Benefits and Challenges of Antivirulence Antimicrobials at the Dawn of the Post-Antibiotic Era

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Abstract: In April 2014, the World Health Organization announced the beginning of a post-antibiotic era and declared antimicrobial resistance (AMR) a public health priority demanding global action. If no action is taken, by 2050 AMR will kill more people each year than cancer, with 10 million estimated annual deaths at a cost of $100 trillion to the global economy. New therapies to tackle multidrug resistant bacterial pathogens are urgently needed. Unlike traditional antibiotics, antivirulence drugs inhibit bacterial virulence instead of growth promising to offer a new class of superior therapeutics that will be ‘evolution-proof’ and ‘tailored-spectrum’. This mini-review discusses the latest emerging evidence on the promised benefits of antivirulence drugs over conventional antibiotics, also highlighting the challenges in evaluating these properties for each of the diverse virulence targets that are currently under investigation. The author argues that overcoming such challenges early in the development process constitutes an important step towards successfully progressing each of the expanding number of antivirulence strategies into next-generation therapies for common human and animal infections that are becoming increasingly refractory to all available antibiotics.

Keywords: Activity spectrum, bacteria, drug resistance, evolution, infection, pathogen, selection, virulence factor.

1. INTRODUCTION

In 2014 the World Health Organization (WHO) declared antimicrobial resistance (AMR) a public health priority demanding global action. This coincided with WHO’s release of an extensive AMR surveillance report that offered the first global perspective on the extent of the problem and its worldwide impact [1]. The report clearly highlighted very high rates of antibiotic resistance across all six WHO regions (represented by 114 participating countries) and for most bacteria that cause common infections in different settings (community, hospitals and the food chain). It became obvious that for certain pathogens resistance is already widespread and excessively high (> 50% across at least 5 out of 6 WHO regions), such as resistance to 3rd generation cephalosporins among Gram-negative Escherichia coli and Klebsiella pneumoniae. Worryingly, co-resistance to fluoroquinolones and aminoglycosides, or even last-resort carbapenems and colistin, is frequently encountered in these organisms, leaving health professionals with very few treatment options against multi-drug resistant (MDR) strains.

Antibiotic resistance increases the global health and economic burden, as patients with drug resistant infections experience worse clinical outcomes and death more often than those infected with drug susceptible bacteria [1]. Currently, 2 million people are affected each year by antibiotic resistant infections in the US alone, resulting in 23,000 deaths, >$20 billion in health care costs and $35 billion in loss of productivity [2]. Alarmingly, many common infections that until recently were readily treatable, such as urinary tract infections and foodborne diarrhoea, are becoming resistant to most available antibiotics, heralding the beginning of a post-antibiotic era in which common infections could become virtually untreatable and lethal. If no action is taken, AMR is predicted to kill more people than cancer by 2050, with 10 million estimated deaths each year and at a global annual cost of up to 100 trillion USD [3]. Clearly, new therapies to tackle MDR pathogens are sorely needed.

Despite some promising advances in new antibiotic discovery, exemplified by the recent discovery of teixobactin [4], the number of new drugs reaching the clinic or currently under development is unacceptably small and could not provide an adequate solution to the pressing AMR problem. Importantly, antibiotics work by killing bacteria or inhibiting their growth, and this imposes strong pressure for selecting resistant variants in the population; whether resistance pre-
exists or develops as a new mechanism, once it spreads among susceptible bacteria it could render expensive new drugs ineffective shortly after their introduction to the clinic. A new class of drugs designed to disarm (antivirulence) rather than kill (antibiotics) bacterial pathogens is promising to revitalize the antimicrobial development pipeline and provide effective therapeutic alternatives to currently failing antibiotics with the additional advantages of being evolutionary robust and pathogen-specific. This mini-review presents the latest research in antivirulence drug development with a focus on emerging evidence on their promised benefits over traditional antibiotics and discusses the challenges that need to be addressed for their successful development into the next-generation antimicrobials.

2. ANTIVIRULENCE: A ‘DISARM - DON’T KILL’ APPROACH

The idea of inhibiting virulence as a means of controlling bacterial pathogenesis research has been a central concept driving bacterial pathogenesis research for a long time and before antibiotics were discovered [5]. However, the term ‘antivirulence’ has not been widely used in this context until the past 10-20 years, coinciding with a resurgence of research interest in antivirulence therapeutics as effective alternatives to antibiotics for controlling MDR pathogens. Indeed, the number of publications on antivirulence compounds and targets has increased noticeably over the past decade (Fig. 1). Early examples of the antivirulence approach primarily include inactivation of bacterial toxins, such as tetanus, botulinum and diphtheria, by neutralizing antitoxin antibodies administered to patients post-exposure; an approach that has been successfully applied in clinical practice since the early 1900’s [6]. Toxins are obvious targets for antivirulence therapies as they are exclusively produced by pathogenic species (e.g. Clostridium tetani) or by pathogenic strains of a species (e.g. enterohemorrhagic E. coli or EHEC) and are key mediators of severe disease pathology [7]. As toxin inactivation during infection has been proven to be an effective way to prevent or relieve acute disease symptoms, important advances have been made in developing new antitoxin monoclonal antibodies, e.g. for Shiga toxin (Urotaxzumab) [8], anthrax [9] and C. difficile toxins [10], as well as inhibitors of toxin function [11] or expression [12]. In addition to bacterial toxins, many other virulence factors have been shown to constitute promising targets for antivirulence strategies (extensively reviewed [13-18]). Some of the most actively researched antivirulence strategies include inhibition of bacterial adhesion and colonization [19-21], virulence regulation and quorum sensing [22-24], and virulence factor folding and secretion [25-27].

While antitoxin therapeutics have been utilized in clinical practice for decades, drug development for most other virulence targets still remains confined within the realms of academia or small biotech companies. The obvious lack of interest from large pharmaceuticals to invest in antivirulence drug development was to be expected during the golden years of antibiotic discovery, yet the landscape has changed considerably over the last 10-15 years. Widespread drug resistance and the rise of superbugs have turned antibiotics from being considered ‘wonder drugs’ to being seen as ‘worthless drugs’ by big pharma, due to a significant increase in development costs and greatly diminishing profits. This vicious circle, where industry profits fed by antibiotic overuse and misuse resulted in rendering most antibiotics ineffective and subsequently drove large pharma away from investing into new antibiotic development due to unfavorable development-over-profit cost analyses, is known as the antibiotics paradox and is the primary reason for the unacceptable drop in new drug numbers [28].

While the cost of identifying and developing new antibiotics is largely unfavorable to big pharma, the plethora of antivirulence targets that have already been validated in vitro and in vivo and the availability of effective inhibitors at various stages of development should represent excellent opportunities for big pharma investment. Crucially, there is a pressing need for new antimicrobials to replace currently failing antibiotics and antivirulence drugs promise to do so in a superior way (Fig. 2).

3. PROMISED BENEFITS OF THE ANTIVIRULENCE APPROACH

3.1. ‘Evolution-proof’ Antimicrobials

Antibiotics fail when resistance mechanisms develop and spread among bacteria. Resistance to antibiotics develops as
a consequence of their mode of action: antibiotics are designed to kill bacteria (bactericidal) or inhibit their growth (bacteriostatic), which results in strong selection pressure for resistant mutants that can escape killing and propagate in the presence of the drug. The antivirulence approach aims to inhibit virulence without affecting bacterial growth; an approach that has the potential to avoid or minimize resistance development as selection pressure is anticipated to be weaker than in the case of bactericidal or bacteriostatic compounds [29]. While this tenet appears to be frequently indicated in the literature, for many of the antivirulence compounds currently under investigation, it has neither been widely evidenced nor rigorously tested in the lab or clinic.

In fact, the compounds most intensively researched for resistance development to date are quorum sensing inhibitors (QSI), also known as quorum-quenching (QQ) compounds, which do not appear to constitute the most evolutionary robust antivirulence strategy [30]. Resistance to C-30, one of the best characterized QQ compounds, has been shown to develop quickly in Pseudomonas aeruginosa lab strains experimentally and it involved a mechanism of enhanced compound efflux that had not been anticipated [31]. Interestingly, resistance to C-30 was also identified in clinical P. aeruginosa isolates not previously exposed to the compound and novel resistance mechanisms, other than drug efflux, were found to exist naturally [32, 33]. While some QSIs might not appear to be ‘evolution-proof’, it is important to first consider and evaluate the evidence of how strictly they fulfill the central ‘disarm-don’t kill’ criterion of the antivirulence approach. This review argues that assessing this criterion in relevant laboratory systems is an important prerequisite for all antivirulence compounds (discussed in 4.1 below), including QSIs and other virulence inhibitors for which resistance development has already been documented experimentally [12, 34].

For other antivirulence strategies, however, where inhibition of a virulence target does not impact on hundreds of genes like in the case of quorum sensing [35], or where the inhibitor only acts on an extracellular virulence target, resistance might be less likely to develop or develop slower. For example, inhibiting iron sequestration in P. aeruginosa by gallium-mediated quenching of the pyoverdin siderophore, was recently reported to attenuate virulence and growth during Galleria mellonella larvae infection and did not select for resistance development in vitro unlike conventional antibiotic therapy [36]. In addition, only partial resistance was observed to the anti-adhesion group 2 capsule polysaccharide (G2cps) in E. coli biofilm growth and it involved multiple unrelated mutations, suggesting that full resistance development is likely to be a rare phenomenon [37]. Similarly, inhibition of the FimH adhesin, an important colonization factor of uropathogenic E. coli (UPEC), reduced bladder colonization in mice with chronic cystitis and prevented urinary tract infection (UTI) by UPEC, including a multidrug resistant strain that is currently circulating worldwide [38, 39]. FimH inhibitors (mannosides) have also been shown to treat catheter-associated UTI in mice in vivo [40] and prevent Chron’s disease E. coli from adhering ex vivo to colonic tissue from transgenic mice mimicking CD patients [41]. While resistance development to mannosides remains to be tested, it is anticipated that it will be rare given their mode of action: i) mannosides are soluble mimics of the FimH adhesin’s natural ligand on host cells and bind with high affinity to the FimH mannose-binding pocket that is invariant in all E. coli (mutations in the pocket attenuate virulence and would not be under strong selection) and ii) mannosides inactivate an extracellular bacterial target, which also makes them less susceptible to cross-resistance, i.e. less likely to be rendered ineffective by pre-existing resistance mechanisms of MDR bacteria (e.g. permeability porin or efflux pump mutations).
The question, however, of whether antivirulence drug resistance will spread far and wide is perhaps a more important one than whether it will develop in the first place, and one for which there is considerable lack of research data. Answers to this question are primarily drawn from theoretical work and predictions based on few experimental evolution studies to date [29]. Any resistance mechanism to a particular antivirulence drug could be selected for, against, or neither depending on the net benefit the targeted virulence factor confers to the pathogen and the pathogen’s population structure during treatment [29]. In the case of QSIs environmental conditions are an additional parameter proposed to influence the frequency of resistant strains [43].

The landscape of resistance evolution and selection is clearly more complex for antivirulence drugs than for classic antibiotics, where resistance to killing or growth inhibition is always beneficial. In order to better predict the fate of antivirulence resistance selection and spread in clinically relevant environments, we need more insight into the net benefits conferred by different virulence factors to pathogen fitness and at different environmental niches that are relevant to each pathogen’s lifestyle.

3.2. ‘Tailored-spectrum’ Antimicrobials

Inhibiting virulence not only offers a plethora of new pharmacological targets urgently needed to revitalize the drug development pipeline but can also allow the design of therapeutics with uniquely tailored activity spectra. Unlike traditional antibiotics, where ‘narrow-spectrum’ usually refers to selective inhibition of either Gram-negative or Gram-positive bacteria, antivirulence compounds have the potential to be species- or even strain-specific, depending on their virulence target and how conserved it is between and/or within a bacterial species. For example, monoclonal anti-toxin antibodies can neutralise specific toxins produced by a single pathogenic species (e.g. *Bacillus anthracis*) or by pathogenic strains within a species (e.g. Shiga-toxin producing *E. coli* or STEC), while inhibitors of sortase A, a conserved cysteine transpeptidase responsible for anchoring surface virulence proteins to the Gram-positive bacterial cell wall, can have a broader spectrum of activity against multiple species, e.g. the aryl (β-amino)ethyl ketones are active against both staphylococci and bacilli [44]. Similarly, type III secretion systems (TTSS) are a common strategy for delivering bacterial effectors into host cells that is shared by many animal and plant pathogens and TTSS inhibitors have been identified that have activity over multiple pathogenic species [27]. The best studied include a series of salicylidene acylhydrazides with demonstrated activity against *Yersinia pseudotuberculosis* [45], *Chlamydia trachomatis* [46, 47], *Enteropathogenic E. coli* (EPEC) [48], EHEC [49], *Salmonella enterica* [50, 51] and *Shigella flexneri* [52], while another class of compounds (thiazolidinones) with a broader spectrum of inhibition against Gram-negative protein secretion systems (type II and type III) have also been described [53].

In addition to species-specificity, and depending on the expression profile of the virulence factor being targeted, antivirulence drugs could be designed to have very specific activity only at selected host niches during treatment, which would likely include the site of infection where expression of the targeted virulence factor would be beneficial. This would not only preclude undesired inhibition of commensal bacteria but also any off-site drug activity that would select for resistant strains at sites of commensal colonization or other natural reservoirs and environments outside the host. Inhibition of the *Staphylococcus aureus* golden pigment (staphyloxanthin) has been proposed as a good example of narrow-spectrum environmental specificity that can be achieved by antivirulence therapeutics [29]. A cholesterol biosynthesis inhibitor also shown to block staphyloxanthin biosynthesis in *S. aureus* [54] would render cells susceptible to killing by reactive oxygen species (ROS) only at host sites where the presence of *S. aureus* triggers neutrophil-influx and ROS production but not at commensal *S. aureus* niches. Similarly, pilicides and mannosides that respectively target the bio-genesis or adhesion function of Gram-negative chaperonusher fimbiae (also known as pili), such as P and type 1 fimbiae in *E. coli*, could have the potential to display environmental specificity in their activity spectrum as many of their fimbral targets are under phase-variable regulation of expression [57]. This ensures that the fimbral target will only be expressed in a fraction of the bacterial population at any given time and only under specific environmental cues that are often encountered at the host infection site [58].

From the various studies that have evaluated the promised benefits of different antivirulence drugs to date, it is becoming increasingly apparent that outcomes are heavily dependent on the nature of the virulence target under investigation. In addition, where evidence is provided that likely disproves an anticipated benefit of a particular antivirulence approach, research activity immediately intensifies around this target-benefit combination, as seen with the development of resistance to QSIs for example. While this is providing invaluable insight and sparked research interest in the QSI field, similar research studies are also needed for most of the antivirulence targets currently under investigation. Given the complexities of the antivirulence approach, there are several challenges that need to be addressed when evaluating the benefits of any given antivirulence target and/or compound (Fig. 2). These challenges are discussed in the section below and should ideally be considered early in the development process of an antivirulence strategy.

4. CHALLENGES IN EVALUATING THE BENEFITS OF THE ANTIVIRULENCE APPROACH

4.1. Antivirulence drug effects on virulence attenuation and pathogen fitness and growth

The distinct mode of action of antivirulence antimicrobials necessitates a new approach to compound testing that is different to antibiotics. Evaluating the central ‘disarm-don’t kill’ criterion early upon target selection and using relevant assays, needs to be a priority in every proof of concept antivirulence target study and for every new compound identified through high-throughput antivirulence screens for which the exact target might not be known. Importantly, the conditions of the *in vitro* assays to be used for initial evaluation of both virulence and growth need to closely reflect the lifestyle of the pathogen and mimic the conditions it encounters at the various niches it might occupy within and/or outside the
host. The importance of using biologically relevant conditions to test pathogen growth inhibition was nicely demonstrated for the synthetic furanone QSI C-30. Several studies had previously reported virulence attenuation without growth inhibition in *E. coli*, *P. aeruginosa* and *Proteus mirabilis* using rich complex media [59-61] but in a study assessing *P. aeruginosa* growth in minimal media containing 0.1% adenosine (a clinically relevant carbon source requiring functional quorum sensing for utilization) C-30 decreased *P. aeruginosa* growth 5-fold [31]. This was the first study to demonstrate that resistance to QSIs could evolve and highlighted the importance of testing antivirulence drug effects on bacterial growth in physiologically relevant conditions. A follow-up study by the same group also demonstrated the importance of testing the antivirulence criteria in more than one bacterial strain, and preferably in several clinical isolates of the species, as C-30 displayed highly variable quorum quenching activity and growth inhibition in 50 *P. aeruginosa* isolates from cystic fibrosis patients [33]. The particularities of specifically evaluating QSIs have been recently reviewed by Defoirdt and colleagues, who proposed a set of best-practice guidelines for future QSI studies [62].

It is reasonable, thus, to expect that if an antivirulence drug inhibits pathogen growth in physiologically relevant conditions or in conditions where expression of the virulence target is beneficial for the pathogen, treatment with this drug will impose strong selection for resistance. Fitness and virulence are two concepts that are often intricately linked for pathogens during infection and which also depend on the host’s genetics and immune status. This is elegantly discussed in an opinion article by Allen et al., where the authors predict the fate of resistance evolution to antivirulence drugs based on the benefit conferred by the virulence factor to be targeted [29]. It was argued that resistance could even be selected against in situations where an antivirulence drug targets ‘locally non-beneficial’ virulence factors, more commonly encountered in opportunistic pathogens [29], but this remains to be demonstrated. Another area that requires rigorous testing is evaluating the net benefits of antivirulence drug candidates afforded to different hosts, *e.g.* with diverse genomics for infection susceptibility or immunocompetent versus immunocompromised hosts. While it could be argued that such data will start to become more widely available as more antivirulence drugs enter clinical trials and engage diverse patient cohorts, *in vivo* studies in animals, particularly relevant mouse models, can offer a powerful means of generating this critical insight on antivirulence therapeutic potential at earlier stages of drug evaluation and at a much lower cost. Overall, a thorough understanding of the fitness costs and benefits associated with expression of different virulence factors for each pathogen during infection and in different hosts would be hugely insightful in effectively progressing antivirulence drug development.

4.2. Evaluating Antivirulence Drug Resistance Development and Spread

Experimentally testing for resistance development should be an integral part of the early stages of antivirulence drug development. Resistance to antivirulence antimicrobials is defined as the recovery of the virulence phenotype that is being inhibited by the drug. If reversal to the virulence phenotype is observed either *in vitro* or *in vivo* following treatment with a virulence inhibitor, several methods could be employed to identify the exact mechanism of resistance. Looking for mutations in the actual virulence target gene is the obvious starting point but it might not always be sufficient. Unanticipated mutations in the antibiotic efflux pump MexAB-OmrM were found to enhance the export of the C-30 compound from *P. aeruginosa* cells and mediate resistance to its quorum quenching activity both *in vitro* and *in vivo* [31]. While spontaneous *P. aeruginosa* mutants were also obtained in this study, the actual resistance mechanism was delineated by utilizing a random transposon mutant library screen [31]. This approach was also used to identify the mechanism of partial resistance to the antibiotic G2cps compound in *E. coli* [37]. Similarly, Hung and colleagues, utilizing a plasmid mutant library of ToxT, the virulence regulator of cholera toxin in *Vibrio cholerae* and target of the antivirulence inhibitor virstatin, to identify virstatin-resistant mutants [12]. An alternative approach that offers great power for generating invaluable insight into the evolutionary robustness of antivirulence drugs is to utilize relevant infection treatment models (cell- and/or animal-based) in combination with whole genome and/or transcriptome sequencing of spontaneous mutants or pathogen populations recovered following an antivirulence treatment regime. While global gene expression has been used in some cases to explore the full antivirulence potential of new compounds, *e.g.* virstatin and TTSS inhibitors [12, 49], future studies should focus on using this approach to investigate resistance evolution to antivirulence drugs.

Investigations into the antivirulence resistance mechanisms that develop under *in vitro* or *in vivo* conditions can not only impart important insight into the probability of resistance developing upon clinical use, but also, most crucially, into whether the mechanism of resistance will have the potential to spread among bacteria *via* horizontal gene transfer. It could be argued that resistance spread is a more crucial clinical question than resistance development itself. This was exemplified by the latest emergence of plasmid-mediated polymyxin resistance in Gram-negative bacteria [63]. Before this very recent report, polymyxin resistance was conferred by chromosomal mutations that could not spread among bacteria, affording clinicians the use of colistin as a last resort therapy for difficult to treat infections caused by carbapenem-resistant *Enterobacteriaceae* (CREs). The emergence however of the plasmid-encoded MCR-1 resistance gene in *E. coli* isolates of animal and human origin in China and the plasmid’s ability to move and be maintained by *Klebsiella* and *Pseudomonas* is alarming as it heralds the loss of another class of last-resort antibiotics [63].

4.3. Evaluating Antivirulence Selectivity and Off-target Effects

Evaluating the species- or environmental-specificity (as defined in section 3.2) of antivirulence drugs is optimally performed when the virulence target is known. However, even if the exact target is not known, the spectrum of an inhibitor’s antivirulence activity could be evaluated against one or multiple pathogens that share the virulence phenotype originally used in the high-throughput screen, and then ideally extend into a broader examination of virulence attenua-
tion in these pathogens. This approach was nicely demonstrated by Felise and colleagues in a study that identified a novel broad-spectrum inhibitor that was active against diverse virulence protein secretion systems in different plant and animal pathogens [53]. Such studies can also advance our understanding of the molecular basis of action of promising antivirulence compounds and help identify their exact virulence targets.

In cases where the antivirulence drug target is known (e.g., structure-based inhibitor design studies), consideration should be given to how conserved the target is across different species or within different strains of a species, in order to predict compound selectivity and incorporate a tuneable activity spectrum into the inhibitor design. For example, the sensor kinase QseC mediates virulence gene regulation and homologues exist in some important human and plant pathogens. However, the small molecule QseC inhibitor LED209 inhibited QseC signaling in homologues sharing less than 60% similarity and displayed antivirulence activity across EHEC, S. enterica and Franciella tularensis [64] constituting a promising broad-spectrum inhibitor lead. Other antivirulence targets under investigation, such as the virulence factor foldase DsbA [65], are even more diverse but can be found more widely across bacterial phyla offering exciting opportunities for designer-spectrum inhibitor development [26]. Interestingly, many important animal and plant pathogens have multiple copies of DsbA that are diverse and can be involved in specific virulence phenotypes, as for example S. enterica with three chromosomal and one plasmid-encoded DsbA copy [66], and this is something to be taken into consideration in future anti-DsbA drug selectivity assays.

Lastly, the potential of antivirulence drugs to minimize off-target effects, in particular harming the host’s resident microbiota, is a very attractive property that needs, however, to be established in vivo. Microbiome changes in the host need to be established during short and long-term antivirulence drug administration using relevant animal infection-treatment model combinations. In the case of some FDA-approved compounds with demonstrated antivirulence activity, such as phosphonosulphonates against S. aureus staphyloxanthine [54] and the polycylic antidepressant maprotiline against F. novicida QseC [67], microbiome studies could already be performed in human patient cohorts receiving the drug for their originally intended purpose. Intriguingly, gut microbial profiling studies on healthy vs. patient populations show a frequent association between reduction in microbial diversity and diseases (dysbiosis), with some studies identifying pathogen communities of low-diversity emerging in the gut of critically ill patients [68]. As these patients are often in need of antimicrobial interventions, it will be important to evaluate the potential of antivirulence antimicrobials in eliminating pathogens from the gut or other commensal sites before they have the opportunity to cause local or systemic infections.

CONCLUSION

The past 10-20 years have seen a resurgence of research interest into the antivirulence approach with many new candidate compounds identified and validated in vitro and in vivo. The plethora of virulence targets amenable to pharmacological inhibition offers exciting prospects for the future of antivirulence drug development but also constitutes a major challenge in the field. The fate of each antivirulence strategy is highly dependent on the virulence factor to be inhibited and this will ultimately determine whether any of the developed inhibitors will successfully deliver the promised benefits of the antivirulence approach. Given the complexities of appropriately evaluating each unique antivirulence strategy and the critical lack of research data for many of these, it is important to not be tempted to draw generalized conclusions yet for the fate of all antivirulence compounds currently under investigation. In an era where we have run out of most ‘off-the-self’ antibiotics and are critically running out of last-resort options, it is imperative to continue to develop and accurately evaluate alternative antimicrobials. With an ever-expanding list of virulence targets and antivirulence compounds currently under investigation, it is a matter of time before antivirulence drugs widely enter clinical practice.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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