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 cepacia [30], and Francisella tularensis [31].
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 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.
**Host pathogens**

*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-throughput 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 habitats. 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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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as
• of special interest
  • of outstanding interest

This was the first study to use the model organism C. elegans in an antimicrobial screen. The authors demonstrated that C. elegans can be used to identify both pathogen-targeting antimicrobials and anti-infective compounds.
Host pathogens


In a follow-up study to their initial C. elegans anti-infective screen, the authors developed an automated, high-throughput screening platform and used it to screen ~37 000 compounds and natural extracts. They identified multiple antimicrobials and anti-infectives.


In a high-throughput screen for molecules that protect C. elegans from bacterial pathogenesis, the authors identified circlopox olamine, an iron chelator. Using it as a chemical probe, they showed that iron starvation is a key virulence determinant of P. aeruginosa in C. elegans.


The authors showed that the small molecule, R24 or RPW-24, protects nematodes during bacterial infection by boosting innate immune defenses. This study demonstrated that C. elegans-based high-throughput screens can uncover immunostimulatory compounds.


The authors used the immunostimulatory small molecule R24 as a probe compound to identify the conserved mediator subunit MDT-15/MED15. They showed that MDT-15/MED15 is a key regulator that links toxin sensing to the activation of innate immune defenses and detoxification responses.


The authors identified the antibiotic colistin in a screen for immunostimulatory compounds. They showed that colistin protects C. elegans infected with Y. pestis by boosting innate immune defenses.


The authors showed that 5-fluorouridine is an anti-virulence compound that protects C. elegans from P. aeruginosa infection by inhibiting pseudomonal pyoverdin production.


The authors identified inhibitors of the pseudomonal protease LasB, which is a key virulence determinant in P. aeruginosa. They identified a group of compounds, mercaptoacetamides, which inhibit LasB and provide C. elegans protection against P. aeruginosa infection.

43. van Tilburg Bernardes E, Charron-Mazeno L, Reading DJ, Reckseidler-Zenteno SL, Lewenza S: Exoplyosaccharides-

From a high-throughput screen in C. elegans for compounds that protect against methicillin-resistant S. aureus infection, the authors identified synthetic retinoid antibiotics and showed that they are rapidly bactericidal against persistor populations. These antibiotics were effective in a murine abscess model, particularly when used in combination with gentamicin.


The authors utilized C. elegans to identify a secreted protein from E. faecalis (EntV) that inhibits C. albicans hyphal growth and biofilm formation. EntV was an effective antifungal treatment in a murine model of oropharyngeal candidiasis.


