Published in final edited form as:

Curr Med Chem. 2009 ; 16(13): 1588–1595.

Antifungal drug discovery through the study of invertebrate model hosts

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Abstract

There is an urgent need for new antifungal agents that are both effective and non-toxic in the therapy of systemic mycoses. The model nematode *Caenorhabditis elegans* has been used both to elucidate evolutionarily conserved components of host-pathogen interactions and to screen large chemical libraries for novel antimicrobial compounds. Here we review the use of *C. elegans* models in drug discovery and discuss caffeic acid phenethyl ester, a novel antifungal agent identified using an *in vivo* screening system.

C. elegans bioassays allow high-throughput screens of chemical libraries *in vivo*. This whole-animal system may enable the identification of compounds that modulate immune responses or affect fungal virulence factors that are only expressed during infection. In addition, compounds can be simultaneously screened for antifungal efficacy and toxicity, which may overcome one of the main obstacles in current antimicrobial discovery.

A pilot screen for antifungal compounds using this novel *C. elegans* system identified 15 compounds that prolonged survival of nematodes infected with the medically important human pathogen *Candida albicans*. One of these compounds, caffeic acid phenethyl ester (CAPE), was an effective antifungal agent in a murine model of systemic candidiasis and had *in vitro* activity against several fungal species. Interestingly, CAPE is a potent immunomodulator in mammals with several distinct mechanisms of action. The identification of CAPE in a *C. elegans* screen supports the hypothesis that this model can identify compounds with both antifungal and host immunomodulatory activity.

Keywords

novel antifungal compounds; compound screens; caffeic acid phenethyl ester (CAPE); *Candida albicans*; *Cryptococcus neoformans*; *Caenorhabditis elegans*

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Conflicts of Interest

The other authors report no potential conflicts of interest.

Introduction

The significant morbidity and mortality associated with invasive fungal infections combined with the growing problem of drug resistance in pathogenic fungi mandate the identification of novel antifungal therapies [1–6]. However, progress in drug development has been hindered for several reasons. Cultivable microorganisms have been over-mined for natural products with antifungal activity and we have nearly exhausted the obvious cellular targets for new drug design. Furthermore, most compounds with antifungal activity in the synthetic compound libraries used for new drug development are toxic to mammalian cells. As a result, there is an increasing interest to discover therapies with novel mechanisms of action. One such approach is to look for compounds that exert antifungal activity by modulating the host immune response [7]; however, such agents are usually missed in traditional *in vitro* screens of compound libraries. To address this challenge, investigators are examining the interaction of fungi with their environmental predators, such as nematodes, predatory amoeba and insects, to develop a facile, *in vivo* system for the identification of novel antifungal therapies [8–11]. We hypothesize that killing of invertebrate hosts by pathogens mimics key features of mammalian pathogenesis. Thus, the study of these interactions permits the identification and characterization of microbial virulence mechanisms, host response elements and novel antifungal compounds of fundamental relevance to pathogenesis, independent of the model system used (reviewed in [12]).

The use of the microscopic nematode *Caenorhabditis elegans* in a novel *in vivo* compound screen could solve some of the main obstacles in antifungal discovery. Two key observations support the use of this system to model systemic mycoses in mammals. First, the expression and regulation of inducible virulence traits in some pathogenic microorganisms likely evolved as a consequence of environmental interactions with natural predators, such as free-living amoeba and *C. elegans* [13, 14]. In fact, the ability of some fungi to establish a lethal infection in humans may simply be a product of this environmental interaction. The molecular characterization of these host-pathogen interactions supports the conclusion that a common set of molecular mechanisms are employed by different pathogens against a widely divergent array of metazoan hosts [14]. Second, many human bacterial and fungal pathogens cause a persistent, lethal infection in *C. elegans* by employing the same virulence characteristics required for infection in mammals (reviewed in [15]).

Here, we update medicinal chemists on the significance of fungal infections, the new approaches that can be used for the identification of novel antifungal compounds and discuss one compound, caffeic acid phenethyl ester (CAPE), identified in a novel *in vivo* screen.

Significance of candidiasis and cryptococcosis

C. elegans-based assays have focused on candidiasis and cryptococcosis, two medically important fungal infections. Candidiasis is the most common fungal infection and *Candida* spp. have become the fourth leading cause of bloodstream infections [16–22]. Nosocomial bloodstream infections due to *Candida* spp. are associated with a mortality rate that can reach 40% [23–27]. In a large study that included 10,038 patients from 1,417 intensive care

units in 17 European countries, invasive candidiasis accounted for 17% of hospital-acquired infections [28, 29]. In the United States, the overall excess cost attributable to candidemia is estimated to be \$1 billion per year, while the average cost of candidemia for a single episode is \$34,123–44,536 (1997 U.S. dollars) [25, 26]. Risk factors for systemic candidiasis (such as neutropenia, surgery, indwelling catheters, renal failure, total parenteral nutrition, diabetes, and the use of steroids) are difficult to modify [30–34]. In addition, localized candidal infections are also a significant health issue. *Candida* spp. are the second most common cause of urinary tract infections in critically ill patients [35]. Furthermore, approximately 70% of women experience vaginal infections caused by *Candida* spp., 20% develop recurrence and up to 50% of this later group have 4 or more episodes per year [36–38].

C. neoformans is an encapsulated fungus that can cause a life-threatening infection in patients with intact and compromised immune systems. The organism has a worldwide distribution and its importance as a human pathogen has increased substantially secondary to the expanding numbers of patients with acquired immunosuppression from HIV infection and therapies such as corticosteroids, cytotoxic treatments for malignancies, and medications to prevent organ transplantation rejection [1]. Although the incidence of cryptococcosis has decreased in the developed world as a result of the widespread adoption of effective antiretroviral therapy to treat HIV infection, cryptococcal disease is still a significant cause of morbidity and mortality in resource limited settings [39]. In parts of Africa, *C. neoformans* is the first or second most common cause of culture-proven meningitis [40, 41]. In a cohort of 1,792 HIV-positive gold miners in South Africa, cryptococcosis was the third leading reason for hospital admission and led to 44% of deaths [42, 43]. Recently, cryptococcal disease has garnered public attention owing to a dramatic outbreak of *Cryptococcus gattii* infections in seemingly immunocompetent hosts on Vancouver Island, British Columbia [44–49].

The *C. elegans* model of microbial pathogenesis

The study of pathogenesis in mammals is challenging owing to their long reproductive cycles, small brood sizes and genetic complexity. Furthermore, mammalian experiments can be expensive and are often fraught with ethical challenges [50]. These shortcomings have prompted researchers to identify alternate hosts for the study of pathogenesis (reviewed in [51] and [52]). The microscopic nematode *C. elegans* is an attractive model system for several reasons. Adult nematodes are small hermaphrodites capable of self-fertilization, which permits the creation of genetically identical populations simply by allowing one animal to produce progeny. Furthermore, the entire cell lineage has been described in detail and is identical in every *C. elegans* adult, which greatly facilitates genetic experiments. Specific gene silencing and genome-wide screens are possible through the use of RNA interference (RNAi) libraries, which employ double-stranded RNA to “knock down” gene expression. Lastly, decades of experience with the genetic manipulation and molecular dissection of this organism has created a rich, collaborative scientific environment for *C. elegans* research. In fact, the 2002 and 2006 Nobel Prizes in Physiology or Medicine and the 2008 Nobel Prize in Chemistry were awarded for studies conducted in this model host.

C. elegans are readily propagated in the laboratory using non-pathogenic bacteria as a food source and they can be easily infected with both bacteria and fungi after transfer of the worms to a plate containing the pathogen of interest. This model has been used to identify novel virulence determinants in a remarkable number of human pathogenic bacteria including *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Serratia marcescens*, *Salmonella enterica*, *Acinetobacter baumannii*, *Yersinia pestis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (reviewed in [52]). In 2002, Mylonakis and colleagues observed that *C. elegans* are killed when fed the human pathogenic fungus *C. neoformans*, but can thrive when offered non-pathogenic cryptococcal strains as a food source [14]. The killing of *C. elegans* by *C. neoformans* in these experiments was dependant upon genes previously shown to be involved in mammalian virulence. These data argue that the interaction with natural predators in the environment, such as free-living amoebae and nematodes, provides selection pressure on *C. neoformans* to express phenotypes that allow survival in invertebrate hosts.

To further characterize cryptococcal virulence mechanisms, the Mylonakis laboratory developed two separate tools in the *C. elegans*-*C. neoformans* system to screen a library of cryptococcal mutants for those with attenuated virulence [53, 54]. These studies identified novel cryptococcal virulence genes important for pathogenesis in mammalian models, providing evidence that molecular studies in this system can yield insights into the host-pathogen interaction.

The evolutionarily ancient environmental interactions between natural predators, such as amoeba and nematodes, and fungi also provide selection pressure for the development of a host immune system [55]. Elegant *in vivo* studies have uncovered several conserved innate immune responses in *C. elegans*. Homologs of the mammalian mitogen-activated protein (MAP) kinase kinase kinase (*nsy-1*), MAP kinase kinase (*sek-1*) and MAP kinase (*pmk-1*) were recovered using forward and reverse genetic screens of worms with enhanced susceptibility to infection and are required for pathogen resistance [56]. Importantly, the MAP kinase encoded by *pmk-1* is a homolog of mammalian p38 MAP kinase, a key component of the Toll-like receptor (TLR) signaling pathway. Furthermore, the nematode ortholog of the human protein SARM, a TLR 1 receptor domain protein (*tir-1*), controls the expression of multiple genes involved in pathogen response including the elaboration of the antimicrobial peptides NLP-29 and NLP-31 [57]. It also seems that programmed cell death in the worm gonad [58] and the synthesis of other antimicrobial peptides (*abf-1* and *abf-2*) [59] are important defense responses in this organism.

In summary, the molecular characterization of a fungal infection in *C. elegans* can offer insight not only into fungal pathogenesis and host immunity, but also the evolution of these conserved responses.

C. elegans bioassays as a screen for novel antimicrobial compounds

Fungal infection in *C. elegans* offers a tractable model of the host-pathogen interaction of direct relevance to higher order species. It therefore follows that this system can be used for the *in vivo* study of chemical compounds to identify novel antimicrobial agents.

Like *C. neoformans*, *C. albicans* establishes a lethal infection in the *C. elegans* digestive tract [Fig. (1)] by employing several virulence traits that are also required for virulence in mammals. *C. albicans* cells develop filaments that differentiate into hyphae (long continuous germ tubes separated by septin rings) and pseudohyphae (chains of distinct cells). These filaments facilitate adhesion to host cells, contribute to biofilm formation, allow direct invasion through host tissues and are thus required for the full virulence potential of *Candida* spp. in mammals and in *C. elegans* [60, 61]. During *C. elegans* infection, wild-type *C. albicans* create a network of true hyphae that pierce the worm cuticle and are easily visible using a dissecting microscope. In a pilot experiment, the death of the nematode and the presence of filaments breaking through the worm were used as hard endpoints in the *C. albicans*-*C. elegans* model to screen a library of chemical compounds with known biological activities using a *C. albicans* strain that constitutively expresses green fluorescent protein (GFP) [61]. Nematodes exposed to compounds with significant antifungal activity moved normally and had no green fluorescence in the intestine.

Conversely, wells containing worms with active *C. albicans* infection had high levels of green fluorescence and contained non-mobile worms engulfed in a filament network. Using this reporter method in a screen of 1,266 compounds with known pharmaceutical properties, 15 were identified that both prolonged survival and inhibited filamentation of *C. albicans*. Three of these compounds were selected for further study and two were found to prolong survival in a murine model of *C. albicans* infection. One of these compounds, caffeic acid phenethyl ester (CAPE) strongly inhibited *C. albicans* filament and biofilm formation *in vitro* at a minimum inhibitory concentration of 64 µg/ml, which interestingly was higher than the concentration used in the initial screen (33 µg/ml) [61]. Moreover, CAPE prolonged the survival of nematodes in the *in vivo C. elegans*-*C. albicans* assay at concentration as low as 4 µg/ml. It is therefore tempting to speculate that CAPE prolongs the survival of nematodes in this assay predominantly through immunomodulation of the host, but further studies are needed to prove this hypothesis. Interestingly, a *C. elegans* live-animal infection model for novel antibacterial agents also identified several compounds that may have immunomodulatory activity. 6,000 synthetic compounds and 1,136 natural product extracts were screened to identify agents that promoted the survival of *C. elegans* infected with the gram positive bacteria *Enterococcus faecalis* [62]. Sixteen compounds and 9 extracts were found to promote nematode survival. As was observed in the antifungal screen, the *in vivo* effective dose of many of these compounds was significantly lower than the minimum inhibitory concentration that prevented *E. faecalis* growth *in vitro*.

Features of the C. elegans screening system

C. elegans whole-animal bioassays for novel antifungal therapies have several attractive features that complement traditional *in vitro* screens. The experiments can be performed in liquid media using standard 96-well plates, allowing the simultaneous screening of

thousands of compounds and the utilization of automated robotics to fill the assay plates, sort the worms into the wells and pin transfer compounds directly from dimethyl sulfoxide (DMSO) stocks to the assay plates. Traditionally, the transition from *in vitro* to *in vivo* models requires additional development to create optimized dosing formulations that are acceptable for a variety of delivery modalities (e.g. intravenous, oral or subcutaneous). In the *C. elegans* bioassay, the compounds may be administered directly to the screening plate from the traditional DMSO stock plates, bypassing additional formulation development. Furthermore, the statistical power of this assay can be adjusted by increasing the number of worms used to screen each compound. For each assay, uniformity and reliability can be quantified mathematically by comparing Z-scores, a statistical tool that evaluates assay performance using compounds with known antifungal activity, such as amphotericin or ketoconazole [63].

The *C. elegans* whole-animal bioassay for novel antifungal therapies may also improve the efficiency of a primary screen in identifying quality lead compounds. By incorporating a broader array of desired compound characteristic filters into the initial screening process (e.g. potency, solubility, permeability and toxicity), higher quality and more refined hits may emerge [Fig. (2)]. The whole-animal *in vivo* assay examines some of these compound characteristics in parallel and avoids inefficiencies associated with the sequential optimization of the individual properties, which must occur in a traditional optimization cycle.

In addition, this screening model permits data-driven decisions regarding the quality of a hit and eliminates preconceived biases about certain chemical classes or motifs. Compounds identified in an *in vivo* screen may not comply with the Lipinski rule of five (an empirically derived set of rules used to predict oral bioavailability of a compound), but have acceptable physicochemical properties, which the Lipinski rules indirectly evaluate [64]. Indeed, many of the chemical phenotypes inferred by the rule of five, such as solubility and compound permeability, must be satisfied in order to be efficacious in this whole organism screening system.

The *C. elegans*–pathogenesis model can also be used to screen host toxicity. In the *C. albicans* infection model, fluconazole was effective in prolonging survival of nematodes exposed to a fluconazole-susceptible *Candida* strain up to a concentration of 32 µg/ml, but at higher concentrations (100 µg/ml) nematode survival was diminished, even compared to the nematodes in an untreated control group. We hypothesize that this toxicity is present at lower drug concentrations, but the beneficial effect of the drug at this concentration in promoting worm survival outweighs its toxic effects [61]. In other studies, *C. elegans* has been used as an indicator of toxicity from heavy metals, environmental pollutants, organic solvents, and neurotoxins [65]. Williams and Dusenbery showed that toxicity of heavy metals against *C. elegans* correlates well with toxicity against mice or rats in rank order tests [66]. Additionally, a separate study showed that there was a significant correlation between the toxicity observed in *C. elegans* and rodents following organophosphate exposure [67].

Lastly, these *in vivo* bioassays may identify compounds with novel mechanisms of action. Antifungal compounds, such as CAPE, may exert activity through immunomodulation of the

host or by altering the expression of fungal virulence factors that are induced only during infection. These agents would be missed in traditional screens, but can be identified in the *C. elegans* system.

We note that the *C. elegans* bioassay cannot replace the *in vivo* study of novel compounds in higher order hosts, but rather submit that this model may provide a more refined and informative approach than traditional *in vitro* high-throughput screening models. However, we also recognize that this system has several limitations. First, the relative simplicity of the nematode innate immune system may restrict the number and types of immunomodulatory compounds that can be identified in the *C. elegans* bioassays. Furthermore, the small size of the nematode makes it difficult to accurately assess important pharmacodynamic properties that influence a compound's systemic absorption, protein binding and volume of distribution. Lastly, these bioassays likely will not screen out all toxic compounds from a compound library. As such, interesting compounds identified in the *C. elegans* bioassays must be studied further using traditional mammalian models [Fig. (2)].

Caffeic Acid Phenethyl Ester (CAPE)

Caffeic acid phenethyl ester [CAPE, Fig. (3)], a structural relative of flavonoids (plant pigments that are synthesized from phenylalanine [68]), belongs to a large family of caffeic acid natural products and analogs which are produced in many plants. CAPE is one of the major components of propolis, a natural resinous product used by honeybees to seal cracks or large potentially infectious objects, such as carcasses, that the insects are unable to remove from the hive [69]. The composition of propolis is based on their plant source [70] and contains amino acids, phenolic acids, phenolic acid esters, flavonoids, cinnamic acid, terpenes and caffeic acid [71]. Interestingly, propolis has been used for thousands of years in folk medicine for several purposes [69, 71–75] and has attracted much attention owing to its multiple reported biological properties and its inherent structural simplicity.

The NF- κ B pathway is a key mediator of many genes involved in cellular proliferation, apoptosis and cytokine production. This transcription factor also activates the expression of several genes involved in the host immune and inflammatory responses, including acute-phase reactants and the cytokines IL-1, IL-2, IL-6, IL-8 and tumor necrosis factor (TNF) [76]. Interestingly, the activation of NF- κ B by TNF is completely blocked by CAPE. CAPE also inhibits NF- κ B activation induced by other inflammatory agents including phorbol ester, ceramide, hydrogen peroxide and okadaic acid [77]. Additionally, CAPE also targets the nuclear factor of activated T cells (NFAT) signaling pathway response [78]. The NFAT proteins are a family of Ca²⁺/calcineurin-responsive transcription factors primarily recognized for their central roles in T lymphocyte activation that plays a critical role in the immune response, myogenesis, chondrocyte differentiation and the development of the cardiovascular system [79–81]. Lastly, CAPE can block the production of reactive oxygen species (ROS) in human neutrophils, induce apoptosis in transformed rat fibroblast cells and suppresses lipid peroxidation in low density lipoproteins [82–85].

Studies indicate that CAPE has cytostatic, neuroprotective, immunomodulatory, anti-inflammatory, hepatoprotective, cardioprotective, vasorelaxant and antioxidant activities.

Furthermore, CAPE can inhibit replication of viruses (including HIV) and bacteria [86]. Data on the antifungal activity of CAPE are limited, but propolis extracts were shown to inhibit *C. albicans* hyphal formation [87] and treat oral candidiasis [88]. The low toxicity and broad antifungal activity may make CAPE an important addition to the antifungal armamentarium. Taken together, these data suggest that CAPE may demonstrate antifungal efficacy by augmenting the antifungal responses of the host.

In summary, *C. elegans* can be used to study fungal pathogenesis on a genome-wide scale and perform high throughput bioassays for novel antifungal compounds that may otherwise be missed in traditional *in vitro* screens targeting fungal growth.

Acknowledgments

This work was supported by NIH grant R01 AI075286 (to E.M.) and a Fellowship in General Immunology from the Irvington Institute (to R.P.W.). E.M. has served as a consultant for Biogen Idec., received research support from Astellas Pharma Inc. and is a member of the Speaker's Bureau for Pfizer Inc.

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Figure 1. *C. albicans* establishes infection in the *C. elegans* intestine

Intact yeast cells are seen within the (a) proximal and (b) distal intestine. White arrows in panel (a) point to the pharyngeal grinder organ. Black arrows point to the intestinal lumen. This figure was published by Breger et al. and used here with permission [61].

Figure 2. Idealized Approach to drug discovery

A schematic of traditional drug discovery models and the *C. elegans* whole organism-based screening systems are presented. IC₅₀: inhibitory concentration₅₀; ADME-PK: absorption, distribution, metabolism, excretion- pharmacokinetics.

Caffeic acid phenethyl ester (CAPE)

Figure 3.
Chemical structure of caffeic acid phenethyl ester (CAPE).