated significant activity against a variety of bacterial species [89,90], but their antiviral activity remains undetermined.

Cathelicidins from other species

Cathelicidins have been characterized in multiple different species, including (but not restricted to) rabbits, cows, horses, sheep, monkeys and fish (the reader is referred to other publications for further information on these peptides [91–96]). Although not within the primary remit of this review, it is worth noting the extensive body of research characterizing the bovine cathelicidins. These were among the first cathelicidin peptides to be identified in mammals [97–100]. Similarly to pigs, cows possess multiple unique neutrophil-derived cathelicidins with considerable structural

variability, including indolicidin, Bac5 and Bac7, the 12-amino acid bovine dodecapeptide and BMAP-27, BMAP-28 and BMAP-34. Indeed, studies have indicated the presence of 11 distinct cathelicidin genes in cattle [101]. These bovine peptides have well-characterized antibacterial activity [91], with BMAP-28, indolicidin and the Bac5/Bac7 peptides demonstrating significant bacterial membrane binding and/or disrupting properties [102–104]. Indolicidin has also recently been shown to reduce parasitic sporozoite infectivity and viability [105]. In addition, bovine cathelicidins also have a range of complementary properties, with BMAP-28 capable of modulating inflammatory gene expression in macrophages [106,107] and apoptosis in *Leishmania* parasites [108], and reducing lethality in murine models of sepsis [109], while indolicidin is capable of inhibiting LPS-induced TNF but inducing chemokine production *in vitro* [110]. The presence of cathelicidins across diverse species attests to their importance, while their variability, perhaps resulting from the diverse microbial challenges these species face, represents a valuable resource in the development of novel therapeutics.

Antiviral properties of cathelicidins

Research on the antiviral properties of CHDP dates back to the discovery of these peptides and is most prominent in the study of defensins (reviewed in [111]). However, this area has been significantly under-researched and, until recently, the antiviral properties of cathelicidins had received little attention, despite their bactericidal potential, pleiotropic immunomodulatory and inflammomodulatory properties, regulation

by inflammatory stimuli and the existence of genetically modified *Camp*^{-/-} mice. Nevertheless, a number of recent studies conducted in humans, mice and *in vitro* have stimulated this field and suggest that cathelicidins may prove to be significant to host defense against viral infections (Table 1).

Vaccinia virus

Vaccinia virus (VV) is an enveloped poxvirus utilized in the vaccination against smallpox infection and its eradication. Infection with VV is normally asymptomatic or may cause a mild rash. However, individuals with atopic dermatitis (AD) have a predisposition to the development of a serious, disseminated rash, called eczema vaccinatum, in response to smallpox vaccination [112]. The expression of hCAP-18 and β -defensins is reduced in AD, and has been proposed to be involved in the increased predisposition to skin infections in these patients [34]. These observations raised the possibility that these peptides may play a role in preventing eczema vaccinatum. Consistent with this hypothesis, expression of LL-37 was found to be induced by VV exposure in normal and psoriatic skin biopsies, but not in those from AD skin [113]. Addressing this initially in cell culture-based studies, both LL-37 and mCRAMP were demonstrated to have antiviral properties against VV (~1 log decrease at 25 μ M) by inducing VV envelope damage [58]. Further research demonstrated that LL-37 removed the outer membrane of VV in a manner consistent with the carpet model for peptide-mediated membrane disruption, but did not damage the inner membrane [114].

Table 1. Antiviral effects of cathelicidins.			
Virus	Genome	Capsid/family	Antiviral activity
Vaccinia virus	DNA	Enveloped poxvirus	Viral envelope damage (LL-37) Exposure of new antigens (LL-37) More pox lesions in <i>Camp</i> ^{-/-} mice (mCRAMP)
RSV	RNA	Enveloped paramyxovirus	Antiviral when premixed with virus (LL-37) Protective effects on epithelial cells (LL-37)
Influenza	RNA	Enveloped orthomyxovirus	Binds virus/membrane disruption (LL-37) Protection in infected mice (LL-37 and mCRAMP) Decreased cytokines in infected mice (LL-37)
HIV	RNA	Enveloped lentivirus	Suppression of HIV reverse transcriptase (LL-37)
HSV	DNA	Enveloped herpesvirus	Antiviral <i>in vitro</i> (LL-37 and protegrin-1)
Dengue	RNA	Enveloped flavivirus	Inhibition of dengue serine protease (protegrin-1)
Adenovirus	DNA	Nonenveloped adenovirus	Antiviral <i>in vitro</i> (LL-37)
mCRAMP: Murine CRAMP; RSV: Respiratory syncytial virus.			

Interestingly, loss of the outer membrane resulted in viral susceptibility to antibody neutralization owing to exposure of antigens that are normally sequestered under the envelope [114]. The opportunity for *in vivo* mechanistic studies represented by *Camp*^{-/-} mice then enabled the demonstration of the antiviral effects of cathelicidins *in vivo*, with mCRAMP-deficient mice developing significantly more pox skin lesions than controls following infection with VV [58]. Thus, these studies clearly demonstrate a nonredundant, direct, antiviral host defense role for cathelicidins against VV infection.

Respiratory syncytial virus

Respiratory syncytial virus (RSV) is the most common viral pathogen causing acute lower respiratory tract infection in young children worldwide [115], and is a leading cause of morbidity and mortality in infants, the elderly and immunocompromised individuals. It has also been implicated in the later development of asthma [116]. There is currently no vaccine or effective antiviral treatment for RSV [117] and novel therapeutics are required. Airway epithelial cell expression of LL-37/hCAP-18 is induced *in vitro* by RSV infection [118], suggesting a possible role in host defense. Interestingly, this upregulation was further enhanced in the presence of the 1:25 OH metabolite of vitamin D, raising the possibility of a role for seasonal vitamin D insufficiency in innate antiviral defenses. A recent evaluation of children presenting with RSV bronchiolitis also discovered significantly lower levels of hCAP-18 expression in the serum of those with RSV bronchiolitis than in children presenting with bronchiolitis induced by human rhinovirus infection [119]. In addition, lower than median hCAP-18 levels in RSV-infected children were found to correlate with more prolonged hospitalization. Furthermore, our recent data demonstrate that LL-37 has effective antiviral activity against RSV *in vitro*, retained by a truncated central peptide fragment [120]. LL-37 prevented virus-induced cell death in epithelial cultures, significantly inhibited the production of new infectious particles and diminished the spread of infection, with antiviral effects observed whether the peptide was premixed with viral particles or used to pretreat the epithelial cells. These data implicate hCAP-18/LL-37 as an important, targetable component of the innate host defense against RSV and suggest future potential in strategies

aimed at prophylactic modulation of cathelicidin expression in vulnerable individuals and/or the development of synthetic peptide analogs for use in postexposure prophylaxis.

Influenza A virus

In addition to RSV, LL-37 has antiviral effects against another common respiratory viral pathogen; influenza A virus (IAV). Infection with IAV is a significant cause of morbidity, and is responsible for 2000–6000 deaths per year in the UK, with >30,000 deaths in the 1989–1990 epidemic [121]. Globally, the H1N1 pandemic in 2009 was estimated to have caused over 200,000 respiratory deaths [122]. Although vaccination can help protect vulnerable individuals against prevalent subtypes, new emergent strains represent a global pandemic threat, and the potential for the emergence of resistance to neuraminidase inhibitors (the current first-line therapy) is of serious concern. Interestingly, maintenance of serum 25-OH vitamin D levels over autumn and winter months in the northern hemisphere has been found to reduce the incidence of acute viral respiratory tract infections [123] and a small prospective clinical trial in Japan suggested that vitamin D3 supplementation during the winter could significantly reduce the incidence of IAV infection in children [124]. These studies raised the possibility that vitamin D-regulated cathelicidin expression could have a role in influenza susceptibility. We subsequently demonstrated that human and murine cathelicidins have antiviral effects against IAV, both *in vitro* and *in vivo* [46]. LL-37 and mCRAMP, but not PG-1, demonstrated antiviral properties when preincubated *in vitro* with IAV (~1 log decrease at 10 µg/ml against A/PR/8/34 [H1N1], but somewhat less effective against A/Udorn/307/72 [H3N2]). A recent publication went on to demonstrate that LL-37 can bind directly to IAV [125]. Although maximal *in vitro* antiviral activity required preincubation of the peptide and virus, antiviral properties were also observed with peptide addition delayed until after viral infection of the cells, and even after the cells were treated with peptide and then washed before infection [125]. Surprisingly, LL-37 was found not to alter the binding or initial uptake of virus by cells, but electron microscopic evaluation demonstrated peptide-mediated disruption of viral membranes, suggested to impair viral survival or propagation within the infected cells [125]. We demonstrated therapeutic cathelicidin-mediated

protection against infection in a murine model, using an aerosolized therapeutic regimen of LL-37 or mCRAMP administration, consisting of a preinfection dose and daily administration for a week after infection [46]. These treatments resulted in significantly greater survival and less weight loss compared with control IAV infected animals. The efficacy of this regimen was similar to treatment with aerosolized zanamivir (a neuraminidase inhibitor currently used in humans) in this model. In addition to somewhat lower viral loads, the animals treated with LL-37 demonstrated strikingly lower levels of inflammatory lung cytokines. This observation raises the possibility that inflammomodulatory properties could be fundamental to the protection against systemic illness and lethality, irrespective of virucidal properties. Interestingly, the D-enantiomers of LL-37 and mCRAMP were similarly protective, but scrambled control peptides had no activity, indicating that the effects of these cathelicidins are probably more dependent upon amphipathicity than overall charge. The lack of protective effects in mice treated with PG-1, suggests species specificity, but the very different structural characteristics of this peptide must be noted when interpreting these data.

HIV

HIV is the lentiviral causative agent of AIDS. Infection with HIV has been radically transformed over the last two decades, from a fatal disease to a manageable chronic infection, by the development and successful use of anti-retroviral drugs that can inhibit multiple steps in the viral lifecycle [126]. However, continued drug development is necessary to combat the emergence of drug-resistant strains. Initial observations with host defense peptides and synthetic analogs suggested antiviral potential against HIV, with varying 50% inhibitory concentration (IC_{50}) estimates of 1–4 $\mu\text{g/ml}$ [127]. Subsequent research, utilizing a lentiviral vector to consider peptide inhibitory potential in the early stages of the replication cycle, demonstrated dose-dependent LL-37- and PG-mediated inhibition of lentiviral vector transfection (with IC_{50} of ~30 and ~16.8 $\mu\text{g/ml}$, respectively) [128]. Although the inhibition of vectors containing the HIV-1 envelope required higher concentrations of LL-37 [128], this cathelicidin and its derivative fragments were also later demonstrated to inhibit the replication of HIV-1

isolates in primary CD4⁺ T cells and cell lines [129,130], with the action of LL-37 independent of HIV-1 receptor expression alteration in these cells. A more recent study also demonstrated that LL-37 could suppress HIV reverse transcriptase activity in a dose-dependent manner and that this function was retained by a central fragment of the peptide (amino acids 17–32) [131]. In addition, the bovine peptides BMAP-18 and indolicidin have been shown to have anti-HIV activity *in vitro* [130,132]. These studies suggest promise for anti-HIV activity of cathelicidins; however, clinical studies are somewhat less clear. Constitutive expression of hCAP-18 has been demonstrated in human epididymal epithelium and attached to spermatozoa in seminal plasma [133] and hCAP-18 is also expressed in cervicovaginal secretions [134]. This expression of hCAP-18 is upregulated in cervicovaginal secretions from individuals with bacterial sexually transmitted infections [134]. In addition, expression levels in cervicovaginal secretions from HIV-negative individuals who were in HIV serodiscordant relationships were highest in those whose HIV-positive partners had the highest viral load [135]. Furthermore, HIV-1 neutralizing activity was demonstrated in the cationic peptide fraction of the cervicovaginal secretions and could be further enhanced by the addition of recombinant LL-37. These data suggest that upregulation of cathelicidin expression in response to HIV exposure may provide a degree of protection from HIV infection. However, HIV-1 neutralizing activity of the cervicovaginal secretions was not found to correlate with the detected levels of endogenous LL-37 [135], and increased expression of hCAP-18/LL-37 has also been correlated with increased HIV acquisition in Kenyan sex workers [134]. Although the latter observation could be a consequence of a higher prevalence of sexually transmitted infections in those individuals acquiring HIV, the *in vivo* significance of LL-37 expression in protection against HIV infection remains to be determined.

HSV

The first evidence for antiviral properties of CHDP came during the earliest characterization of the properties of α -defensins, with the demonstration of direct antiviral activity of HNP-1 against the enveloped virus HSV-1 [136]. This was shown to be dependent upon both pH and temperature, and to be inhibited

by the presence of serum. Despite further studies on the antiviral effects of defensins on this virus, the activity of cathelicidins is less well characterized. One study demonstrated antiviral activity of LL-37 against HSV-1 (~2 log decrease) *in vitro* using A549 cells, but used very high concentrations of peptide (500 µg/ml; ~100 µM) in this study [137] and the mechanisms of action were not explored experimentally. Another study also described antiviral activity against strains of HSV-1 and HSV-2, with 30–50% protection in a quantitative microplate screening assay at 44.5 µM LL-37, using ME-180 cells [138]. A similar magnitude of protection was described for PG-1 using the same assay (40–70% protection at 92.8 µM). Strikingly, the PG-1 D-enantiomer demonstrated increased antiviral activity against HSV-1 and -2, whereas removal of the intramolecular disulfide bonds inactivated the peptide [138]. In addition, the bovine peptides indolicidin and BMAP-28 (but not BMAP-27) have been shown to have some antiviral activity against HSV-1 and/or HSV-2 [138–140].

Dengue virus

Dengue is the most prevalent mosquito-borne viral infection of humans. Dengue virus, an RNA virus that is part of the *Flaviviridae* family, has four major serotypes (DENV-1 to -4), and can result in a number of clinical syndromes including dengue fever, dengue shock syndrome and dengue hemorrhagic fever. A total of 50–100 million instances of dengue fever are estimated to occur annually and effective therapeutics or vaccines for the dengue virus are lacking.

A recent study demonstrated that PG-1 could inhibit DENV-2 serotype replication in MK2 cells (at IC₅₀ of 11.7 µM) *in vitro* at concentrations that were shown to be of low toxicity to the cells [141]. PG-1 had a highly effective capacity to inhibit dengue serine protease NS2B-NS3pro *in vitro*. NS2B-NS3pro is responsible for the cleavage of viral polyproteins to produce structural and nonstructural proteins, and inhibition of this activity has inhibitory effects on dengue virus replication. Whether this effect extends to cathelicidins from other species, such as humans, remains to be determined.

Nonenveloped viruses

All of the antiviral properties of cathelicidins described above have been against enveloped

viruses, with viral envelope disruption implicated as the mechanism of action in the case of VV and IAV [58,114,125]. In addition, viral envelope disruption has been described in a substantial proportion of the reports evaluating the antiviral activities of defensins (reviewed in [111]). However, defensins have also been found to have antiviral activity against adenovirus (Ad) in a serotype-dependent manner by inhibiting an early step in viral entry [142]. The extent to which cathelicidins are active against nonenveloped viruses remains largely unstudied. Whereas expression of retrocyclin (cyclical θ-defensin) analogs in the chloroplasts of tobacco plants conferred resistance against Tobacco Mosaic Virus, expression of functional PG-1 failed to do so [143]. Nevertheless, one group has demonstrated LL-37 activity against Ad19 (~2 log decrease at 500 µg/ml LL-37) [137]. These data demonstrated slower antiviral kinetics when compared with activity against HSV, perhaps implicating a different mode of action against nonenveloped viruses. By contrast, the same study found no significant activity against Ad3, Ad5 or Ad8 [137]. However, our preliminary, previously unpublished studies, suggest LL-37 activity against Ad5 at more physiological peptide concentrations, demonstrating significant inhibition of cellular transfection with Ad5–GFP after 1-h preincubation at ≥10 µg/ml LL-37 peptide (**Figure 1**). While these studies need to be extended, and definitive, mechanistic studies remain to be performed on a range of nonenveloped viruses, our preliminary data and the peer-reviewed, published research suggest that cathelicidins have antiviral properties that extend beyond damaging viral envelopes.

Mechanisms & immunomodulation

The mechanisms underpinning the antiviral properties of cathelicidins remain largely unknown (**Figure 2**). The *in vitro* studies described above for VV, RSV and IAV all suggest that direct interaction with the viral particle may be one possible antiviral mechanism [58,114,120,125] and optimal effectiveness is generally shown following pretreatment of the virus with peptide, prior to infection of cells. However, the effects on nonenveloped viruses indicate additional mechanisms of antiviral activity. It is interesting to note that antiviral activity was also observed when cells were treated with peptide and then washed before infection with the virus [120,125]. This might be

Figure 1. Antiviral activity of LL-37 against adenovirus. A total of 2×10^6 plaque-forming units Ad-GFP (E1-deleted, replication-deficient adenovirus encoding GFP; ViraQuest Inc., IA, USA) was preincubated for 1 h at 37°C over a concentration range of either LL-37 or scrLL-37 prior to addition of this virus and peptide mix to A549 cells at a multiplicity of infection of 100. Alternatively, A549 cells were preincubated for 1 h over a concentration range of LL-37 (0–50 μg/ml) before removal of the media containing the peptide, washed twice with phosphate-buffered saline, and media containing 2×10^6 plaque-forming units of Ad-GFP was added to the cells, resulting in a multiplicity of infection of 100. In all cases, treated cells were then incubated in phenol-red free Dulbecco's modified Eagle's medium supplemented with 1% Ultrosor™ G serum substitute (Pall Biopharmaceuticals, France) at 37°C, 5% CO₂ for 18 h before the fluorescence intensity of each well was measured using a BioTek® (Bedfordshire, UK) multiwell plate reader. Figure shows mean fluorescent intensity as a percentage of untreated transfected cells $\pm$ standard error of the mean, from $n \geq 5$.

* $p < 0.01$; ** $p < 0.001$.

Ad-GFP: GFP-expressing adenovirus; scr: Scrambled.

an effect of peptide retained on the cell surface or internalized by the cells. Indeed, we have previously demonstrated that LL-37 can interact with epithelial membranes and be actively internalized in a microtubule-dependent manner [144]. Furthermore, membrane-associated human β -defensin-2 has been shown to destabilize the RSV envelope during cell entry by the virus [145] and the retrocyclin RC2 can crosslink and immobilize surface glycoproteins to block influenza hemagglutinin-mediated fusion of the viral and endosomal membranes during viral internalization [146]. These precedents for the antiviral properties of CHDP being mediated via interactions with the cell membrane

suggest a need to evaluate such mechanisms for cathelicidins. However, alternatively, the effects observed after peptide treatment of the cells could represent the induction of an antiviral state resulting from the immunomodulatory properties of these peptides. Indeed, the dramatic effects of LL-37 on cytokine production and survival in IAV-infected mice, compared with the relatively modest effect on viral loads *in vivo* [46], also suggest that peptide-mediated modulation of inflammation and immunity may be an important component of their antiviral function.

Cathelicidins have a broad repertoire of inflammomodulatory and immunomodulatory activities, which might be expected to affect viral infection and the host response. In relation to LL-37-mediated antibacterial defenses, this peptide has antiendotoxic properties resulting from its lipopolysaccharide binding capacity [147]. However, in addition, we demonstrated that LL-37 can modulate downstream signaling and actually induce certain chemokines while also inhibiting proinflammatory cytokine production [147]. Indeed, further work has subsequently demonstrated that this peptide has a complex interaction with Toll-like receptor (TLR) signaling pathways [148–152], with the potential to modify the nature of host responses to viral and bacterial infection. Interestingly, these effects are observed at peptide concentrations that are lower (typically 1–5 μg/ml) than those required for microbicidal activity. Mammalian cells respond to a range of different microbial components or pathogen-associated molecular patterns via innate pattern recognition receptors including TLR, RIG-I-like receptors and nucleotide-binding domain leucine-rich repeat containing receptors (reviewed in [153]). Antiviral responses depend, at least in part, on TLR recognition of the viral genome, with ssRNA and dsRNA viruses recognized by TLR7/8 and TLR3 and viral DNA by TLR9 [154]. Thus, the ability of cathelicidins to modulate cellular responses to TLR-3, -7, -8 and -9 agonists [32,39,150–152,155] may be of considerable significance, although some of the literature remains contradictory. LL-37 has been shown to enhance viral dsRNA signaling via TLR3 [150], colocalizing with TLR3 and dsRNA and promoting cytokine responses in rhinovirus-infected human airway epithelial cells and activated

peripheral blood mononuclear cells. LL-37 was also shown to promote IL-8 release by a human airway epithelial cell line in response to polyinosinic–polycytidylic acid, a synthetic analog of dsRNA [151]. However, by contrast, LL-37 and mCRAMP have been reported to complex with polyinosinic–polycytidylic acid and inhibit TLR3 binding and signaling in APCs [152]. A recent study demonstrated that LL-37 upregulated dsRNA-induced TLR3 responses in human cells, but downregulated these responses in murine cells, with mCRAMP downregulating the responses in cells from both species [39]. Although the authors proposed that this represents species specificity, the human and murine cell lines used in this study were not ideally matched, raising the possibility that the nature of the responses may alternatively depend upon the cell lineage studied (e.g., leukocytes vs epithelial cells). LL-37 has been known for some time to be capable of transferring nucleic acids into mammalian cells [156]. This may underpin the enhanced response to nucleic acids, given the intracellular location of their sensors. However, it is also possible that nucleic acids complexed with cathelicidin may elicit modified responses through the additional involvement of LL-37 receptors, such as FPR2, or the engagement of different trafficking pathways [39]. Indeed, otherwise nonimmunogenic self-DNA and self-RNA has also been shown to induce TLR-7, -8 and -9-dependent inflammatory responses when complexed with LL-37 [32,155,157]. This has been shown to lead to the induction of type I interferons from dendritic cells, monocytes [32,155,157] and keratinocytes [158]. If a similar effect should occur in response to viral nucleic acids complexed to cathelicidin, this could potentially underpin the induction of an enhanced antiviral state in response to peptide exposure. Although clearer mechanistic studies are required, and the effect of cathelicidins upon RIG-I-like receptors and nucleotide-binding domain leucine-rich repeat containing receptors pathways needs to be determined, these processes may play a key role in cathelicidin-mediated antiviral responses.

In addition to their capacity to modulate pattern recognition receptor signaling pathways and cytokine responses, additional modulatory properties of cathelicidins may modify host responses to viral infections. These include chemotactic properties that could influence the

nature and magnitude of the cellular inflammation response [38], the ability to modify neutrophil function [55,159] and the capacity to promote apoptosis in infected epithelial cells [160]. We have also demonstrated that LL-37 can induce the secondary necrosis of apoptotic neutrophils and the release of neutrophil granules proteins [16]. This might be expected to result in an increase in viral exposure to neutrophil α -defensins, which also have antiviral properties against a range of pathogenic human viruses (reviewed in [111]). Finally, in addition to altering dendritic cells responses to viral nucleic acids, we have shown that LL-37 can modulate the differentiation and function of these cells, with the capacity to alter the nature and magnitude of subsequent adaptive immune responses [161]. In that context, it is interesting to note that the weight loss observed

Figure 2. Possible mechanisms of cathelicidin-mediated antiviral activity. The mechanisms underpinning cathelicidin-mediated antiviral activity remain largely undefined. However, studies suggest that these peptides may modulate infection with different viruses in a number of different ways, as indicated by the green boxes.

PRR: Pattern recognition receptor.

in IAV-infected mice largely stabilized in the LL-37-treated infected animals around the time of adaptive immune response onset [46], raising the possibility that the nature of this response was altered. The extent to which any of these mechanisms are fundamental to cathelicidin-mediated antiviral activity remains to be determined. Nevertheless, the capacity of these peptides to modulate recognition of viral pathogen-associated molecular patterns, downstream inflammatory signaling, innate cellular responses, cell death and adaptive immunity, make their modulatory properties of clear interest in the development of novel antiviral therapeutic strategies.

Conclusion & future perspective

The discovery of novel approaches to prevent and treat viral diseases remains a significant challenge for the medical and scientific communities. Recent developments in our understanding of key naturally occurring innate antimicrobial agents, immune recognition and signaling offer new opportunities to target critical components of antiviral host defenses. New insights into the antiviral potential of CHDPs, such as cathelicidins, suggest that these peptides may be important targetable components of this antiviral defense system and may additionally represent templates to inform the development of novel synthetic antiviral analogs.

The evidence that cathelicidins have key antiviral roles is only just starting to emerge; however, the antiviral potential of other CHDPs is already more clearly established [111]. Naturally occurring α - and β -defensins have activity against IAV, RSV, HIV, HPV, HSV and Ad *in vitro*, and in various *in vivo* models of infection with some of these viruses. In addition, synthetic analogs based on θ -defensins (not expressed in humans due to a premature stop codon [162]) have shown promise, including in a murine model of severe acute respiratory syndrome infection [163]. Similarly to the studies with cathelicidins, research conducted using defensins suggests that peptides may target the virus directly, affect multiple different points in the viral lifecycle and also modulate innate immune signaling and the nature and/or magnitude of the inflammatory and immune responses to infection. It is tempting to speculate that the diversity of mechanisms reported for different peptides may represent

many facets of the innate host defense, targeting the same virus simultaneously via multiple different approaches by utilizing a range of different peptides. With that in mind, it may be important that future approaches study the synergy between peptides, rather than examine them only in isolation. It is also possible that the capacity of CHDPs to modulate the nature and magnitude of host defense responses against infection may offer the potential to develop therapeutics with broader applicability against multiple viruses and minimize the potential to promote the emergence of resistant strains by avoiding direct peptide targeting of the virus. Indeed, the recent development of innate defense regulator peptides, initially engineered to remove direct antibacterial activity while retaining antimicrobial function, is demonstrating promise against a range of infectious diseases [164–166]. Such peptides hold exciting potential as future therapeutics and may also be applicable to viral infections.

The extent to which CHDPs may prove to be maximally efficacious given therapeutically, used for postexposure prophylaxis or targeted in an entirely prophylactic manner (such as aiming to enhance native peptide production via winter season vitamin D administration) remains to be determined. The effective targeting of such approaches will be dependent upon the development of an enhanced understanding of the mechanisms by which these peptides have antiviral effects, the extent to which these are directly virucidal or via modulation of the innate immune system, and the physiological relevance of *in vitro* observations in animal models and human disease. Although this research is at an early stage, the potential for the development of novel interventions targeting viruses and the innate host responses to these pathogens has clear appeal.

Financial & competing interests disclosure

SM Currie is supported by a University of Edinburgh College of Medicine and Veterinary Medicine Studentship, DJ Davidson is supported by a MRC Senior Non-clinical Research Fellowship (G1002046). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

EXECUTIVE SUMMARY**Cationic host defense peptides**

- Cationic host defense peptides (CHDPs) are critical components of the innate immune system.
- CHDPs have broad-spectrum antibacterial potential and the capacity to modify inflammation and immunity.
- CHDPs are emerging as antiviral components of innate immune responses with possible therapeutic potential.

Cathelicidins

- Cathelicidins (e.g., human CAP-18/LL-37, murine CRAMP and porcine protegrins/PMAps/prophenins/PR39) are multifunctional CHDPs.
- Cathelicidins are expressed primarily by neutrophils, epithelial cells and macrophages.
- Cathelicidin expression can be regulated by vitamin D metabolites and can be upregulated by vitamin D and/or phenyl butyrate.
- Cathelicidins have nonredundant roles in defense against infection in multiple systems *in vivo*.

Antiviral activity of cathelicidins

- Cathelicidins have antiviral activity against vaccinia virus, respiratory syncytial virus, influenza virus, HIV, HSV, dengue virus and adenovirus.
- The mechanisms of antiviral activity include direct damage to viral envelopes, inhibition of viral protein function and modulation of host cell responses to infection.

Future perspective

- Evaluating the antiviral potential of cathelicidins and the direct and indirect mechanisms involved is at an early stage.
- Innate immune defense may involve synergies between multiple CHDPs acting via different mechanisms.
- CHDP-mediated modulation of host defense responses against viral infection may have therapeutic potential with broader applicability against multiple viruses and may minimize the potential to promote the emergence of resistant strains by avoiding direct peptide targeting of the virus.
- Targeting upregulation of endogenous CHDP expression (e.g., via winter season vitamin D administration) may be a prophylactic antiviral strategy.

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