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Using *C. elegans* for antimicrobial drug discovery

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Abstract

Introduction—The number of microorganism strains with resistance to known antimicrobials is increasing. Therefore, there is a high demand for new, non-toxic and efficient antimicrobial agents. Research with the microscopic nematode *Caenorhabditis elegans* can address this high demand for the discovery of new antimicrobial compounds. In particular, *C. elegans* can be used as a model host for *in vivo* drug discovery through high-throughput screens of chemical libraries.

Areas covered—This review introduces the use of substitute model hosts and especially *C. elegans* in the study of microbial pathogenesis. The authors also highlight recently published literature on the role of *C. elegans* in drug discovery and outline its use as a promising host with unique advantages in the discovery of new antimicrobial drugs.

Expert opinion—*C. elegans* can be used, as a model host, to research many diseases, including fungal infections and Alzheimer's disease. In addition, high-throughput techniques, for screening chemical libraries, can also be facilitated. Nevertheless, *C. elegans* and mammals have significant differences that both limit the use of the nematode in research and the degree by which results can be interpreted. That being said, the use of *C. elegans* in drug discovery still holds promise and the field continues to grow, with attempts to improve the methodology already underway.

Keywords

Caenorhabditis elegans; *Candida albicans*; drug discovery; high throughput; model host

1. Introduction

Sydney Brenner first introduced *C. elegans* in research in the 1960s and 1970s^[1]. *C. elegans* is a free soil-living nematode with length of 1 mm and a diameter of 50 µm. Each *C. elegans* hermaphrodite is made up of 959 cells, including a nervous system which consists of 302 nerve cells, 213 hypodermis cells, and 34 intestine cells. *C. elegans* has an average life span of approximately 2–3 weeks and a reproduction time of 3.5 days^[2]. Hermaphrodite nematodes produce both oocytes and sperm.

In the laboratory setting, *C. elegans* can be easily maintained on nematode growth medium agar, fed on the nonpathogenic bacterium *Escherichia coli* OP50 strain, and preserved at –80 °C. For experimental purposes, *C. elegans* is easy to store and handle with relatively small cost, and can be utilized with both solid and liquid medium. The nematode is

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Declaration of Interest

The authors declare no other conflict of interest.

transparent, allowing fluorescent compound tracing inside its body. Because of their small size, *C. elegans* nematodes can be placed in 96- or 384-well plates for high throughput experiments. Moreover, *C. elegans* has a fully sequenced genome^[3, 4] and there are decreased ethical concerns involved in their use compared to mammals. Also, *C. elegans* can be genetically modified easily^[5]. There is a website that provides the genetic data of *C. elegans*^[5–10] and the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota provides many strains at minimal charge.

2. *C. elegans* in drug discovery

2.1 Antifungal drugs identified using *C. elegans*

Candida albicans is the most common pathogenic fungal infection and the fourth most common blood-stream infection^[11, 12]. The cost of infections reaches \$1 billion per year, and the average cost of dealing with a case of systemic candidiasis in 1997 was estimated at \$34,123–44,536^[13, 14]. The development of resistant strains to conventional antifungal agents, and even newer generations of antifungal agents such as echinocandins^[15], have made research for new antifungal compounds essential^[16–18]. Interestingly, patients who have been under antifungal therapy for a long time, such as those with immunodeficiency, are most likely to be the sources for resistant strains^[18–20].

C. albicans can cause a lethal infection in *C. elegans*^[21]. The infection process in *C. elegans* involves the formation of filaments, the same process as *C. albicans* infection in humans^[22, 23]. Conventional human antifungal agents against *C. albicans* increase the survival of the nematodes when they are infected with *C. albicans*. Importantly, assays have been designed to utilize the *C. albicans*-*C. elegans* system in high throughput screening to test libraries of compounds for their activity as antifungal agents^[21, 24].

To create the *C. albicans*-*C. elegans* assays, pre-infection^[21] or co-inoculation methods^[24] can be used. These two methods have different features. The co-inoculation method uses 96-well non-binding half area plates, on which we first transfer liquid screen medium and the specific compound that we want to examine for its antifungal activity. Next we transfer the nematodes, which have been grown earlier, and finally we place *C. albicans*. In other words, the infection of the nematode by *C. albicans* takes place in the plates, not before their placement in the plates. This method differs from pre-infection, in which the nematodes are infected with *C. albicans* and then are transferred to the plates, where they will be exposed to the compound. In the pre-infection method, the nematodes are transferred on *C. albicans* lawns for 2 hours before being transferred to the plates.

The co-infection method is superior to the pre-infection method for several reasons. First, the pre-infection method is disadvantageous because the tool which is used to move the nematodes that are contaminated with *C. albicans* becomes contaminated with *C. albicans*, forming a biofilm which is difficult to remove and complicates cleaning the tool. Moreover, a possible automated dispensing system will improve the reliability and uniformity of the assay. In addition, less time is required for co-infection experiments, as no extra time is required for the infection. Third, some materials which are used with the pre-infection methodology are not necessary with the co-infection methodology, such as those required for the infection of the nematodes by *C. albicans*, and for their collection and transfer into other plates. Finally, the similarity between the survival of the nematodes in both experiments has been established: in experiments with both methodologies the nematodes died after 4 days or more, and the development of filamentations by *C. albicans* in the dead nematodes was observed, indicating that the co-inoculation method can be used in place of the pre-infection method.

Using these assays, 1266 chemical compounds were screened and 15 compounds identified that can increase the survival of *C. elegans* infected with *C. albicans* and prevent *in vivo* filamentation^[23] of *C. albicans*^[21]. Also the compounds were evaluated for their toxicity on the *C. elegans*. An example of these compounds is Caffeic acid phenethyl ester (CAPE), a component of honeybee propolis, which was further tested in a murine model of candidiasis and showed antifungal activity in experimental mice with systemic candidiasis. CAPE inhibits the NF-kappaB protein (nuclear factor kappa-light-chain-enhancer of activated B cells) in mammals and has immunomodulatory features. However, because *C. elegans* does not have an NF-kappaB homologue, it is possible CAPE affects the immune system of *C. elegans* via a different mechanism. Hence, the *C. elegans* has many differences with the mammals but this is not a major drawback to the use of the nematode system for initial steps of drug discovery.

In subsequent research, the *C. elegans*-*C. albicans* assay was modified and improved and an extra 3228 compounds were tested^[24]. Of these, 1948 were FDA-approved drugs and the remaining 1280 were small molecules from a diverse group of chemicals that have various actions. 19 compounds were detected which increased the survival of the nematodes after infection with *C. albicans*. Some of these were already known as antifungal agents, confirming the reliability of the assay. Additionally, some compounds may have had antifungal activity, but could not be detected because either they have nematocidal activity apart from their antifungal activity, or they have limited solubility in water, which was used in the experiment. Another possibility is that some compounds may not have had activity against *C. albicans* but have activity against other types of fungi. Out of the 19 compounds which were found to have an antifungal activity, some of these were immunomodulators, such as cyclosporine A, ascomycin, and FK-506 (tacrolimus). Their antifungal activity was found to be weak, but in high quantities they had a considerable action and could offer 50% survival to the nematode. It has been shown that immunomodulator compounds synergistically act with known antifungal compounds in curing fungal infections. Their mechanism of action is through disrupting the action of calcineurin, which is involved in *C. albicans* survival in the serum and in the development of resistance to azoles. Another compound which was identified was triadimeron which acts through blocking the synthesis of ergosterol (the fungi cell wall component). Furthermore, the compound dequalinium chloride was identified, which is an anti-tumor and protein kinase C inhibitor. This compound prevents the integrity of the cell wall of *C. albicans* through the Rho 1-PKC (protein kinase c) pathway. Finally, the compound concanamycin A was also detected, which is a Vacuolar-type H⁺-ATPase inhibitor (Table 1).

The same modified assay has been used to test various natural products for their antifungal activity^[25]. Saponins are natural products isolated from plants. 12 saponins were identified as antifungal agents (Table 1). Two of the saponins were studied further. From this study it has been found that the *C. albicans* hyphae and biofilm formation are interrupted by the saponins. To further test the toxicity of these saponins, a hemolysis assay was conducted and no hemolysis was detected for any of the saponins. Therefore the saponins prefer to bind to ergosterol rather than cholesterol. Furthermore, it has been noted that treating the *C. albicans* cells with saponins can make them more susceptible to osmotic stress applied by salt. Notably the mechanism of action of saponins is to increase the cell permeability. Therefore, it increases the penetration of other compounds such as photosensitizers and increases the susceptibility of fungi to photodynamic therapy. This therapy utilizes a dye, the photosensitizer, that is not toxic, and a low intensity light. When oxygen is present, the combination of these is cytotoxic.

Interestingly, the comparison of MIC (minimum inhibitory concentration) and EC50 (half maximal effective concentration) can be used in *C. elegans* assays to detect compounds that

have a greater antimicrobial activity when they are used *in vivo*. This means that the particular compound acts not only against virulence factors but may alter the immune response of the organism against the infection. An example of this is the compound caffeic acid phenethyl ester (CAPE), as mentioned above. CAPE inhibits the development of *C. albicans* both *in vivo* and *in vitro*. This compound was found to have higher MIC than EC50, 64 mg/ml and 4mg/ml respectively, indicating that this compound has a greater effectiveness *in vivo* than *in vitro*^[21]. This led to the hypothesis that these compounds may have immune modulatory properties and may alter the response of the nematode toward *C. albicans*.

The immune system of *C. elegans* is different from the immune system of mammals. Therefore, it might be difficult to identify drugs which affect the immune system of the mammals. However, the innate immune response is evolutionarily conserved in some extent between invertebrates and vertebrates. Moreover, with the use of *C. elegans* as a model host the host defense against pathogen can be studied. For example, studies using the *C. elegans* receptors CED-1 and C03f1.1.3 and the orthologues mammalian scavenger receptors SCARF1 and CD36 identified for their role in the immune response of the organisms against fungal pathogens^[26].

Importantly, there is a need for toxicity testing of various compounds and drugs^[27]. *C. elegans* is a promising model for estimating the toxicity of compounds^[28]. *C. elegans* has been used as an indicator of toxicity from heavy metals, environmental pollutants, organic solvents, and neurotoxins^[29]. Toxicity against nematodes has been quantified based on nematode survival, growth, reproduction, expression of stress response proteins, feeding behavior, and movement. The utility of *C. elegans* in toxicology testing greatly depends on how it correlates to toxicity in mammalian models. Williams and Dusenbery determined that toxicity of heavy metals against *C. elegans* as measured by the LC₅₀ values (concentration resulting in 50% death) correlates well with toxicity against mice or rats in rank order tests^[30]. Additionally, Cole et al. reported a significant correlation from rank order toxicity tests from organophosphates between *C. elegans* and rodents^[31].

In antimicrobial discovery it is possible to estimate the toxicity of the compounds on *C. elegans*. For example, in the case of the *C. albicans* infection model, fluconazole up to 32 µg/ml was effective in prolonging survival of nematodes exposed to a fluconazole-susceptible *Candida* strain, but at higher concentrations (100 µg/ml) nematode survival was diminished, even compared to the nematodes in an untreated control group. Most likely, this toxicity is present at even lower concentrations, but the beneficial effect from the antifungal activity outweighs the toxic effect^[21]. Another example is the malachite green carbinol base which is metabolized to the leucomalachite green form^[24, 32]. It was observed that this compound does not inhibit the development of *C. albicans* in a small concentration of 0.95 mg/ml—the nematodes died from infection and hyphae protruded through the cuticle of nematodes. With concentrations greater than 0.95 mg/ml up to EC50 (7.62 mg/ml), the compound rescued the nematodes from infection with *C. albicans* and the survival of the nematode was increased. However, using concentrations of more than 7.62 mg/ml decreased nematode survival.

2.2 Antibacterial compounds identification using *C. elegans*

Many human pathogens can cause infection in *C. elegans*, including *Pseudomonas aeruginosa*, *Salmonella enterica*, *Enterococcus faecalis*, and *Staphylococcus aureus*. All of these human pathogens can be a food source for *C. elegans*. The pathogen and the mechanism of infection can be studied with the use of *C. elegans* as a model of infection^[33, 34]. For example, *C. elegans* has been used as a model host in the creation of the *C. elegans*-*E. faecalis* infection assay. A high throughput screen of 6,000 synthetic

compounds and 1,136 natural extracts for their activity as antimicrobial was applied. 16 synthetic compounds and 9 natural extracts were recognized for their activity in increasing nematode survival^[35]. Some of the compounds demonstrated a greater effectiveness *in vivo* than *in vitro* which suggests that these compounds target bacterial virulence factors or change the host immune system. 15 of 16 synthetic compounds have no toxicity and only one compound has a growth retardation effect on the nematodes.

A new screen was done, with the improved method of co-inoculation. 33,931 compounds and 3,283 natural extracts were tested, and 28 compounds and extracts were identified for their antimicrobial activity^[36]. 6 compounds increased the survival of *C. elegans* but did not inhibit the growth of *E. faecalis* *in vitro*. Therefore, the mechanism of action possibly is different from current antibiotics and they may act as immune system modulators or inhibitors of virulence factors.

2.3 Anthelmintic drugs discovery using *C. elegans*

Anthelmintic drug discovery with the use of *C. elegans* as a model host can be carried out. A great number of compounds have been tested so far but the use of the model for this purpose has diminished considerably. Recently, the activity of the KSI-4088 compound against the *C. elegans* was studied and the results show that the compound is an active anthelmintic drug^[37].

2.4 Possible antimicrobial target identification with *C. elegans*

In addition to compound screening, *C. elegans* can be used to study microbial virulence and host-pathogen interactions^[38–50]. Understanding the pathways and these mechanisms of infections will enable us to identify novel targets for antimicrobial agents. For example, the disaccharide trehalose plays an important role as an energy source and stress protectant in many organisms like bacteria and fungi^[51, 52]. It has been found that the trehalose pathway is important for the survival of *C. albicans* in the host and it plays a significant role in infection^[53]. *C. neoformans* tps1 (trehalose-6-phosphatase synthase 1) strain is the mutant strain of the trehalose-6-phosphate (T6P) synthase. In a *C. elegans* model of *C. neoformans* infection, the importance of the role of the trehalose pathway in the infectious process was confirmed, with the *tps1* mutant showing attenuated virulence in *C. elegans*^[54]. Therefore, the trehalose pathway could be a potential target for a new class of antifungal drugs in the *C. elegans* model (Table 2).

Another example is the phosphatidylinositol-specific phospholipase C (PI-PLC). Phospholipase C 1 gene (*PLC1*) is the encoding gene of PI-PLC^[55]. *PLC* is a principal modulator of virulence of *C. neoformans* that acts through the protein kinase C/MAPK (mitogen-activated protein kinase) pathway and controls the release of Phospholipase B1 from the plasma membrane^[56–59]. Using the *C. elegans* model of *C. neoformans* infection, it has been established that *PLC1* is essential for virulence of *C. neoformans in vivo*^[55]. Hence the *Plc1* is possibly a novel target for new antifungal drugs (Table 2).

The study of polymicrobial infections and *in vivo* pathogen-pathogen interactions also can be accomplished using *C. elegans* as host. These interactions are very important medically. The *in vivo* interaction between the prokaryote *Acinetobacter baumannii* and the eukaryote *C. albicans* using *C. elegans* as a model host was studied^[60]. It was found that *A. baumannii*^[61] competes with *C. albicans* and inhibits the ability of *C. albicans* to form hyphae and biofilm, the two important virulence factors of *C. albicans*. This was proved by the enhanced survival of nematodes when *C. elegans* was infected with these two pathogens compared to infection by *C. elegans* alone (Table 2). Therefore, understanding the

mechanism of *in vivo* interaction between pathogens can uncover new targets for antimicrobial compounds^[62, 63].

2.5 The use of *C. elegans* in drug discovery beyond antimicrobial agents

In addition to antifungal and antibacterial compounds, drug discovery with the use of *C. elegans* can be accomplished for a variety of medication classes including anesthetics, antidepressants, antineoplastic, and antihypertensive drugs^[35, 64–69]. For example, the use of *C. elegans* could help to learn more about the mode of action of anesthetics and in the discovery of novel anesthetics^[70]. Also, the cAMP-response element binding protein (CREB)-mediated pathway and the complete neuronal circuit which this pathway affects was studied using *C. elegans*^[71] and this model could be used for the study of mental disorders and the discovery of new drugs^[72].

In another study, 14100 compounds were screened for their ability to change phenotypes of the *C. elegans*^[66]. 308 compounds were detected that can change nematode characteristics such as morphology, growth and egg laying. Nematopine A was identified for its effect on the nematode morphology and egg laying. It was found that nematopine A targets the *egl-19* (EGG Laying defective) gene that encodes the L-type calcium channel α_1 -subunit in the *C. elegans*^[73, 74]. It is similar to 1, 4-dihydropyridines class of hypertensive drugs that antagonize α_1 subunit of L-type calcium channels. Also nematopine A can act against the L-type calcium channel of vertebrates, hence it can act as a new calcium channel antagonist. As a result, with the help of *C. elegans*, a new active compound that could act as anti-hypertensive drug was identified.

The *C. elegans* can be used for antineoplastic drug discovery. Research has shown that the *abl-1* gene of *C. elegans* antagonizes the germline apoptosis, after ionizing irradiation, that is mediated by p-53 and that the *C. elegans* might be used as a model for the development of new antineoplastic medications^[75].

Expert opinion—In this review, we present the advantages of utilizing *C. elegans* in antimicrobial drug discovery. *C. elegans* has many advantages, including low cost, short life cycle, and well-studied genetics. Moreover, the nematodes can be used in high throughput screens and this model allows for the detection of new compounds with antimicrobial efficacy that can be later studied in detail. The initial testing of compounds in *C. elegans* before testing in mammals provides economic, time and ethical advantages.

One of the unique properties of *C. elegans* is its utility as a tool for drug discovery. Because the screen happens during a true infection process, the nematode system can help in detecting compounds which act by blocking virulence factors of pathogens and compounds that have an immune modulator effect on the host can be identified. Furthermore, it is possible to estimate the toxicity of compounds. This is very important because compounds that have a toxic effect on *C. elegans* could be toxic to mammalian cells. Despite large differences in anatomical and biochemical properties between mammals and invertebrates, it has been shown that there are enough similarities and correspondences to use *C. elegans* as a model host for research of a variety of diseases and medications. Many *C. elegans* mutant strains can mimic human diseases such as Alzheimer's disease, Duchenne muscular dystrophy, diabetes, and cancer^[76–80]. A very important factor in utilizing *C. elegans* in antimicrobial discovery is that there are similarities between microbial pathogenesis in mammals and *C. elegans*; therefore, compounds with antimicrobial property detected in *C. elegans* are highly likely to be compounds which can be used in humans. Searching for new compounds is facilitated by the utilization of the high-throughput technique for screening chemical libraries^[36, 81]. This technique uniquely provides the tools for screening thousands of compounds^[82–84]. Both *in vitro* and *in vivo* methods can be utilized in the high-throughput

search. *In vitro* studies can provide information about the activity and the efficacy of the compounds against microbes. However, *in vivo* studies are desirable and more practical, because they add information about the host immune response and the toxicity of the compounds at the same time. As a result, this whole animal screen can identify new classes of drugs that up-regulate the immune system of the host.

The initial testing of compounds in *C. elegans* before testing in mammals provides economic, time and ethical advantages. The life cycle of the nematode is short and the time that is needed for infection and death of the nematode by a pathogen is less in comparison with a mouse model of infection. Moreover, high throughput screening with mammals, however, raises ethical issues.^[85, 86] These invertebrates include *C. elegans*, which has been studied and used intensively in the field of microbiology and high throughput screening. Differences between *C. elegans* and mammals pose some limitations. Verifying the antifungal properties of compounds using mammals remains necessary. Testing the compounds for their toxicity at mammalian level is required, and the study of drug pharmacodynamics and pharmacokinetics, like the distribution, the absorption and the protein binding of the compound, is important. *C. elegans* does not have all of the organs and genes of a mammal, but some features and functions are evolutionarily conserved^[87–89]. Overall, these differences do not limit the use of *C. elegans* as a model host. Moreover, *C. elegans* has mechanisms of defense that have similarities with mammals. It has a four layered cuticle^[90] that lines its exterior, rectal, and oral cavities^[91]. In addition to the physical defense mechanisms, *C. elegans* has an enzymatic xenobiotic defense mechanism. For example, it has xenobiotic detoxification enzymes like cytochrome P450s and xenobiotic efflux pumps like ATP-binding cassette transporters. Therefore, many chemical compounds cannot accumulate easily in active concentrations within the nematode. Lately, a structural based accumulation model has been developed that can detect compounds^[92] with a better accumulation within the nematode and increased bioavailability and bioactivity. Therefore, in the future it will be possible to choose compounds that have a better likelihood of reaching a target within the nematode.

There are efforts underway to improve the high throughput methods that utilize *C. elegans* in discovery of new antimicrobial agents, to become more effective and more accurate. These include a trial of a quantifiable automated scoring assay, a better nematode dispensing system, and an automated simultaneous *C. elegans*-*C. albicans* dispensing system. Moreover, the improvement of jointly dispensing *C. albicans* and *C. elegans* in each well in an automated way will save time and effort. Recently, a new automated detection system has been developed. It can recognize the developmental stage of the nematode from the size of the nematode. Also, a new imaging system can measure the fluorescence patterns. Therefore, it helps assessing the biological processes^[93].

Drug discovery is a challenging research field. Research with the use of *C. elegans* has demonstrated great results and holds a promising future. The nematode is an excellent tool for the researcher and provides many valuable experimental elements. The many advantages of their use, including easy handling, the option for high throughput assays and the possibility of study of the interactions between the host and the pathogen have made the *C. elegans* a promising tool for preclinical drug discovery.

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Table 1Compounds identified using *C. elegans* for preclinical drug discovery

Compound name	Active against	Reference	Compound name	Active against	Reference
Enoxacin	<i>C. albicans</i>	[21]	Triadimeron	<i>C. albicans</i>	[24]
CAPE	<i>C. albicans</i>	[21]	Dequalinium chloride	<i>C. albicans</i>	[24]
Cyclosporine A	<i>C. albicans</i>	[24]	Concanamycin A	<i>C. albicans</i>	[24]
Ascomycin	<i>C. albicans</i>	[24]	A2 Sakurasaponin	<i>C. albicans</i>	[25]
FK-506	<i>C. albicans</i>	[24]	A8	<i>C. albicans</i>	[25]
A16 Agmoside	<i>C. albicans</i>	[25]	A19	<i>C. albicans</i>	[25]
A24	<i>C. albicans</i>	[25]	A25	<i>C. albicans</i>	[25]
A11	<i>C. albicans</i>	[25]	A17	<i>C. albicans</i>	[25]
A20	<i>C. albicans</i>	[25]	A21	<i>C. albicans</i>	[25]
A7	<i>C. albicans</i>	[25]	Nemadipine A	<i>E. faecalis</i>	[34]

Table 2Targets for potential anti-microbial drugs identified using *C. elegans*

Target	Reference
Trehalose pathway	[54]
Phosphatidylinositol-specific phospholipase C	[55]
<i>C. albicans</i> – <i>A. baumannii</i> interactions	[60]