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Review

Novel peptide therapeutics for treatment of infections

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As antibiotic resistance increases worldwide, there is an increasing pressure to develop novel classes of antimicrobial compounds to fight infectious disease. Peptide therapeutics represent a novel class of therapeutic agents. Some, such as cationic antimicrobial peptides and peptidoglycan recognition proteins, have been identified from studies of innate immune effector mechanisms, while others are completely novel compounds generated in biological systems. Currently, only selected cationic antimicrobial peptides have been licensed, and only for topical applications. However, research using new approaches to identify novel antimicrobial peptide therapeutics, and new approaches to delivery and improving stability, will result in an increased range of peptide therapeutics available in the clinic for broader applications.

Introduction

The early years following Fleming's widely heralded discovery of penicillin in 1929, and Florey's subsequent demonstration of the chemotherapeutic activity, were followed by a golden era of discovery. This was, however, soon followed by the spectre of antibiotic resistance. This has led to a constant race between researchers developing new compounds, and bacteria developing resistance.

The majority of antibiotics have been identified from natural sources, produced by other microbes. The first synthetic antibiotic was Prontosil, the forerunner of the sulfonamides (Domagk, 1935). However, the heyday of antibiotic discovery in the last century has given way to the present situation where we have a plethora of derivatives of the old classes, but few new leads in the biopharmaceutical pipeline. It has been estimated that it takes 12 years and over £250 million to bring a drug to market, and only a third of those that are successful yield a profit (Billstein, 1994). Therefore it is unsurprising that pharmaceutical houses have turned their attention to the more lucrative antifungal and antiviral drugs, where the market is less crowded. In the past 40 years, only a handful of new classes of antibiotics have appeared, and these new drugs are only effective against Gram-positive bacteria.

Traditionally, clinicians have used a range of polypeptide antibiotics, which fall into two basic classes. Firstly there are the cyclic decapeptides, including the tyrocidins and gramicidin S, and secondly the polymyxins. Both classes work in a similar way, in that the primary action is due to the binding of the compound to bacterial membranes, which subsequently disturbs membrane integrity and function. These peptide antibiotics are especially active against Gram-negative bacteria.

There will always be a place for the traditional serendipitous screening for microbial compounds with an ability to inhibit growth of other microbes, and the rational synthesis of analogues, but new classes of compounds are being evaluated, many of which are peptides or proteins. A potentially rich source of peptide therapeutics that is being investigated by researchers is the innate immune response, the effectors of which are produced by eukaryotes to defend themselves against microbial attack. Examples discussed in more detail below are cationic antimicrobial peptides (CAMPs) and peptidoglycan recognition proteins (PGRPs). In addition, systems biology will also facilitate the development of novel antibiotics, by an understanding of the interaction of microbe and host cell at the molecular level. The example of cyclic peptides is discussed below, as a way of producing entirely new peptide antibiotics against targets identified in this way.

Novel approaches to develop CAMPs as therapeutics

Over the last 20 years, around 1500 CAMPs have been identified (Wang *et al.*, 2009). CAMPs are innate immune effectors produced by a broad range of different organisms, which demonstrate antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, parasites and enveloped viruses. Some have even been reported to possess anti-tumour properties. CAMPs are positively charged, between 12 and 100 amino acids in length, and form an amphipathic structure (for reviews see Jenssen *et al.*, 2006; Zaiou, 2007).

Despite their similar general physical properties, individual cationic peptides have very limited sequence homologies although there are four prominent structural classes of

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CAMPs: α-helical peptides, the disulfide-bonded β-sheet peptides, the loop-structured peptides and the extended peptides (Fig. 1) (Jenssen *et al.*, 2006; Llobet *et al.*, 2008). The α-helical peptides are linear molecules with no rigid structure in aqueous media but that form amphipathic helices in proximity to hydrophobic membranes, e.g. magainins (Zasloff, 1987) and melittins (Steiner *et al.*, 1981). β-Sheet peptides have a more ordered structure normally maintained by intramolecular disulfide bonds, e.g. defensins (Ganz & Lehrer, 1994) and protegrins (Steinberg *et al.*, 1997). In contrast, the loop-structured and extended peptides are relatively unstructured, with the exception of the loop structure itself.

Although the exact mechanism of action of CAMPs has yet to be established, it is believed that the initial phase is via electrostatic interaction with the target cell. Positively charged peptides are attracted to negatively charged surface components, such as heparin sulfate found in viral envelopes, charged LPS moieties in Gram-negative bacterial outer membranes, and lipoteichoic acids of Gram-positive bacteria (Jenssen *et al.*, 2006). In the case of Gram-negative bacteria, their survival is dependent on the integrity of their outer membrane, which is highly dependent on LPS. LPS is polyanionic and forms bridges with other LPS molecules via divalent cations. CAMPs readily form complexes with LPS in preference to cations and traverse the outer membrane via the 'self-promoted uptake' mechanism (Hancock, 1984; Vaara, 1992). For some peptides, lethality is caused via subsequent cytoplasmic membrane disruption; however, there are data suggesting that some CAMPs also cross into cells where there may be intracellular targets (Brogden, 2005). The possibility that peptides have multiple targets, as well as their fundamental interaction with the bacterial membrane, means the chances of resistance by target modification are slim, as this would require the complete alteration of the membrane and/or several biochemical pathways to be bypassed (Marr *et al.*, 2006). Resistance to conventional antibiotics can emerge at rates of 10^{-7}–10^{-10}, whereas resistance to antimicrobial peptides has not been reported to arise naturally, although repeated subculture on subinhibitory concentrations of CAMPs can induce resistance, which is linked to production of proteases or alteration of membrane composition (Marr *et al.*, 2006). Resistance required over 10 passages with 50 % of the MIC of the peptide present and only increased the MIC at most fivefold (Hancock, 2001). There are also a few bacteria that are naturally resistant to CAMPs, such as *Burkholderia*, due to unique membrane composition.

As well as reacting directly against micro-organisms, CAMPs have recently been described as mediators of host immune response. This change in understanding as to the way peptides may function has led to the term 'host defence peptides' (HDPs) being used, as antimicrobial activity is likely due at least in part to immunomodulatory effects. This proposed mechanism would explain how some peptides can be present *in vivo* at suboptimal concentrations, yet still bring about an antimicrobial response, especially when some peptides have been reported as losing direct killing activity under physiological salt and serum conditions (Bowdish *et al.*, 2005; Goldman *et al.*, 1997). HDPs can alter cellular functions such as apoptosis, chemotaxis, gene transcription and cytokine production, as well as enhance angiogenesis and wound healing (Allaker, 2008). LL-37, the only reported human cathelicidin, has been reported both as having direct antimicrobial activity and as an initiator of host response via Toll-like receptors (Yoshioka *et al.*, 2008). HDPs have also been developed synthetically that show no *in vitro* antimicrobial action, but are very effective in bringing about a selective immune response *in vivo* with no toxicity problems, e.g. IMX00C1 (Bowdish *et al.*, 2005) and IDR-1 (Scott *et al.*,

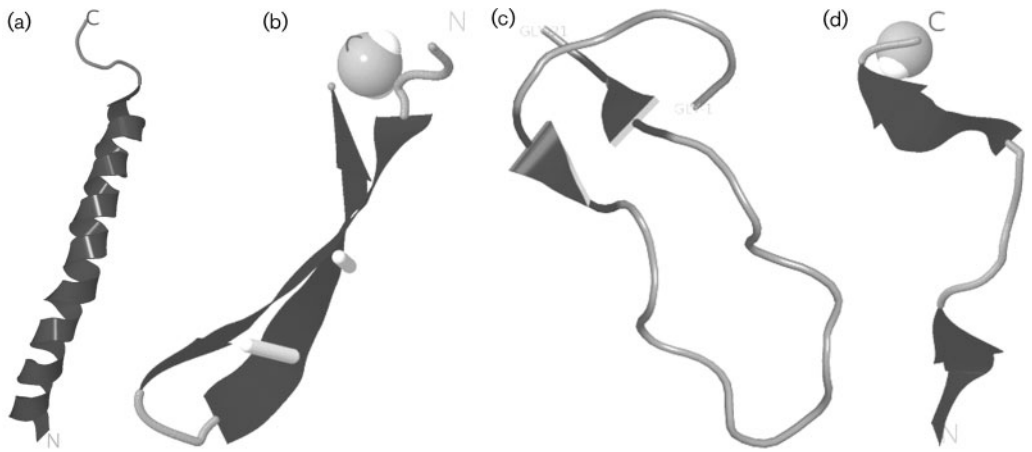

Fig. 1. The four structure classes of CAMPs. (a) α-Helical (LL-37), (b) disulfide-bonded β-sheet (Protegrin-1), (c) loop-structured (Microcin J25) and (d) extended (Indolicidin) (Hühne *et al.*, 2007; Reichert & Sühnel, 2002). Figures taken from Jena Library of biological macromolecules (www.fli-leibniz.de/IMAGE.html) with permission.

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2007). HDPs have also been demonstrated as potent adjuvants used in conjunction with vaccines, reducing the amount of inoculations needed (Li *et al.*, 2008). This is an exciting development, particularly with a number of charitable immunization programmes in action in the third world, and could lead to a considerable financial saving, without compromising immunity.

Some CAMPs have been evaluated in clinical trials, but with little success. Pexiganan (a Magainin II homologue), the first CAMP to enter clinical trials, was evaluated as an antibiotic cream for foot ulcers. However, it was refused a licence by the FDA in 1999 after two studies failed to demonstrate efficacy. Iseganan (a Protegrin I homologue) was tested against oral mucositis, but also failed to demonstrate efficacy. However, Omiganan (an Indolicidin homologue) is pending licensing, formulated as a gel to prevent catheter infections (Giuliani *et al.*, 2007; Marr *et al.*, 2006). In addition to using CAMPs as sole therapeutics, there is also the potential for using CAMPs alongside existing antibiotics, which may lead to a synergistic effect (Marr *et al.*, 2006).

PGRPs as potential therapeutic agents

Peptidoglycan is an essential component of the cell wall of the vast majority of bacteria. As such it has been a target for antibiotics and for the innate immune system. PGRPs are innate immunity molecules conserved from insects to mammals which recognize peptidoglycan. First identified in the silkworm (Yoshida *et al.*, 1996), insects have subsequently been found to possess up to 19 PGRPs, including splice variants (Werner *et al.*, 2000), which are involved in activation of proteolytic cascades or have amidase activity that acts as a scavenger function for peptidoglycan fragments. Subsequently, PGRP orthologues were identified in both humans and the mouse (Kang *et al.*, 1998). This review will focus on mammalian PGRPs, which do vary in activity and structure somewhat from their insect counterparts. Mammals have four PGRPs (Liu *et al.*, 2001) currently designated PGLYRP-1, PGLYRP-2, PGLYRP-3 and PGLYRP-4 (Table 1).

All mammalian PGLYRPs are secreted and form homodimers, although PGLYRP-3 and PGLYRP-4 preferentially produce heterodimers over homodimers if co-expressed. With the exception of PGLYRP-2 homodimers, the dimers are disulfide-linked (Depauw *et al.*, 1995; Lu *et al.*, 2006; Zhang *et al.*, 2005). The bactericidal activity of PGLYRPs requires physiological concentrations of Zn^{2+}, and protein glycosylation is also essential for activity (Wang *et al.*, 2007). Each PGLYRP has a different spectrum of antibacterial activity (Table 1). Gram-negative bacteria are killed most efficiently by PGLYRP-1 and PGLYRP-3, and least efficiently by PGLYRP-4 (Lu *et al.*, 2006; Wang *et al.*, 2007). Bactericidal concentrations *in vitro* are around 0.1–1 μM, which makes them more active than most antibacterial peptides (Royet & Dziarski, 2007). However, their high molecular mass makes them much less potent when considered by weight, which is what is clinically relevant. Interestingly, the PGLYRPs are less active against normal flora commensal organisms, although the reason for this is not known (Lu *et al.*, 2006).

PGRPs possess several structurally distinct sites. Crystal structures of both insect and human PGRPs have revealed a peptidoglycan-binding groove; the minimum fragment that binds is a muramyl-tripeptide, whereas a muramyl dipeptide or peptide lacking *N*-acetylmuramic acid do not bind (Guan *et al.*, 2004a, b, 2005, 2006; Kumar *et al.*, 2005; Swaminathan *et al.*, 2006).

The bactericidal proteins PGLYRP-1, PGLYRP-3 and PGLYRP-4 are active against a range of bacteria (Lu *et al.*, 2006; Wang *et al.*, 2007). The mechanism for the bactericidal activity of PGRPs is not known; however, it is not caused by cell wall lysis, as with peptidoglycan-lytic enzymes. The mechanism is most likely to be similar to the effect of antibiotics that inhibit cell wall synthesis. PGRPs may bind to peptidoglycan fragments or its metabolic precursors preventing cell wall synthesis (Lu *et al.*, 2006). PGLYRP-1 is able to inhibit growth of both Gram-positive and Gram-negative bacteria, although it is more active against Gram-positive bacteria. PGLYRP-1 has higher bactericidal activity at pH 6.4 than in neutral or alkaline conditions, which is consistent with production in PMN granules (Wang *et al.*, 2007) which are acidic following phagocytosis. In contrast, PGLYRP-3, PGLYRP-4 and PGLYRP-3 : 4 are most bactericidal at pH 7.6, consistent with conditions found in the intestines, saliva and sweat where they are mainly produced (Wang *et al.*, 2007). Mice without the PGLYRP-1 gene are more susceptible to

Table 1. Site of production and activity of human PGRPs

PGRP	Site of production	Activity
PGLYRP-1	Polymorphonuclear leukocyte granules, M cells of Peyer's patches	Bactericidal for both Gram-positive and Gram-negative bacteria, but most active against Gram-negatives
PGLYRP-2	Liver (secretion into blood); skin, mouth and intestine (epithelial cells)	Amidase
PGLYRP-3	Skin, mouth, intestine, eyes; sebaceous and sweat glands	Bactericidal, most effective against Gram-negative bacteria
PGLYRP-4	Skin, mouth, intestine and eyes; sebaceous, sweat and salivary glands	Bactericidal against Gram-positive bacteria

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intraperitoneal infection by Gram-positive bacteria: the phagocytes are able to ingest bacteria but are not then able to kill the bacteria (Dziarski *et al.*, 2003), indicating that PGLYRP-1 has a function in intracellular killing and degradation of bacteria. Interestingly, mutation of the genes encoding PGLYRP-3 and PGLYRP-4 may be a contributory factor in the development of psoriasis, being located in the epidermal differentiation gene cluster in the psoriasis sensitivity locus (Sun *et al.*, 2006). This implies that the role of PGRPs in innate immunity is more complex that merely bacterial killing.

PGLYRP-2 is rather unusual among the PGLYRPs, being an *N*-acetylmuramoyl-L-alanine amidase which is able to degrade peptidoglycan by hydrolysing the bond between the *N*-acetylmuramic acid and L-alanine (Wang *et al.*, 2003). The C-terminal region containing the three conserved PGRP domains is required and sufficient for amidase activity but the activity is reduced compared to that of the full-length protein (Wang *et al.*, 2003). PGLYRP-2 is able to degrade single-layered peptidoglycan from *Escherichia coli*, but not intact multi-layered peptidoglycan from *Staphylococcus aureus*, which has to be predigested by lysozyme in order for it to be cleaved effectively (Gelius *et al.*, 2003). It is possible that PGLYRP-2 plays a role in elimination of peptidoglycan, resulting in a reduction in the ensuing pro-inflammatory response, and thus prevention of overactivation of the immune response which would result in excessive and detrimental inflammation (Royet & Dziarski, 2007), similar to the role of PGRP-SC1B in *Drosophila* (Mellroth *et al.*, 2003).

It has previously been found that killing of bacteria is enhanced by the synergistic action of PGRPs with antimicrobial peptides such as PLA_2, defensins, BPI or peptidoglycan-lytic enzymes (Wang *et al.*, 2007). PGLYRP-1 has also been shown to act synergistically with lysozyme, showing an increase in bacterial lysis and growth inhibition *in vitro* with *E. coli* (Cho *et al.*, 2005). Therefore, when considering therapeutic applications of PGRPs, it is worth considering them as adjuncts to improve activity of other compounds, such as CAMPS (Wang *et al.*, 2007), as well as antimicrobials in their own right. In addition, it has been proposed that PGRPs may have secondary immunomodulatory functions that may also enhance their activity *in vitro*. For example, porcine PGLYRP-2 acts as an amidase, but has also been implicated in regulating β-defensin expression (Sang *et al.*, 2005). The resistance of commensal bacteria to human bactericidal PGLYRPs has significance for medical countermeasures as it means that PGRPs could potentially have fewer side effects than traditional broad-spectrum antibiotics.

Novel cyclic peptide antimicrobials identified in biological systems

Traditional combinatorial chemistry approaches can generate extensive small-molecule libraries, yet despite the opportunity afforded by almost unlimited functional group diversity, synthetic small-molecule inhibitor libraries generated in this way can be difficult to decode. Libraries of up to 10^5 compounds can be routinely produced by 'split and pool' protocols (Tan, 2005; Webb, 2005), but identifying the active members of the pools is time-consuming and can be challenging (Irwin, 2006). In comparison, libraries synthesized in biological systems are often more extensive, and allow straightforward identification of the active compounds (Falciani *et al.*, 2005; Ulrich *et al.*, 2006; Uttamchandani *et al.*, 2006). Taking this approach one step further, combining the molecule production in a biological system with an *in vivo* screen makes the biological approach even more powerful, with the added benefits of speed and ease of identification of a compound active against the target of choice (Horswill *et al.*, 2004; Matsumoto *et al.*, 2006; Tavassoli & Benkovic, 2007; Uttamchandani *et al.*, 2006).

The compounds synthesized in biological approaches are often small polypeptides. However, small linear peptides are often unstable, and susceptible to degradation by the cell's proteases, a process which involves the ubiquitin–proteasome system (Goldberg *et al.*, 1997; Hilt & Wolf, 1996). Various approaches to improve intracellular stability have been evaluated, based on constraining peptide structures by a variety of means. For example, attaching short dimerizing peptides to both the amino and carboxyl termini of several 18-mer peptides appeared to create stable monomeric tertiary structures (Gururaja *et al.*, 2000). Alternatively, embedding peptides within the scaffold of a larger protein such as thioredoxin (Colas *et al.*, 1996) has also been employed. However, both these types of approaches can have variable success. Cyclic peptides, however, are significantly more resistant to proteolysis than their linear analogues, and their structure can result in a higher binding affinity for cognate binding proteins, because of a reduced entropic cost of binding (Gururaja *et al.*, 2000).

Peptides have been examined as an approach to the development of antivirals. For example, the naturally occurring cyclic cystine-knot small proteins MCoTI-I and MCoTI-II have been shown to act as protease inhibitors. Synthetic analogues specific for foot-and-mouth disease virus 3C protease have been created which may be an avenue for development of novel therapeutics (Thongyoo *et al.*, 2008).

The human immunodeficiency virus type 1 (HIV-1) has shown itself able to rapidly mutate to resistance against antiretroviral drugs, although combination therapy with a cocktail of active compounds has had a huge impact on disease progression and mortality. The majority of currently available drugs target only three steps in the viral replication cycle, namely virus fusion, reverse transcription and proteolytic processing of viral proteins (De Clercq, 2005), limiting the number of possible combinations and increasing the likelihood of cross-resistance. Targets based on host cellular proteins, rather than viral targets, are less

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likely to develop resistance. Therefore a host protein, CypA, with an essential role early in the HIV-1 replication cycle was identified as a possible target for intervention. CypA, a member of the immunophilins, binds specifically to the viral Gag polyprotein (Luban *et al.*, 1993). This interaction is essential for viral replication and inhibition impairs viral replication (Hatziioannou *et al.*, 2005; Sokolskaja *et al.*, 2004; Steinkasserer *et al.*, 1995). Cyclosporins, a class of cyclic undecapeptides known for their immunosuppressant activity, can bind to CypA, with a resulting anti-HIV-1 effect. Synthetic derivatives, particularly a modification to substitute the undecapeptide at position 4, were created which did not have immunosuppressive effects, while maintaining the antiretroviral activity (Papageorgiou *et al.*, 1994; Wenger, 1986; Zenke *et al.*, 1993). The most promising lead reported to date, Debio-025, is D-methylAla3-ethylVal4 cyclosporin, which has shown promising activity *in vivo*, being orally bioavailable in a suitable formulation, and having a long half-life in the circulation. Debio-025 selectively inhibits replication of HIV-1 in $CD4^+$ cell lines and peripheral blood mononuclear cells, but not HIV-2 or simian immunodeficiency virus (Ptak *et al.*, 2008). Naturally occurring isolates of HIV-1 resistant to Debio-1 have been identified, which surprisingly have evolved to replicate in human cells without interaction with CypA, following mutation of the capsid protein (Ptak *et al.*, 2008). Resistance was found in approximately 15 % of strains, including some multidrug-resistant isolates, although the rate of resistance was subgroup-specific. Debio-025 is rather narrow in its activity: of a panel of viruses tested, only HIV-1 and hepatitis C virus (HCV) were susceptible. However, initial clinical trials in HIV-1- and HCV-infected patients yielded promising results (Flisiak *et al.*, 2006, 2008; Herrmann *et al.*, 2007), particularly in coinfected individuals. The Debio-025 example illustrates that if the target of the novel antimicrobial can be bypassed, resistance can be an issue, whether the target is in the organism or in the host. However, if the right target can be identified, interference of host–pathogen interactions may be a rich new seam to mine for antimicrobial development.

An elegant approach integrating intracellular production of libraries of cyclic peptides and a biological screening system has been reported (Scott *et al.*, 1999; Tavassoli & Benkovic, 2007). This method, using split-intein circular ligation of peptides and proteins (SICLOPPS), can generate libraries in *E. coli* of up to 10^8 different compounds, much larger than chemically synthesized pools. An additional advantage of biological small molecule libraries is that it is relatively easy to identify the active members of the library (Falciani *et al.*, 2005; Ulrich *et al.*, 2006; Uttamchandani *et al.*, 2006). In SICLOPPS, the *Synechocystis* DnaE split intein is exploited in combination with degenerate oligonucleotide technology to generate the libraries of high complexity. Briefly, degenerate oligonucleotides encoding the peptides are inserted between the two parts of the split intein, while maintaining the correct reading frame. This is done using a PCR-based technique whereby the random nucleotides are incorporated into the forward primer, termed the 'library primer', which binds the 3′ end of the C-terminal intein and the 5′ end of the N-terminal intein (Fig. 2). The

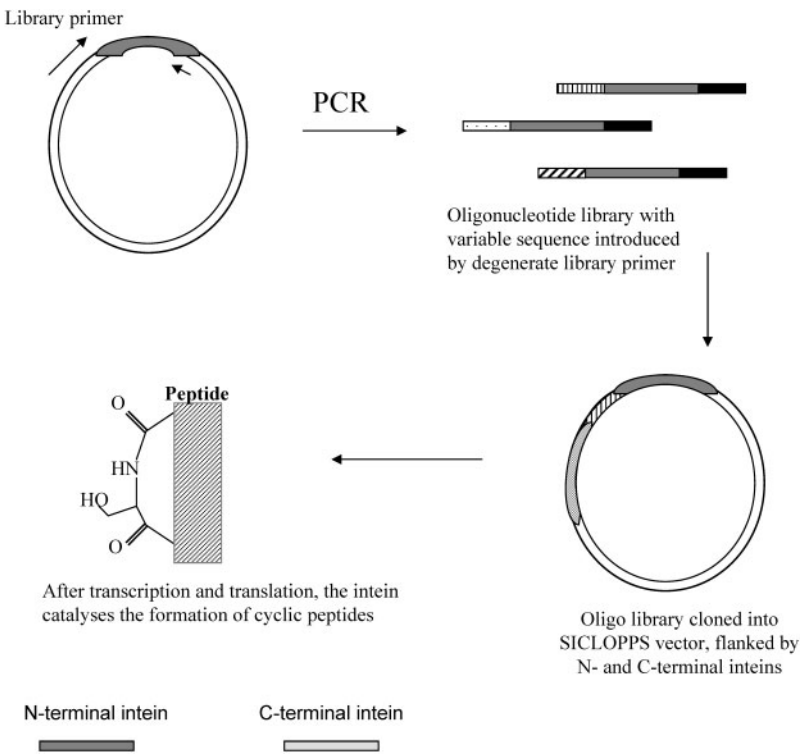

Fig. 2. Construction of SICLOPPS libraries. A degenerate library primer is used in combination with a non-variable primer to amplify the N-terminal intein region by PCR. This is cloned into the SICLOPPS vector so that the random sequence is flanked by C- and N-terminal inteins. The expressed fusion protein folds to form an active intein. Splicing results in cyclization of the target peptide.

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reverse primer is not degenerate, and binds outside the intein. Although there is no limit on the number of amino acids in the target peptide, five variable amino acids are used in most screens as the theoretical number of products (34 million) (Tavassoli & Benkovic, 2007) is within the number of transformants that can easily be produced. This oligonucleotide library is then cloned into a shuttle vector and used to express cyclic peptides. Inhibitors of protein–protein interactions can then be identified in the library in bacterial reverse two-hybrid screening systems. For example, this *in vivo* screening approach was employed to identify inhibitors of the ClpXP protease (Cheng *et al.*, 2007). ClpXP belongs to a group of enzymes in Gram-negative bacteria termed the 'ATPases associated with diverse cellular activities plus' (AAA^+) with ATP-dependent proteolytic functions involved in regulatory mechanisms controlling gene expression (Neuwald *et al.*, 1999). The tmRNA is a specialized RNA that can enter the ribosome and tag a nascent protein with a peptide (Keiler *et al.*, 1996). This tag is a target for intracellular proteases, such as the ClpXP protease, so the tagged protein is rapidly degraded. The recognition of the tag is facilitated by a specificity factor, such as SspB, the cognate factor for ClpXP (Levchenko *et al.*, 2000; Moore & Sauer, 2005). One downside to this as an antimicrobial target is that ClpXP is not essential for survival of all bacteria, including *E. coli*. However, inactivation of ClpXP by mutagenesis is lethal in some bacterial species, and the protease has been shown to be involved in control of expression of virulence factors. For example, in *Pseudomonas aeruginosa*, mutation of the genes encoding ClpXP resulted in abolition of the mucoid phenotype (Qiu *et al.*, 2008). Clinically this makes ClpXP an interesting antimicrobial target, as the mucoid phenotype is associated with chronic lung infection in cystic fibrosis patients.

In addition to inhibitors of protein–protein interactions, the method has also been modified to identify inhibitors of protein–DNA interactions; for example, DNA adenine methyltransferase (Dam) was identified using this system. Dam plays a number of key roles in bacterial processes, including mismatch repair, the timing of DNA replication and the transcription of certain genes (Noyer-Weidner & Trautner, 1993). Inactivation of *dam* genes in a range of pathogens resulted in attenuation (Heithoff *et al.*, 1999; Julio *et al.*, 2001; Robinson *et al.*, 2005; Taylor *et al.*, 2005), making Dam an attractive target for novel antimicrobial development. A SICLOPPS library of 2.4×10^7 clones was screened in an *in vivo* assay, and three promising inhibitors were identified based on their facilitating transposition by inhibiting Dam activity. The Dam inhibitor screening exploited the fact that Tn*5* transposase binds to specific recognition sites in DNA, but only if they are unmethylated: following methylation by Dam, the transposase cannot bind to the recognition sequence (Goldberg *et al.*, 1997; Hilt & Wolf, 1996; Naumann *et al.*, 2008). These three candidates all possessed a conserved motif, $[K/R]_3$-X_4-M_5, which could serve as the basis for modified candidates.

Challenges to the *in vivo* use of peptides

To date, the vast majority of clinical trials have focused on the use of peptides via the topical route, permitting the direct application of the therapeutic at the required site on the body. There are a number of challenges when considering other routes of administration, such as oral dosing. The main issue associated with the use of therapeutic peptides is improving the stability of the molecule from degradation by intestinal, tissue and serum proteases (peptidases).

There are several traditional methods for improving the resistance of peptides to proteases involving the modification of the N-terminus and C-terminus of the peptides through acetylation and amidation, respectively, and/or the replacement of amino acids at predicted cleavage sites with non-natural residues (i.e. D-amino acids). These methods have been proven successful and are now routinely offered by commercial peptide synthesis companies. One well-studied example is the glucagon-like peptide GLP-1-(7-34)-amide being developed as a potential treatment for type II diabetes. During *in vitro* studies, the *N*-acetylation of this peptide improved stability in the presence of an endogenous protease (dipeptidyl peptidase IV) from mere minutes to at least 2 h whilst retaining the protein's biological activity (John *et al.*, 2008).

The reduction of the size of a peptide down to its key biologically active domains is a strategy that has also been adopted to improve stability, which may also be combined with the approaches mentioned above, such as incorporation of D-amino acids. An early, well-characterized example of a synthetic peptide is octreotide, an analogue of somatostatin, used in the treatment of gastrointestinal tumours. A combination of reducing the overall length of the peptide and the replacement of labile amino acids with D-amino acids resulted in octreotide having a plasma half-life of up to 1.5 h in comparison to minutes for somatostatin (Harris, 1994). The position of amino acids that can be substituted in this way has been shown to be critical in order for α-helical antimicrobial peptides to retain their secondary structure and also biological activity (Hong *et al.*, 1999). D-Amino acid substitutions in the central portion of the amino acid sequence of the synthetic KSLK antimicrobial peptide deleteriously affected the biological activity. However, the diastereomer peptides with D-amino acid substitution at the terminal ends retained biological activity and had a vastly improved stability in mouse serum (Hong *et al.*, 1999). Similar findings have recently been published for an antimicrobial peptide (EFK17) based on the sequence of LL37. In a comprehensive study, the substitution of amino acids at four known protease cleavage sites produced enantiomers that were highly stable but exhibited little antimicrobial activity (Stromstedt *et al.*, 2009). However, substituting the same protease sites with tryptophan residues markedly reduced susceptibility to enzymic degradation whilst retaining antimicrobial activity of the peptide. By also

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modifying the terminal ends of the tryptophan-substituted peptides via *N*-acetylation and C-amidation the peptide was protected from degradation by an increased range of human and bacterial proteases. This study hints at the possibility that peptide therapeutics can be engineered to overcome proteolytic degradation to make them more useful as therapeutic agents by non-topical routes.

Protease resistance can also be engineered into a particular peptide sequence through directed evolution, the use of biological systems to create large libraries of variant peptides based on a specific sequence. Phage display, or other *in vitro* expression systems such as CIS display, are techniques that have also been used to engineer in protease resistance (Eldridge *et al.*, 2006; Odegrip *et al.*, 2004; Wirsching *et al.*, 2003). Phage display libraries have also been used to generate synthetic peptides with little sequence homology to the original protein yet can have the same biological activity or properties (Wrighton *et al.*, 1996). Based on these studies, the product Hematide, an erythropoiesis stimulating agent, has been developed that appears to be stable in serum and non-immunogenic (Woodburn *et al.*, 2009).

As described above, cyclic peptides are inherently resistant to proteolytic cleavage. Thus the cyclization of peptides is an alternative strategy where resistance to proteases can be engineered by linking the amino- and carboxy-terminus of the polypeptide sequence ('head-to-tail' cyclization). This type of modification has been shown to improve the stability of a therapeutic peptide in human plasma for up to 24 h, in comparison to the linear form, which was degraded in minutes (Pakkala *et al.*, 2007). Synthetic cyclic peptides have been rationally designed that not only have favourable *in vitro* and/or *in vivo* stability profiles but also show a greater selectivity for bacterial rather than mammalian cell membranes (Dartois *et al.*, 2005; Fernandez-Lopez *et al.*, 2001; Oren & Shai, 2000).

Bioavailability is an important consideration in developing effective therapeutics. The bioavailability of peptides can be markedly improved through the attachment of polyethylene glycol (PEG) moieties. The benefits of PEGylating peptides are well established and have previously been comprehensively reviewed (Harris & Chess, 2003). The addition of PEG increases the overall molecular mass of the peptide, which in turn can reduce renal clearance from the circulatory system. PEGylated peptides have also been shown to have an improved stability from proteolytic degradation (Ramon *et al.*, 2005), and the attachment of PEG can provide protection from detection by the immune system (He *et al.*, 1999). Examples of therapeutic peptides in the clinic with good pharmacokinetic profiles are PEG-Intron and Pegasys, PEGylated interferon α-2b and α-2a, respectively, used in the treatment of hepatitis C infections. However, the site of attachment and size of the PEG chosen to be added to interferon α-2b is an important consideration that can have deleterious effects on biological activity if not done correctly (Grace *et al.*, 2005). Similar data have been presented for PEGylated antimicrobial peptides where the size of the PEG moieties can negatively affect the antimicrobial properties of the peptide (Jacob *et al.*, 2008).

The use of an efficient targeting or slow release drug delivery system in conjunction with a therapeutic peptide may reduce or mitigate the need for the extensive modifications outlined above. Liposomal systems offer a number of benefits including increased circulation time of the encapsulated therapeutic whilst permitting targeting, as a result of natural leakage of the circulatory system, at sites of tumour growth or infection. This has been exploited for delivery of anticancer drugs, such as daunorubicin and doxorubicin, products named DaunoXomes and Doxil, respectively, and may have benefits for delivery of peptide therapeutics as well. The liposome encapsulation of peptides is still at the research phase; however, modest improvements in the half-life of encapsulated insulin relative to the free protein have been reported (Khaksa *et al.*, 2000). PEG ('stealth' liposomes) and ligands (e.g. antibodies, mannose) can be attached to liposomes to enhance the circulation time of the peptide–liposome formulation and improve targeting of the therapeutic cargo to a specific cell type (Irache *et al.*, 2008; Samad *et al.*, 2007).

In order to aid the clearance of intracellular infections, various strategies are under development to induce liposomes to burst upon endocytosis through the incorporation of pH-sensitive elements (e.g. lipids, cationic peptides) into the membrane bilayer. As the pH drops in the endosome, a conformational shift in the active (fusogenic) lipid or peptide in the membrane results in the enforced release of the encapsulated cargo (Simoes *et al.*, 2004). Therefore, as well as providing protection to the encapsulated peptides, liposomes with pH triggers may also be able to target therapeutic peptides intracellularly for the successful clearance of intracellular infections. Cationic (cell-penetrating) peptides have also been extensively studied for the delivery of molecules to intracellular compartments (Vives *et al.*, 2008). However, despite being able to deliver a variety of molecule types *in vitro* (e.g. peptides, nucleotides), their *in vivo* use requires further development and the mechanism(s) by which these peptides enter cells remains unclear.

The use of microspheres composed of polymers such as polylactic acid or polylactic-glycolic acid may have some utility in maintaining the circulation time of peptides in the blood. This approach has produced some promising results in vaccine research and in reducing the frequency of dosing of therapeutic peptides (Elvin *et al.*, 2006; Flick-Smith *et al.*, 2002; Kemp *et al.*, 2004). As a critical concentration of therapeutic peptide is often required for the molecule's antimicrobial activity, these slow-release mechanisms may have a limited utility in this context. Therefore smart polymers that release their cargo under specific physiological conditions (e.g. temperature, pH) may be required to provide a coordinated, targeted release

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of peptide. Some of the emerging patented technologies using these approaches with microspheres have been reviewed recently (Fogueri & Singh, 2009).

In summary, for an antimicrobial peptide to effectively treat an infection, it is likely to require some level of physical (structural), chemical or composition modifications in order to improve its overall pharmacokinetic properties. When used in conjunction with an appropriate delivery system that can aid longevity of the therapeutic peptide and/or improve targeting to the site of infection, a therapeutic peptide is likely to be more effective *in vivo*. Until suitable delivery systems are identified, it is likely that the application of peptide therapeutics will mainly be in the topical treatment of local infections where the peptides can be directly applied. However, with a lot of research in this area currently under way, including the application of nanomedicine to the issue of drug delivery, the utility of therapeutic peptides is likely to expand to include a wider range of infections.

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