Cationic host defense peptides; novel antimicrobial therapeutics against Category A pathogens and emerging infections

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Cationic Host Defense Peptides (HDP, also known as antimicrobial peptides) are crucial components of the innate immune system and possess broad-spectrum antibacterial, antiviral, and immunomodulatory activities. They can contribute to the rapid clearance of biological agents through direct killing of the organisms, inhibition of pro-inflammatory mediators such as lipopolysaccharide, and by modulating the inflammatory response to infection. Category A biological agents and materials, as classified by the United States National Institutes for Health, the US Centers for Disease Control and Prevention, and the US Department of Homeland Security, carry the most severe threat in terms of human health, transmissibility, and preparedness. As such, there is a pressing need for novel frontline approaches for prevention and treatment of diseases caused by these organisms, and exploiting the broad antimicrobial activity exhibited by cationic host defense peptides represents an exciting priority area for clinical research. This review will summarize what is known about the antimicrobial and antiviral effects of the two main families of cationic host defense peptides, cathelicidins, and defensins in the context of Category A biological agents which include, but are not limited to; anthrax (*Bacillus anthracis*), plague (*Yersinia pestis*), smallpox (*Variola major*), tularemia (*Francisella tularensis*). In addition, we highlight priority areas, particularly emerging viral infections, where more extensive research is urgently required.

Keywords: CHDP, Antimicrobial peptide, Cathelicidin, Defensin, Emerging pathogen, Host defense

Introduction

Cationic host defense peptides (HDP), also known as antimicrobial peptides, play a crucial role in the innate host defense system.

HDP are evolutionarily conserved and categorized into two main families in humans; defensins and cathelicidins. The defensin family of peptides can be further subdivided into α -, β -, and θ -defensins.

Numerous studies have shown that HDP are present in a range of species including animals, plants, and humans,¹ where they exhibit diverse activities that modulate the innate immune system including; the recruitment of inflammatory cells to the site of infection,^{2,3} wound healing and angiogenesis,^{4,5} differentiation and maturation of dendritic cells,⁶ regulation of cell death pathways,⁷⁻⁹ and broad spectrum antimicrobial activity against numerous bacteria, viruses, and fungi.¹⁰ Most HDP are small (<100 amino acids) and contain a high proportion of arginine and lysine residues that contribute to their positive charge. They also tend to have an amphipathic structure which makes them highly effective at incorporating into biological membranes.¹¹

The broad spectrum antimicrobial and immunomodulatory activities of HDP have led to a significant number of studies investigating the utility of naturally occurring or synthetic derivatives of HDP as novel therapeutics. This is of particular relevance in the context of emerging infections which require rapid clinical intervention, examples of which include the recent Ebola outbreak in Guinea, Liberia and Sierra Leone which has claimed in excess of 10,000 lives, and the Zika virus outbreak in Latin America.

This article summarizes current knowledge of the microbicidal and virucidal potential of cathelicidins and defensins against several highly pathogenic organisms (detailed in Table 1). These organisms, classified as Category A by the US Centers for Disease Control and Prevention (CDC), The US Department of Homeland Security, and the National Institute for Allergy and Infectious Diseases (NIAID) represent organisms and biological agents that pose the highest risk to public health due to their transmissibility and high rates of mortality. The pathogens and agents that are currently placed in the Category A priority list include Anthrax (caused by the bacterium *Bacillus anthracis*), Botulinum toxin (produced by the bacterium *Clostridium botulinum*), Plague (caused by the bacterium *Yersinia pestis*), Smallpox (caused by the viruses *Variola*

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Table 1 The antimicrobial activities of naturally occurring and fragments of naturally occurring host defense peptides against Category A pathogens and emerging infections

Peptide	Sequence	Effect
LL-37	LLGDFFRKSKKEIGKEFKRIVQRIKDFLRNLPRTES	<ul style="list-style-type: none"> • Activity against Influenza virus^{30,39} • Activity against vegetative form of <i>B. anthracis</i>^{76, 78} • Inhibitory against endospore form of <i>B. anthracis</i>^{78, 85} • Activity against <i>Y. pestis</i> bacteria⁸⁹ • Activity against <i>F. tularensis novicida</i> bacteria¹⁰² • Anti-biofilm activity against <i>F. tularensis novicida</i> bacteria¹⁰² • Short term protection of mice during lethal infection with <i>F. tularensis</i> live vaccine strain¹⁰¹ • Reduces bacterial load of macrophages infected with <i>F. tularensis</i> live vaccine strain¹⁰¹ • Activity against Vaccinia virus^{106,107} • Activity against <i>F. tularensis novicida</i> bacteria¹⁰³
hBD-1	DHYNCVSSGGQCLYSACPIFT-KIQGTCYRGKAKCKK	
hBD-2	GIGDPVTLCKSGAICHVPVFCPR-RYKQIGTCGLPGTKCKCKP	<ul style="list-style-type: none"> • Activity against <i>F. tularensis novicida</i> bacteria¹⁰³ • Activity against <i>F. tularensis</i> live vaccine strain¹⁰³
hBD-3	GIINTLQKYCYRVRGGRCVAVLS-CLPKEEQIGKCSRGRKCCRRKK	<ul style="list-style-type: none"> • Activity against vegetative form of <i>B. anthracis</i>⁷⁶ • Activity against <i>F. tularensis</i> live vaccine strain¹⁰³
Protegrin-1	RGGRLCYCRRRFVCVGR	<ul style="list-style-type: none"> • Inhibitory against endospore form of <i>B. anthracis</i>⁷⁷ • Activity against vegetative form of <i>B. anthracis</i>⁷⁹
mCRAMP	GLLRRKGGEKIGELKKIGQKI-KNFFQKLVPQPEQ	<ul style="list-style-type: none"> • Activity against Influenza virus³⁰ • Activity against vegetative form of <i>B. anthracis</i>⁷⁹ • Activity against <i>Y. pestis</i> bacteria⁹² • Activity against Vaccinia virus^{106,115}
C1-C15 fragment (chicken cathelicidin-2)	RFGRFLRKIRFRPK	<ul style="list-style-type: none"> • Activity against vegetative form of <i>B. anthracis</i>⁸⁴ • Activity against <i>Y. pestis</i> bacteria⁸⁴
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS	<ul style="list-style-type: none"> • Inhibitory against endospore form of <i>B. anthracis</i>⁸⁵ • Activity against vegetative form of <i>B. anthracis</i>⁸⁵ • Activity against <i>F. tularensis novicida</i> bacteria⁸⁵
HNP-1-3	ACYCRIPACIAGERRYGTCTIYQGRL-WAFCC (HNP-1) CYCRIPACIAGERRYGTCTIYQGRL-WAFCC (HNP-2) DCYCRIPACIAGERRYGTCTIYQGRL-WAFCC (HNP-3)	<ul style="list-style-type: none"> • Rescues macrophages from death induced by endospore form of <i>B. anthracis</i> (HNP-1)⁸⁶ • Protects macrophages from <i>B. anthracis</i> Lethal Toxin mediated cytolysis (HNP1-3)⁸⁶ • Protects mice against <i>B. anthracis</i> Lethal Toxin intoxication (HNP1-3)⁸⁶ • Dimerizes with <i>B. anthracis</i> Lethal Factor (HNP-1)^{88,89} • Inhibitory against endospore form of <i>B. anthracis</i> (HNP1-3)⁸⁶ • Activity against vegetative form of <i>B. anthracis</i> (HNP1-3)⁸⁷ • Activity against vegetative form of <i>B. anthracis</i>⁷⁷
Rhesus θ -defensin 1-3	GFCRCLCRRGVCRICITR (θ -defensin-1) GVCRCLCRRGVCRICIRR (θ -defensin-2) GFCRCICTRGFCRCICTR (θ -defensin-3)	<ul style="list-style-type: none"> • Inhibitory against endospore form of <i>B. anthracis</i>⁷⁷ • Inhibitory against <i>B. anthracis</i> Lethal Factor⁷⁷
NA-CATH (King Cobra cathelicidin)	KRFKKFFKLLKNSVKKRAKKFFK-KPKVIGVTFFP	<ul style="list-style-type: none"> • Rapid binding to <i>B. anthracis</i> spores resulting in enhanced phagocytosis and killing by macrophages⁹⁰ • Activity against <i>F. tularensis novicida</i> bacteria¹⁰²

major and to a lesser extent, *Variola minor*), Tularemia (caused by the bacterium *Francisella tularensis*), and all viral hemorrhagic fevers including Marburg, Ebola (*Filoviridae*), Lassa, Junin virus, Lymphocytic choriomeningitis virus or LCMV, Guanarito virus, and Machupo virus (*Arenaviridae*), Hantaviruses and Rift Valley Fever (*Bunyaviridae*), and Dengue (*Flaviviridae*).

We suggest that there is a significant need for a greater understanding of the antimicrobial roles of HDP in the innate immune response to infection, and with that knowledge there is huge potential for the development of HDP as novel therapeutics for the frontline treatment of emerging infections.

Cathelicidins

Cathelicidins take their name from ‘Cathelin’, a protein sequence first identified in porcine leukocytes¹² and for

their ability to inhibit the protease cathepsin-L.¹³ All cathelicidins contain an N-terminal signal peptide, a Cathelin-like domain, and a variable C-terminal antimicrobial domain.¹⁴ Cathelicidins tend to be linear peptides which can either fold into an amphipathic α -helical structure such as myeloid antimicrobial peptides (MAP) e.g. PMAP-23 (pig), SMAP-29 (sheep) and BMAP-27 (cattle) or have a proline rich structure e.g. Bactenecins (Bac5, Bac7) and PR-39.¹⁵ The exception to these general cathelicidin structures are the protegrins, which contain the amino acid, cysteine and have a β -hairpin structure that is stabilized by two or more disulfide bonds.^{16,17}

Cathelicidins are first synthesized as prepropeptides, and, following removal of the signal peptide, the inactive propeptide is generally stored in neutrophil granules.¹⁸ Upon neutrophil recruitment and degranulation, the propeptides are cleaved allowing the active c-terminal

component to exert effects at a site of infection or inflammation.^{10,19} Although it is generally recognized that cathelicidins are stored in neutrophils and are upregulated during the inflammatory response,²⁰ they have also been identified in several other cell types, tissues, and biological fluids such as macrophages, lymphocytes, eosinophils, NK cells, mast cells, B cells, T cells, keratinocytes, epithelial cells, sweat, breast milk, and plasma.^{21–24} Humans contain only one cathelicidin gene, termed *CAMP*, which codes for LL-37/hCAP-18 but a wider variety have been found in cattle, rodents, and pigs.^{15,25,26}

Under certain conditions, cathelicidins from multiple species have been demonstrated to have substantial antimicrobial activity against a wide range of bacteria, viruses, and fungi,^{27,28} albeit in some cases at significantly higher superphysiological *in vitro* concentrations than those that have been observed *in vivo*. However, the potential therapeutic opportunities of increased cathelicidin concentrations in disease models cannot be understated, as it has been demonstrated that exogenous delivery or overexpression of these peptides could enhance clearance and improve survival against respiratory pathogens such as influenza virus, or the opportunistic pathogen *Pseudomonas aeruginosa* using *in vivo* infection models.^{29,30}

Conversely, the importance of cathelicidins in the context of human host defense is evidenced through a patient group called morbus Kostmann who suffer from a rare congenital neutropenia. These individuals are deficient in neutrophil specific granules, and therefore lack neutrophil-sourced LL-37, consequently rendering them more susceptible to infection.³¹ In addition, it has been shown that mice deficient in mCRAMP (murine Cathelicidin Related Antimicrobial Peptide), the murine orthologue of LL-37, have increased susceptibility to skin, respiratory, urinary tract, and gut infections.^{32–35}

The mechanisms underlying the potent antimicrobial potential of HDP have been the subject of a large number of studies, and it is clear that many host defense peptides exhibit both immunomodulatory and direct antimicrobial activities that can contribute to the innate host response to invading pathogens. In the context of direct antimicrobial activity, the carpet model for antimicrobial peptide activity suggests that instead of forming transmembrane pores, the peptides cover a membrane in a ‘carpet-like’ manner and dissolve it through detergent-like action.³⁶ Cathelicidins such as LL-37 have been shown to rapidly permeabilize pathogen membranes by forming discreet toroidal pores (Reviewed in³⁷) The peptides may then also be subsequently able to enter the pathogen and interfere with transcription and other essential processes, as has been indicated in a study with the proline rich cathelicidin Bac71-35.³⁸ In the context of viral infection, LL-37 has been shown to directly damage the viral membrane of influenza³⁹ and to target the HIV-1 reverse transcriptase,⁴⁰ suggesting that cathelicidins exert their activity through a multitude of targeted effects on pathogens. While this

article is predominantly focused on their antimicrobial activity, the immunomodulatory activities of HDP are also of fundamental importance in host defense against infection, and are described in detail elsewhere.⁴¹

Defensins

Defensins are small host defense peptides that are found in vertebrates, invertebrates, plants, and in fungal species. In vertebrate organisms, they are typically 18 to 45 amino acids in size and are cationic, amphipathic molecules that are cysteine-rich in composition. Defensins exhibit a predominantly β -sheet structure that is stabilized by three intramolecular disulfide bonds.⁴² There are three major subfamilies, termed α -defensins, β -defensins, and θ -defensins. Humans only possess functional α - and β -defensins; θ -defensin mRNA has been found in humans, but it is not translated due to a premature stop codon on the θ -defensin (DEFT) gene. θ -defensins are only found in non-human primates.⁴³ In humans, 6 α -defensin and 31 β -defensin peptides have been identified.⁴⁴ In similarity to the cathelicidin family, defensins were initially shown to be potent antibacterial molecules,⁴⁵ but latter studies revealed that they exhibit significant antiviral and immunomodulatory activities, including the circumstantial ability to mediate proinflammatory responses and contribute to suppression or resolution of inflammation.^{46,47}

In terms of expression and production, α -defensins can be split into two groups. Human neutrophil peptides (HNP) 1–4 are primarily produced by neutrophils, but can also be produced to a lesser extent by cells of myeloid origin such as macrophages, NK cells, and some T-cells, B- cells, and dendritic cells.^{42,48} Human defensins 5 and 6 (HD-5 and HD-6) are predominantly expressed by the epithelium of the gut, specifically Paneth cells, and the reproductive tract.^{49–52} They are produced as precursor pre-peptides that require proteolytic cleavage to remove partial fragments. Human β -defensins (HBD) are more widely expressed at mucosal surfaces and skin epithelium, although in other mammals such as the cow, they are produced to a greater extent in neutrophils.^{53–56}

The immunomodulatory roles of defensins in host defense have also been extensively described in the literature, particularly in relation to the modulation of infection-related inflammation. The two human families of defensins have been shown to bind to a number of cell surface receptors exerting immune regulatory effects such as chemotaxis of T-cells, macrophages, and dendritic cells,^{57–60} induction of proinflammatory cytokine production by macrophages, mast cells, and keratinocytes,^{61–63} and regulation of cell death pathways and cell survival.^{64,65} In addition, defensins have also been described to have regulatory roles in inflammation resolution, through the suppression of pro-inflammatory gene expression and cytokine production in response to bacterial infection.^{65–67} It is likely however, that a substantial number of factors contribute to the immune outcomes elicited by defensins,

including concentration and microenvironment specific effects.

Loss of antimicrobial defense due to dysregulated defensin production can result in enhanced pathophysiological observations in a range of diseases. For example, one study revealed that mice with reduced α -defensin expression in the gut showed greater susceptibility to *Escherichia coli* infection in a challenge model.⁶⁸ Furthermore, it was shown that a loss of mouse β -defensin-1 results in impaired clearance of *Haemophilus influenzae* from the lung,⁶⁹ while other studies have revealed an important regulatory role for α -defensins in the regulation of gut microbiota and in the urinary tract.^{70–72}

The antimicrobial activity of cathelicidins and defensins against Category A bacterial pathogens

Anthrax (*Bacillus anthracis*)

Anthrax results from cutaneous contact, inhalation or ingested exposure to endospores of the causative agent of the disease, *B. anthracis*. *B. anthracis* is a Gram-positive endospore-forming bacterium which can be found in the dormant form in soil, predominantly in areas that are warm and damp.⁷³ Animals ingest endospores while grazing on contaminated pastures, and once ingested spores return to the vegetative form of the organism to cause infection. Human infection with *B. anthracis* is rare, but primarily occurs when humans are exposed to infected animals or animal products. Several reports have also described ‘injectional anthrax’ a form of the disease associated with drug users injecting substances contaminated with *B. anthracis*.⁷⁴ Initial symptoms of *B. anthracis* exposure can vary dependent upon the route of exposure, but can result in pain, respiratory difficulties, fever, septicemia and, without appropriate treatment, can result in death.

Treatments for cutaneous or contact anthrax are available in the form of antibiotics to treat the infection, such as penicillin, ciprofloxacin, erythromycin, or tetracycline.⁷⁵ Inhalational anthrax is significantly more difficult to treat. Those at high risk of exposure can be vaccinated against *B. anthracis*, but in the case of a deliberate and public exposure, treatment rather than prophylaxis would be the primary option.

The inhibitory potential of naturally occurring cathelicidins and synthetic derivatives toward *B. anthracis* has been documented in several studies. Initial studies demonstrated that unidentified peptides produced by lung epithelium, (which were likely to be LL-37 and/or hBD-3) had inhibitory effects toward the vegetative form *in vitro*,⁷⁶ while the porcine cathelicidin Protegrin-1 displayed inhibitory activity toward *Bacillus* spores.⁷⁷ Subsequently, it was shown that relatively high concentrations of LL-37 (40–60 $\mu\text{g/ml}$) inhibited the growth of the vegetative form *B. anthracis* by 50% and that the peptide was bactericidal at >125 $\mu\text{g/ml}$.⁷⁸ This also showed demonstrable activity of LL-37 toward newly germinated *B. anthracis* spores.

It should be noted that while the concentrations used are considered to be superphysiological, there may be a therapeutic role in the context of exogenous cathelicidin delivery, or enhancement of the innate cathelicidin response to infection.

One study has suggested that cathelicidins sourced from human (LL-37) murine (mCRAMP) and porcine (PG-1) sources have antimicrobial activity toward the vegetative form of *B. anthracis* at physiological (micromolar) concentrations.⁷⁹ Additionally, the same study demonstrated that exogenous intraperitoneal (i.p.) administration of human, porcine, and murine cathelicidins could protect against subsequent i.p. challenge with *B. anthracis* spores in a murine model. In the case of porcine cathelicidins, this was attributed to direct killing of the bacteria. In contrast, the mechanism proposed for human and murine cathelicidins was enhanced recruitment of neutrophils to sites of infection, which contributes to clearance of the pathogen. Importantly, post-infective administration of cathelicidins four hours after an initial sub-cutaneous challenge significantly increased survival, but this was not observed when utilizing an intra-tracheal route of administration. This could be attributed to the challenge model using the spore form, which does not germinate in the lung. However, further evidence that cathelicidins are an important component of the host response to *B. anthracis* was detailed in a study demonstrating significantly decreased survival of CRAMP-/- cathelicidin-deficient mice compared to wild type in a vegetative *B. anthracis* subcutaneous challenge model.⁸⁰

B. anthracis has evolved a substantial number of immune evasion strategies and can avoid destruction by HDP. For example, extracellular proteases produced by *B. anthracis* can degrade LL-37.⁷⁸ It has been suggested that the activity of these proteases is modulated by expression of ClpX, an ATP-dependent chaperone that can recognize protein substrates by binding to protein degradation tags; vegetative *B. anthracis* species lacking ClpX are hypersensitive to killing by cathelicidins.⁸⁰ As such, pharmacological inhibition of the ClpX gene has been investigated as a potential therapeutic target.⁸¹ Most recently, studies have identified tellurite-resistance genes yceG and yceH involved in resistance to the antimicrobial cathelicidins, although the mechanism underlying the resistance is unclear.⁸² It should be noted that other physical mechanisms of *B. anthracis* resistance to HDP and subversion of the innate immune response have been identified, such as membrane-incorporated lysylphosphatidylglycerols, produced by expression of the *mprF* gene.⁸³

In addition to the sensitivity of *B. anthracis* to naturally occurring HDP, the effectiveness of synthetic cathelicidin derivatives has also been explored. The antibacterial activities of several variants of a cathelicidin-derived peptide (C1–15), which demonstrated decreased salt sensitivity and eukaryotic cytotoxicity compared to naturally occurring cathelicidins have been investigated.⁸⁴ The C1–15

peptide and derivatives exhibited substantially enhanced antimicrobial activity against the vegetative form of *B. anthracis*. A separate study utilized sequences derived from LL-37, from the silk moth peptide, cecropin A, and from the *Xenopus* peptide magainin II, to form novel hybrid α -helical structures. The novel fusion peptides were found to exhibit potent antimicrobial effects, in some cases substantially greater than the parent peptides, particularly against the vegetative form of *B. anthracis*, where it was shown that several of the fusion peptides displayed significantly more anti-endospore activity than either of the native forms of LL-37 or cecropin A.⁸⁵

Paralleling the antimicrobial activities of cathelicidin peptides, the inhibitory potential of defensin families against *B. anthracis* have also been evaluated in several studies. An initial study revealed that human alpha-defensins exhibited the ability to neutralize the activity of *B. anthracis* lethal factor, an enzymatic component of anthrax toxin that is implicated in the pathogenesis of anthrax. Furthermore, it was revealed that mice exposed to a lethal dose of *B. anthracis* lethal factor were protected by intravenous administration of purified human neutrophil peptides 1–3, members of the human alpha defensin family.⁸⁶ In contrast to the studies described above, administration of purified LL-37 peptide in the same mouse model failed to confer protection against a lethal dose of anthrax exotoxin. Similarly, it was demonstrated that the alpha defensin fraction of PMN granules was the component responsible for killing of both the vegetative and spore forms of *B. anthracis* in an *in vitro* neutrophil infection model.⁸⁷ Subsequent studies offered further detail on the underlying mechanism by revealing the contribution of dimerization to HNP-1-mediated inhibition of *B. anthracis* lethal factor.^{88,89}

It is interesting that the antimicrobial activities of defensin peptides extend beyond those of human origin. For example, θ -defensins of nonhuman primate origin exerted several distinct antimicrobial effects on *B. anthracis* *in vitro*.⁷⁷ In this study, θ -defensins exhibited antimicrobial activity against the vegetative form of *B. anthracis*, as well as inhibitory effects against the endospore form. Additionally, the enzymatic activity of anthrax lethal factor was inhibited by incubation with θ -defensin, a protective effect that was extended to increased survival of a murine macrophage cell line (RAW264.7) when exposed to anthrax lethal toxin. A more recent study has indicated that the immunomodulatory activity of θ -defensins may also play a key role in host defense against *B. anthracis*.⁹⁰ While the antimicrobial effects of θ -defensins are particularly sensitive to serum, *in vivo* administration of the peptides in murine challenge models with *B. anthracis* spores could protect against lethality. It was further shown that the θ -defensins rapidly bound to *B. anthracis* spores, resulting in increased phagocytosis and killing by macrophages, likely enhancing clearance.

Evidence of evasion strategies by *B. anthracis* toward the action of defensin peptides has also been uncovered. In similarity to findings observed with cathelicidin resistance, loss of the *ClpX* gene was revealed to increase sensitivity to human alpha-defensin-2.⁸⁰ In addition, functional loss of the *mprF* gene renders *B. anthracis* hyper-susceptible to defensin peptides through lack of membrane lysylphosphatidylglycerols.⁸³

Taken collectively, it is clear that both cathelicidins and defensins can exhibit potent and specific activity against the endospore and vegetative forms of *B. anthracis*, thus delivery of exogenous peptide merits further exploration as a therapeutic or preventative strategy.

Plague (*Yersinia pestis*)

Plague is caused by the enterobacteria *Yersinia pestis*, which primarily causes disease in rodents and is transmitted via bites from infected fleas. This zoonotic pathogen is also able to cause disease in humans, which manifests in three clinical states; (1) Bubonic plague, which is the most common form of the disease, attributed to flea bites and resulting in lymphadenitis developing in the drainage lymph nodes causing pain and swelling known as buboes. (2) Pneumonic plague, which can arise as a secondary infection following spreading of *Y. pestis* to the lungs from other infected sites resulting in a severe pneumonia. Transmission of this form to other individuals is extremely common by transfer of respiratory droplets. (3) Septicemic plague which can develop from bubonic plague in the absence of lymphadenitis, resulting in an infection of the bloodstream such as meningitis or endotoxic shock.^{91,92} According to the World Health Organisation (WHO), the mortality rates of each of these forms of the disease without prompt and effective treatment would be bubonic plague (50–60%) and septicemic and pneumonic (up to 100%).

Y. pestis infection is commonly treated with three main antimicrobial agents, streptomycin, tetracycline, and chloroamphenicol, and is curable if treatment is given promptly. This has resulted in the disease being relatively dormant for decades in many parts of the world.⁹¹ However, the plague has been categorized as a re-emerging disease, since data collected by the WHO between 1987 and 2009 saw 47,516 human plague cases, and 4060 deaths that were reported by 26 countries.⁹³ More worryingly, has been the reports of several distinct resistant strains of *Y. pestis*. In 1995, two strains were isolated from two different districts of Madagascar, *Y. pestis* 17/95, that is resistant to eight of the recommended antimicrobials for treatment and prophylaxis and *Y. pestis* 16/95, that was resistant to streptomycin.^{94,95} Between 1996 and 1998, three further resistant strains were isolated from different regions in Madagascar,⁹⁶ and the frequency of resistant strains of the bacterium has been attributed to horizontal gene transfer.⁹⁷ Studies have revealed that *Y. pestis* co-incubated in the

flea mid-gut with *E. coli* (pIP1203) containing an antimicrobial resistant plasmid, transfer of resistance to *Y. pestis* occurred after only three days of co-incubation.⁹⁵ This emphasizes the need for alternative therapies to treat this re-emerging disease.

There is a relative paucity of research examining the antimicrobial potential of HDP toward *Y. pestis* strains, and this needs to be addressed. Interestingly, the primary focus has been on the synthetic cathelicidin C1–15. This is a highly active antimicrobial region of the chicken cathelicidin-2 (CATH-2) that shows great promise for the development of novel antimicrobials due to its broad spectrum antimicrobial activity but low level hemolytic activity against erythrocytes. Molhoek and colleagues generated variations of the peptide sequence by replacing phenylalanine (Phe) residues with tryptophan (Trp), enhancing the peptide's activity.⁸⁴ It was found that a single Phe-Trp substitution resulted in peptide reducing the *Y. pestis* CFU/ml by a factor of four, whereas multiple Phe-Trp substitutions resulted in eight times more potent antimicrobial activity against the bacterium. More recently, two other engineered synthetic cationic peptides, termed WLBU2 and WR12, were shown to have potent activity against *Y. pestis* in an *in vitro* bacterial killing model.⁹⁸

It has also been demonstrated that the native human cathelicidin, LL-37, was able to reduce *Y. pestis* CFUs by more than 10^3 at concentrations of 160 µg/ml of the peptide.⁹⁹ While this concentration is considered to be superphysiological in the context of the innate response to infection, it highlights the potential for cathelicidin-derived peptides to be developed as a front-line therapy against this infection. Importantly, *Y. pestis* has the capacity for resistance against native HDP, therefore the generation of novel, synthetic peptides is even more important in a therapeutic context.

The plasminogen activator outer membrane protein (Pla) that is involved in bacterial binding to extracellular matrix proteins, appears to be involved in the increased resistance of *Y. pestis* to HDP *in vitro*. This is primarily through inhibition of the activity of LL-37, potentially via proteolytic degradation of the peptide.¹⁰⁰ Another group have shown that a mutant of *galU*, believed to be responsible for glucose-1-phosphate uridylyltransferase, is able to reduce resistance of *Y. pestis* to the actions of the murine cathelicidin mCRAMP, and that the Minimum Inhibitory Concentration (MIC) was reduced by more than eight times that of the control.⁹²

The relative scarcity of existing data on the antimicrobial activity of HDP toward *Y. pestis* is surprising, however the studies mentioned above further support the idea that native or synthetic cathelicidin peptides could represent a novel avenue of treatment.

Tularemia (Francisella tularensis)

The facultative intracellular bacterium *Francisella tularensis* is responsible for causing the zoonotic disease

Tularemia. While the organism predominantly infects rabbits, hares, and rodents, resulting in large rates of mortality within these animal populations, it can also cause disease in humans via tick and deer fly bites, or by contact with infected animals. Symptoms in humans include a high fever and other 'flu-like' symptoms, which can be problematic for diagnosis. Disease severity depends on the transmission method of the organism; skin contact can result in ulcers at the site where an infected tick or fly bites. Pneumonia can also develop; this is the most severe form of the disease and results from inhalation of the bacterium or direct inoculation of the lungs.¹⁰¹

F. tularensis is regarded as a Category A bioterrorism agent as it is extremely infectious, requiring less than fifty viable infectious organisms to cause disease and as few as 10 in the most pathogenic of strains,¹⁰² which means it could easily be disseminated throughout a human population. Furthermore, if exposure to the bacterium occurred via an aerosol, this would result in the more severe pneumonic forms of the disease which is associated with up to 35% mortality if left untreated.¹⁰³ Without treatment tularemia pneumonia can be fatal, but if appropriate antibiotics are administered the majority of patients will recover, although symptoms can persist for several weeks. While antibiotic resistance in *F. tularensis* is relatively uncommon, strains that are resistant to erythromycin are prevalent in Europe.

There are two well characterized strains of *F. tularensis*, namely *F. tularensis novicida* (also known as *F. novicida*)¹⁰² and another strain developed from *holartica*, which is a subspecies of *F. tularensis* that is less virulent to humans. This is used as a model in mice due to its similarities with the most pathogenic human strain of *F. tularensis* (Schu 54), and is known as the live vaccine strain (LVS).^{101,103} Studies have revealed that LL-37 was found to possess microbicidal activity against *F. novicida*, interestingly to a greater extent than NA-CATH, a helical cathelicidin found in venom from the snake, *Naja atra*. LL-37 and NA-CATH demonstrated EC₅₀ values of 0.24 and 1.54 µg/ml, respectively. Further studies have also shown that LL-37 was able to effectively inhibit biofilm formation of the bacterium at concentrations as low as 3.8 ng/ml, even though bacterial growth in the Tryptic Soy Broth (TSB-C) was not inhibited.¹⁰² However, it is likely that the lack of antimicrobial activity in the TSB-C could be attributed to the NaCl content of the broth. This is particularly important as it has previously been documented that the antimicrobial activity of LL-37 is significantly inhibited in the presence of NaCl. For example, it has been shown that high concentrations of Na⁺ ions (~100 mM) can reduce the antimicrobial activity of LL-37 up to eightfold against *Staphylococcus aureus* and *Salmoella typhimurium* serovar *enterica*. A separate study revealed that the MIC of LL-37 against *E. coli* increased from 16 µg/ml at 20 mM NaCl up to >500 µg/ml at 300 mM NaCl, reflecting the high salt sensitivity of this peptide.^{104,105}

Using *in vitro* bacterial killing studies, it has been demonstrated that novel synthetic peptides, CaLL and MALL (hybrid host defense peptides comprised of partial peptide sequences from Cecropin A, LL-37 and magainin II) showed a 99% and 90% reduction in the bacterial population of *F. tularensis*, respectively, over a 4 h period. Similarly, magainin II showed ~90% reduction in bacterial colonies, although interestingly neither LL-37 nor Cecropin A appeared to display any antibacterial activity against this strain.⁸⁵ The mechanism of action of these novel peptides is unclear, unlike LL-37, they are not expected to form alpha helical structures. It was subsequently demonstrated that LL-37 (20 µg/ml) was able to significantly reduce the intracellular bacterial load of LVS (live vaccine strain) infected J774A murine macrophage-like cells after 24 h. In addition, exogenous delivery of LL-37 was shown to provide a short-term protective effect against *F. tularensis* as multiple doses of 200 µM of LL-37 significantly prolonged the life expectancy of mice infected with a lethal dose of LVS. However, it is notable that all mice eventually succumbed to the infection, highlighting the importance of clearly characterizing the mechanism of action involved.¹⁰¹

The human beta-defensins, hBD-1, 2 and 3 are upregulated in the lung in response to bacterial infection. Therefore, in the case of more severe pneumonic forms of tularemia, it is likely that these HDPs would be involved in the initial innate immune response toward the organism. However, in experimental studies using the alveolar epithelial cell line A549, it was demonstrated that an increase in expression of hBD-1 and hBD-2, but not hBD-3, occurred when cells were exposed to *Francisella* *in vitro*.¹⁰³ hBD-1 showed no antimicrobial potential against *F. novicida* *in vitro*, but did result in a 70% reduction in the bacterial load of LVS at the highest concentrations of peptide tested (100 µg/ml) while hBD-2 showed effective antimicrobial activity against *F. tularensis*. Interestingly, and despite there being no increase in expression, hBD-3 showed an EC₅₀ value of 0.84 and 0.39 µg/ml for *F. novicida* and LVS, respectively, indicating that it had the strongest bactericidal activity of the three human beta-defensins tested.

Taken collectively, it is clear that HDP can play a key role in the innate response to this infection, and should be considered for development as therapeutics. One recent study utilizing engineered cationic host defense peptides (WLBU2 and WR12) has already demonstrated *in vitro* bacterial killing activity toward *F. tularensis*, both by direct exposure, and in an *ex vivo* in-cell infection model utilizing J774 macrophage cells.⁹⁸ While the incidence of antibiotic resistance in *Francisella* is significantly lower than other common pathogens, there is still a need to develop novel antimicrobials to prepare for emergence of antibiotic resistance in the future.

The antiviral activity of cathelicidins and defensins against Category A viral pathogens *Smallpox (Variola major)*

The eradication of smallpox in 1979 was the celebrated success of the WHO vaccination program. Smallpox was a serious infectious disease caused by the virus *Variola major*, with transmission occurring through airborne droplets and a mortality rate of approximately 30%. Infection with the less common form, *Variola minor*, did not generally have a high mortality rate. Diseased individuals presented with characteristic raised blisters that would then go on to produce scarring in survivors. In the decade preceding the official eradication of the disease, it was estimated that approximately two million people died of smallpox per year. However, this is far from being the end of the smallpox story and there is still concern over the potential use of the virus in bioterrorism. For this reason, vaccination has been reintroduced in many parts of the world particularly among individuals working in military and medical fields. The laboratory prototype for the orthopoxvirus family is the vaccinia virus and this is also the virus that has been used for the successful vaccination against smallpox for over 200 years. Vaccinia virus has a double-stranded DNA genome (>180 kb) and replicates in the cytoplasm.

It has been demonstrated that HDP are an important component of innate immunity against vaccinia virus.¹⁰⁶ Studies have determined that the cathelicidin LL-37 inactivates vaccinia virus by a carpet-based mechanism.¹⁰⁷ It was shown that HDP, including LL-37, were efficient at removing the outer membrane of vaccinia and that they might also, without the requirement for membrane removal, alter the exposure of antigens allowing susceptibility to neutralizing antibody.¹⁰⁷ This suggests that the peptides may simultaneously induce direct killing by the carpet-based mechanism, but could also reveal sequestered antigens for antibody binding, and subsequent clearance by the adaptive immune response.

A serious complication of vaccination has been identified as eczema vaccinatum (EV), a potentially life-threatening dissemination of vaccinia virus.^{108–110} Vaccination has been contraindicated in patients with atopic dermatitis, a chronic skin condition that affects approximately 20% of children in the USA,¹¹¹ and a lack of upregulation of LL-37 in atopic dermatitis lesions has been linked to development of EV.¹¹² While animal models have been investigated in an attempt to understand the biological and molecular basis of EV, the degree of reaction to vaccinia virus would appear to be a combination of a number of factors including young age, allergen sensitization, and mast cell function.¹¹³ In this context, mast cells are known to be a source of mCRAMP, the rodent equivalent of human LL-37.¹¹⁴ mCRAMP has been shown to have the potential to kill vaccinia virus directly with deficient mice being

less able to clear the virus.¹⁰⁶ Studies have also revealed that mast cells can degranulate and kill the vaccinia virus using host defense peptides.¹¹⁵

Taken collectively, these findings suggest an intimate link between LL-37 and EV. Therefore, we would suggest that not only should cathelicidin-based peptides be considered as a front line antiviral therapy, but as an adjunct treatment to ameliorate the possibility of EV development following vaccination. It is clear that strategies to minimize or prevent adverse reactions to smallpox vaccination will depend on further research into the resistance or susceptibility to vaccinia virus infection, including the important contribution of HDP.

Emerging viral infections

Perhaps the biggest gap in the scientific literature is the role of HDP in emerging viral infections that are responsible for hemorrhagic fever outbreaks, such as Ebola, Marburg, Dengue, and Lassa. These pathogens emerge rapidly, can spread easily, and there is often no existing vaccine. Recent history has included two outbreaks of high significance (Ebola and Zika) that have had a major clinical impact in countries across the world.

We suggest that focus should be placed on the development of novel peptide therapeutics, based on cathelicidins and defensins; the demonstrable activity of cathelicidins and defensins against other viruses such as influenza is a good indication that development of these peptides will be of considerable value. There is also promise for the development of other peptide-based therapeutics against hemorrhagic viruses, a recent study has revealed that an innate immune collectin, scytovirin from cyanobacteria, displays potent antiviral activity against Ebola Zaire virus *in vitro* and *in vivo*.¹¹⁶ Using a lethal mouse model of Ebola infection, the authors demonstrated that repeated administration of scytovirin substantially increased mouse survival rates, therefore this lectin could represent a novel antiviral therapeutic for this infection. Interestingly, the antiviral activity of scytovirin also extended toward Marburg virus. A more recent study has also revealed that COOH-terminal peptides of CXCL9 and CXCL12 γ display antiviral activity toward Dengue virus by competing for binding sites on host cells.¹¹⁷

Conclusions

With the continued and serious threat posed by emerging, re-emerging, or modified pathogens, in addition to the potential for such pathogens to be released as part of a biological incident, the development of novel therapeutics based upon naturally occurring host defense peptides will be a key area of future research. Antimicrobial peptides for clinical use are already in commercial development, and are currently being tested in several infectious and inflammatory disease settings (Reviewed in^{118,119}). In addition, the use of an additive combination of HDP molecules, or indeed synergistic HDP pairings or groupings,

could provide extremely powerful therapeutic avenues for investigation.

However, there are several questions that must be addressed before antimicrobial peptides could be safely used as effective frontline therapies for Category A pathogens and emerging infections. Issues such as their relatively large size, stability of antimicrobial or immunomodulatory activity, and questions surrounding immunogenicity and cytotoxicity require greater understanding, with multiple reports of resistance mechanisms in several pathogens having already been presented (Reviewed in¹²⁰). In addition, the pluripotent immunomodulatory activities of host defense peptides remain to be fully understood. While a relatively recent study has highlighted how the structure of LL-37 can be modified into a more stable and potent peptide,¹²¹ in this regard, a substantial effort must be devoted to the development of smaller more stable synthetic peptides that can mimic the biological activities of naturally occurring peptides while avoiding any unwanted side effects in order to fully exploit their potential.

Transparency declarations None to declare.

Funding FF is supported by an Edinburgh Napier University Research Studentship.

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