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MINIREVIEW

Beyond conventional antibiotics for the future treatment of methicillin-resistant *Staphylococcus aureus* infections: two novel alternatives

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Abstract

The majority of antibiotics currently used to treat methicillin-resistant *Staphylococcus aureus* (MRSA) infections target bacterial cell wall synthesis or protein synthesis. Only daptomycin has a novel mode of action. Reliance on limited targets for MRSA chemotherapy, has contributed to antimicrobial resistance. Two alternative approaches to the treatment of *S. aureus* infection, particularly those caused by MRSA, that have alternative mechanisms of action and that address the challenge of antimicrobial resistance are cationic host defence peptides and agents that target *S. aureus* virulence. Cationic host defence peptides have multiple mechanisms of action and are less likely than conventional agents to select resistant mutants. They are amenable to modifications that improve their stability, effectiveness and selectivity. Some cationic defence peptides such as bactenecin, mucroporin and imcroporin have potent *in vitro* bactericidal activity against MRSA. Antipathogenic agents also have potential to limit the pathogenesis of *S aureus*. These are generally small molecules that inhibit virulence targets in *S. aureus* without killing the bacterium and therefore have limited capacity to promote resistance development. Potential antipathogenic targets include the sortase enzyme system, the accessory gene regulator (*agr*) and the carotenoid biosynthetic pathway. Inhibitors of these targets have been identified and these may have potential for further development.

Introduction

Serious infections caused by *Staphylococcus aureus* are important globally in the hospital setting and in the community. These range from minor infections of the skin and soft tissue to life-threatening systemic infections, such as bloodstream infections and endocarditis. Methicillin-resistant *S. aureus* (MRSA) is resistant to most conventional β-lactam antibiotics because of the carriage of the *mecA* gene encoding an alternative penicillin-binding protein, PBP2a, for which β-lactams have low affinity (Hartman & Tomasz, 1984; Reynolds & Brown, 1985). The majority of MRSA isolates are resistant to drugs in the other antibiotic classes including aminoglycosides and macrolides (Fluit *et al.*, 2001). Our diminishing arsenal of anti-infectives for the treatment of systemic MRSA infections highlights the need for alternative antimicrobial agents with superior properties in terms of efficacy, reduction in toxicity and resistance.

Among the agents currently recommended by the Infectious Diseases Society of America for the treatment of MRSA infections are vancomycin, clindamycin, daptomycin, linezolid, trimethoprim, tetracycline and streptogramins (Liu *et al.*, 2011). However, increasingly *in vitro* resistance to currently used agents is reported and clinical failures have occurred (Soriano *et al.*, 2008; Yoon *et al.*, 2008; Baltz, 2009; Prabhu *et al.*, 2011; Gould *et al.*, 2012; Ruiz de Gopegui *et al.*, 2012). As summarized in Fig. 1, new members of existing antibacterial classes in the late phases of clinical trials, with potential for the treatment

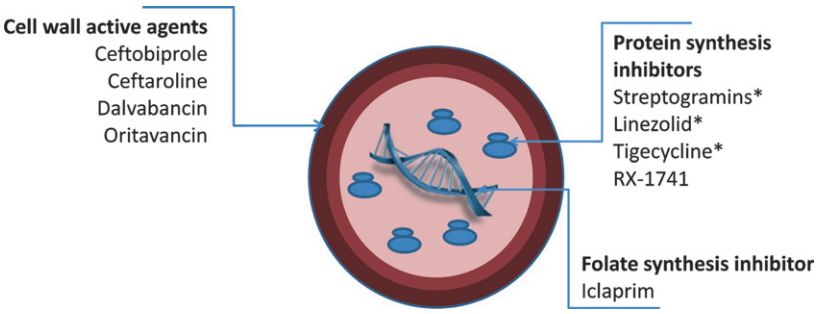

Fig. 1. New MRSA agents in clinical use (*) and MRSA agents in development.

of MRSA infections include ceftobiprole, ceftaroline, dalbavancin, oritavancin (peptidoglycan synthesis inhibitors) and iclaprim (folate synthesis inhibitor). Ceftobiprole and ceftaroline are novel advanced generation cephalosporins with a broad activity spectrum and strong affinity for PBP2a with ceftobiprole showing stability to β-lactamases (Zhanel *et al.*, 2008; Dauner *et al.*, 2010). Dalbavancin and oritovancin are semi-synthetic lipoglycopeptides with a heptapeptide core similar to vancomycin. In addition to effects on the cell wall, these agents also disrupt cell membrane integrity through membrane depolarization. They also have longer half-lives allowing for less frequent dosing compared with vancomycin and teicoplanin (Zhanel *et al.*, 2010). The success of these newer agents remains to be assessed clinically. It is clear that large pharmaceutical companies preferentially appear to favour the development of new generation classical antibiotic classes, with improved properties. This may be because, compared with new agents with alternative mechanisms of action, their safety and efficacy is well established *in vivo* and they are amenable to pharmaceutical preparation. However, in view of the propensity to develop resistance associated with conventional current antibiotics and their derivatives, the long-term future of anti-staphylococcal agents may involve an exploration of agents with alternative and multiple modes of antibacterial activity. Additional properties such as antipathogenic or immunomodulatory activity would also be desirable in novel MRSA drugs. Such adjunct properties would be particularly important for the treatment of community-associated MRSA (CA-MRSA) which is associated with enhanced virulence that may be toxin mediated (Voyich *et al.*, 2005). The investigation of alternative therapeutic agents with novel mechanisms of action remains largely an activity for academic researchers and small biotechnology companies. This type of research has resulted in preclinical developments in the areas of innate immune defence peptides and antipathogenic agents with potential as novel anti-MRSA therapeutics. For example, cationic peptides offer multiple and alternative modes of action that may circumvent the problem of antimicrobial resistance. Significant improvements, to the chemistry of such peptides, have increased their attractiveness in terms of pharmacokinetics, toxicity and cost. Antipathogenic agents can potentially attenuate the virulence of MRSA, and therefore, this therapeutic approach may have significantly less propensity to contribute to antimicrobial resistance. These novel approaches to the treatment of MRSA infections, though in their infancy in terms of pharmaceutical development, may provide alternative or complementary therapy in the future. Recent developments in these areas and their future potential as novel anti-infectives are discussed.

Cationic host defence antimicrobial peptides and their therapeutic potential

Cationic antimicrobial peptides (CAMPs) are a group of ubiquitous peptides that are part of the host innate immune system of animals and plants, and these molecules have several properties that make them promising candidates for development as agents for the treatment of microbial infections including those caused by MRSA (Hancock & Patrzykat, 2002; Zhang & Falla, 2006). Native CAMPs are structurally diverse, varying in size, sequence, content of α helical or β-sheet motifs, disulphide bridges and linear extended structures. Despite their structural diversity, CAMPs are all polycationic and amphipathic, two features thought to facilitate their antimicrobial mechanism (Dathe *et al.*, 1997). The main mechanism of antimicrobial action of host defence peptides (HDPs) is biophysical rather than biochemical, where the target is the cytoplasmic membrane structure itself (Fig. 2). In Gram-positive and Gram-negative organisms, the antimicrobial activity of CAMPs is initiated through electrostatic interactions with the anionic phospholipid head-groups of the cell envelope that may lead to either membrane perturbations as has been shown for human β-defensins (Yeaman *et al.*, 1998) and magainins (Westerhoff *et al.*, 1989) or translocation across the membrane and interaction with various intracellular targets as occurs for cathelicidins such as LL-37 and bactenecin (Sadler *et al.*, 2002).

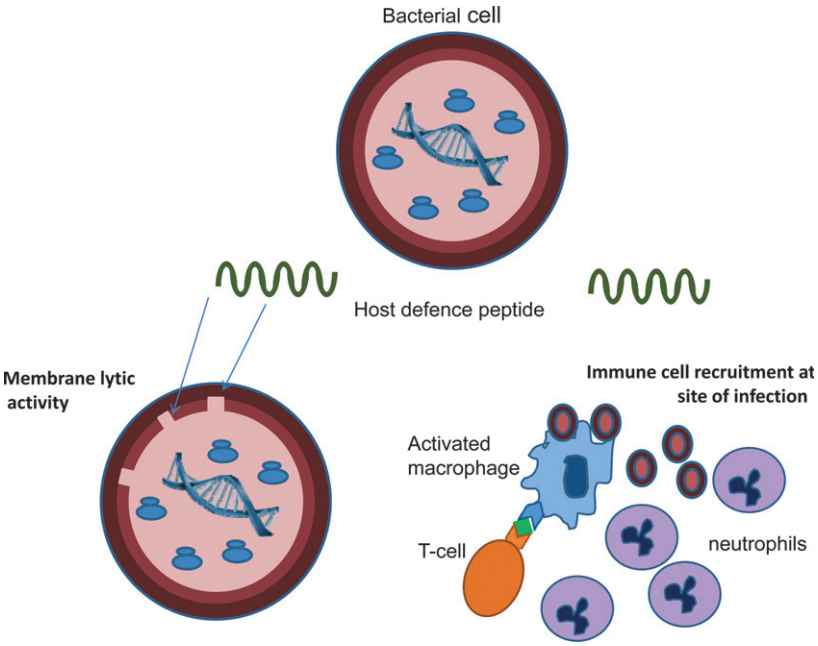

Fig. 2. Dual effects of host defence peptides. Host defence peptides can exert antibacterial effects directly by forming pores in the cell membrane or can modulate the immune response to infection by inducing transcription of cytokines or directing cellular components of the immune system such as neutrophils, dendritic cells, monocytes and macrophages to the site of infection.

Three host defence peptides have completed or are in phase III clinical trials: the magainin 2 analogue pexiganan (MSI-78) for the prevention of diabetic foot ulcers; iseganan, from pig protegrin, for the treatment of oral mucositis; and omiganan for the prevention of catheter infections and acne. Pexiganan failed to be approved by the FDA because of nonsuperiority to approved agents but it remains one of the best studied CAMPs. Clinical trials involving HDPs have to date mainly been limited to topical applications although some, such as the human lactoferrin fragment hLF1-11, for bacteraemia and fungal infection, being developed for systemic applications are in early clinical trials. The sequences, properties, *in vitro* activities and phase of development of some of these peptides that may also have potential as *S. aureus* anti-infectives are outlined in Table 1.

Classic antibiotics target biochemical properties such as folate, peptidoglycan, nucleic acid and protein synthesis, which are often mediated through enzyme inhibition or inhibition of binding to intracellular targets. However, the ability of HDPs to kill multi-resistant bacteria and to poorly select resistant mutants may be related to the contribution of additional alternative and multiple pathways to their mechanism of action, such as depolarization of the bacterial membrane, pore formation and the induction of degradative enzymes and disruption of intracellular targets (Hadley & Hancock, 2010).

The potential direct antimicrobial activity of mammalian host defence peptides can be complemented by a chemotactic activity for phagocytes and memory and effector T cells (Fig. 1). Additionally, they mediate the recruitment of immature dendritic cells, by direct chemotactic activity or by upregulation of chemokine production in macrophages, and promote maturation of these dendritic cells directly or indirectly by inducing production of inflammatory cytokines (IL-1β, TNFα) (Bowdish *et al.*, 2005, 2006; Yeung *et al.*, 2011). Although these latter activities result in the local release of pro-inflammatory cytokines, host defence peptides can also reduce the systemic production of TNFα, IL-1β and IL-6, as has been demonstrated for LL-37 (Mookherjee *et al.*, 2006). Therefore, HDP modulation of the immune response to bacteria appears to involve not only enhancement of specific pro-inflammatory responses, but also suppression of other elements of the pro-inflammatory response, the additive effects of which contribute to a more controlled inflammatory response after the initial potent cytokine response (Yeung *et al.*, 2011). Some of these immunomodulatory properties alone are sufficient to prevent or clear infection. This was demonstrated by the efficacy in a mouse model of infection, of an immune defence regulator peptide, IDR1, which is devoid of direct antimicrobial activity, but which can selectively activate innate immune responses (Scott *et al.*, 2007). This peptide has recently entered phase I clinical safety trials and is intended for use in the prevention of infection in chemotherapy-induced immune-suppression. More recently, another immune defence regulator, derived from the sequence of the bactenecin peptide IDR-1002, has shown enhanced chemokine induction with a stronger protective effect in an *in vivo* model of *S. aureus* infection (Nijnik *et al.*, 2010; Turner-Brannen *et al.*, 2011). The combination of

Table 1. Examples of natural cationic peptides with potential for development as *Staphylococus aureus* anti-infectives

Peptide name	Source	Amino acid sequence	Proposed mechanism	MIC in mg L^{-1}*	Stage of development
Buforin II	Asian Toad (*Bufo bufo gargarizans*) stomach	TRSSRAGLQWPVGRVHRLLRK	Translocation and interaction with nucleic acids (Park *et al.*, 1998)	8† (Giacometti *et al.*, 2000)	Pre-clinical
LL-37	Human (neutrophils and epithelial cells)	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Translocation and interaction with intracellular target. Monocyte, T-cell, neutrophil chemotaxis	31 (Bals *et al.*, 1998)	Pre-clinical
Bac8c	Synthetic derivative of bactenecin from bovine neutrophils	RIWVIWRR	Membrane depolarisation and cytoplamic permeabilization (Spindler *et al.*, 2011)	2 (Hilpert *et al.*, 2005)	Pre-clinical
Temporin10a	Frog (*Rana ornativentris*) skin	FLPLASLFSRLL	Pore formation, membrane depolarization (Kim *et al.*, 2001)	0.014‡(Kim *et al.*, 2001)	Pre-clinical
Syphaxin (SPX1-22)	Frog (*Leptodactylus syphax*) skin	GVLDILKGAAKDLAGHVATKVINKI	Not elucidated	31.9‡ (Dourado *et al.*, 2007)	Pre-clinical
Iseganan (IB-367)	Derivative of protegrin from porcine neutrophils	RGGLCYCRGRFCVCVGR	Pore formation, membrane depolarizaton (Sokolov *et al.*, 1999)	4 (Mosca *et al.*, 2000)	Treatment of oral mucositis. Phase III clinical trials
Pexiganan (MSI-78)	Magainan analogue	GIGKFLKKAKKFGKAFVKILKK	Cell membrane disruption and pore formation	16–64§ (Fuchs *et al.*, 1998)	Topical treatment of diabetic foot ulcers. Phase III clinical trials
PMX-30063D	Defensin peptide mimetic	n/a	Membrane disruption	$\leq$ 2¶	Acute SSTI. Phase II clinical trials
Omiganan (MBI-266)	Derivative of indolicidin from bovine neutophils	ILRWPWWPWRRK	Membrane depolarization. Inhibition of DNA, RNA and protein synthesis	16† (Sader *et al.*, 2004)	Prevention of catheter infections and acne. Phase III clinical trials
IDR-1	Derivative of bactenecin from bovine neutrophils	KSRIVPAIPVSLL	Chemokine induction and reduction of pro-inflammatory cytokines (Scott *et al.*, 2007)	n/a	Prevention of infections in the immunecompromised Phase I clinical trials
IDR-1002	Derivative of bactenecin from bovine neutrophils	VQRWLIVWRIRK	Chemokine induction and enhanced leukocyte recruitment	n/a	Pre-clinical

*Clinical Laboratory Standards Institute (CLSI) broth microdilution method with modifications, unless indicated otherwise.
†90% inhibition, standard CLSI methods.
‡Units have been converted from μM to mg L^{-1}.
§Mean MIC at which 90 % of *S. aureus* (n = 10) or MRSA (n = 15) isolates were inhibited.
¶http://irgnews.com/sites/default/themes/publisher/images/companies/PYMX/PYMX-PMX-30063_fs.pdf.

selective recruitment of effector cells and suppression of inflammatory cytokines found for these peptides would result in a balanced anti-infective response with reduced risk of uncontrolled inflammation.

Cationic host defence peptides with potential as MRSA anti-infective agents

Although approximately 17 cationic peptides are in clinical trials to date (though not all in the MRSA therapeutic area) (Yeung *et al.*, 2011), most of these are for topical application. While alternative topical agents may be useful for skin and soft tissue infections, the potential of cationic peptide or HDPs as dual immunomodulatory/bactericidal agents in *S. aureus* infections may be realized through their development as systemic agents. Two of the best studied natural human HDPs are the cathelicidin, LL-37, and human beta defensin (HBD). These HDPs are released from a variety of cells in response to bacterial challenge. However, it has been suggested that their relatively low *in vivo* levels and their inactivation by serum constituents are inconsistent with an effective direct killing activity *in vivo* (Bowdish *et al.*, 2005). As described above, their immunomodulatory activities have been demonstrated and these may be more important than their direct killing properties (Fig. 2). In the area of *S. aureus* anti-infectives, both LL-37 and HBDs have served as templates for the development of derivatives with improved potential for therapeutic application and lower potential for toxicity than the natural peptides. For example, the combination of HBD with a specific immune-modulatory peptide (mannose-binding lectin) has recently proved effective in a MRSA mouse wound infection model (Li *et al.*, 2010a, b). LL-37 and its synthetic derivatives have shown both *in vitro* antibacterial activity and inhibition of *S. aureus* biofilm formation, and no significant haemolysis of erythrocytes (a marker of cell toxicity) was reported up to 100 μg mL^{-1} of each derivative (Dean *et al.*, 2011). A nonpeptide structural mimetic of defensin, with low toxicity, PMX-30063D, is currently in clinical development for infections involving *S. aureus*.

CAMPs from a wide range of nonhuman sources including pig protegrin, temporins and syphaxins from frog skin and buforin from toad have been investigated for their *in vitro* activity towards *S. aureus* including MRSA (Table 1). However, there is further merit in the discovery of peptides of nonhuman and ancient origin because evolutionary dynamics may have driven the modification of effector molecules in early organisms while largely conserving the signalling pathways and pattern recognition systems that respond to infection. Therefore, they have unique structures that may potently activate specific immune responses that contribute to a more measured inflammatory response, with limited possibility of cross-resistance to natural HDPs. Candidate peptides that may be exploited for specific systemic application for MRSA infections include peptides derived from ancient organisms such as mucroporin and imcroporin from the venom of the scorpion and the recently described c-arminin1a from the eumetazoa *Hydra*. Mucroporin is a 17 amino acid peptide from the venom of *Lychas mucronatus* that rapidly kills bacteria by membrane disruption. The native peptide is active against MRSA (MIC = 25 μg mL^{-1}) and other multi-resistant organisms and an improved MIC of 5 μg mL^{-1} and a broader spectrum of activity have been reported for an amino acid substituted derivative, mucroporin 1 (Dai *et al.*, 2008). Imcroporin is an immune defence peptide from the venom of *Isometrus maculates*, and *in vitro* activity has also been demonstrated against MRSA strains (MIC = 20–50 μg mL^{-1}). The peptide demonstrated less than 10 % haemolysis of erythrocytes at the MIC and was comparable to vancomycin in survival studies on mice infected with *S. aureus* (Zhao *et al.*, 2009). A recombinant 31 amino acid peptide, c-arminin 1a, from the ancient fresh water animal of the Eumetazoa species, *Hydra magnipapillata*, has been recently shown to have potent anti-MRSA activity *in vitro* (0.4 μM), does not demonstrate haemolytic activity and its activity is independent of the salt concentration (Augustin *et al.*, 2009). In sequence, this peptide does not resemble any known protein and it lacks cysteine residues, which would facilitate its synthesis and production in large quantities. These properties make c-arminin an attractive molecule for further exploitation. The search for ancient cationic peptide structures with potent activity towards multi-resistant clinically important bacteria such as MRSA is on-going but has already revealed potential candidates that may serve as lead compounds.

Challenges in developing host defence peptides as therapeutic agents

The major obstacles to the development of cationic peptides as systemic therapeutics are concerns about their potential toxicity or immunogenicity and their poor stability. In addition, concerns about development of peptide resistance and unknown effects of synthetic HDPs on the natural innate response to infection have been raised. Host defence peptides are expensive to produce in commercial quantities, and this issue has also affected their potential for development.

The relative lack of negatively charged lipids on mammalian cell surfaces and their weak membrane potential gradient may selectively protect eukaryotes from the action of cationic peptides. However, some cationic

peptides such as LL-37 can translocate across mammalian cell membranes because their sequence resembles that of nuclear signalling peptides. Limited data are available on the cytotoxic effects of cationic peptides on mammalian cells. LL-37 does not show significant haemolytic activity at concentrations greater than its antimicrobial activity but *in vitro* cytotoxic effects have been reported that are dependent on the nature and metabolic state of the target cells and on the evolutionary form of the mature peptide. (Tomasinsig *et al.*, 2009). Because of their small size and linear structure, the majority of host defence peptides are considered to be weakly immunogenic but antibodies have been successfully raised against some cationic peptides such as defensins, hCAP-18 and lactoferrin (Panyutich *et al.*, 1991; Shimazaki *et al.*, 1996; Sorensen *et al.*, 1997). *In vivo* toxicity is an area that has not been systematically assessed for cationic host defence peptides, and this may be because so few have proceeded to this level in clinical trials.

It has been suggested that HDPs, if developed as MRSA anti-infectives, would have low propensity to select resistant mutants compared with classical antibiotics. This is based on the multiple mechanisms of action of HDPs. However, bacteria and the human host have co-evolved, and *S. aureus* adaptations have been described for a small number of host defence peptides. For example, reduced susceptibility to defensin and protegrins has been demonstrated in *S. aureus* which is mediated by the incorporation of positively charged L-lysine into the cytoplasmic membrane and is catalysed by the product of the *mprF* gene (Peschel *et al.*, 2001; Ernst *et al.*, 2009). Interestingly, this membrane modification also contributes to *S. aureus* resistance to the CAMP-like agent daptomycin, which is currently in clinical use for MRSA infections. An investigation of the evolution of CA-MRSA shows that USA 300 and USA500 strains are more resistant to the innate immune defence peptides, dermicidin and indolicidin than isolates from the epidemic clones from which they originated (Li *et al.*, 2009). Despite these reported resistances, the immune-modulatory properties of HDPs, which may arguably be more important than their direct antimicrobial therapeutic properties, are not influenced by conventional resistance mechanisms and this is where HDPs may offer a real advantage over conventional antibiotics.

Natural HDPs may be released either locally at the site of infection or systemically in response to infection (Yang & Oppenheim, 2004). Some authors have argued that the augmentation of these triggers or the provision of analogous triggers of host immunity may dampen the natural innate or adaptive responses to infection or may cause excessive stimulation of inappropriate immune responses. Inappropriate antibody responses to the administration of self-proteins have been infrequently reported. The possibility of unpredictable effects on the natural host immune response highlights the importance of detailed characterization of the innate immune response. These investigations would include characterization of signalling pathways of pattern recognition agonists, regulatory elements of innate immunity and selective immunomodulatory effects of HDPs.

Development of host defence peptide-based agents for systemic administration will require considerable efforts to overcome some of the limitations mentioned above. However, improvements that address some of the limitations of promising candidate peptides have been reported. Substitution of *D*-amino acids into the peptide sequence of LL-37 derivatives was shown to minimize proteolysis and increase antibacterial activity (Stromstedt *et al.*, 2009), and the *in vitro* cytotoxic effects of LL-37 have been reduced by truncation of the sequence while antibacterial activity is retained (Nell *et al.*, 2006). Modifications that increase overall charge or amphipathicity increase potency, allowing lower concentrations to be used (Chen *et al.*, 2005). Pharmacokinetic properties have improved with the conversion of some host defence peptides to peptidomimetic or peptoid forms, use of *D*- or β- amino acids and PEGylation (Hong *et al.*, 1999; Hamamoto *et al.*, 2002; Hancock & Sahl, 2006; Imura *et al.*, 2007). Some of these host defence mimics, in addition to their excellent drug-like properties, failed to generate resistant derivatives of *S. aureus in vitro* compared with ciprofloxacin or norfloxacin (Tew *et al.*, 2006).

Targeted delivery of host defence peptides to the site of infection may further improve the therapeutic potential of these molecules. The increased local concentrations that could be reached with this approach could potentially remove constraints because of higher relative MICs for some HDPs. Improved delivery has had some success in the area of host defence peptides as candidates for anticancer therapy, including conjugation to a 'tumour-homing' motif, peptide hormone or antibody, bioconversion to an active agent by tumour-specific enzymes and liposomal technology (Ellerby *et al.*, 1999; Marks *et al.*, 2005; Mader & Hoskin, 2006; Chakrapani *et al.*, 2008; Jia *et al.*, 2008; Song *et al.*, 2009a). The identification and assessment of similar targeting approaches for delivery of defence peptides to sites of infection is in its infancy with antibody conjugation of a synthetic derivative of a salivary host defence peptide, histatin serving as an example. While pro-peptide inactivity in this case has not been clearly demonstrated, with improved design, the approach has clear therapeutic potential (Szynol *et al.*, 2006). In the MRSA field, the further development of cationic peptides for systemic use as targeted candidates against MRSA will depend on the selection of appropriate

effective candidates that are amenable to chemical modification and the design of bacterial or site of infection-mediated targeting approaches.

Another limitation to the therapeutic application of peptide-based anti-infectives is the high cost associated with chemical synthesis in large quantities. Synthetic mimics of antimicrobial peptides that have an unnatural backbone but maintain the biophysical characteristics of CAMPs offer a cost advantage (Rotem & Mor, 2009). Recently, the economic feasibility of chemical synthesis on a multi-tonne scale has been demonstrated for the biomimetic antiretroviral agent, enfuvirtide (Bray, 2003). Large-scale recombinant production of the fungal defensin, plectasin, has been achieved at commercially viable yield and purity, from cultures of the yeast, *Aspergillus oryzae* (Mygind *et al.*, 2005). Methodologies for large-scale industrial production of seven recombinant host defence peptides representative of those that are currently undergoing clinical trials have recently been developed by fusion to sumoase protease (SUMO), cloning into *Escherichia coli* and a two-step purification of the fusion product from the culture. This expression system gave high yields of intact and biologically active peptides and has demonstrated a cost-effective means of HDP production under good laboratory manufacturing processes that would be required for human therapeutic applications (Bommarius *et al.*, 2010).

Therapeutic approaches that target MRSA virulence

Another novel approach to the development of anti-staphylococcal agents with reduced capacity to elicit bacterial resistance is the development of 'antipathogenic' agents. These agents are designed to interfere with bacterial virulence mechanisms including binding to host tissues, evasion of phagocytosis, biofilm production and the production of toxins. The limited antibacterial activity of such agents may minimize the development of resistance while controlling the pathogenic process through diminished bacterial virulence. Controlling pathogenic processes in this way may allow the host immune response to more effectively overcome the infection. However, these agents could serve as adjuncts for immunocompromised patients. This antipathogenic approach, which relies on the identification and characterization of appropriate virulence targets, has been an academic research pursuit for over two decades. Promising targets that may be disrupted among *S. aureus* in the development of novel antipathogenic drugs include the accessory gene regulator (*agr*), sortase enzyme system, the carotenoid biosynthetic pathway and other recently discovered regulatory pathways. These systems contribute to the ability of *S. aureus* to effectively invade and damage the host, and therefore, their modulation represents a novel strategy in the anti-infective field and should be further explored.

The quorum sensing response

The quorum sensing response in *S. aureus* describes the coordinated expression of virulence genes in response to bacterial cell density and is modulated by complex regulatory systems, the best characterized of which is the accessory gene regulator (*agr*). *Agr* modulation contributes to the expression of a variety of virulence genes at different stages of infection through quorum sensing auto-inducing peptide (AIP) signals. (Novick, 2003; Cheung *et al.*, 2004). This role for *agr* in the inverse coordinated expression of genes that promote colonization and invasion has prompted many researchers to pursue *agr* as an antivirulence target. Specific molecules in the *agr* system, AIP and RNAIII (the effector molecule), have been investigated as potential targets for inhibition (Dell'Acqua *et al.*, 2004; Qazi *et al.*, 2006; Balaban *et al.*, 2007; George *et al.*, 2008). A global inhibitor of *S. aureus* AIPs was designed based on structure–function analysis and consists of a truncated thiolactone region of AIP-II (Lyon *et al.*, 2000), and more recently, investigations of a series of synthetic mimetics of this region have revealed the minimum structural requirements for inhibition (George *et al.*, 2008). Early administration of an AIP analogue attenuated abscess formation in a mouse subcutaneous abscess model but based on their findings, the authors suggest that administration of such quorum sensing inhibitors for *S. aureus* infections may be only of prophylactic value based on the kinetics of AIP activation (Wright *et al.*, 2005).

The potential of targeting *agr* for the treatment of device-related infections, which are difficult to treat with conventional antibiotics because of biofilm production, has been demonstrated by inhibition of this regulatory system with RNAIII-inhibiting peptide (RIP). This peptide caused a significant dose- and duration-dependent reduction in bacterial load in MRSA graft infections in rats, which was further reduced when RIP was administered in combination with teicoplanin (Balaban *et al.*, 2007; Simonetti *et al.*, 2008). The therapeutic efficacy and safety of RIP and two synthetic analogues of RIP have also been shown in histopathological studies in a mouse model of *S. aureus* sepsis (Ribeiro *et al.*, 2003). Although the target of RNAIII activating peptide has controversially been shown not to function in *S. aureus* pathogenesis (Shaw *et al.*, 2007), RIP has been shown to reduce staphylococcal infection in several *in vivo* models of infection and no toxicity has been noted. With regard to biofilm dispersal, however, it has conversely been shown *in vitro*

that *agr* inhibition is required for biofilm formation, and biofilm dispersal has been demonstrated with the addition of AIP to up-regulate *agr*-induced protease production (Boles & Horswill, 2008).

More recently, the nonribosomal secondary metabolite, aureusamine, was reported to regulate virulence gene expression, and the isogenic *ausA* mutant, which failed to haemolyse blood agar, had attenuated virulence in a mouse model of infection compared with the wild-type strain (Wyatt *et al.*, 2010). This reported role for aureusamine in virulence gene regulation was later found to be because of an inadvertent mutation in the *SaeR* two component regulator system (Sun *et al.*, 2011). The controversies surrounding the genetic stability of the *agr* locus in laboratory strains and the complexity of the roles of RIP, AIP and aureusamine in *S. aureus* pathogenesis have hampered progress in targeting quorum sensing systems for the discovery of novel anti-infectives. Nonetheless, these studies have been important in demonstrating the therapeutic potential of targeting virulence mechanisms and have prompted the study of other pleiotrophic regulators. With regard to novel therapeutic agents to inhibit *agr*-mediated virulence expression, the discovery of new molecules may be advanced due to the development of a simple, inexpensive assay to allow screening of large numbers of molecules for their effects on *S. aureus* virulence. This system is based on the observation of colour changes in response to the candidate molecule, in the growth media of *S. aureus* strains with *lacZ* fusions to the *agr*-regulated genes, *spa* and *hla*, in the presence of a beta-galactosidase substrate (Nielsen *et al.*, 2010).

Staphylococcus aureus sortase enzymes

Attachment of *S. aureus* to host endothelial tissue is facilitated by proteins that recognize specific tissue components such as fibrinogen, fibrin and collagen. The activity of these so-called microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) is dependent on their covalent attachment to bacterial peptidoglycan. The anchoring of these molecules to the cell wall is catalysed by a group of cysteine transpeptidases called the sortase enzymes (Fig. 3), which in *S. aureus* include two isoforms, SrtA and SrtB (Mazmanian *et al.*, 1999, 2002). SrtA is constitutively expressed, while SrtB is expressed in response to low iron conditions. Deletion of the sortase A gene (*srtA*) in *S. aureus* results in failure to display MSCRAMMs and therefore attachment to host components including IgG, fibronectin and fibrinogen. In a mouse model of *S. aureus* infection, mutants lacking *srtA* had at least a 2 log reduction in bacterial growth in multiple organs and a 1.5 log increase in lethal dose compared with the wild type (Mazmanian *et al.*, 2000). Later investigations demonstrated that *srtA* knockout mutants showed reduced virulence in models of septic arthritis and endocarditis (Jonsson *et al.*, 2003; Weiss *et al.*, 2004).

It has been recently shown that disruption of *srtA* in five biofilm-producing clinical isolates of MRSA results in significant reduction (up to sixfold) in glucose-induced biofilm formation which can be reversed by complementation (O'Neill *et al.*, 2008). The SrtB enzyme has a role specifically in the attachment of iron acquisition proteins such as IsdA, isdB, etc, and mutants that lack the *SrtB* gene are also associated with reduced virulence in the

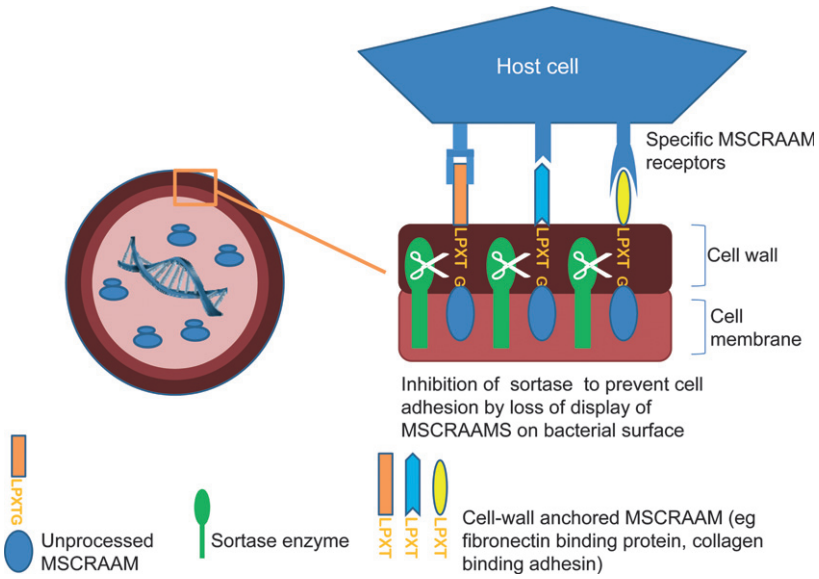

Fig. 3. Mechanism of sortase processing of MSCRAAMs. Sortase B cleaves at the LPXTG motif to allow display of MSCRAAMs at the cell surface. Their display facilitates adhesion to host cells. If sortase is inhibited, the bacterial cell has reduced adhesion to the host cell as the surface adhesins are not displayed.

mouse model of septic arthritis but only in the later stages of infection when iron is limited in the environment (Jonsson *et al.*, 2003).

The pathogenesis of *S. aureus* in persistent infections is linked to its ability to survive within macrophages where it is protected from the host immune response. Expression of *SrtA* has also been shown to be critical to phagosomal survival of *S. aureus* as *SrtA* mutants are efficiently killed by macrophages (Kubica *et al.*, 2008). These studies suggest that SrtA specifically may be a potential target for the development of novel anti-infective agents and may have specific application for complicated or persistent *S. aureus* infection including those involving biofilms. Selective toxicity by sortase inhibition is possible as there is no related sortase homologue in eukaryotic cells. The localization of SrtA within the cell membrane of *S. aureus* and other Gram-positive organisms offers an advantage in terms of the ease of access to this target where the activity of potential inhibitors will not rely on transport across the cell envelope. It has been speculated that bacterial resistance to sortase inhibition would be reduced compared with classical antibiotics given that *SrtA* mutants have similar growth rates to the wild type (Weiss *et al.*, 2004). The lack of disruption to essential gene function by *SrtA* mutation or inhibition, together with significantly attenuated virulence potential associated with loss of sortase activity, suggests that selective pressure would not be as significant for these possible agents as it is for antibiotics such as penicillin or aminoglycosides where the target is essential for cell survival and where selective pressure would favour the development of resistance. Numerous molecules have been investigated as potential inhibitors of sortase enzymes. Some of the most promising of these have been discovered by small molecule screening and were selected based on their 'drug-like' structures. For example, Oh and colleagues discovered a novel class of *S. aureus* sortase inhibitors, the diarylacrylonitriles, from a library of 1000 small molecules. Modification of the lead compound from the initial screen resulted in a reduction in IC_{50} from 231 to 9.244 μM (Oh *et al.*, 2004). These authors have further shown that this molecule, (Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile, was effective in an *in vivo* mouse model of *S. aureus* infection. Survival rates increased and joint and bone infections decreased in the treated animals compared with controls (Oh *et al.*, 2010). The aryl (β-amino) ethyl ketones were also selected from a large screening library of small molecules. These are mechanism-based enzyme inhibitors that have selectivity for *S. aureus* SrtA with IC_{50} and K_i values in the low micromolar range (lead compounds IC_{50} 15–47 μM) (Maresso *et al.*, 2007). More recently, pyridazinone and pyrazolethione analogues, selected from over 300 000 small molecules, have been shown to reversibly inhibit SrtA with IC_{50}s in the high nanomolar range (Suree *et al.*, 2009).

Sortase remains an attractive candidate as an antivirulence target, and the discovery of several distinct sortase inhibitors with activities in the nano- to micro-molar range and with drug-like properties is encouraging. However, challenges remain that require further investigation. The inhibition of sortase enzymes, by preventing the display of surface antigen, may dampen the host immune response which is required for bacterial clearance. Furthermore, bacterial clearance, even for virulence attenuated bacteria, requires active opsonophagocytic killing which may be impaired in the immunocompromised patient. A pharmacological evaluation of sortase inhibitors should be carried out, to assess therapeutic efficacy and toxicity. Further discoveries are needed to increase the pool of molecules available for further investigation as potential therapeutic agents. The further advancement of these discoveries will be initially guided by their properties in *in vivo* models of infection.

Staphyloxanthin biosynthesis

The antioxidant properties of the carotenoid pigment, staphyloxanthin, responsible for the golden colour of *S. aureus*, protect the organism from reactive oxygen species produced by neutrophils (Liu *et al.*, 2005). This finding suggests that modulation of this metabolic pathway may have antipathogenic effects. In a mouse subcutaneous model of infection, mice infected with *S. aureus* mutants lacking this pigment have significantly reduced bacterial loads and no visible lesions compared with the wild-type strain (Liu *et al.*, 2005). Increased bacterial clearance of staphyloxanthin mutant compared with the wild type was also shown by these authors in a murine model of nasal colonization (Liu *et al.*, 2008).

One of the key enzymes in staphyloxanthin biosynthesis is *S. aureus* dehydrosqualene synthase (SQS or CrtM) which catalyses the condensation of two molecules of isoprenoid farnesyl disphosphate to form dehydrosqualene. Interestingly, there is overlap between the early steps of staphyloxanthin biosynthesis and human cholesterol biosynthesis. Human SQS and the bacterial enzyme CrtM have 30 % sequence identity but have been shown to share significant structural features (Liu *et al.*, 2008). Furthermore, compounds originally developed as cholesterol-lowering agents have been shown to inhibit *S. aureus* CrtM in the nanomolar range and have been investigated as potential antipathogenic agents (Liu *et al.*, 2008). Two cholesterol-lowering agents, lapaquistat acetate and squalestatin, interact with both human squalene synthase and *S. aureus* CrtM at specific common residues (Kahlon *et al.*, 2010). Among the most potent inhibitors of CrtM

that also prevent staphyloxanthin formation in cellular assays are the phosphosulphonates with K_i in the range 1.5–135 nM and the diphenyl ether phosphonoacetamides with K_i in the range 30–70 nM. The most potent of the phosphosulphonates (designated BPH652) was further tested because it had advanced through preclinical animal testing and two human clinical trials as a cholesterol-lowering agent (Sharma *et al.*, 1998a, b). No inhibition of the growth of three human cell lines was found up to a concentration of 300 μM BPH652. The *in vivo* activity of BPH652 has also been determined in a mouse model of systemic *S. aureus* infection, and 98% reduction in *S. aureus* colony forming units was achieved in the treated group (Liu *et al.*, 2008). The question of selectivity for the *S. aureus* CrtM over human SQS has also been addressed by these authors and several halogen-substituted derivatives show selectivity for the bacterial enzyme (Song *et al.*, 2009b).

The diphenyl ether phosphonoacetamides have further improved properties in terms of their uptake into cells (IC_{50} = 8 nM) while retaining their selectivity for the bacterial enzyme and their negligible toxicity in human cell lines (Song *et al.*, 2009c). The inhibition of the staphyloxanthin pathway in *S. aureus*, as antivirulence agents, is attractive, because many cholesterol-lowering agents have previously undergone clinical trials, and their toxicities and pharmacokinetic properties are already known (Liu *et al.*, 2008). Further testing of the improved molecules described above in animal infection models will be eagerly awaited.

The pigmentation of *S. aureus* because of staphyloxanthin can be exploited in the development of technologies for rapid screening of candidate inhibitory molecules and one such system has been used successfully to identify at least four known inhibitors of lipid metabolism that reduce staphyloxanthin pigmentation, from a natural compounds library (Sakai *et al.*, 2012).

Conclusion

The anti-infectives industry appears to rely on the development of further generations of conventional antibiotics which have improved properties but do not offer new modes of action. Here, we have highlighted areas where basic and applied research has demonstrated the potential of novel anti-MRSA therapies. It is clear, however, that further research is required to determine when and how these compounds can be administered. Investment in generating convincing *in vivo* data that support a protective role for novel therapeutic agents with minimum side-effects is required. Given that the majority of patients requiring therapeutic intervention for *S. aureus* infection are immunocompromised, it appears that both of the approaches discussed here would have potential as adjuvant therapies rather than their exclusive use as anti-infectives. It is interesting therefore that synergistic *in vitro* and *in vivo* effects have been reported using a combination of two HDPs and vancomycin (Cirioni *et al.*, 2006), and a potential advantage of the administration of pexiganan with β-lactam antibiotics has also been demonstrated (Giacometti *et al.*, 2005). These combined applications would potentially extend the therapeutic effectiveness of current antibiotics. Antivirulence approaches, aimed at modulating the pathogenic effects of *S. aureus* infection, could also be investigated in conjunction with conventional antibiotics.

Transparency declaration

HH has had recent research collaborations with Steris Corporation, 3M, Inov8 Science, Pfizer & Cepheid. He has also recently received lecture & other fees from 3M, Novartis & Astellas. DF, MD none to declare.

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