Targeting Virulence not Viability in the Search for Future Antibacterials

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ABSTRACT

New antibacterials need new approaches to overcome the problem of rapid antibiotic resistance. Here we review the development of potential new antibacterial drugs that do not kill bacteria or inhibit their growth, but combat disease instead by targeting bacterial virulence.

INTRODUCTION

In the ongoing battle between people and pathogens, the pendulum seems to be swinging in favour of the bugs. The rapid increase in resistance to antibiotics combined with the slowing to a trickle of new antibiotics progressing through the pipeline over the past decades has led to this point. The situation has been described by the Infectious Diseases Society of America as a looming "public health crisis" [1].

There are any number of reasons why pathogenic bacteria acquire antibiotic resistance, and why resistance is growing at such an alarming rate. The question is, given where we are now, how can we ensure that the pendulum swings back in our favour? One school of thought is that we need to change the way we discover new antibiotics. Historically, antibiotics have been identified by their ability to kill or inhibit growth of bacteria. A prime example is penicillin, originally identified by Fleming's serendipitous discovery that a penicillium mould inhibited bacterial growth on an agar plate. Ever since, screening approaches have been engineered to find chemicals that do the same thing, and molecular approaches have focused on identifying essential genes to target for drug intervention. The problem with therapeutic approaches that

target viability is that they induce a high selection pressure. A bacterium exhibiting resistance to the antibiotic will have an enormous selective advantage over its competitors in a bacterial population, so that resistance will develop rapidly in the presence of that antibiotic.

An alternative to killing bacteria or stopping their growth, is to search for drugs that disarm bacteria. This idea focuses on developing drugs that inhibit bacterial virulence [2-4] rather than bacterial viability (Figure 1). Targeting virulence offers several potential advantages including:

(i) an increased repertoire of pharmacological targets

(ii) generating antimicrobials with new mechanisms of action

(iii)reducing resistance development due to decreased selective pressure [3]

(iv)and potentially preserving gut microbiota.

On the other hand, development of antivirulence therapies presents its own unique challenges. We can no longer use established screening systems that identify compounds that kill or inhibit growth of bacteria. And minimal inhibitory concentration measures are obsolete in this scenario. Specific *in vitro* and *in vivo* assays will need to be developed to screen for compounds that inhibit specific virulence processes. And, given that virulence mechanisms vary from one bacteria to another, antivirulence drugs are likely to have a narrow spectrum of activity. Their success in the clinic may well depend on development of real time diagnostics that identify the causative organism and enable therapy personalised to the infectious agent.

In this review we highlight several virulence pathways currently being targeted for the development of antivirulence drugs, including adhesion, secretion and toxin production (Figure 2, summarised in Table 1). We also highlight two master virulence targets that coordinate deployment of entire arsenals of virulence factors either by communicating information (quorum sensing) (Figure 2, Table 1) or by assembling an armoury of bacterial weapons (oxidative folding) (Figure 3). Drugs that block these master systems may have a broader spectrum of activity.

TARGETING OCCUPATION

A crucial first step in colonization by bacteria is adhesion to host cells; blocking this process may prevent establishment and maintenance of infection [4]. Adhesion is mediated by surface proteins (adhesins, autotransporters *etc*) and multi-protein scaffolds (*eg* pili) protruding from bacteria that interact specifically with carbohydrates on the host cell surface.

In Gram-positive organisms, adhesion depends on sortases, a family of cysteine transpeptidases that covalently anchor adhesin proteins to the bacterial cell wall [5, 6]. Sortases have been targeted in several antibacterial drug discovery programs and screening against *S. aureus* and *Bacillus anthracis* have identified hits which could potentially be developed into potent sortase inhibitor drugs [7-11].

By contrast, cell adhesion and invasion in Gram-negative organisms generally relies on the production of pili [4]. Two strategies have been developed to block pilus-mediated adhesion. One is the identification of pilicides, molecules that prevent pili biogenesis by interfering with the underlying usher-chaperone pathway [12]. Pilicides

have been shown to reduce production of several components of this pathway in uropathogenic *E. coli* including type I and P pili fimbrial proteins [13]; Dr family adhesins [14]; and curli [15], with some also preventing biofilm formation [12, 15]. Significantly, the usher-chaperone pili assembly machinery is present in many species including *Escherichia, Salmonella, Klebsiella, Yersinia* and *Pseudomonas* [16] suggesting that pilicides may have a broad spectrum of activity [17].

A second strategy to inhibit pili-based adhesion relies on physically blocking the interaction between the adhesin and the host cell. The carbohydrate binding site is localised at the very tip of the pili. Carbohydrate derivatives and molecules mimicking mammalian glycans dramatically reduce the adhesive properties of bacterial pili [18-20]. Importantly, one such inhibitor prevented acute infection *in vivo* and also treated chronic cystitis caused by a multi-resistant *E. coli* in an animal model [18].

TARGETING WEAPONS DELIVERY

Bacteria have evolved complex machineries to deliver proteins and toxins into a host cell across membranes and cell walls, and these machineries play a central role in pathogenesis. The system attracting most attention is the Type III secretion system (TTSS). This syringe-like multiprotein apparatus injects bacterial effector proteins and toxins directly into the host cell cytosol, and thereby hijacks a wide range of cellular processes [21]. Many components of the TTSS are specific to prokaryotes and several studies have explored TTSS inhibitors as potential therapeutics (recently reviewed in [22]). Importantly, the TTSS machinery is present in many pathogens including *Escherichia, Shigella, Salmonella, Pseudomonas, Chlamydia* and *Yersinia*

spp., so that targeting common elements could result in broad-spectrum TTSS inhibitors [23].

Indeed, high-throughput screening identified thiazolidinone derivatives that block TTSS from Gram-negative pathogens including *S.* Typhimurium and *Yersinia enterocolitica*, reduced the virulence of *Pseudomonas syringae* and inhibited other secretion systems such as the Type II in *Pseudomonas* and the Type IV in *Francisella* [24]. Similarly, small molecule screening identified a series of salicylidene acylhydrazides capable of inhibiting the TTSS of intracellular (*Chlamydia trachomatis*) and extracellular pathogens (*Yersinia* ssp.) [25-27]. Some of these compounds showed protective activity against the sexually transmitted pathogen *C. trachomatis* in mouse infection models [28].

TARGETING TOXINS

Toxins are the primary virulence factors of many bacterial pathogens. Examples include botulinum and tetanus neurotoxins, cholera, anthrax, diphtheria and Shiga toxins. All are proteins delivered into the host to cause mass cell destruction and tissue damage [29]. Their extreme toxicity and critical role in pathogenesis makes inhibition of toxin production an obvious approach for development of antivirulence antimicrobials. This can be achieved by targeting toxin transcription and expression: virstatin inhibits the transcription factor ToxT that regulates expression of cholera toxin and cholera co-regulated pilus, and blocks intestinal colonization by this pathogen in murine models [30]. Similarly, a small molecule inhibitor of toxin TcdA and TcdB expression by *Clostridium difficile*, has shown efficacy in a hamster model of gastrointestinal infection [31].

Antibodies have been developed to neutralise toxins and are already used to treat bacterial diseases such as tetanus, diphtheria and botulism [32]. For example, botulism toxin neutralizing antibodies from horse sera are used to treat adult botulism and a human-derived botulism antitoxin has been used to treat infants [33]. These outcomes provide clinical evidence validating the use of antitoxin drugs after infection.

Other antibody therapies are at different stages of development [34]. For example, antibodies against Shiga toxin were shown to protect against Shiga toxin-producing *E. coli* (STEC) in a piglet model of acute gastroenteritis [35]. Similarly, efficacy was demonstrated in mouse and hamster infection models by combining human antibodies against *C. difficile* toxins A and B [36]. The potential use of *B. anthracis* as a bioweapon has made this and other high threat pathogens the focus of intense efforts to develop antibodies and vaccines [37]. Antibodies that inhibit anthrax toxins (ABthrax, Valortim among others) have shown promising protection in a range of animal models and are now in clinical development [38-41].

TARGETING COMMUNICATION SYSTEMS

Bacterial cell-to-cell communication is essential for microbes to adapt to changing environments and this communication is regulated by Quorum Sensing (QS) networks. Gram-positive and Gram-negative bacteria both use complex regulatory QS circuits to sense their population densities and regulate the expression of virulence factors, allowing successful establishment of infection [42]. The canonical QS pathways consist of secreted signal molecules known as autoinducers (AI, *e.g.* acyl-homoserine

lactones (AHLs) in many Gram-negative bacteria, autoinducing peptides (AIPs) in Gram-positive bacteria). Upon reaching a threshold concentration, AI molecules interact with cognate sensor receptors (*e.g.* LuxR and LuxS receptors) to induce the expression of virulence genes.

Given the central role of QS systems in bacterial pathogenesis, many efforts have focused on interfering with these pathways (recently reviewed in [43-48]). Quorum quenching is a term that has been used to describe "any approach that interferes with microbial QS signalling" [49]. QS networks have been quenched or modulated at three points (reviewed in [49]) by: (1) inhibiting signal generation (*e.g.* by blocking synthesis of AHL *in vitro* using AHL analogues [50, 51]), (2) degrading the signal molecule (AHLs can be destroyed chemically by increasing the pH [2] or by use of "quorum quenching" enzymes [47], or inactivated with antibodies [52]), and (3) blocking the interaction of the QS signal molecule with the receptor. The last is the most popular approach. Screening of natural and synthetic compounds has produced potent antagonists of sensing receptors for many bacteria (*e.g.* enterobacteria, *Pseudomonas*, *Staphylococci*) with some antagonists being protective in animal models of infection (reviewed in [53]). Furthermore, inhibitors capable of blocking QS networks in several Gram-negative pathogens, open the possibility of QS inhibitors with broad spectrum activity [54].

The increasing number of patent applications for QS inhibitors clearly reflects the interest in this approach [55]. Notably, targeting QS has yielded potent molecules that prevent biofilm formation, a major hurdle in treating many bacterial infections [47]. Although a lower risk of resistance development was predicted for QS-regulating

molecules, recent data indicate that bacteria can develop resistance to these compounds [56]. For example, the QS inhibitor C-30 [57] had no effect on the growth of *Pseudomonas aeruginosa* in rich media, but in minimal media it did affect bacterial growth and selected for resistance [58].

TARGETING WEAPONS ASSEMBLY

Virulence factors produced by bacteria are generally proteins, and these virulence proteins need to be assembled correctly to function. An important feature of many virulence factor proteins produced by Gram-negative bacteria is the requirement for structural bracing in the form of disulfide bonds. Disulfide formation between pairs of cysteine residues increases the chemical and physical stability of proteins. Conversely, failure to form native disulfide bonds results in degradation and loss of activity.

Oxidative protein folding, the process of introducing disulfide bonds into folding proteins, is a rate-limiting step in the assembly of many virulence factors and requires the activity of specific enzymes [59]. The classic bacterial disulfide bond (DSB) machinery, first characterised in *E. coli* K-12[60], comprises a soluble periplasmic enzyme DsbA and an integral membrane protein DsbB (Figure 3). DsbB and its quinone cofactor together generate disulfides *de novo,* and transfer them to DsbA[61] which introduces disulfides directly into folding proteins[62].

Whilst some variation exists in the DSB enzymes in different bacteria (reviewed in [63]), there is now overwhelming evidence that the DSB oxidative protein folding machinery is a master regulator of bacterial virulence. Recent compelling evidence

comes from a study using an animal model of melioidosis in which mice infected with the causative agent of melioidosis, *Burkholderia pseudomallei,* all died within 42 days whereas mice infected with *B. pseudomallei* lacking the gene for DsbA all survived[64]. Similarly, animal infection models have demonstrated that deletion of *dsbA* or *dsbB* in uropathogenic *E. coli* (UPEC) severely attenuated its ability to colonize the bladder[65], and that *dsbA* mutants in *Salmonella enterica* serovar Typhimirium were avirulent[66].

Indeed, many bacteria lacking a functional DsbA have been shown to have reduced virulence, increased sensitivity to antibiotics and diminished capacity to cause infection. These include uropathogenic *E. coli* (UPEC), enteropathogenic *E. coli* (EPEC), *Bordetella pertussis* (whooping cough), *Vibrio cholerae* (cholera), *P. aeruginosa* (opportunistic human pathogen), *Haemophilus influenzae* (opportunistic human pathogen), *S. flexneri* (diarrhoea) and *Neisseria meningitidis* (bacterial meningitis) amongst others [67-74].

The loss of virulence can be attributed to the misfolding of a (normally) disulfide-containing protein substrate of DsbA. For example, *E. coli dsbA* mutants are non-motile. The loss of motility is a consequence of the misfolding of protein FlgI, a component of the periplasmic ring of the flagellar motor, and a DsbA substrate[75]. DsbA is also required for the correct folding of virulence proteins involved in bacterial adhesion, secretion and toxicity. Similarly, mutational inactivation of *dsbB* also affects bacterial virulence[76].

These observations point to a major regulatory role in virulence and identify the DSB

enzymes as key targets for the development of anti-virulence agents. The DSB machinery offers a number of advantages as antibacterial drug targets including:

(i) DSB inhibition affects multiple bacterial virulence pathways;

(ii) DSB enzymes are localised to the outer compartment of bacteria making drug delivery more amenable than cytoplasmic targets;

(iii) DsbAs are more highly conserved than the virulence factors they assemble, so that inhibitors are likely to be effective against multiple pathogens; and

(iv) Structures of DSB enzymes are in the public domain, so that structure-based approaches for drug discovery are supported.

However, whereas DSB systems are conserved and required for pathogenicity in Gram-negative bacteria, the link between DSB systems and virulence in Gram-positive organisms is not confirmed. Moreover, to our knowledge, there are no reports of small molecule inhibitors of DSB enzymes that are effective *in vivo*. Indeed, they represent challenging targets for drug design. Structures of DsbA and DsbB reveal that their interaction surfaces lack deep binding cavities, which is often an impediment to inhibitor design. Nevertheless, there are an increasing number of examples of small molecules designed to block protein-protein interactions against other targets [77]. Furthermore, screening of small molecule "fragments" identified compounds that interacted with DsbB and led to a series of compounds capable of inhibiting DsbB *in vitro* [78]. If we can develop inhibitors of DSB-mediated oxidative protein folding, these would have enormous value as antivirulence agents by potentially blocking the assembly of multiple bacterial virulence factors.

SUMMARY

In the search for future antibacterials to overcome antibiotic resistance, antivirulence agents promise more than a glimmer of hope. Several strategies have been put forward, and target validation and preliminary screening have been performed to identify important virulence pathways and master virulence machineries. However, aside from antibodies that inactivate specific bacterial toxins, none of these compounds with new mechanisms of actions have yet reached the clinic. So it remains to be seen whether all or some of these antivirulence approaches will live up to expectations. We eagerly await studies showing how new generation antivirulence antibacterials perform, whether they will reduce resistance development, whether they will need to be combined with traditional antibiotics, or whether they can resurrect antibiotics made obsolete by bacterial resistance mechanisms.

Table 1. Selected examples of virulence factor inhibitors

Mode of action		Selection of studied pathogens	Ref.
Adhesion Inhibitors			
Aaptamines	Natural product sortase A inhibitors	*S. aureus*	[8]
Pyridazinone and pyrazolethione derivatives	Synthetic sortase A inhibitors	*S. aureus*, *B. anthracis*	[11]
Pilicides	Regulate pilus biogenesis by blocking the chaperone/usher assembly pathway	*E. coli*	[12]
Toxin Inhibitors			
Virstatin	Inhibits ToxT transcription factor blocking expression of cholera toxin	*V. cholerae*	[30]
ABthrax, Valortim	Antibodies; inhibit anthrax toxins	*B. anthracis*	[38-41]
TTSS Inhibitors			
Thiazolidinone derivatives	Prevent translocation of effector molecules	*Yersinia*, *Salmonella*, *Francisella*, *Pseudomonas*	[24]
Salicylidene acylhydrazides	Prevent translocation of effector molecules	*Escherichia*, *Yersinia*, *Chlamydia*, *Pseudomonas*, *Salmonella*, *Shigella*	[28]
QS Inhibitors			
Furanone derivatives	Inhibitors mimic AHLs; bind LuxR receptor and inhibit QS-regulated gene expression	*E. coli*, *P.aeruginosa*, *Proteus mirabilis*, *Staphylococci*	[53]
Lactonase and acylase	"Quorum quenching" enzymes degrade AHL, to block the quorum sensing response	*Bacillus ssp*, *Erwinia carotovora*, *P. aeruginosa*, *Pectobacterium carotovorum*	[47, 53]

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CONFLICT OF INTEREST STATEMENT

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare:

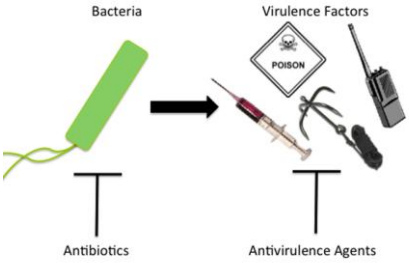
JLM had support from the Australian Research Council for the submitted work; MJS and JLM had funded grants from the Australian Research Council in the previous 3 years; BH and MJS had support from the Australian National Health and Medical Research Council in the previous 3 years; MJS and JLM had grants and non-financial support from Biota Pty Ltd, over the past 3 years. MJS had grants and non-financial support from Biota Pty Ltd outside the submitted work over the past 3 years.

FIGURE LEGENDS

Figure 1. Antibiotics and anti-virulence agents. Antibiotics (left) kill bacteria or prevent their growth. Antivirulence agents (right) render bacteria harmless by blocking the activity of virulence factors. Virulence factors can include toxins (denoted by poison sign), secretion systems (syringe), adhesion factors (grappling hook), or quorum sensing (walkie-talkie), amongst others.

Figure 2. Examples of bacterial virulence pathways that have been targeted for antimicrobial development. ***Bacterial adhesion to the host cell***; 1. Inhibitors of pili biosynthetic machineries (e.g. chaperone/usher pathway); 2. Inhibitors of the carbohydrate-binding sites in the adhesin molecules. ***Bacterial secretion systems***; 3. Inhibitors of the Type Three Secretion system (TTSS) to block injection of effector proteins to the host cell. ***Toxin production***; 4. Toxin neutralisation to inhibit damage to the host. ***Acyl-homoserine lactone (AHL) mediated Quorum Sensing (QS)***: 5. Inhibitors of AHL synthase LuxI. 6. AHL degrading enzymes (e.g. lactonase and acylase). 7. Inhibitors of AHL binding to transcriptional regulator LuxR.

Figure 3. Schematic representation of the DSB catalytic cycle. Quinones (labeled Q) generate disulfides in DsbB (orange), which are transferred to DsbA (green), which catalyses oxidative protein folding in substrate virulence factors (blue; and indicated above). In concert, the disulfide in DsbA (labeled S-S) is reduced to two thiols (labeled SH) to complete the catalytic cycle.



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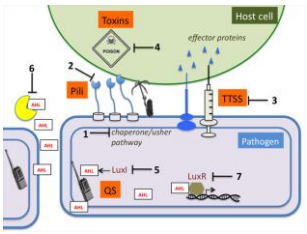

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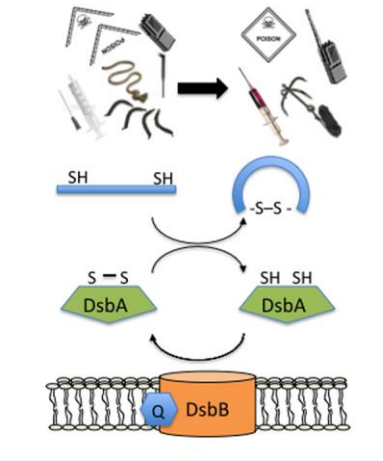

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