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Peptide Design for Antimicrobial and Immunomodulatory Applications

Evan F. Haney and **Robert (Bob) E.W. Hancock***

Abstract

The increasing threat of antibiotic resistance in pathogenic bacteria and the dwindling supply of antibiotics available to combat these infections poses a significant threat to human health throughout the world. Antimicrobial peptides have long been touted as the next generation of antibiotics capable of filling the anti-infective void. Unfortunately, peptide based antibiotics have yet to realize their potential as novel pharmaceuticals, in spite of the immense number of known antimicrobial peptide sequences and our improved understanding of their antibacterial mechanism of action. Recently, the immunomodulatory properties of certain antimicrobial peptides have become appreciated. The ability of small synthetic peptides to protect against infection *in vivo* has demonstrated that modulation of the innate immune response is an effective strategy to further develop peptides as novel anti-infectives. This review focuses on the screening methods that have been employed to assess novel peptide sequences for their antibacterial and immunomodulatory properties. It will also examine how we have progressed in our ability to identify and optimize peptides with desired biological characteristics and enhanced therapeutic potential. In addition, the current challenges to the development of peptides as anti-infectives are examined and the strategies being used to overcome these issues are discussed.

Keywords

Antimicrobial peptides; immunomodulatory peptides; host defense peptides

Introduction

With the increasing emergence of antibiotic resistant pathogens[1] and the dwindling supply of antibiotics capable of combating these infections, coupled with a reluctance from pharmaceutical companies to invest in infectious disease research[2], the need for novel antibiotics has never been more urgent. Since their discovery in the 1980s, antimicrobial peptides (AMPs) have been lauded for their potential as novel antibiotics[3,4]. Their broad spectrum antimicrobial activity and selectivity for bacterial over eukaryotic cells make them attractive candidates for novel drugs compounds. Indeed, attempts have been made to harness this potential and a handful of peptides have been developed as novel pharmaceuticals and evaluated in clinical trials[5]. Countless more novel AMP sequences, with broad spectrum antibacterial activity, are reported in the literature on an almost daily basis. While antibiotics derived from naturally occurring AMPs have yet to supplant the most effective antibiotics on the market, significant advances have been made in the field of AMP research both in the identification of novel peptide sequences and in assessing their potential as anti-infectives.

*Corresponding author Centre for Microbial Diseases and Immunity Research University of British Columbia 2259 Lower Mall Research Station Vancouver, British Columbia, V6T 1Z4 Canada bob@hancocklab.com.

More recently, the immunomodulatory properties of AMPs have also become appreciated and many of these peptides are now known to stimulate the immune system while suppressing the inflammatory response. Consequently, the term host defense peptide (HDP) is a better descriptor for these molecules as it encompasses both the direct antibacterial activity as well as their capacity for immunomodulation. As our understanding of the complex mechanisms underlying these immunomodulatory peptides has improved, it has become apparent that this represents a promising new route for expanding the therapeutic potential of HDPs[6]. This review will focus primarily on the strategies that have been developed or accessed in our research program to identify novel AMP sequences and how we have progressed in our understanding of the immunomodulatory properties of HDPs leading to the design of synthetic innate defence regulator peptides (IDRs) with desirable anti-infective and anti-inflammatory properties. For more comprehensive descriptions of the field of novel peptide design we refer the readers to overview references[5–7]. We will also address the current state of developing HDPs as antibiotics and immune modulators and discuss the challenges and strategies that are being used to optimize peptides for clinical use.

Antimicrobial Peptides

AMPs are ubiquitous throughout nature and the significant role they play in preventing and combating infectious pathogens is well established[7]. They play a major role in the immune defence mechanisms of insects and plants and are an important component of the innate immune response of animals, including crustaceans, mammals and humans. Traditionally, AMPs have been isolated from natural sources and their antimicrobial potency has been established *in vitro*. For instance, one of the first reported AMPs was magainin 2 isolated from the skin secretions of *Xenopus laevis* frogs, and the purified peptide had broad spectrum antimicrobial activity against an array of bacterial species[8]. Reports of novel AMP sequences isolated from natural sources are still commonplace in the literature. In fact, there are now over 2100 AMPs derived from natural sources listed in the Antimicrobial Peptide database[9,10] and this number continues to grow.

AMPs are characterized as short peptide sequences typically between 12 and 50 residues in length[11]. There are exceptions to this as antimicrobial activity has been observed for synthetic peptides as short as 6 residues[12] and some larger cationic proteins have direct antibacterial and immunomodulatory properties such as lysozyme[13] and lactoferrin[14]. AMPs are typically rich in hydrophobic residues, including Leu, Ile, Val, Phe and Trp, and they usually have an excess of cationic amino acids which confers a net positive charge, on the order of +2 to +9[7]. These properties allow AMPs, in the presence of phospholipid membranes, to adopt diverse amphipathic structures that can be separated into four broad structural classes: α-helical, β-sheet, extended conformation and looped peptides containing disulphide bridges[7]. The amphipathic nature of these structures is an important component of their mechanism of action against bacteria since amino acid changes that perturb amphipathicity reduce antimicrobial activity. Some AMPs have strong lytic effects on bacterial membranes resulting in direct killing of bacterial cells. Others interact with the cytoplasmic membrane to inhibit events dependent on this membrane including cell wall biosynthesis, energy generation and cell division. Alternatively, some peptides traverse the phospholipid bilayer and enter the bacterial cell where they ultimately interfere with intracellular processes by binding to DNA, RNA and certain proteins. A number of models have been described to explain the various mechanisms of action for AMPs and these have been discussed in detail in recent reviews[5,15–17].

The classical approach to studying AMPs involves identifying and purifying the peptides from a natural source and then measuring the antibacterial potency of a highly pure sample *in vitro*. Some peptides are still identified in such a manner, such as three cysteine-rich

peptides recently isolated from dandelions[18]. Unfortunately, purifying large quantities from a natural source is often impossible, and therefore synthetic peptides are made using solid phase peptide synthesis methods[19], and are obtained at a high level of purity. Synthetic peptides can also be mutated at specific residues to examine the effect that this has on antimicrobial activity. If the peptide loses activity, then that residue is in some way necessary for the AMPs biological function. On the other hand, if the antibacterial potency improves, then this provides important information regarding the structural determinant of activity and elevates the potential of this peptide as a novel anti-infective. This iterative process has been successfully applied to a number of peptides including: indolicidin[20], polyphemusin[21] and bactenecin[22].

Another strategy involves examining the antimicrobial activity of truncated versions of a larger peptide to see if activity is retained. An early study examined N-terminal truncations of magainin 2 and found that the first three residues of the native peptide could be removed without dramatically decreasing the activity while removing the Lys residue at position 4 dramatically decreased the potency of the amphibian peptide[23]. Such a strategy can be used to isolate the residues necessary for the bactericidal effect while reducing the costs required to synthesize longer peptides. For instance, a six residue fragment of bovine lactoferricin was shown to have equivalent antibacterial activity compared to the full length 25 residue lactoferricin B peptide[24] and critically this hexamer is considerably less expensive to produce synthetically (although in our hands[25] it is difficult to make peptides smaller than 8 amino acids with significant antimicrobial activity as measured by a modified CLSI method[26]). This strategy can also be employed to remove regions of peptides that have undesirable characteristics. For example, BMAP-18, a truncated version of the potent bovine myeloid antimicrobial peptide (BMAP-27), was shown to have antiparasitic activity against trypanosomatid parasites, but was significantly less toxic compared to the longer peptide[27].

These iterative approaches to evaluating and improving antimicrobial activity, while ultimately successful, are time consuming and large amounts of synthetic peptides are required to evaluate large numbers of derivatives with the observed increases in antimicrobial activity being oftentimes modest, at best. In fact, it is highly likely that many peptide sequences have been examined in this way and are underreported in the literature because the resulting peptides displayed reduced antibacterial potency. As a result, many researchers have developed methods to identify promising AMP sequences while decreasing the number of peptides that need to be produced synthetically to evaluate their *in vitro* activity.

Early efforts to screen large numbers of peptide sequences involved using combinatorial libraries of short peptides[12]. This method succeeded at identifying short peptide sequences with significant antimicrobial activity. Unfortunately, this method is not amenable to examining longer peptide sequences because the number of permutations and combinations of peptide sequences increases exponentially with the length of the polypeptide chain. With the increasing number of sequenced genomes available, other groups have used genomic approaches to search for novel peptide sequences in the DNA sequences of various organisms. Recent successes describe the identification of novel cathelicidin-like AMPs in pandas[28] as well as monotremes and marsupials[29]. However, such a methodology is limited to scanning for molecules with homology or at least analogy to known AMP sequences and these peptides usually need to be optimized to enhance their therapeutic potential. More high-throughput approaches to examine large numbers of peptide sequences involve phage display[30] and ribosome display[31] with subsequent enrichment for peptides that bind to bacterial membranes. In these cases, researchers are limited by the choice of the immobilized interacting partner and the inherent technical complexity associated with these peptide display technologies. The current bottleneck in developing AMPs as promising

pharmaceuticals lies in our ability to screen novel peptide sequences in a high-throughput fashion while synthesizing sufficient quantities of these peptides to evaluate their antimicrobial activity.

Recently, our lab described a method of peptide screening[25] whereby peptides are synthesized on cellulose sheets and then their activity is screened against a luminescent strain of *Pseudomonas aeruginosa* constitutively expressing a luciferase gene cassette incorporated into the bacterial chromosome[32]. This method uses SPOT-synthesis[33] and standard Fmoc (Fluorenyl methoxy carbonyl) chemistry to generate a series of cellulose tethered peptides with known sequences. The peptides are then cleaved from the cellulose sheets and their ability to inhibit growth of the *P.aeruginosa* strain is measured as inhibition of luminescence. This method was successfully used to generate a complete substitution library of Bac2a (RLARIVVIRVAR-NH_2), a linear variant of the bovine peptide, bactenecin[25]. The single amino acid substitutions of Bac2a that resulted in increased activity were then combined to generate optimized 12-mer and 8-mer sequences with potent and broad spectrum antibacterial activities[25]. Peptide synthesis on cellulose sheets was also used to examine the sequence requirements of Bac2a[34]. In this case, 49 Bac2a derivatives were generated with scrambled amino acid sequences to examine if sequence specificity was required for the antimicrobial activity. Based on the luminescent *P.aeruginosa* killing assay, the peptides fell into 6 different activity classes varying from significantly more active than Bac2a, of equivalent activity to Bac2a or weak to no killing activity at the highest peptide concentration tested. This result supports the idea that AMPs lack a sequence-specific interaction to exert their bactericidal effect and represents a promising approach that can be used to generate novel candidate peptides. One of the most active scrambled peptides, Bac034, was further optimized through a complete substitution analysis and then combining the most active mutations to arrive at peptides with substantially better MIC values compared to Bac2a[34], exactly as had been done previously for Bac2a itself[25]. It should be noted that any active AMP sequences identified using this technique still need to be synthesized in larger quantities to confirm the increased antimicrobial activity against other bacterial species as well as elucidate the mechanism of action and activity in animal infection models. However, the SPOT-synthesis technique of generating multiple peptide sequences on cellulose sheets is a relatively simple and inexpensive way to screen and identify large numbers of novel AMP sequences with potential pharmacological applications.

More recently, computer aided design of AMPs has been used to predict the antimicrobial activity of novel peptide sequences prior to synthesis. These methods rely on the chemo-informatic method of quantitative structure-activity relationship (QSAR) modelling to relate the measured antimicrobial activity to the structural characteristics associated with the equivalent peptide sequences, as defined through the use of dozens of physico-chemical “descriptors” (including inductive parameters such as contact energy between neighbouring amino acids that assess how the properties of amino acids change along the length of the peptide)[35]. Using a test set of peptides derived from Bac2a peptide, novel peptides with significant activity against *P.aeruginosa* were used to predict structure-activity relationships and test the validity of QSAR descriptors[36,37]. These developed QSAR descriptors were then used, along with pattern recognizing artificial neural networks, to predict the antimicrobial activity of a virtual library of 100,000 9 amino acid peptides[38]. A total of 200 peptides from this virtual screen were synthesized to validate the models generated by the QSAR descriptors. This approach proved remarkably accurate, since 94% of the peptides predicted to be better than Bac2a were actually found to be more active, while all of the peptides predicted to be worse than the linear bactenecin derivative had lower MIC values[38]. This strategy successfully identified optimized peptide sequences with antimicrobial activity more than ten-fold better than a peptide that showed efficacy in Phase III clinical trials and

comparable to or better than conventional antibiotics against a broad spectrum of multidrug resistant “Superbugs”; the peptides were also active systemically in mouse infection models[38]. The use of such *in silico* methods has the potential to dramatically increase the number of candidate peptides with antimicrobial activity and is capable of predicting which peptides will be active *in vitro*. These methods save time and resources, by lowering the number of peptides that need to be synthesized (e.g. 100,000 peptides would cost $400,000 to synthesize on peptide arrays and at least $1.2 million for conventional synthesis) as well as decreasing the number of time-consuming MIC measurements that need to be performed.

Immunomodulatory Peptides

HDPs are important components of the immune response as evidenced by the fact that animals defective in production of the mouse cathelicidin CRAMP are more susceptible to infections[39,40]. However, researchers have critically re-examined the role that these biomolecules play in host defense against bacterial infections. Often, the reported MIC values for a given peptide are measured in minimal media or phosphate buffer but it is known that the antimicrobial activities of peptides are highly sensitive to salt concentrations and the presence of divalent cations, serum components and polyanionic glysoaminoglycans[41]. For example, the human cathelicidin peptide LL-37 has MICs against *E.coli* in the low μg/ml range under conditions of low ionic strength but these MIC values go up with increasing NaCl concentration[42] and the antibacterial activity of LL-37 is virtually abolished when tested in tissue culture media[41]. Interestingly, under the latter more-physiological conditions, LL-37 exhibits a wide range of immunomodulatory properties *in vitro* and these activities can be recapitulated in animal models. For example, LL-37 is known to suppress pro-inflammatory cytokines in response to bacterial lipopolysaccharides and lipoteichoic acids[43], prevents activation of macrophages by these bacterial components[44], upregulates the production of chemokines and chemokine receptors[44], promotes angiogenesis[45] and wound healing[46]. These immunomodulatory properties are not limited to LL-37 since other peptides, such as mammalian defensins[47,48], other cathelicidins[49], and synthetic derivatives have immunomodulatory properties (For recent reviews, see [6,17,50]).

One of the greatest obstacles in the development of immunomodulatory peptides as therapeutics is identifying how the peptides interact with and stimulate the cells of the immune system. There is evidence that immunomodulatory peptides target multiple receptors and processes within cells, depending on both the cell type and the amino acid sequence of the peptide. For instance, LL-37 indirectly stimulates the P2X(7) receptor in human embryonic kidney cells[51] and transactivates epidermal growth factor receptor in epithelial cells[52], interacts with formyl peptide receptor-like 1 in many cell types[53] and enhances TLR3 signalling in response to viral dsRNA[54]. Most HDPs share characteristics with cell penetrating peptides and they can translocate into eukaryotic cells, which appears to be necessary for many of their activities. For example, biotinylated LL-37 is actively internalized into epithelial cells through endocytosis and accumulates in the perinuclear region of the cell[55]. Once inside the cell, these peptides are free to bind to intracellular targets, such as LL-37 binding to GAPDH [56] or synthetic IDR-1 peptide (see below) binding to sequestosome-1/p62[57] leading to signal transduction (e.g. through p38 mitogen-activated protein kinase [MAPK]) and chemokine induction.

Ultimately, HDPs affect multiple signalling pathways within a cell. A systems approach was used to examine the effect of LL-37 on the immune response of CD14+ monocytes. In total, 475 differentially expressed genes were detected by microarray analyses, and linked to the involvement of several signalling pathways in the activities of LL-37[58]. Some of these pathways including the MAPKs, p38, JNK, extracellular signal-regulated kinase-1/2

(ERK1/2), as well as Src-family kinases and PI3 kinases[58]. Evidently, the interactions between immunomodulatory peptides and cells of the immune system are complex and the response of the immune system to the stimulation of a peptide depends on the sequence of the peptide, the receptors that it interacts with, the cell type, and the other endogenous and pathogen related signals present.

Similar to the approaches used in optimizing the antimicrobial activity of peptides, iterative approaches have been used to try and understand the mechanisms of immune cell stimulation. For example, truncated versions of LL-37 were tested for their ability to induce IL-8 production in keratinocytes and the response to the peptide was different depending on whether the peptide was shortened from the N- or C- terminus[59]. The endotoxin neutralizing capacity of a truncated 18-mer of LL-37 was also optimized through amino acid substitution to generate a peptide that protected mice against endotoxin shock[60].

Natural HDPs with inherent immunomodulatory activity have served as templates to generate synthetic IDR peptides with a remarkable ability to modulate the immune response in cell cultures and *in vivo*[61–63]. The potential of immunomodulatory peptides as novel therapeutics was first illustrated by the peptide IDR-1 (KSRIVPAIPVSLL-NH_2)[61]. IDR-1 was generated from Bac2a by designing a sequence with two internal Pro residues that was incompatible with antimicrobial activity. It was screened for its ability to enhance chemokine induction and suppress LPS-stimulated pro-inflammatory cytokines such as TNF-α in human peripheral blood mononuclear cells and for efficacy in mouse infection models. As expected, IDR-1 displayed absolutely no direct antimicrobial activity, even in buffer, but protected mice from methicillin-resistant *S.aureus*, vancomycin resistant *Enterococcus* and *Salmonella* infections and influenced several signalling pathways in human monocytes leading to the production of certain immune cell-recruiting chemokines and suppression of inflammatory responses (as confirmed in the animal model studies)[61]. The discovery of IDR-1 and its effectiveness in preventing and treating infections provided the important discovery demonstrating that modulation of the innate immune response provides an effective strategy to combat antibiotic resistant infections as an effective complement to current therapeutic options.

Compared to the better-described structure-activity relationships concerning the direct antibacterial activity of AMPs, relatively little is known regarding the structural and sequence requirements underlying the immunomodulatory properties of HDPs. This is likely due to the multiple targets with which HDPs interact to eliciting cellular responses and the different requirements for peptide uptake into cells, making it complicated to isolate specific structural characteristics responsible for the stimulation or suppression of a specific signalling pathway; in addition the assay systems are more labour intensive making high throughput analyses difficult. Despite this, a synthetic library approach using QSAR methodology was recently undertaken to iteratively examine the effect of point substitutions, scrambling and deletion variants of Bac2a and how these affected the immune stimulating properties of the resulting peptides. Using this methodology, IDR-1002 (VQRWLIVWRIRK-NH_2) was identified as a much stronger inducer of chemokines production than IDR-1 and was able to more effectively protect mice from invasive *S.aureus* infection[63]. It was found that IDR-1002 both induces chemokines[63] and enhances monocyte migration towards chemokines on fibronectin[64] suggesting that optimizing peptides that modulate chemokine production and immune cell migration is a promising avenue for the generation of peptides with improved *in vivo* protective properties. In addition, the benefits of immunomodulatory peptides may extend beyond their anti-infective activities as IDR-1002 has demonstrated potential as a treatment option to control chronic inflammation in arthritis[65] and may incorporated into microparticle vaccine formulations to improve the immune response to vaccines[66,67].

Another promising immunomodulatory peptide was identified from the above-described Bac2a screen. IDR-1018 (VRLIVAVRIWRR-NH_2), has modest antibacterial activity but showed considerable promise as a novel immunomodulatory peptide by strongly inducing MCP-1 and MCP-3 chemokine expression and suppressing the LPS induced production of TNF-α in peripheral blood mononuclear cells[62]. More recently, it has been shown that IDR-1018 modulates the differentiation of human macrophages[68], promotes wound healing[69], protects against invasive *S.aureus* infections of mice and shows promise as an adjunctive treatment for malaria[70] and protects against lung infections and pneumonia caused by multi-drug resistant strains, cf. IDR-1002[71]. Structural studies were performed on IDR-1018 to better understand the structure-activity relationships responsible for its immunomodulatory properties. IDR-1018 was unstructured in phosphate buffer, adopted an α-helical conformation in DPC micelles and formed a predominantly β-turn structure in the presence of SDS micelles and anionic vesicles[62]. This structural plasticity, depending on the nature of the environment, indicates that the structural requirements for immunomodulatory and antibacterial activity are complex and that modest alterations in the sequence of the peptide can have dramatic impacts on the biological activity of a peptide. Intriguingly our preliminary QSAR studies have indicated that the descriptors for antimicrobial and immunomodulatory activity do not strongly overlap indicating that they are independently structurally determined (Jenssen and Hancock, unpublished). These studies provide an important first step in furthering our appreciation of the structural aspects that govern the activities of IDRs and as our understanding of these features improves, it can be applied to future QSAR studies to generate novel IDR sequences with optimized immunomodulatory characteristics.

Judging from the examples presented for AMPs and synthetic IDR peptides, it appears that we are at a point where we can reasonably identify and screen peptides for their direct antibacterial activity and immunomodulatory properties. Our understanding of the characteristics that contribute to direct antibacterial activity is quite extensive owing to years of research from many research groups that correlates the AMP sequence and structure to its potency, although the complexity of descriptors means that no simple relationship between structure and activity can be drawn. Nevertheless we can use combinations of these descriptors as inputs for QSAR modelling to potentially test hundreds of thousands of sequences *in silico* and predict novel peptides with excellent therapeutic potential as antibacterial agents. The structural requirements underlying immunomodulation are comparatively poorly understood, owing to the complexity of the cellular response to the presence of an immunomodulatory peptide. However, initial semi-random and iterative design studies have successfully generated synthetic IDRs with excellent *in vivo* activity, emphasizing that such an approach is a viable method for generating novel immunomodulatory peptides. As mentioned earlier, the immunomodulatory activities of novel synthetic IDRs are difficult to predict due to the many different responses that can occur depending on the cell type. As a result, specific tests that correlate with a desired immune response, such as increased chemokines release from peripheral blood mononuclear cells[61] or anti-inflammatory activity reducing LPS-stimulated TNFα production[62], can be used to screen and asses the immunomodulatory activity *in vitro*. Ultimately, since the innate immune response is inherently complex and dependent on other underlying stimuli (e.g. from the infection itself), the immunomodulatory activity of each new IDR peptide needs to be confirmed *in vivo*. This is typically labour-intensive and involves significant cost[6]. Regardless, efforts are currently underway to understand which cellular responses are the best predictors of immunomodulatory activity and improved QSAR modeling of synthetic IDR peptide using updated descriptors should generate novel sequences with applications towards improving human and animal health.

Therapeutic Applications of Peptides – Successes and Challenges

Despite the very substantial number of AMPs that have been identified and their recognized potential as antibacterials and immunomodulators, a relatively small number of peptides have made it to clinical trials. Examples of peptides at their most advanced stages of clinical development and their targeted clinical applications are shown in Table 1. Many of the antimicrobial HDPs are being considered for topical application either because of systemic toxicity or lability to proteases in the blood. Peptides are rapidly metabolized within the body and it has been suggested that high doses of peptide are required to achieve the desired antibiotic effect *in vivo*, which is much easier to achieve through topical application. It is worth mentioning that the protective effects of IDR-1 were observed in mice with intravenous, intraperitoneal or subcutaneous administration and when the peptide was administered either 48 hours prior to or 6 hours after infection[61], suggesting that the immunomodulation induced by peptides continues even after the peptide is cleared from circulation. Regardless, several strategies have been used to overcome these issues associated with peptide stability and toxicity.

The most obvious obstacle to the administration of AMPs as therapeutics is their inherent susceptibility to proteolytic degradation. If administered orally, the peptides will encounter the hydrolytic activities of pepsin, trypsin and chymotrypsin as they travel through the digestive tract. Alternatively, if administered systemically by IV, they can be degraded by proteases present in the blood or taken up by cells and rapidly distributed throughout the body. Additionally, some bacterial species are also known to produce proteases that inactivate certain AMPs[72,73] leading to their enhanced survival in the presence of peptide. Consequently, while no formal pharmacokinetic studies have been published to date, peptides are likely to have an inherent short half life *in vivo* and several strategies have been employed to improve the proteolytic stability of peptide based drugs[74].

One simple strategy to block proteolytic degradation involves acetylation of the N-terminus to block the activity of aminopeptidases[75], although this does remove one positive charge, which might impact on activity. Peptide cyclization, through a disulphide bridge or joining the backbone at the N- and C- terminus, has also been shown to improve serum stability of short synthetic AMPs[76]. A popular strategy to improve proteolytic stability of peptides is to incorporate non-natural D-isomers of amino acids, altering the stereochemistry of the peptide backbone and inhibiting susceptibility to proteases. The D-enantiomer of a peptide, which is a mirror image of the native L-peptide, often retains the antimicrobial activity of the native sequence because the interactions with the bacterial membrane are not dependent on interactions with a specific receptor[77,78]. Interestingly, it was recently reported that a protease resistant D-enantiomer of peptide M33 (KKIRVRLSA) was more active against Gram-positive pathogens than the L-amino acid enantiomer[79], indicating that D-isomers of AMPs might be further optimized beyond the simple conversion of the peptide sequence from the natural L-form. A similar approach uses the retro-inverso (RI) D-isoform of a peptide, in which the peptide is synthesized with the opposite N- to C- sequence using only D-enantiomers. Such an approach maintains the spatial orientation of the amino acid side chains found in the native peptide after folding, while protecting the backbone from proteolytic degradation. This strategy was recently used to generate protease resistant D- and RI- forms of bovine myeloid antimicrobial peptide 28 (BMAP28) that retained much of the antimicrobial activity of the parent peptide. Interestingly, the D and RI forms of BMAP28 also retained their immunomodulatory properties but the RI peptide was significantly less toxic towards epithelial cells and monocytes[80]. The immunomodulatory properties of D-peptides have not been examined in detail, but their immense potential is highlighted in the observation that D-LL-37 is a more potent stimulator of IL-8 in keratinocytes compared the natural L-isoform of LL-37[59]. These studies clearly demonstrate that peptide enantiomers

have the potential to give rise to protease resistant HDPs with desirable immunomodulatory properties.

Incorporation of unnatural amino acids into peptide sequences also provides improved metabolic stability and increases the range of physicochemical properties that can be used to optimize peptides as antibacterial agents. Because of the importance of positive charge to the antimicrobial activity, several conservative substitutions can be made for the cationic residues, Lys and Arg, that change the length of the side chain but preserve the positively charged amino or guanidino group. For instance, replacement of one arginine residue in the apidaecin 1b analog, Api88, by L-ornithine or L-homoarginine increased the peptide stability in serum without dramatically affecting the activity against *E.coli*[81]. Tryptophan residues are also considered important residues for determining the antimicrobial activity of AMPs[82] and are another amino acid that is commonly substituted to modulate the activity of peptides. The Trp residues in peptide P-113 were replaced with β-naphthylalanine and β-(4,4′-biphenyl)alanine resulting in peptides that retained their potency at physiological salt concentrations[83]. Other groups have optimized for simple characteristics of AMPs, such as cationicity and amphipathicity, and applied these traits to the design and synthesis of novel peptides containing unnatural amino acids. A recent report describes the screening of 36 sequences that incorporate tetrahydroisoquinolinecarboxylic acid (Tic) and octahydroindolecarboxylic acid (Oic) residues and the resulting peptides were found to have MICs as low as 6.25μg/ml against the clinically relevant ESKAPE pathogens[84]. While the reported activities were relatively modest, as our understanding of the use of non-natural amino acids improves, they can be used to make test sets of peptides and then used in computational QSAR studies to optimize the antimicrobial potency of AMPs containing non-natural amino acids. This would expand the tool box for synthesizing AMPs from the 20 naturally occurring amino acids to an almost endless supply of amino acid derivatives that are only limited by the organic chemistry required to generate them.

Peptidomimetics are polymeric molecules that mimic peptides but have altered backbone structures to improve peptide stability while maintaining the biological properties of the parent peptides (for recent reviews see [85,86]). The principle behind using peptidomimetics is to preserve the spatial orientation of the side chain residues while altering the peptide backbone to make it impervious to the activity of proteases. Peptidomimetics are used in a variety of biological applications and many examples of peptidomimetics based on AMP sequences have been described including: β-peptides[87], peptoids[88] and oligoacyllysines[89]. Peptidomimetics have not been widely studied for their immunomodulatory activities but it is conceivable that many of the immunomodulatory properties seen in IDR peptides could be engendered in mimetics provided that they are still able to translocate into cells and/or interact with above-described cellular receptors that influence the immunomodulatory response. Several peptidomimetics based on HDPs as well as non-natural amino acid substituted peptides are in various stages of clinical development[5] demonstrating that these are viable approaches for harnessing the therapeutic potential of HDPs while addressing the issue of stability *in vivo*.

Various drug delivery systems have been designed to improve the stability of peptide based drugs, improve their bioavailability *in vivo* and target them to specific sites within the body. Since AMPs are known to interact with biological membranes, lipid based formulations are a logical extension of this to improve the biological properties of peptides[90]. An interesting example used melittin-loaded perfluorocarbon nanoemulsion particles to target the cytotoxic peptide to tumor cells while blocking the extremely hemolytic activity of the melittin peptide[91]. In addition to liposomal formulations, other nanoparticles have been examined as potential AMP carriers including: dendridite polymers, solid core nanoparticles, carbon nanotubes and DNA cages[92]. PEGylation, the process of covalently adding polyethylene

glycol chains to polypeptides, is another relatively common practice used to improve stability bioavailability of protein and peptide drugs[93]. Indeed, PEGylated versions of synthetic AMPs have been shown to retain their antimicrobial activity while improving their biocompatibility and protease stability[94,95]. However, care needs to be taken when covalently attaching large PEG moieties to peptides as these may negatively impact the interactions between the peptide and bacterial cells, resulting in lowered antimicrobial activity[96].

Another obstacle to the development of peptides as pharmaceuticals is the relatively high cost associated with generating synthetic peptides on a large scale[7]. Recombinant expression of peptides could be used to generate large quantities of peptides with low materials costs. However, there are drawbacks to using bacterial heterologous expression of AMPs. Firstly, the overexpressed peptide is often toxic to the bacterial cell as these molecules have inherent antibacterial activity. This can be blocked by expressing the peptide bound to a large (anionic) fusion protein which masks the toxic effects of the peptide inside the cell. A recent method, appropriate for large scale and cost-effective production of HDPs, successfully produced seven different recombinant HDPs as SUMO (Small Ubiquitin-like Modifier protein) fusions, including LL-37 and IDR-1[97]. However, it should be pointed out that no AMPs currently being evaluated in clinical trials are made recombinantly, although plectasin that has been developed pre-clinically is indeed made recombinantly. Additionally, when using recombinant methods to generate peptides, one must neutralize the carboxyl terminus chemically and one is limited to using the 20 naturally occurring amino acids as building blocks, precluding the incorporation of non-natural amino acids or peptidomimetics to improve peptide activity and stability.

The potential of HDPs as novel antibacterial treatment options for human pathogens continues to drive much of the research into these natural molecules. Interestingly, their use may extend beyond topical and systemic antibiotics and their potential applications in other areas are currently being evaluated. For instance, biofilms are multicellular communities of bacteria that grow on surfaces with enhanced resistance to antibiotics and disinfectants, making them difficult to eradicate[98,99]. Increasing evidence demonstrates a clear link between biofilms and a negative impact on human health[100] and it is estimated that as many as 80% of infections in the body are due to bacteria in biofilms[99]. Specific HDPs have been shown to inhibit the formation of biofilms, even at peptide concentrations below the MIC for planktonic bacteria[101,102], suggesting that HDPs may be useful anti-biofilm agents. Intriguingly such peptides have broad spectrum anti-biofilm activity and this activity appears completely independent of activity against free swimming (planktonic) bacteria[101]. Additionally, tethering of AMPs to surfaces has generated non-toxic antimicrobial and anti-biofilm surfaces for use in implant devices[103]. It appears that the mechanism of bacterial killing by tethered peptides may be different from that of peptides in solution[104], since a largely independent series of peptide descriptors define optimized tethered peptide sequences.

Conclusions

The increasing prevalence of antibiotic resistant pathogenic bacteria and the burden that this places on health systems throughout the world[105,106] highlights the desperate need to develop novel antibiotic compounds. HDPs have long been touted for their potential to fill the current void in novel antibiotic discovery, but this potential has yet to be realized and only a handful of anti-infective peptides have entered clinical trials with no approved drugs to date. Much of the research thus far has focused on optimizing the direct antibacterial activity of HDPs but this strategy has yet to yield novel therapies and clinical trials have been largely limited to topical applications. Recently, the immunomodulatory properties of

HDPs have garnered significant attention and many peptides are now known to stimulate the innate immune response while suppressing potentially harmful inflammation. Our group has developed several peptide screening techniques to evaluate the antibacterial and immunomodulatory properties of peptides in an attempt to quickly and effectively identify synthetic IDR peptides with therapeutic potential. Ideally, the peptides with the greatest pharmaceutical potential might be those that posses both immunomodulatory and antibacterial (or anti-biofilm) activities (Figure 1). Several strategies have also been developed to improve the stability of HDPs *in vivo* which should lead to better pharmacokinetic and pharmacodynamic profiles for HDP based drugs and this is a research area of great importance to the field. Evidently, more work is required to completely understand HDPs but the outlook for HDPs as novel antimicrobial and immunomodulatory therapeutics remains promising and we anticipate that in the near future their potential as anti-infectives will finally be realized.

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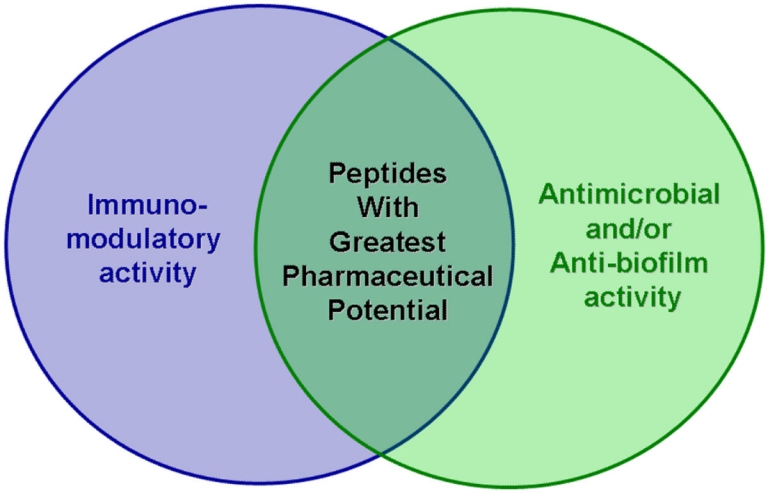

Figure 1.

HDPs can have direct antimicrobial (or anti-biofilm) activity and/or immunomodulatory properties. Peptide sequences can be optimized for their direct antimicrobial activity, or they can be optimized for their ability to modulate the immune response. Those peptides that possess strong immunomodulatory properties and have potency in inhibiting biofilms or killing bacteria are likely to have the greatest potential to be developed as novel anti-infective drugs.

Table 1

Examples of host defence peptide in clinical trials. Table adapted from Acafan et al.[17] and Fjell et al.[5]

Peptide	Sequence	Company	Application	Progress	Reference
Pexiganan acetate	GIGKFLKKAKKFGKAFVKILKK-NH_2	Access Pharmaceuticals	Topical antibiotic	Phase III	NCT00563433 & NCT00563394
Omiganan (MX226/MBI-226)	ILRWPWWPWRRK-NH_2	Migenix/BioWest Therapeutics	Prevent catheter infections, topical antiseptic, severe acne and rosacea	Phase IIIb/II	NCT00027248 & NCT00231153
hLF1–11	GRRRRSVQWCA-NH_2	AM-Pharma	Bacteraemia and fungal infections in immunocompromised haematopoetic stem cell transplant recipients	Phase I/II	NCT00509938
Iseganan (IB-367)	RGGLCYCRGRFCVCVGR-NH_2	Ardea Biosciences	Oral mucositis	Phase III	NCT00022373
PAC-113	AKRHHGYKRKFH-NH_2	Pacgen Biopharmaceuticals	Oral candidiasis	Phase IIb	NCT00659971
IMX942	5 amino acid peptide derived from IDR-1	Inimex	Nosocomial infections, neutropenia	Phase II	http://www.inimexpharma.com/prod_tech_profile.html
OP-145	IGKEFKRIVERIKRFLRELVRPLR-NH_2	OctoPlus; Leiden University	Chronic middle ear infections	Phase I/II	ISRCTN84220089
Plectasin (variant NZ2114 in development)	GFGC_1NGPWDEDDMQC_2HNHC_3KSIKGYKGGYC$_1$AKGGFVC$_2$KC_3Y	Novozymes	Broad spectrum antibiotic	Pre-clinical	http://www.novozymes.com/en/news/news-archive/Pages/45873.aspx