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Considerations and caveats in anti-virulence drug development

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Abstract

As antibiotic resistance remains a major public health threat, anti-virulence therapy research is gaining interest. Hundreds of potential anti-virulence compounds have been examined, but very few have made it to clinical trials and none have been approved. This review surveys the current anti-virulence research field with a focus on the highly resistant and deadly ESKAPE pathogens, especially *Pseudomonas aeruginosa.* We discuss timely considerations and caveats in anti-virulence drug development, including target identification, administration, preclinical development, and metrics for success in clinical trials. Development of a defined pipeline for anti-virulence agents, which differs in important ways from conventional antibiotics, is imperative for the future success of these critically needed drugs.

Introduction

Antibiotic resistance is an ever-growing public health concern, exacerbated by the recent appearance of bacteria resistant to all available antibiotics [1]. The US government has described this concern as a major unmet need of the 21st century and called for the development of alternative antibacterial strategies [2]. In response, researchers have made advances toward such strategies, the most promising of which are antimicrobial peptides, immunotherapy, phage therapy, nanoparticles, and anti-virulence drugs (reviewed in [3••]).

Here we focus on anti-virulence approaches, which disrupt pathogen virulence, but not pathogen growth or viability. The goals of the anti-virulence approach are to reduce antibiotic use and, ultimately, decrease the occurrence of antibiotic resistance, while preserving beneficial flora. Anti-virulence agents do not impose strong selective pressures on bacteria that favor the evolution of resistance and persistence mechanisms and because they do not affect viability, they should not disrupt beneficial microbiota.

Candidate anti-virulence compounds have been identified via screening of natural products, structural modification of native ligands, *in silico* docking, and high-throughput screening (HTS) of chemical libraries. Although anti-virulence research literature has grown

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exponentially in recent years (Figure 1), the first anti-virulence drug has yet to come, begging the question: *What is holding up the anti-virulence drug pipeline?*

Anti-virulence strategies for ESKAPE pathogens

The so-called ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) 'escape' killing by antibiotics and defy eradication by conventional therapies [4]. ESKAPE bacteria are particularly concerning because they represent the largest group of nosocomial pathogens with growing incidences of antibiotic resistance [4]. The frequencies of vancomycin resistance among *Enterococci* and methicillin resistance in *S. aureus* (MRSA) have reached 61% and 60%, respectively [5,6]. Furthermore, their antibiotic resistance makes them especially deadly, with mortality rates being 14% for methicillin-resistant *S. aureus* [2], 25% for vancomycin-resistant *Enterococci* [7], 39% for *P. aeruginosa* [8], and 50% for hospital-acquired *A. baumannii* [9].

Anti-virulence strategies for ESKAPE pathogens tend to target (1) specific virulence factors [e.g., type three secretion systems (T3SS), enterotoxins] [10,11], (2) master virulence regulators [e.g., two-component systems, quorum sensing (QS)] [12,13••], or (3) resistance to host defenses and antibiotics [e.g., capsule, staphyloxanthin, biofilm] [14,15•,16]. Representative examples of ESKAPE anti-virulence targets and their inhibitors are listed in Table 1. Well-tolerated natural virulence inhibitors, including garlic, menthol, clove, and black pepper have shown promise against enterotoxins, T3SS, and biofilm [11,17–19], can be applied topically, but often lack the specificity or efficacy required for systemic infections. Hla, a β-barrel pore-forming toxin, has been targeted for MRSA anti-virulence because of its effects on skin necrosis and lethality [20]. For example, morin hydrate, which inhibits Hla self-assembly and thereby prevents pore formation, and was shown to be protective in a pneumonia mouse model [21].

Multicellular, surface-associated communities, known as biofilms, can increase pathogen antibiotic tolerance up to 1000-fold [22]. *A. baumannii*, MRSA, and *P. aeruginosa* biofilms form quickly and are extremely tolerant of antibiotics [23]. 2-Aminoimidazole and triazole-derived compounds are promising biofilm inhibitors [16,23–25], but have not yet been subjected to large-scale *in vivo* studies.

Anti-virulence strategies for *P. aeruginosa*

Most anti-virulence strategies for *P. aeruginosa* target virulence systems (protein secretion, biofilm) or master virulence regulators (c-di-GMP, QS) (Table 1). *P. aeruginosa* T3SS is critical for delivery of toxins into host cells [31]; drug discovery has focused especially on targeting the T3SS effector ExoU/S, PscF/PcrV needle proteins, and the regulator ExsA [10,32]. The T3SS apparatus is well-conserved among pathogens, broadening the application of T3SS inhibitors to multiple pathogens [32] and polymicrobial infections. *P. aeruginosa* type II (T2SS) and type V secretion systems have been targeted to a lesser extent [33,34], though the similarities between T2SS and T3SS may reveal T2SS inhibitors incidentally [35].

Anti-biofilm inhibitors targeting carbohydrate-binding lectins show good potency *in vitro* and *in vivo*, but might disrupt host lectins [27,36]. Type IV pili [37] are unsuitable targets because they are not well-conserved in *P. aeruginosa* isolates. Global biofilm regulators such as cyclic di-GMP signaling and QS systems are appealing anti-virulence targets [38,39]. Cyclic-di-GMP signaling is important for motility and biofilm formation in multiple pathogens [38]. Screens have identified cyclic di-GMP inhibitors that reduce *P. aeruginosa* biofilm formation by interfering with the cyclic di-GMP synthetase WspR or its target PelD, some with low IC_{50} values [29], but *in vivo* studies are lacking.

The LasR, RhlR, and MvfR QS systems rely on their respective synthetases LasI, RhlI, and PqsABCD, which produce the respective cognate activating ligands C12-HSL, C4-HSL, and HHQ/PQS [39]. Most LasR and RhlR inhibitors are ligand analogues [40,41]. Natural compounds identified by screening exhibit good *in vivo* potency but have been shown to be cytotoxic and subject to efflux-mediated resistance [42,43•]. QS synthetase inhibitors have also been described [44].

The MvfR QS system is critical for acute and chronic/relapsing infections [45,46]. HTS and ligand-based approaches have been employed to identify MvfR inhibitors [13••,47,48]. Inhibitors of the MvfR-regulated gene product PqsA have been shown to have good efficacy in mice [12]. Modified PqsD ligands [49] and PqsD-related enzyme ligands and inhibitors [50,51] have also been developed as potential inhibitors. Whole-cell HTS has revealed highly potent *P. aeruginosa* QS inhibitors (IC_{50} 200–300 nM) with efficacy against acute and relapsing infections in mice [13••]. They are the first inhibitors of antibiotic-tolerant cell formation [13••]. Combined with agents that kill antibiotic tolerant/persister cells [52•], they may provide successful therapies against cells that survive antibiotic/host killing, a critical unmet need.

Resistance to anti-virulence drugs

As mentioned above, to prevent selective pressure toward resistance development, as occurs with classical antibiotics, anti-virulence drugs should not affect pathogen viability. *In vitro* studies have suggested resistance to some anti-virulence molecules [43•,53,54]; however, *in vivo* studies are needed to confirm this. Nonetheless, by definition, virulence contributes to pathogen fitness *in vivo*. That is, an anti-virulence resistant mutant would outcompete sensitive cells during treatment. However, some important microbial population genetics and ecological concepts modulate this assumption.

Firstly, selective pressure depends on whether the targeted virulence factor is communal (public good) benefiting sensitive and resistant subpopulations alike, or individualized (private good) benefiting only the resistant subpopulation. Recent *in vitro* studies indicate that only individualized factors become dominant, whereas communal factors do not provide a competitive advantage to resistant cells [43•,55–57]. Secondly, virulence factors may have distant benefits manifested away from the infection site (e.g., commensals in reservoir). Virulence factors such as staphyloxanthin are dispensable in non-infection sites [58], reducing their selective advantage [59]. *A priori* assessment of whether a factor yields a distal benefit, an aspect currently overlooked, could significantly improve target evaluation

efforts. Thirdly, bacterial population structure plays an important role in resistance spread. Physical separation between resistant and sensitive cells, as it occurs in biofilms, or low diffusion rate conditions create microenvironments where the so-called 'cheater' cells cannot access communal goods, leads to individualized benefit and resistance spread within the microenvironment [55,59]. However, this effect is dampened when factors can be shared [55,59]. Overall, given the above considerations for target selection, direct *in vitro* comparisons indicate that the potential selective advantage for resistance to anti-virulence agents is, at most, very weak relative to that for antibiotics [57].

Clinical use of anti-virulence drugs, obstacles, and considerations

In theory, a single inhibitor targeting a well-conserved virulence factor could treat multiple pathogens in polymicrobial infections. Highly conserved targets such as T3SS and Hla are promising because they are specialized, critical virulence factors. For generalized virulence functions like QS or capsule formation, however, attention must be paid to the issue of specificity with the aim of minimizing effects on native microbiota. Understanding the function of putative targets and their regulatory interactions is critical for development of a well-designed reporter system and thorough efficacy validation. Moreover, to enable clinical translation, knowing the impacts of target inactivation in infection is necessary for the design of appropriate *in vivo* readouts and clinical trials. Whole-cell screens that permit the identification of cell-permeable compounds and elimination of cytotoxic compounds are advantageous for the development of potent anti-virulence inhibitors [13••].

Questions regarding the optimal timing and appropriate infection type for use of virulence inhibitors can be infection and target specific. Prophylactic administration, such as in preoperative or prolonged hospitalization situations, may reduce nosocomial infections [60]. Regarding infection type, chronic infections may be the most important application for anti-virulence therapies because they often involve antibiotic-tolerant or multi-drug resistant pathogens [61]. Combinatorial therapy with antibiotics, as in the case of the QS MvfR inhibitors, may help limit the development of tolerance to antibiotics and disrupt multiple virulence functions in pathogens [13••].

Perhaps the most unique challenge for preclinical anti-virulence studies is defining metrics of success. Because immediate bacterial death is unlikely to occur, studies must probe salient features, such as cell damage, inflammatory response, disease severity, or other aspects of pathophysiology. This consideration is especially important for those infections in which the immune response can be more detrimental than the infection itself, as in septic shock [62]. Infection clearance can still be a parameter, though clearance would rely on host immunity and thus may take several days or longer. These factors become even more relevant when transitioning from preclinical to clinical trials due to differences between murine and human immunity [2,63].

Despite the large pool of potential anti-virulence therapeutics discovered thus far, the low potential for resistance, and other immunological positives, few clinical trials have been attempted with virulence inhibitors. T3SS inhibitors have been subjected to clinical trials, but failed [64,65]. *P. aeruginosa* T3SS and *S. aureus* toxin antibodies are currently in phase-

two trials [66]. Lectin inhibitors were reported to be effective in a small trial with cystic fibrosis patients [67], but no follow-up studies have been done. Based on the paucity of compounds currently in trials, one major hurdle may be planning preclinical studies that consider anti-virulence metrics appropriately, as discussed above. Perhaps, another equally important aspect to consider is the integration of predictive analytics into the clinical trials of anti-virulence drug discovery [68,69•] to avoid preemptive trial failure. Finally, moving agents into preclinical and clinical studies is costly, and prohibitively so for academic research laboratories. Industry collaborations may help with experimental studies and funding, but both industry and regulatory agencies should be prepared to pursue different avenues of assessing anti-virulence therapy efficacy. Indeed, academia–industry partnerships could be invaluable for advancing the anti-virulence agent pipeline [3••]. A productive pipeline would also benefit plant and animal food production given that both contribute to the emergence, persistence, and spread of resistant bacteria.

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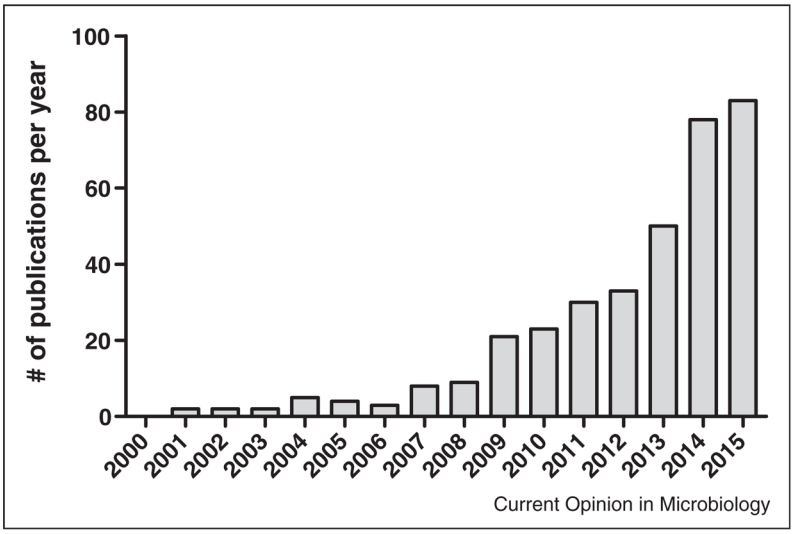

Figure 1.

Growth in publications with the keywords ‘anti-virulence’ or ‘antivirulence’ in PubMed. Some relevant publications without these keywords were likely not included.

Table 1

Selected examples of anti-virulence targets and inhibitors for ESKAPE pathogens

Target	Pathogen	Example inhibitor	*In vivo* model employed	Reference
Hla	*S. aureus*	Morin hydrate	Mouse (lung)	[21]
Staphlyoxanthin	*S. aureus*	Phosphonoacetamide derivative	Mouse (intraperitoneal)	[15•]
Enterotoxins	*S. aureus*	Menthol	None	[11]
Sortase A	*S. aureus*	Chlorogenic acid	Mouse (sepsis)	[26]
Biofilm	*S. aureus*	Black pepper oil	*C. elegans*	[18]
	A. baumannii	TAGE-triazole conjugates	None	[16]
	K. pneumoniae	GarO (garlic ointment)	None	[17]
	P. aeruginosa	Mix of sugars	Mouse (lung)	[27]
QS	*S. aureus*	C14-TOA (3-acyltetronic acid)	Mouse (arthritis)	[28]
	P. aeruginosa	M64	Mouse (burn and lung)	[13]
C-di-GMP	*P. aeruginosa*	Ebselen	None	[29]
Protein secretion	*P. aeruginosa*	Anti-PcrV antibody	Mouse (lung)	[10]
Capsule	*S. aureus*	Fascioquinol E	None	[14]
	K. pneumoniae	Triazines	*Tetrahymena pyriformis*	[30]