A Versatile Group of Molecules, Can Defensins Make an Impact in Medicine?

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Antimicrobial peptides are an ancient form of innate defense and are present in all ways of life. In humans they are present as cathelicidins and defensins. Both are important for the immune system and they exhibit activity against viruses, bacteria and fungi. Defensins exhibit less cytotoxicity and are better characterized and are thus more easily developed as therapeutic tools. Defensins are apt at doing a multitude of things, from inhibiting Herpes simplex virus replication and preventing anthrax lethality to helping with wound closure and acting as biomarkers for a variety of ailments. Defensins have consistently shown good results in a laboratory setting but have less than exemplary in vivo results. Defensins’ multifunctionality as well as the complex environment in living organisms makes characterizing why defensins are not performing as well in vivo difficult. They can also exhibit negative side-effects such as increasing the infectivity of the HIV and inhibiting anti-viral molecules of the innate immune system. Nevertheless, they exhibit big potential as complementary drugs, adjuvants, biomarkers, wound treatment and much more. Further characterization and development is absolutely necessary in these times of increasing antibiotic resistance.

Defensin, Adjuvant, Biomarker, Virus inhibitor, Application, Potential
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1 Abstract

Antimicrobial peptides are an ancient form of innate defense and is present in all ways of life. In humans they are present as cathelicidins and defensins. Both are important for the immune system and they exhibit activity against viruses, bacteria and fungi. Defensins exhibit less cytotoxicity and are better characterized and are thus more easily developed as therapeutic tools. Defensins are apt at doing a multitude of things, from inhibiting Herpes simplex virus replication and preventing anthrax’ lethality to helping with wound closure and acting as biomarkers for a variety of ailments. Defensins have consistently shown good results in a laboratory setting but have less than exemplary in vivo results. Defensins’ multifunctionality as well as the complex environment in living organisms makes characterizing why defensins are not performing as well in vivo difficult. They can also exhibit negative side-effects such as increasing the infectivity of the HIV and inhibiting anti-viral molecules of the innate immune system. Nevertheless, they exhibit big potential as complementary drugs, adjuvants, biomarkers, wound treatment and much more. Further characterization and development is absolutely necessary in these times of increasing antibiotic resistance.
2 Introduction

Large organisms defend themselves from small organism-based threats in many ways. One of the oldest defences against microbes is the production of antimicrobial peptides (AMPs) which are present in all lifeforms\(^1\). There are two main groups of AMPs in humans, cathelicidins and defensins. This review will deal with defensins and their potential use in a therapeutical setting.

Defensins are known to have antibacterial, antiviral and antifungal properties, as well as the ability to modulate the innate and adaptive immune systems in various organisms\(^2\). Structurally all defensins are small cationic peptides with many conserved cysteine residues. These cysteine residues form a so called “defensin fold” by stabilizing a Beta-sheet through disulphide bonds. Other than the cysteines there is great sequence diversity amongst defensins\(^3\).

Defensins have been found in animals, fungi and plants. It has been suggested that all defensins evolved from a singular progenitor, but the exact evolutionary relationship is uncertain\(^4\). In insects there is a single defensin group, conveniently called insect defensins. They are used as an acute phase reaction to infectious threat and are secreted into the blood stream, in contrast to mammalian defensins that fulfil a more static role\(^5\). In mammals there are three distinct defensin families that all evolved from the same precursor gene, they are called Alpha-, beta- and theta-defensins\(^6\). Alpha and beta defensins are the main groups and are present in almost all mammals. Theta defensins were first isolated in rhesus macaques and are found in non-human primates\(^7,8\). The gene for theta-defensin has a stop-codon within the signal sequence in humans, rendering it inert\(^8\). There is great variety between mammal species as to what defensin exists where. In humans, there are six different alpha defensins. Four of them (HNP1-4) are primarily found in neutrophil granules and the two other types (HD5 & 6) are produced and distributed by Paneth cells in the gut. Beta-defensins are present in epithelial tissues and secretions all over the body\(^9\).
3 Structure and mechanism of interaction

All defensins are classified as small amphipathic, cationic peptides of roughly 29-42 amino acids in length. They have conserved cysteine residues that stabilizes a Beta-sheet through three disulphide bonds\textsuperscript{3,10}. This basic structure is the layout for alpha and beta defensins, the difference between them being the order of cysteine linkages. Theta-defensins have a different layout compared to the others, with the cystine linkages stabilizing a circular structure\textsuperscript{7}. Insect defensins differ from mammalian defensins by containing a beta-sheet disulphide-linked with an alpha-helix and having different cysteine links\textsuperscript{3}.

![Figure 1. A 3D rendering of human beta defensin 2 (hBD2) with the characteristic defensin fold. By Emw - Own work, CC BY-SA 3.0,](https://commons.wikimedia.org/w/index.php?curid=8767138)
Defensins’ direct activity includes forming pores in negatively charged lipid bilayers. This makes them active against bacteria’s and viruses’ anionic membranes, but not against the hosts own cells\(^\text{11}\). This was discovered in tests with human alpha-defensin 1 (HNP-1) where it was found to permeabilize the outer and inner membrane of \textit{E. coli}, leading to cell death\(^\text{12}\). Bacteria can acquire resistance to the defensin mechanism of lipid interaction, it is however limited to specific strains of bacteria\(^\text{13}\).

HNP1-3, HD5 and human beta defensin 3 are lectins, which makes them capable of binding to glycoproteins and glycolipids found on the surface of pathogenic organisms and initiating immune responses\(^\text{14,15,16}\).

Because defensins are cationic and amphipathic they can also interact with DNA and other proteins through charge-charge or hydrophobic interactions\(^\text{10,17,18}\).

Defensins can be found as oligomers or multimers which has important, but very poorly understood, implications for their varied capabilities\(^\text{10,19,20}\).

### 4 Defensins in various therapeutical areas

Human beta-defensin (hBD) 1 and 2 in combination with each other has been shown to reduce the growth of \textit{Salmonella typhimurium} by 85-96% \textit{in vitro}. The survival rate of mice treated with hBD-1 and -2 was roughly 50% at 206 hours after inoculation. Compared to the 0% survival rate at 24 hours after inoculation for the \textit{Salmonella} infected control this is a marked improvement\(^\text{21}\).

Defensins are potent against pathogenic organisms but they can also perform other functions, in HNP-1’s case it can inhibit anthrax toxin. Tests on mice revealed that the lethal toxin produced by \textit{Bacillus anthracis}, which causes anthrax, was neutralized effectively by human alpha-defensin 1. Anthrax is hard to treat with antibiotics if treatment is not initiated promptly after inhalation of \textit{B. anthracis} due to the secreted toxin left in the victim. HNP-1 could effectively be used to complement the antibiotic treatment as a toxin inhibitor. When there is such a potent inhibitor of the toxin
present in our bodies, the reason why \textit{B. anthracis} toxin is toxic to humans is unclear. It is theorized that the endogenous concentration of HNP-1 is not sufficient to reach the neutralizing threshold. Alternatively, \textit{B. anthracis} could have a suppressive effect on human leukocytes’ secretion of defensins\textsuperscript{22}.

These kinds of results indicate the potential of defensins in a therapeutical setting and the supposed applications are many and diverse. Some of the most interesting ones are outlined below.

\textbf{4.1 Defensin usage as biomarkers and cancer suppressors}

Measuring the concentration of defensins in circulation or relevant tissues can indicate infectious activity indirectly with the body upregulating defensin production when it is facing infectious threat. Using this principle, defensins have been suggested to work as biomarkers for diseases such as inflammatory bowel disease\textsuperscript{23}, cirrhosis\textsuperscript{24} and idiopathic pulmonary fibrosis\textsuperscript{25}.

Patient infected by HIV receive antiretroviral therapy (ART) to prevent the infection reaching AIDS-status. The same patients are more susceptible to opportunistic infection and by extension the development of cancer in the oral cavity\textsuperscript{26}. hBD-2 concentration correlates directly with development of oral squamous cell carcinoma (OSCC) and can thus be used as a biomarker for malignant transformation\textsuperscript{27}. Monitoring patients receiving long-term ART for changes in hBD-2 concentration could be a useful strategy in discovering OSCC as early as possible\textsuperscript{28}. Defensins have been suggested to work very well as biomarkers for colorectal cancer, with different expression levels both in tissue and plasma being indicative of different stages in cancer progression\textsuperscript{29,30,31}. Beta-defensins have been used in fusion proteins that targets the epidermal growth factor receptor (EGFR) which is overexpressed in a lot of cancers. \textit{In vivo} tests have shown that this type of fusion protein suppresses cancer cell proliferation and induces mitochondrial-mediated apoptosis of the cancer cells\textsuperscript{32}.

Alfa-defensins are useful as a biomarker when testing for periprosthetic joint infection. Using a simple commercially available test-kit that measures alpha-defensin concentrations, a specific and sensitive result could be produced. This method is more
accurate than current diagnostic methods which do not use alpha-defensins as markers\textsuperscript{33,34}.

Psoriasis is an autoimmune disease that lacks good quantitative markers for disease activity. hBD-2 concentration in serum is directly correlated with the disease activity of psoriasis, enabling monitoring of the disease activity over time and treatment\textsuperscript{35}.

4.2 Defensins as antiviral agents

It has been shown that all three families of defensins have antiviral activity \textit{in vitro}. Theta- and beta-defensins erect networks of crosslinked surface glycoproteins to block membrane fusion by viruses. They are effective in stopping viral entry even if the virus has started to fuse with the host membrane\textsuperscript{16}. Alpha-defensins also inhibit viral entry at multiple steps but the mechanism is not known. Both alpha and beta defensins interact with the herpes simplex virus at multiple steps in pre- and post-entry, inhibiting its life cycle\textsuperscript{18,36}. All types of defensins also interact with influenza virus A to aggregate the virus and promote uptake by neutrophils\textsuperscript{37}.

\textit{In vivo} testing has revealed that HNP-1 plays an important part in antiviral immunity that is not related to their direct antiviral activity \textit{in vitro}\textsuperscript{38}. HNP-1 has also proved to inhibit influenza virus replication post-infection through cell-mediated pathways, inhibiting protein kinase C in the process which suggests PKC pathway involvement\textsuperscript{39}.

HNP-1 has the ability to inhibit HIV-1 infection in many ways, this ability is however diminished greatly in human and bovine serum. The proposed reason for this is that the oligomeric form of defensins is the main contributor to the antiviral activity, and this form is disrupted in serum\textsuperscript{40}.

4.3 Defensins aiding in wound healing

Maggot therapy has been used worldwide to treat wounds for a very long time, and it has been seeing a surge of use in modern times\textsuperscript{41}. It works by debridement (removal of dead tissue), disinfection and an accelerated rate of wound healing\textsuperscript{42}. It is useful in treating non-healing wounds and has been used to treat infections by methicillin-resistant \textit{Staphylococcus aureus} (MRSA)\textsuperscript{43}. The insect defensin lucifensin is the main
disinfecting component found in maggots from the green bottle fly (*Lucilia sericata*) which is the most common larvae used\textsuperscript{44,45}. This was discovered as recently as 2010, which means that defensins have been used therapeutically as alternatives to antibiotics without knowing of their involvement\textsuperscript{44}.

Coprisin is a defensin-like AMP discovered in the dung beetle\textsuperscript{46}. It has potent antimicrobial activity against both gram-negative and gram-positive species of bacteria and uses the same mechanism as defensins and other AMPs, disrupting the bacterial membrane through a strong net positive charge\textsuperscript{47}. Coprisin has a similar effect to the antibiotic ampicillin when treating wounds infected by *Staphylococcus aureus* and promotes reepithelization and neovascularisation of the wound tissues. Compared to controls, coprisin- and ampicillin-treated wounds have more leukocytes and fibroblasts which indicates an accelerated rate of healing\textsuperscript{48}.

Similarly to coprisin, hBD-3 has a positive effect on wound closure. It also inhibits bacterial growth of *S. aureus* in infected diabetic wounds in preclinical tests on pigs as well as on epithelial skin grafts on nude rats\textsuperscript{49}.

Bee defensin-1, a peptide found in royal jelly, has demonstrated an ability to promote reepithelization and wound closure in uninfected excision wounds. It does so by promoting MMP-9 secretion from keratinocytes and increasing keratinocyte migration\textsuperscript{50}.

### 4.4 Defensins in vaccination

Beta-defensins show potential as viral vaccine adjuvants\textsuperscript{51}. Murine beta-defensin 2 (mBD2) increases specific immune response in the host when used as an adjuvant in a DNA-construct vaccine. The vaccines using mBD2-antigen DNA constructs were more successful at eliciting an immune response than those with just the antigen\textsuperscript{52}.

Bacillus Calmette-Guérin (BCG) is the standard vaccine for tuberculosis (TB) currently in use. An issue with the BCG vaccine is that the protection against TB is very inconsistent, made even more problematic by the fact that revaccination does not increase the protection. This makes it hard to gain consistent protection against tuberculosis and if increased risk of infection is suspected, immunization is hard.
Revaccination has therefore been discontinued in many developed countries\textsuperscript{53,54}. It has been shown that when combined with different antigens, two mBD2 DNA vaccines can provide similar protection against tuberculosis infection as BCG. The DNA-vaccines when used together with the BCG vaccine offers a greater protection against tuberculosis infection than just BCG alone. This dual vaccination strategy could prove a very effective way of boosting the immunization against TB\textsuperscript{52}.

5 Potential negative effects
A major consideration when designing therapy with the respiratory tract in mind is Surfactant protein (SP)-D. It is an important antiviral protein of the innate immune system, unsurprisingly found in lung surfactant. Alpha defensins bind to SP-D which reduces SP-D’s antiviral properties substantially. Beta defensins do not bind into SP-D with the same affinity, however they have much lower antiviral activity compared to alpha defensins. Theta defensins bind into SP-D with strong affinity, however they do not reduce its antiviral activity\textsuperscript{55}.

Alpha-defensins promote epithelial cell proliferation at low concentrations and conversely are cytotoxic at higher concentrations. Note that a relatively high concentration of defensins are required for their antimicrobial effect. Thus, alpha defensins are poor candidates for antimicrobial therapy. However, beta-defensins do not affect epithelial cells at any concentration, making them a better option against bacteria\textsuperscript{56}. Theta-defensins are not cytotoxic or hemolytic. Acyclic porcine protegrins are however, as a representative of the cathelicidin family of AMPs, toxic to human fibroblasts and leads to lysis of erythrocytes\textsuperscript{57}. This makes defensins a far easier AMP to work with in therapeutical development.

6 Conclusion
It seems clear that defensins have the potential to work in a multitude of different therapeutical applications, while already working effectively in maggot therapy. However, \textit{in vivo} testing is still lacking good data and clinical trials have shown poor results\textsuperscript{58}. 
Alpha-defensins have in vitro properties that allow it to inactivate several different viruses, these are however lost in vivo\textsuperscript{59}. There is also conflicting data on whether HD5 and HD6 increases the infectivity of HIV in physiological conditions\textsuperscript{60,61}.

Because of the complex nature of the in vivo environment it is not yet fully know why the results are poor. It is partly because of weak binding to the host cells which reduces the effectiveness of pathogen-defensin interaction. It is theorized that finding or synthesizing defensins that have lost the ability to interact with the host cells would result in minimal loss of antipathogen activity in vivo. This would be a crucial step towards making defensins viable in a therapeutical setting\textsuperscript{62}.

Given that defensins play a large role in a lot of processes, from regulating the microbiotic homeostasis in the small intestine\textsuperscript{63}, to preventing HIV-1 entry or even enhancing HIV infectivity, the in vivo implications for therapy are extensive and needs to be characterized properly before effective treatment options can become available. This also means that defensins have the potential to be very versatile therapeutic tools that can be used in a wide variety of situations. Because of their ancient origin and widespread usage in the tree of life as well as their obvious usefulness it would be foolish not to try to apply them therapeutically as treatment to various ailments. Will they reach the point where they can be clinically relevant? Only time will tell.

7 Societal relevance

With antibiotic resistance ever increasing, more methods are required to stem the flood of infectious threats. These methods could be alternatives to the currently overused antibiotics, or they could take part in the antibiotic process and minimize resistance development. Defensins could work as both alternatives to antibiotics as well as a method of helping the process. And defensins could do much more, showing potential in wound healing and combating viruses among other things. The only thing seemingly preventing them from working therapeutically is poor characterization. With an increased knowledge of defensins and the way they work inside our bodies comes a great therapeutical value. This knowledge could lead to a vast array of new methods to not only combat infection but also control regulation of the immune
system and bodily microbial homeostasis. This is in turn can lead to advances in treatment of autoimmune diseases and allergies among other things.

8 References


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