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Considerations and caveats in anti-virulence drug development

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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.

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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.

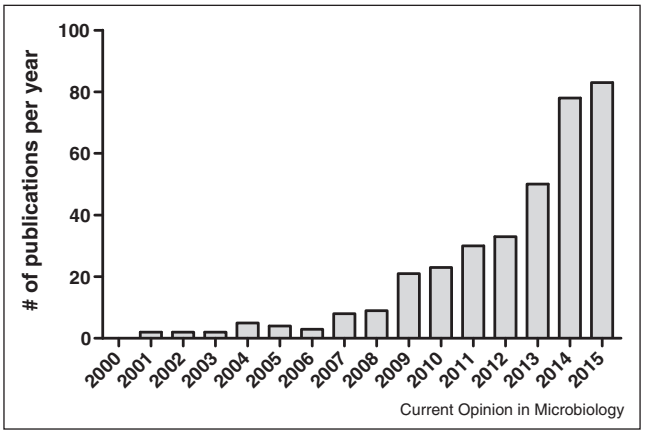
Candidate anti-virulence compounds have been identified via screening of natural products, structural modification of native ligands, *in silico* docking, and high-throughput screening (HTS) of chemical libraries. Although anti-virulence research literature has grown exponentially in recent years (Figure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

Figure 1



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

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].

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]