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Caenorhabditis elegans in high-throughput screens for anti-infective compounds

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Abstract

New classes of antimicrobials that are effective therapies for infections with multi-drug resistant pathogens are urgently needed. The nematode *Caenorhabditis elegans* has been incorporated into small molecule screening platforms to identify anti-infective compounds that provide protection of a host during infection. The use of a live animal in these screening systems offers several advantages, including the ability to identify molecules that boost innate immune responses in a manner advantageous to host survival and compounds that disrupt bacterial virulence mechanisms. In addition, new classes of antimicrobials that target the pathogen have been uncovered, as well as interesting chemical probes that can be used to dissect new mechanisms of host–pathogen interactions.

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Introduction

Antimicrobial-resistant pathogens are one of the greatest threats to human health. Infections with these microbes are particularly common and devastating in hospital settings where the widespread use of broad-spectrum antibiotics and a multitude of susceptible hosts create a veritable breeding ground for resistance [1]. Of particular concern are the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), which are intrinsically resistant to several different classes of antibiotics and can also efficiently acquire antibiotic-resistant determinants. Thus, these organisms are often able to 'escape' treatment with currently available therapies [2]. Moreover, ESKAPE pathogens can cause deep-seated infections, such as endocarditis and osteomyelitis, which require several weeks of antibiotic therapy [1]. In this setting, patients often suffer from debilitating side effects of the antibiotics themselves and are at risk for infection relapse, even with standard-of-care approaches. Compounding these problems is the striking absence of novel antibiotics in the development pipeline that have potent antimicrobial activity against the ESKAPE pathogens. In addition, all of the currently available antibiotics target some aspect of bacterial growth or metabolism [3]. New antimicrobials that promote rapid, sterilizing cure of infections with ESKAPE pathogens and exert minimal selection pressure on bacteria to develop resistance are urgently needed.

Antimicrobial drug discovery platforms often utilize high-throughput screens of synthetic compound libraries and natural product extracts for small molecules with direct antimicrobial activity. This approach, though previously effective at identifying novel drug classes, has several limitations [3]. Lead compounds identified by assaying for bacterial growth inhibition or bactericidal properties *in vitro* often lack the same effectiveness *in vivo*. Problems with compound bioavailability, metabolism in the host, and toxicity are often encountered during lead optimization. Perhaps most concerning is the possibility that all of the obvious bacterial targets amenable for the design of bactericidal antibiotics have been exhausted, which decreases the likelihood that these traditional screens can identify new classes of anti-infectives [4].

To address these challenges, investigators have been developing screening platforms that will identify new classes of anti-infective molecules. One idea that has gained traction in the last 10 years is to incorporate a pathogen-infected live animal into screening systems and assay for molecules that prolong the life of the host. Because the endpoint of these screens is a live animal, rather than a dead pathogen, anti-infective compounds with new mechanisms of action can potentially be identified that may have been missed in traditional screens. In addition, the incorporation of a live host into the screening system offers an in-assay counter-screen against molecules that are generally toxic. Screening platforms for novel anti-infective compounds have been developed using the nematode *Caenorhabditis elegans* and the zebrafish *Danio rerio* [5••,6–8]. In this review, we focus on *C. elegans*-based screens, which have been used to mine hundreds of thousands of compounds for those with

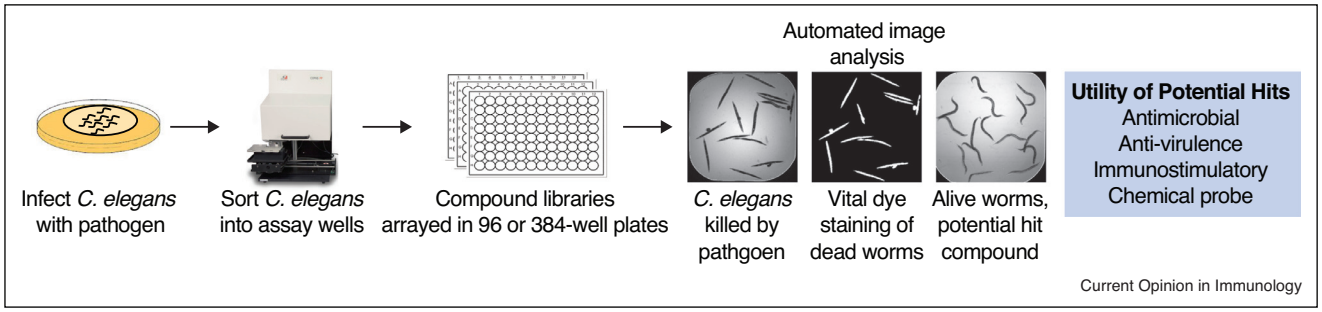
activity against a wide range of human bacterial and fungal pathogens, including many of the ESKAPE organisms. These efforts have uncovered several types of interesting compounds, such as *(i)* molecules that boost innate immune responses in a manner advantageous to host survival *(ii)* compounds that disrupt bacterial virulence mechanisms without affecting pathogen growth *(iii)* new classes of antimicrobials that target the pathogen and *(iv)* chemical probes that can be used as tools to dissect new mechanisms of host–pathogen interactions (Figure 1). Finally, we will discuss some of the remaining challenges; most notably, the demonstration that a compound with novel antimicrobial activity identified using a heterologous host can be effective at treating an infection in humans.

Advantages of *C. elegans* in anti-infective screening platforms

C. elegans can be infected with multiple human pathogens and have emerged as a powerful system to study host–pathogen interactions [9–19]. Interestingly, many of the same virulence determinants used by pathogens to establish infection in humans are also involved in killing nematodes [20–22]. In addition, *C. elegans* mount pathogen-specific immune defenses that involve conserved innate immune regulators [9–18]. Pioneering work in the laboratory of Frederick Ausubel demonstrated that the microscopic nematode *C. elegans* can be incorporated into a screening platform for new antibiotics [5••,23••]. Nematodes have several advantages that make them particularly amenable for use in high-throughput screens (Table 1). *C. elegans* is a microscopic organism about 1 mm in length and therefore 10–20 pathogen-infected nematodes can fit comfortably in a single well of a 384-well plate. Their generation time is short (3 days) and a single hermaphrodite animal can produce approximately 300 progeny. Thus, large numbers of nematodes can be grown in the laboratory quickly. In addition, this assay can be fully automated through the use of a modified flow cytometry machine that can dispense pathogen-infected nematodes into assay plates, and robots to pin compounds into the wells. Finally, dead *C. elegans* can be identified with a cell permeable dye, which can be quantified in the assay wells in an automated fashion using image analysis platforms [23••,24] (Figure 1).

In the first utilization of this approach, Moy *et al.* used *C. elegans* in a relatively low-throughput screen of 7136 synthetic compounds and natural product extracts for those with activity against the opportunistic human pathogen *E. faecalis* [5••]. Sixteen compounds and 9 extracts protected *C. elegans* during *E. faecalis* infection. Interestingly, 12 of these hits provided protection *in vivo* at concentrations that were far lower than the concentration, which inhibited bacterial growth *in vitro* [minimal inhibitory concentration (MIC)]. Thus, these compounds likely confer protection by modulating pathogen virulence or stimulating host immune defenses. The authors called these molecules 'anti-infectives,' a terminology we use here. Subsequently, this group of investigators developed an automated, high-throughput screening platform and mined a library of 37 200 synthetic compounds and natural product extracts [23••]. This effort identified 108 hits, of which 80 were either known or suspected antimicrobials. However, 28 of these compounds do not have any structural relationship with known antibiotics and nine of these molecules are anti-infectives. Since these seminal studies, *C. elegans* has been used in screens for compounds that provide protection from *Candida albicans* [25], methicillin-resistant *S. aureus* [26], *P. aeruginosa* [27••], *A. baumannii* [28], *Burkholderia pseudomallei* [29], *Burkholderia cenocepacia* [30], and *Francisella tularensis* [31].

Figure 1



A schematic of high-throughput screens for small molecule anti-infectives in *C. elegans*. *C. elegans* can be infected with a pathogen of interest by letting them feed on a lawn of the microbe. Infected animals are then sorted in an automated fashion into the wells of a 96 or 384-well plate that contain individual compounds arrayed from a small molecule library. Pictured is the COPAS BioSort for sorting *C. elegans* (Union Biometrica). Pathogen-infected animals are killed in control wells and attain a rod-like morphology. A vital dye is used to stain the dead animals, which can be quantified in an automated fashion using an image analysis platform. Wells with a potential hit compound will contain living nematodes with a sinusoidal shape that will not be stained by the vital dye. Images of *C. elegans* in the assay wells are from [23••] and used here with permission from the authors and the journal.

Table 1

Advantages and limitations of *C. elegans* high-throughput screening for anti-infective compounds

Advantages
- Capacity for high-throughput screens
- Offers in-assay screen against generally toxic compounds
- *C. elegans* can be infected with multiple human pathogens
- Ability to identify anti-infective compounds that are immunostimulatory or disrupt pathogen virulence
- Has uncovered new classes of antimicrobials that target the pathogen
- Enables the identification of chemical probes that can dissect mechanisms of immune regulation and host–pathogen interactions
Limitations
- *C. elegans* lack professional immune cells
- *C. elegans* lack canonical pathogen recognition receptors and NF-κB signaling
- Compounds identified may not have activity in humans
- Assessment of bioavailability and organ toxicity is not possible

Immunostimulatory compounds

Modulation of host immunity is an emerging strategy for the development of new anti-infective small molecules [32]. In theory, compounds that augment core defense pathways have the potential to confer protection to a broad range of pathogens. Moreover, anti-infectives that target the host may exert less selection pressure on bacteria to develop resistance than molecules that are directly bactericidal or bacteriostatic. The promise of using *C. elegans* to identify immunostimulatory, anti-infective compounds with broad-spectrum antimicrobial activity was initially demonstrated in a study of 31 compounds that were previously identified in a high-throughput screen for anti-infectives. These molecules protected *C. elegans* from *E. faecalis* infection, but did not have any structural relationship to known antimicrobials. Eight of these 31 molecules also protected *C. elegans* from infection with *P. aeruginosa* at doses several fold lower than their MIC. One of these compounds, R24 (also known as RPW-24), was found to protect *C. elegans* infected with *P. aeruginosa* through its immunostimulatory activity [33••]. R24 does not inhibit growth of the pathogen *in vitro*, but robustly stimulates the transcription of *C. elegans* innate immune defense genes in a manner that provides protection from bacterial infection. Using transcriptional profiling and genetic epistasis experiments, the authors showed that the immunostimulatory activity of R24 is partially dependent on the p38 MAP kinase PMK-1 pathway [33••,34••,35], a conserved signaling cascade of central importance to host defense in *C. elegans* and mammals [9,10].

Subsequently, a *C. elegans* transgenic, GFP-based immune reporter strain was used to screen for immunostimulatory small molecules directly [36•]. Of 1120 compounds screened, 45 were found to activate immune reporter expression, including the polymyxin antibiotic colistin. Although colistin has bactericidal activity against a wide range of pathogens, it provided protection to nematodes infected with *Yersinia pestis*, a gram-negative pathogen that is resistant to colistin. The authors showed that the protection provided by colistin is dependent on the p38 MAP kinase innate immune pathway, as well as the stress-response mediators, DAF-16/FOXO and SKN-1/Nrf [36•]. Another study found that a natural product extract has immunostimulatory activity in *C. elegans* and protects nematodes from *S. aureus* infection [37].

In summary, *C. elegans*-based pathogenesis assays can be used in primary screens of large compound libraries for immunostimulatory compounds that protect nematodes from bacterial infection. However, it remains to be seen whether small molecules identified using this approach will have anti-infective activity in humans. Nematodes lack professional immune cells and a circulatory system. In addition, functional orthologs of key immune regulatory proteins, such as pattern recognition receptors (e.g. Toll-like receptors), and NF-κB signaling pathways are not present in *C. elegans*. The one *C. elegans* ortholog of a Toll-like receptor, *tol-1*, prevents pharyngeal invasion of *Salmonella enterica* and is involved in neuronal control of pathogen avoidance, but it is unclear whether it functions in pattern recognition in these contexts [38,39]. Thus, for an anti-infective identified using *C. elegans* to be efficacious in humans, it would likely have to target a core immune signaling pathway, such as the p38 MAPK cascade, act exclusively in intestinal epithelial cells, and tune immune activation in a manner that does not itself cause collateral injury. If these hurdles prove to be insurmountable, at a minimum, these immunostimulatory compounds can be useful probe compounds for dissecting new mechanisms of the host–pathogen-interactions, which is discussed further below.

Anti-virulence compounds

Disruption of virulence factor expression in pathogens presents an attractive strategy for the development of new anti-infective compounds. Such therapies could be used to disarm pathogens in the host, and may be most useful in combination with bactericidal antibiotics to improve their efficacy. In the first screens for anti-infectives in *C. elegans*, Moy *et al.* suggested that this screening platform is ideally suited to identify these anti-virulence molecules [5••,23••]. In a large screen for molecules that protected *C.*

elegans from *P. aeruginosa* infection, Kirienko *et al.* identified 5-fluorouracil and showed that it both inhibits pseudomonal growth and the production of pyoverdine [40•], an iron-chelating siderophore that is essential for pseudomonal virulence [41]. The inhibition of pyoverdine by 5-fluorouracil occurs via a downstream metabolite, 5-fluorouridine, which the authors show is a *bona fide* anti-virulence compound that itself does not affect bacterial growth. In a separate study, another *P. aeruginosa* virulence factor, LasB, was targeted for design of anti-virulence compounds [42•]. LasB is a zinc metalloprotease that causes tissue damage and nutrient release in the host, which in turn promotes *P. aeruginosa* growth and pathogenesis. Through both *in silico* and *in vitro* studies, the authors found that a group of compounds called mercaptoacetamides inhibit LasB production at low μM concentrations. These LasB inhibitors protected *C. elegans* against *P. aeruginosa* infection.

Biofilm production is another important virulence determinant that has been targeted in a high-throughput screen for anti-virulence compounds [43]. Biofilms are composed of primarily exopolysaccharide and DNA, and play a major role in bacterial pathogenesis. van Tilburg Bernardes *et al.* identified several compounds that inhibit the expression of the major exopolysaccharide-producing enzymes. These compounds demonstrated protective activity in a *C. elegans*-*P. aeruginosa* pathogenesis assay.

Pathogen-targeting compounds

C. elegans-based pathogenesis assays have also been deployed to identify new therapies that directly target particularly problematic bacterial pathogens. Recently, a *C. elegans* screen for compounds active against methicillin-resistant *S. aureus* (MRSA) identified a new class of synthetic retinoid antibiotics [44••]. Kim *et al.* showed that these retinoid antibiotics penetrate and disrupt the lipid bilayer of *S. aureus*, which kills the bacteria. Importantly, these antibiotics are rapidly bactericidal, have activity against stationary phase cultures of *S. aureus* cultures, and synergize with the aminoglycoside antibiotic gentamicin. Additionally, derivatives of these retinoid compounds were relatively non-toxic towards human hepatocytes and significantly reduced bacterial burden in a murine model of *S. aureus* abscess formation, particularly when used in combination with gentamicin.

In addition to *S. aureus*, *C. elegans*-based, high-throughout screening systems have been established for the gram-negative pathogens *A. baumannii* [28], *B. pseudomallei* [29], and *F. tularensis* [31]. To identify antimicrobial compounds against *A. baumannii*, 68 insect-derived antimicrobial peptides (AMPs) were screened, which identified 14 cecropin or cecropin-like AMPs that prolonged *C. elegans* survival [28]. One peptide, BR003, had activity against a panel of gram-negative bacteria and disrupted *A. baumannii* membrane permeability. In a separate study, five of 1760 FDA-approved compounds prolonged the survival of *C. elegans* infected with *F. tularensis* [31]. One hit, diflunisal, a non-steroidal anti-inflammatory, inhibited *F. tularensis* growth *in vitro* and in macrophages.

Complementing antibiotic discovery efforts, *C. elegans* has also been used to identify new antifungal therapies [25,45,46]. In a high-throughput screen of 1266 compounds, 15 compounds were identified that prolonged the survival of *C. elegans* infected with *C. albicans* [25]. Two of these compounds, caffeic acid phenethyl ester and enoxacin, were efficacious in a murine model of systemic *C. albicans* infection [25]. In addition, Graham *et al.* utilized *C. elegans* to identify a secreted protein from *E. faecalis* (EntV) that inhibits *C. albicans* hyphal growth and biofilm formation [47••]. EntV was an effective antifungal treatment in a murine model of oropharyngeal candidiasis, which demonstrates that *C. elegans* models of pathogen-pathogen interactions [48,49] can be co-opted to identify naturally synthesized antimicrobials.

Going forward, another source of naturally synthesized antimicrobial compounds may be the immune effectors that are the functional output of nematode immune defenses. *C. elegans* and other nematodes eat microorganisms for food and live in microbe-rich habits. Thus, their evolution has been shaped by interactions with both nutritious and pathogenic microbes. The immune effectors in *C. elegans* that are mounted to kill pathogens have not been fully characterized, though they presumably have direct antimicrobial activity.

Small molecules as chemical biology tools to dissect mechanisms of the host–pathogen interaction

A major advantage of *C. elegans* pathogenesis models is the ability to dissect both innate immune defense and microbial virulence mechanisms in a system that is amenable for large-scale genetic manipulation of both the host and pathogen. Through these powerful genetic tools, a detailed picture of host–pathogen interactions in *C. elegans* is emerging, which has offered key insights into evolutionarily conserved principals of innate immune activation and pathogen resistance (reviewed in [12,14,17–19]). The anti-infective molecules that are being uncovered in *C. elegans*-based high-throughput screens present interesting probe compounds to dissect these mechanisms in new ways. Because these molecules were identified for their ability to modulate nematode-pathogen interactions in a manner that benefits the host, they may, in an unbiased manner, reveal the specific nodes in either the pathogen or the host that are amenable for therapeutic manipulation. Although work to identify the targets of anti-infective compounds and characterize these mechanisms in detail is still emerging, a few studies illustrate the potential of this approach.

Iron availability and acquisition is a key determinant of host–pathogen interactions [50]. Recently, Kirienko *et al.* identified the iron chelator ciclopirox olamine in a high-throughput screen for small molecules that provide *C. elegans* protection against *P. aeruginosa* infection [27••]. Utilizing iron chelators as chemical probes, they found that iron starvation is an important virulence determinant of *P. aeruginosa* in *C. elegans*. *P. aeruginosa* secretes the iron chelating siderophore pyoverdine, which disrupts mitochondrial homeostasis and induces a protective response in *C. elegans* that involves mitophagy [51] and the activation of innate immune defenses [52,53]. These data support previous studies [54,55], which demonstrate that *C. elegans* couple mitochondrial homeostasis to immune activation.

In addition to robustly activating innate immune defense genes in a manner that protects *C. elegans* from bacterial infection, the small molecule infective R24, which was discussed above, also strongly induces xenobiotic detoxification genes [33••,34••,35]. R24 was used as a chemical probe to explore the link between activation of detoxification responses and innate immune defenses. This study showed that a conserved subunit of the mediator transcriptional regulatory complex, MDT-15/MED15, is necessary for coupling activation of protective immune defenses towards bacterial pathogens and detoxification of both R24 and secreted bacterial toxins [34••]. R24 drives the transcription of immune effectors whose basal regulation requires the canonical p38 MAPK PMK-1 immune pathway, but their induction occurs independently of this signaling cascade [34••,35]. Thus, identifying the target of R24 may reveal a new mechanism by which immune activation is tuned to provide an advantage to the host during infection.

Conclusion

The last decade has seen a number of notable achievements in the use of *C. elegans* to identify anti-infective compounds. High-throughput screening platforms have been established for a wide variety of bacterial and fungal pathogens, and hundreds of thousands of compounds have been screened for novel antimicrobial activity. Notably, several compounds, which appear to directly target the pathogen, are efficacious in mouse models of infection. In addition, *C. elegans*-based screening platforms are demonstrably good at identifying anti-infective small molecules that likely disrupt virulence factor production in bacteria, as well as those that boost immune responses to give the host an advantage during infection. However, it is notably difficult for studies of new therapies in mice to translate into cures that are effective in humans [56]. This may prove even more challenging for discoveries made using invertebrate model hosts. Yet, at a minimum, anti-infective molecules identified in *C. elegans* offer provocative probe compounds for the dissection of key pathways in either the host or the pathogen that modulate the outcome of the host–pathogen interaction.

Conflict of interest statement

Nothing declared.

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