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# Drug discovery: Insights from the invertebrate *Caenorhabditis elegans*

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## Abstract

Therapeutic drug development is a long, expensive, and complex process that usually takes 12–15 years. In the early phases of drug discovery, in particular, there is a growing need for animal models that ensure the reduction in both cost and time. *Caenorhabditis elegans* has been traditionally used to address fundamental aspects of key biological processes, such as apoptosis, aging, and gene expression regulation. During the last decade, with the advent of large-scale platforms for screenings, this invertebrate has also emerged as an essential tool in the pharmaceutical research industry to identify novel drugs and drug targets. In this review, we discuss the reasons why *C. elegans* has been positioned as an outstanding cost-effective option for drug discovery, highlighting both the advantages and drawbacks of this model. Particular attention is paid to the suitability of this nematode in large-scale genetic and pharmacological screenings. High-throughput screenings in *C. elegans* have indeed contributed to the breakthrough of a wide variety of candidate compounds involved in extensive fields including neurodegeneration, pathogen infections and metabolic disorders. The versatility of this nematode, which enables its instrumentation as a model of human diseases, is another attribute also herein underscored. As illustrative examples, we discuss the utility of *C. elegans* models of both human neurodegenerative diseases and parasitic nematodes in the drug discovery industry. Summing up, this review aims to demonstrate the impact of *C. elegans* models on the drug discovery pipeline.

**KEYWORDS**
*C. elegans*, drug discovery, genetic screenings, human disease models, pharmacological screenings

## 1 | INTRODUCTION: THE NEMATODE *Caenorhabditis elegans*

Since its introduction into research by Sydney Brenner in the early 60s, the nematode *Caenorhabditis elegans* has played a pivotal role in different areas of biomedical investigation.[1–9] Studies on *C. elegans* have contributed to fundamental breakthroughs in life science, such as the discovery of genetic regulators of programmed cell death, the use of the green fluorescent protein (GFP) as a protein marker, and the discovery of RNA interference (RNAi).[10–12]

*C. elegans* is a tiny (~1 mm in length), soil, free-living, and bacteria-eating nematode[13,14] which can develop as either of two sexes, self-fertilizing hermaphrodites or males. Males represent a minor population (~0.2% of population).[14,15] Healthy hermaphrodites produce up to 300 self-progeny although they are capable of giving approximately 1000 offspring if they are mated with males.

This nematode typically lives for about 3 weeks at 20°C in the laboratory. Unlike other members of the Nematoda phylum, *C. elegans* is a nonhazardous and nonpathogenic animal that can be manipulated with standard safety rules. A unique benefit of using *C. elegans* in the laboratory is that it can be frozen and stored in liquid nitrogen or −80°C ultra-freezers until needed.[14] Moreover, its use does not raise the ethical issues associated with the use of vertebrates (Figure 1).

*C. elegans´* life cycle, which is very short, takes approximately 2.5–3 days and is divided into embryogenesis (~16 h), four larval stages (L1–L4, ~28 h) and the adult molt, from L4 to adult (~12 h). Adverse environmental conditions (starvation, high population density, high temperature) in the late L1 larval stage trigger entry into a developmental arrest phase called dauer.[16,17] Dauer larvae can survive stressful conditions for months until encountering favorable conditions that permit resuming reproductive development. The presence of separate sexes and the short life cycle permit rapid genetic crosses in the laboratory. It has an invariant number of somatic cells: 959 in hermaphrodites and 1031 in males. *C. elegans* cellular genealogies have been traced from embryo to newly hatched larva. Unlike mammals, the pattern of cell lineages is invariant among individuals, which has been key to understand the roles of developmental control genes.[18–20]

Despite its apparent simplicity, *C. elegans* contains organs and tissues present in more complex organisms, such as muscles, hypodermis, intestine, reproductive and excretory system, and a well-described nervous system (Figure 1). It is also the only organism where the complete neural wiring diagram has been established.[21,22] Its transparency allows the easy visualization of specific cells and subcellular structures through Nomarski (differential interference contrast, DIC) optics using whole-live animals. The visualization of protein expression patterns, protein subcellular localization, and activity of specific cells using transgenic reporters and genetically encoded calcium indicators is feasible in living animals at any point in their lives (Figure 1).[23–25]

The fact that *C. elegans* was the first multicellular organism to have its complete genome sequenced (*C. elegans* Sequencing Consortium 1998) as well as its amenable genetic manipulation helped to turn this worm into a powerful organism for genetic screens.[26,27] 83% of *C. elegans* proteome is predicted to have human homolog genes [28] and it has been estimated that ~50% of *C. elegans* protein-coding genes have a functional ortholog in humans.[29] Essential biological molecules and cell signaling pathways, such as the insulin-signaling pathway, innate immunity regulatory pathways, synaptic machinery, and systems involved in protein homeostasis are highly conserved between worms and mammals.[30–34]

Taken together, all these features make *C. elegans* an excellent model organism that bridges the gap between the simplicity of cultured cells and the complexity of multicellular organisms.
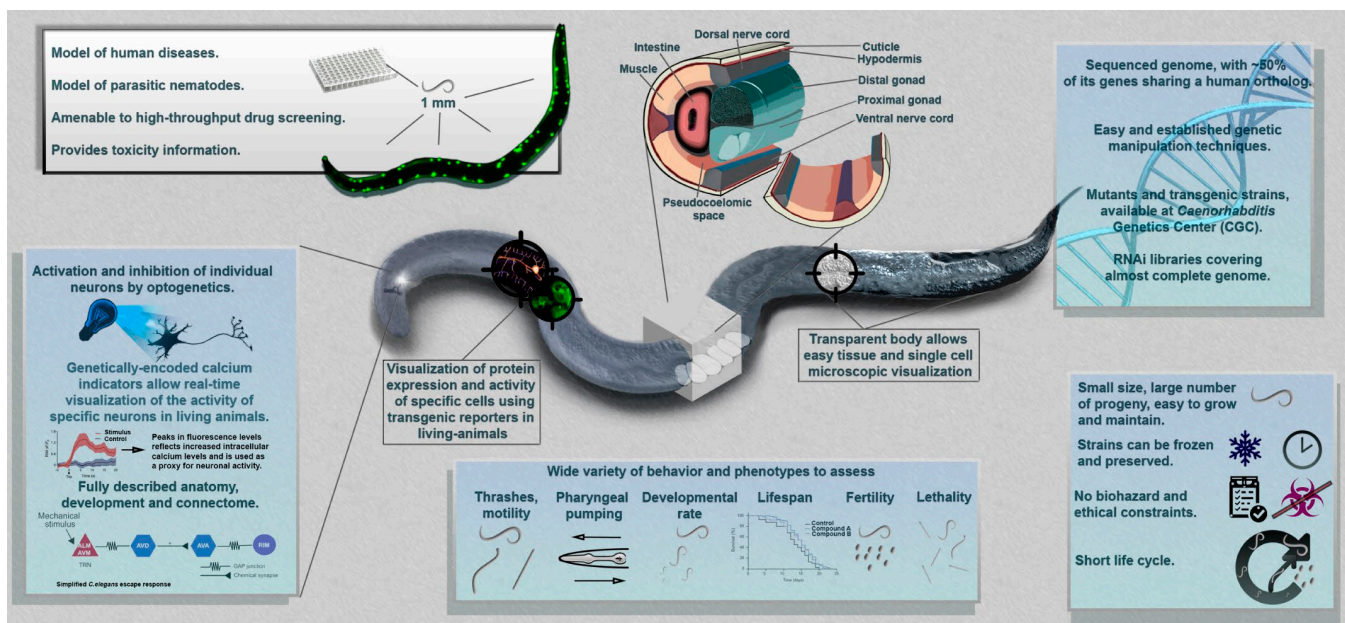


**FIGURE 1** *Caenorhabditis elegans* as a versatile platform for drug discovery. Its genetic amenability and ease of transgenesis allows elucidation of MOAs by forward and reverse genetic screens and generation of "humanized worms" to emulate conditions seen in humans. All this combined with the feasibility for HTS and automation of easily scored phenotypes converts *C. elegans* into a powerful model for pharmacological research. For more detailed anatomy and other *C. elegans* resources visit: Wormatlas (www.wormatlas.org), Wormbase (www.wormbase.org), Wormbook (www.wormbook.org)

## 2 | *C. elegans* IN DRUG DISCOVERY

The advances in molecular biology techniques and genome knowledge allowed drug screening to be carried out directly on target proteins. Therefore, target-based drug screens (TBS) have become the most commonly used approach for drug discovery. In this type of approach, compounds are screened for their capacity to bind or alter the activity of specific target proteins using cell extracts or cell cultures. However, as targets are previously identified and validated, TBS does not permit the discovery of novel targets and the elucidation of mechanisms of action (MOAs) is frequently limited to agonism or antagonism of known receptors/pathways. Moreover, once hits are detected they need to be tested in the context of a whole organism to further analyze its biological efficacy and toxicity. In contrast to TBS, phenotypic-based drug screens (PBS) use cells or animal disease models to identify compounds that rescue or ameliorate the disease phenotype. This animal-based approach includes the effects of cell communications and tissue interactions in the effect of a given compound. This unbiased strategy allows the identification of compounds with novel MOAs. One of the main caveats of PBS is that the identification of targets and MOAs could be complicated, particularly when mammalian animal models are used. This drawback can be significantly mitigated using invertebrates, such as the nematode *C. elegans*, which has lately gained consideration as an excellent platform for the identification of new drug targets and drug discovery.

Automation of worm transfer, image acquisition, and data analysis allows the use of *C. elegans* for high-throughput screening (HTS) assays.[35–39] Several companies (Celescreen, Sunnybiotech, Nagi Biosciences) offer these types of assays to the pharmaceutical industry for the identification of new potential compounds and validation of pharmacological targets. In the last 10 years, HTS in *C. elegans* has become—in fact—a useful tool to identify candidate compounds as potential treatments for several pathological conditions (Table 1).

The fact that *C. elegans* shares the nematode phylum with several parasitic roundworms makes it a straightforward model to be used for the development of anthelmintic drugs. Its use has allowed the MOA elucidation of several nematocidal drugs[75–78] (Table 2). However, its use goes far beyond than merely applying *C. elegans* to the discovery of drugs with anthelmintic potential. *C. elegans* is also exploited as an accessible platform to recapitulate distinctive phenotypes of human diseases, such as cancer, diabetes, neurodegenerative disorders, and pathogen infection.[79–89] The molecular pathways underlying a biological process that, when altered, could lead to physiological diseases are usually conserved throughout the animal kingdom. The alteration of these conserved pathways in *C. elegans* often leads to specific phenotypes and behaviors, such as modifications in lifespan, stress resistance, or locomotion. All these phenotypes are easily measurable using automated protocols and devices, making the screening of drugs that ameliorate or rescue these defects a relatively simple procedure.

Several genetic tools for the manipulation of single genes or groups of genes are readily available in *C. elegans* (chemical mutagenesis, transgenesis, RNAi, and CRISPR/Cas9) (Figure 1). Animals carrying mutations of highly conserved biochemical pathways or expressing exogenous pathognomonic protein of a specific disease can be engineered in few weeks. Mutant strains and animals expressing human transgenes are available at a very low cost at the *Caenorhabditis* Genetics Center (CGC). Moreover, the rapid life-cycle and the high brood size of *C. elegans* permit the analysis of the effects of compounds not only in the exposed animal but also in the progeny in a very short time-frame.

Cell cultures or cell extracts are typically the initial step in drug development. Using invertebrate animal models in these early steps is ideal to reduce research costs and time as they help to identify compounds that, apart from being effective in interacting with the active target, maintain their efficacy even after absorption, distribution, metabolism, and excretion (ADME) processes. They also provide information about potential systemic toxicity and biocompatibility.[96]

Although ADME processes are important sources of information compared with cell cultures, in *C. elegans* they have certain limitations that should be taken into account at the moment of processing results from drug-screening assays. In particular, the thick cuticle, which forms a strong barrier that limits drug absorption, can usually render false-negative results.[97]

*C. elegans´* low-cost maintenance, small size, genetic amenability, and conservation of key molecular pathways with mammalian animals convert this worm into an excellent alternative to significantly reduce drug development costs. Although the use of *C. elegans* as a platform for drug screening is recent, there are compounds that have been identified in this nematode and which, once validated on vertebrate models, are currently being evaluated in human patients. For example, hits obtained in a drug screening performed in a *C. elegans* model of amyotrophic lateral sclerosis were validated in zebrafish and mice. One of these hits, the neuroleptic Pimozide, a $Ca^{2+}$ channel blocker that stabilizes neuromuscular transmission in *C. elegans*, has also shown efficiency in a short randomized controlled trial of sporadic ALS subjects.[43] Another report evaluated the pearl powder (a Chinese medicine) by combining *C. elegans* studies with a clinical trial. The authors have correlated the lifespan extension found in *C. elegans* with the higher antioxidant capacity in blood samples of patients treated with pearl powder.[98] These two examples serve as proofs of concept for the translational potential of drug screenings in the invertebrate *C. elegans*. Since the number of drug screenings in this nematode is increasing year after year (Table 1), more clinical trials for hits identified in *C. elegans* are expected in the near future.

### 2.1 | Genetic screening assays

One of the greatest advantages of using *C. elegans* for biomedical research is undoubtedly its amenability for genetic manipulation. Genetic screens are widely used in *C. elegans* to either discover gene function or find genes involved in relevant biological pathways. Furthermore, the feasibility of rapid genetic crossing permits epistatic analyses to find new players in known genetic pathways.[99]

**TABLE 1** *Caenorhabditis elegans* phenotypic-based drug screenings. The table shows relevant drug screenings assays performed during the last decade in *C. elegans* models for human and animal diseases

| Biological activity | Initial screening set | Identified hits | References |
|---|---|---|---|
| Anti-proteotoxicity Neuroprotection | 10 positive modulators of healthspan | Metformin, lithium, and curcumin | 40 |
| | 18 compounds | α-methyl-α-phenylsuccinimide | 41 |
| | 87 flavonoids and 13 neurosteroids | 12 flavonoids (e.g., isoquercitrin) and 2 steroids (3β-Methoxy-Pregnenolone and 17β-estradiol) | 42 |
| | 3 850 compounds | 13 hits (e.g., pimozide) | 43 |
| | 983 FDA-approved drugs | 4 hits (dronedarone, tofranil, bendrofluazide, buspar) | 44 |
| | 4 polyphenolic compounds | Ferulic acid | 45 |
| | 30 FDA-approved drugs | Tannic acid, bacitracin | 46 |
| | 115 000 compounds | Four tetrahydroquinolinones | 47 |
| Anti-aging | 1386 FDA-approved drugs | Verapamil | 48 |
| | 32 compounds | 1 hit (chalcone like-compound) | 49 |
| | ~100 serine hydrolase inhibitors | JZL184 | 50 |
| | 107 FDA-approved drugs | Tiagabine | 51 |
| | 15 FDA-approved drugs | Captopril | 52 |
| | 33 000 compounds | 57 hits (e.g., nitrophenyl piperazine-containing compounds) | 53 |
| | 1280 compounds | 57 hits (e.g., minocycline) | 54 |
| | normal and disease-associated endogenous metabolites | α-ketoglutarate | 55 |
| Anti-microbial | 69 compounds | 5 hits (phenyl triazine compounds) | 56 |
| | 82 000 compounds | 185 hits (e.g., synthetic retinoid CD437) | 57 |
| | 86 000 compounds | 195 hits (e.g., 5-fluorouracil) | 58 |
| | 21 500 compounds | 318 hits (e.g., phenylsulfonyl pyrazinecarbonitrile) | 59 |
| | 640 FDA-approved drugs | 42 hits (e.g., closantel) | 60 |
| | 1600 compounds | 18 hits (e.g., iron-chelator ciclopirox olamine) | 61 |
| | 1300 extracts (from endophytic fungi) | 4 hits | 62 |
| | 2560 | 12 hits (e.g., natural saponins) | 63 |
| Anthelmintic | 12 benzopyrano pyrazol compounds | 4 hits | 64 |
| | 575 compounds | 29 hits (e.g., arylidene ketones) | 65 |
| | 11 imidazole-derivatives | 2 hits (e.g., diisopropylphenyl-imidazole) | 66 |
| | 400 compounds (Pathogen Box library) | 18 hits (e.g., isoxazole compounds) | 39 |
| | 480 compounds | 20 hits (e.g., dihydrobenzoxazepinones) | 67 |
| | 67 012 compounds | 30 hits (e.g., ethyl benzamide moiety compounds | 68 |
| Anti-tumoral | 4 in vitro anticancer compounds | 4 hits | 69 |
| | 30 plant extracts | Harmine (from the plant *Peganum harmala*) | 70 |
| | ~9000 compounds | 2 hits (an EGFR inhibitor and a MEK inhibitor) | 71 |
| Anti-metabolic disorders (obesity, insulin resistance, type II diabetes) | 24 plants and fungal extracts | 2 hits (extracts of Inonotus obliquus and Gardenia jasminoides) | 72 |
| | 350 natural products | 1 hit (swertiamarin) | 73 |
| | 8 natural plant compounds | 2 hits (isoquinoline alkaloids) | 74 |

To date, two strategies to perform screenings in *C. elegans* have been developed: forward genetics and reverse genetics. Whereas the former uses mutagens to randomly generate mutations that induce or reverse a given phenotype, the latter analyzes the phenotype obtained after altering or knocking down a specific known gene. Forward genetics therefore goes from phenotype to gene, whereas reverse genetics works the other way around, that is, from gene to phenotype.

### 2.1.1 | Forward genetics

Mutations are important tools for gene function discovery. Mutagenesis can be accomplished using a variety of chemical agents, such as ethyl methanesulfonate (EMS), N-ethyl-N-nitrosourea (ENU), trimethylpsoralen (TMP), among others. Hermaphrodites (P0) are

**TABLE 2** Recent contributions in deciphering drug-targets and mechanism of action. Representative compounds for which the use of *C. elegans* has been useful in elucidating their mechanisms of action

| Compound | Field | Identified target/Mechanism of action | References |
|---|---|---|---|
| Resveratrol | Neurodegeneration | Reduces β-amyloid by targeting UBL-5 and XBP-1, proteins implicated in UPR$^{mt}$ and UPR$^{ER}$ | 90 |
| Minocycline | Aging | Increases lifespan by decreasing mARN translation | 91 |
| RPW-24 | Microbial infection | Stimulates innate immune response through pmk-1/p38 MAPK pathway, and the transcription factor, *atf-7* | 92 |
| Monepantel | Helminth infection | Targets ACR-20 and ACR-23 from the DEG-3 subgroup of nAChR subunits | 93 |
| Metformin | Cancer | Induces tumor growth inhibition and lifespan extension by targeting nuclear pore complex (NPC) and acyl-CoA dehydrogenase family member-10 (ACAD10) | 94 |
| Hesperidin | Metabolic disorders | Inhibits lipid accumulation by downregulating lipid metabolism genes (*fat-6 and fat-7*) | 95 |

Abbreviations: ACR, acetylcholine receptor subunit; atf-7, Cyclic AMP-dependent transcription factor 7; MAPK, mitogen-activated protein kinase; pmk-1, p38 MAPK; UBL-5, Ubiquitin-like protein 5; UPR$^{ER}$, unfolded protein response of the endoplasmic reticulum; UPR$^{mt}$, mitochondrial unfolded protein response; XBP-1, X-Box Binding Protein 1.

randomly mutagenized at the late L4/early adult stages and then distributed in Petri dishes. F1 hermaphrodite progeny that are heterozygous for these mutations can be allowed to self-fertilize. The F2 animals, which bring mutations to homozygosis, are isolated based on the phenotype of interest.[27] Several mutations generate clearly altered phenotypes, such as changes in development, lifespan, stress resistance, uncoordinated movement (unc), or dumpy-shaped animals (dpy). Today, mutated genes underlying a given phenotype can be identified by whole-genome sequencing.[100,101]

Another approach, which has proven to be very useful for identifying novel gene interactions, consists in performing mutagenesis directly in strains already carrying mutations that induce a strong phenotype.[102–104] Mutant animals are then mutagenized allowing enhancer or suppressor genes to be detected. For example, a suppressor screening recently performed on a hyperactive mutant in UNC-2, the worm ortholog for calcium voltage-gated channel subunit alpha 1A (CACNA1A) channel, has led to the identification of key modulators of channel function.[104]

Chemical genetic screening is another strategy which involves the use of drugs that induce a strong phenotype. After mutagenesis, which may affect the drug target gene or other genes involved in the same pathway, either resistant or hypersensitive mutants can be identified.[75,105–107]

In *C. elegans*, forward genetic screens have been traditionally used to identify molecular targets and MOAs for anthelmintic agents.[75–77,108] However, more recently, this approach has also proved to be useful for discovering targets and MOAs for drugs with potential relevance in metabolic, neurological, and oncological disorders.[68,94,109–112]

### 2.1.2 | Reverse genetics

In 1998, Fire and Mello found that injection of double-stranded RNA (dsRNA) into worm gonads allows gene knocking down in the progeny.[12] Since then, RNAi knockdown has become a powerful tool in other animals and cultured cells.[113–115] However, in *C. elegans* it can be easily achieved by feeding worms with bacteria expressing dsRNA or just soaking worms in dsRNA solution. Therefore, this technique has served for functional analyses of a plethora of genes in worms.

Genomic RNAi bacterial feeding libraries that cover most of the *C. elegans* genome have been generated,[116,117] avoiding the requirement of in vitro synthesis of dsRNA. *C. elegans*, one of the few organisms for which genome-wide RNAi screens are feasible, has been found to be a useful tool to identify genes involved in essential biological processes[2,118–120] and pathological conditions.[107,121,122]

A historical limitation of RNAi knock-down in *C. elegans* has been the poor penetrance of RNAi in neurons. However, several strategies have been developed to solve this caveat, namely strains expressing the transmembrane proteins required for RNAi transportation SID-1 pan-neuronally,[123] strains deficient in genes coding for ribonuclease enzymes,[124] neurons transformed to an immature and more permeable stage[125] or even a combination of them.[126]

In the last decade, several customized endonucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs),[127,128] have been used in genome editing. Other techniques, such as CRISPR/Cas9 and Mos-1 single copy insertion that allow the introduction of almost any change in any gene, have also been developed, further widening the tool repertoire to perform reverse genetics in the nematode.[129–131]

### 2.2 | Drug screening assays

Drug ingestion is probably the main way through which xenobiotic molecules can gain access to target tissues in wild-type *C. elegans*.[132,133] However, the amphids, a pair of anterior sensory structures opened to the outside environment, and the vulva are also involved in drug and nanoparticles intake, respectively.[134,135] Moreover, the use of animals that have a compromised cuticle can significantly increase drug absorption.[97] *C. elegans* is typically grown in the laboratory on petri-dishes containing NGM agar and *E. coli* as a food source. In the

past, during the first drug screens, compounds were generally dissolved in the agar.[14,136] Although this method has contributed to the elucidation of MOAs for several compounds previously proven to be biologically active,[137,138] the agar-based technique does not permit large scale screenings because it is labor- and time-consuming and needs large quantities of drugs.[14] Even with limitations, these assays are still very valuable for assessing small compound libraries.[66]

The introduction of *C. elegans* liquid cultures in multi-well format together with workflow automation from worm transfer to data analysis enabled the development of HTS assays.[139,140] Moreover, the breakthrough of microfluidic devices for *C. elegans* have improved the strength of HTS mainly thanks to the small amounts of compound (microscale) that are required and to the possibility of observing worms in parallel or in a serial manner.[141]

## 2.3 | Challenges of using *C. elegans* model in drug discovery

In spite of the obvious advantages of using *C. elegans* for drug screening, some caveats need to be considered. First, *C. elegans* cultures require bacterial co-culture as a food source. Many drugs and compounds are metabolized by bacteria and could alter drug efficacy. These limitations can be circumvented using killed bacteria as a food source.[142,143] However, feeding worms with dead bacteria can also affect some phenotypes (e.g., it slows worm development).[144] Therefore, the chosen food source will depend on the phenotype to be assessed, the compounds to be tested, and the bacteria species to be used for worm feeding.[142,145]

A second disadvantage is related to the thick *C. elegans* cuticle that usually affects drug uptake. Some studies revealed that internal tested-drug concentration could be less than half of that applied externally.[146] This implies that some drugs are discarded as hits due to their inefficacy to pass through the cuticle barrier and that some compounds need very high drug concentrations to produce a biological effect. To reduce these limitations, mutant strains with compromised cuticles have been used to enhance drug uptake.[97] The selection of these mutants depends on the trade-off between enhanced cuticle permeability and animal fitness.[97] Although *C. elegans* cuticle impermeability is indeed a limitation, it increases the potential significance of a positive hit in specific drug screening assays. In line with this, hits in anthelmintic search assays that induce death in *C. elegans* are extremely likely to kill parasitic nematodes (which in general have thinner cuticles) at lower concentrations.[68]

Finally, *C. elegans* lacks a circulatory system and many vital organs present in mammals. It has an innate immune system but lacks an adaptive immune system or a myelination system. This is the reason why it is difficult to model diseases affecting these organs/systems in *C. elegans*. In some cases, it is nonetheless possible to study a disease affecting a particular mammal organ nonexistent in the worm using phenologues. The latter are described as a group of overlapping genes defining a pathway that, when disrupted, cause different phenotypes in different species. For example, osteogenesis imperfecta (known as brittle bone disease) is caused by mutations in the human collagen COL1A1 and COL1A2 genes.[147] Although *C. elegans* lacks bones, it expresses collagen genes. When worm collagen genes are mutated, they lead to cuticle defects, easily showing recognized dumpy phenotypes.[148] Thus, the dumpy phenotype can be used to perform screenings to study collagen-associated diseases even in the absence of the affected tissue.

Additionally, even if a disease gene ortholog is not present in the worm, a disease model can be artificially generated by expressing human transgenes.[149,150] Transgenic animals are then used as tools to screen drugs or new genes involved in the disease phenotype with the goal of finding new therapeutic options. For example, mutations in α1-antitrypsin (AT) lead to lung and liver diseases.[151,152] Although *C. elegans* has no orthologs for AT, transgenic expression of human AT gene in worms can replicate in the animal intestine the abnormal misfolded protein accumulation observed in patients´ livers. A drug screen performed using this model allowed to detect drugs capable of decreasing misfolded protein accumulation.[35]

## 3 | RELEVANT *C. elegans* APPLICATIONS FOR DRUG TARGET DISCOVERIES

As illustrative examples of the use of *C. elegans* model in pharmacology research, we will discuss findings that show the advantages of using this nematode in the drug discovery field.

## 3.1 | Neurodegenerative diseases

As a result of the continuous increase in the proportion of the elderly population, age-related disorders have become a major health concern. Neurodegenerative diseases (NDs), in particular, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), are considered as top ten lethal illnesses (G7 Academies´Joint Statements 2017). Despite their growing incidence worldwide and decades of intense research, there is still neither a cure nor a set of effect-mitigation strategies for these diseases. Research efforts are currently aimed at precluding or—at least—delaying their progression. The discovery of curative treatments for the majority of these NDs remains elusive due to several factors. NDs are heterogeneous in etiology (while some of them have a genetic or hereditary origin, others have no related cause). They are also heterogeneous in the collection of neurons affected, leading to an assortment of clinical manifestations ranging from cognitive to progressive motor dysfunction.[153] They nonetheless share some pathological hallmarks. For example, aging is a strong risk factor related to their development.[154] Moreover, the abnormal formation and deposition of misfolded protein aggregates in specific neurons is a trademark of most NDs. Because of this attribute, these disorders are generally referred to as "proteinopathies." Therefore, current research is focused on the above-mentioned common features by targeting pathological aggregation or by delaying physiological aging.[155]

**TABLE 3** List of the main *C. elegans* models of ND, employed for the understanding of disease molecular mechanisms and for performing genetic and pharmacologic screenings

| Model of | Strategy | Expression | Transgene | Phenotype |
|---|---|---|---|---|
| HD | polyQ expression | Constitutive muscle | Punc-54::polyQ-yfp | Age-dependent aggregation and motility defects[82] |
| | | Constitutive pan-neuronal | Prgef-1::polyQ-yfp | Age-dependent aggregation and neuronal dysfunction like trashing and pharyngeal pumping[83] |
| | polyQ-HTT expression | Constitutive muscle | Punc-54::htt513Q128::yfp | Age-dependent aggregation and motility defects[183] |
| | | Chemosensory neurons | Posm-10::gfp+Posm-10::httQ150+dpy-20(+) | Accelerated ASH neuronal degeneration[184] |
| | | Mechanosensory neurons | Pmec-3::htt57Q128::cfp+Pmec3::yfp | Aggregated polyQ, morphological abnormalities and dysfunction of mechanosensory neurons[185] |
| AD | human Aβ expression | Constitutive muscle | Punc-54::$A\beta_{1-42}$ | Age-dependent aggregation and paralysis[178] |
| | | Temperature-induced muscle | smg-1(cc546);ls[Pmyo-3::$A\beta_{1-42}$::letUTR+rol6(su1006)] | Rapid paralysis by temperature upshift[186] |
| | | Constitutive pan-neuronal | Pmyo-2::yfp+Punc-119::$A\beta_{1-42}$ | Impaired locomotion and chemotaxis behavior[187] |
| | | Temperature-induced pan-neuronal | smg-1(cc546);ls[snb-1::$A\beta_{1-42}$+mtl-2::gfp] | Defective chemotaxis, formation of amyloid deposits, and serotonin hypersensitivity[188] |
| | human Tau expression | Constitutive pan-neuronal | Paex-3::h4R1NTau WT, V337M and P301L | Insoluble tau accumulation, neurodegeneration and uncoordinated movement (Unc)[189] |
| | | Single copy knock-in in mechanosensory neurons | Single copy human Tau mutants (T231E and K274/281Q) | Diminished touch response and neuronal morphological abnormalities[190] |
| PD | α-synuclein expression | Constitutive muscle | Punc-54::α-syn::yfp+unc-119(+) | Motility deficits and α-syn aggregation[5,159] |
| | | Constitutive pan-neuronal | Paex-3::α-syn (WT, A53T)+Paex-3::gfp/Pdat-1::gfp | Motility deficits, significant dopaminergic neuron loss[158,191] |
| | | Constitutive dopaminergic neurons | Pdat-1::α-syn (WT, A53T)+Pdat-1::gfp | Dopaminergic neuronal loss and dendritic breaks, α-syn accumulation[158,192] |
| | Neurotoxin-induced | Dopaminergic neurons | [Pdat-1::gfp] subject to 6-OHDA or MPTP | Morphological dopaminergic neuronal defects[193] |
| ALS | SOD-1 models | Temperature-induced muscle | Phsp-16.2::sod-1 (WT, A4V, G37R, G93A) + Pmyo-3::sod-1 (WT, A4V)::GFP +rol-6(su1006) | Increased sensitivity to oxidative stress, and oxidative stress induced- SOD-1 aggregation[194] |
| | | Constitutive muscle | Punc-54::sod=1(WT, G85R, G93A, G127insTGGstop)::yfp | Aggregation of mutant SOD-1 and motility defects[195] |
| | | Constitutive pan-neuronal | Psnb-1::sod-1(WT, G85R)::yfp | Aggregation of mutant SOD-1 and motility defects[196] |
| | | Single copy knock-in | Single copy knock-in expression of mutants forms of sod-1 (A4V, H7Y, L84V, G85R, G93A) | Aggregation of mutant SOD-1 and oxidative stress induced neurodegeneration[180] |
| | TPD-43 models | Constitutive GABAergic motor neurons | Psnb-1::tdp-43 (WT, G290A, A315T, M337V)+snb-1::gfp | Uncoordinated movement and GABAergic motor neuron degeneration[197,198] |
| | | Constitutive pan-neuronal | Punc-47::tpd-43 (WT, A315T) | Age-dependent motility defects and neuronal degeneration[199] |
| | FUS models | Constitutive pan-neuronal | Prgef-1::fus (WT, R514G, R521G, R522G, R524S, P525L) | Aggregation and paralysis[200] |
| | | Single copy knock-in | Single-copy knock-in of R524S and P525L equivalent mutations into fust-1 (FUS orthologous gene) | Impaired neuronal and muscular autophagy[181] |

Abbreviations: ALS, Amyotrophic lateral sclerosis; a-syn, a-synuclein; FUS, RNA-binding protein Fused in Sarcoma; HTT, huntingtin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine;6-OHDA, 6-hydroxydopamine; polyQ, polyglutamine; SOD-1, superoxide dismutase 1; TPD-43, Transactive response DNA-binding protein-43.

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In this respect, *C. elegans* is a powerful in vivo model for the development of targeted therapeutics. Most of the advantages that convert this animal into a superb candidate for drug discovery have already been mentioned in this review and are also applicable to the neurodegeneration research field. Still, there are some topics that deserve special attention. Features that confer *C. elegans* an extra value as a model for age-related proteinopathies research include, among others: (i) the remarkable similarities at the molecular and cellular levels between nematode and vertebrate neurons, (ii) the continuous design of mutant and transgenic *C. elegans* models of human NDs,[82,156–161] (iii) the thoroughly studied whole-animal neuronal connectome which can be individually visualized using fluorescent reporters in the living transparent worm,[11,21,22,162] (iv) its short and genetically tractable lifespan,[7,163–166] and (v) the recently developed high-throughput (even automatized) drug screening platforms.[35,54,167–173] However, the limitations on using a "humanized" worm model for neurodegenerative proteinopathies should be taken into account, particularly its simple nervous system, glia and lack of myelin. Furthermore, genes encoding for voltage-gated sodium channels are absent in the *C. elegans* genome.[174] Unlike vertebrates, the action potentials detected in muscles and neurons of *C. elegans* depend on voltage-gated calcium channels.[175–177] Despite these caveats, *C. elegans* is an excellent whole-animal platform to identify genes, drug targets, and compounds with neuroprotective roles in human NDs.

### 3.1.1 | *C. elegans* models for ND

The first *C. elegans* model of a human ND was built 25 years ago,[178] and, since then, an extensive list of animal models for most of the NDs have been generated (Table 3). The methods that are regularly used include knocking-down or -out the homologous gene involved in the human disease if it is present in the worm genome or, if there is no orthologous, the method of choice involves the transgenic expression of the human gene to mimic a disease-related phenotype. Most of the *C. elegans* models of human NDs are transgenic strains developed by overexpression of human wild-type or disease-associated mutant genes. However, overexpressing genes, even as wild-type, sometimes results in an artificial condition that can be pathogenic. CRISP/Cas9 technology has been fortunately established in *C. elegans* [179] and recent single-copy knock-in models of some NDs have been developed (Table 3).[180–182]

Since *C. elegans* has no orthologous gene for β-amyloid (Aβ), α-synuclein, or huntingtin, models of NDs have been essentially produced by transgenic overexpression of the disease causing-protein in either body-wall muscle cells, in all neurons or in a specific subset of neurons.[82,83,150,158,178,195,197,201] In most cases, the protein is tagged with GFP, which permits monitoring protein aggregation in whole live animals. Furthermore, according to the promoter used, that is, either muscle or neuronal promoters, toxic protein aggregation is reflected by specific pronounced phenotypes which

are indispensable for high-throughput screenings. For instance, muscle-expressing strains usually manifest proteotoxicity as age-dependent locomotion defects. In addition, muscle cells are large, allowing the visualization of protein aggregation more easily than in neurons. Moreover, RNAi is more efficient in muscles than in neurons, making these models more amenable for RNAi screens. On the other hand, transgenic strains with neuronal expression of protein aggregates are more accurate models of NDs. In the latter, proteotoxicity can be assessed by means of indirect phenotypes, such as neurodegeneration or specific morphological and/or behavioral neuronal defects. Thus, according to the selected strain, different read-outs have been described, which can be used in HTS with the aim to evaluate genes and drug modifiers as potential therapeutic interventions.

Genetic and pharmacological screenings have been performed in these models by tracking protein aggregation and assessing a simple phenotype (such as locomotion or neuronal behavior defects) in whole-live animals. In fact, large RNAi-mediated reverse screens, performed in *C. elegans* models of NDs, have identified gene modifiers of protein homeostasis, including genes with roles in protein synthesis, folding, degradation, and vesicle trafficking.[3,5,159,202–205] Forward mutagenesis genetic screens have also been developed in these models. For example, a study using a *C. elegans* model of HD identified a novel gene, called modifier of aggregation 4 (*moag-4*) as an enhancer of protein aggregation.[206] Pharmacological screenings have also contributed with hundreds of neuroprotective compounds, classified in a wide range of categories, including natural and synthetic products, herbal medicines, and also FDA-approved drugs (Table 1). These findings highlight the strength of *C. elegans* in finding novel genetic and drug modifiers for human NDs, reinforcing its potential in preclinical drug discovery.

Despite the complexity of the pathology underlying NDs, most of the pathways involved are conserved or they can be mimicked in the worm. The aim of using *C. elegans* as a platform of NDs is to dig inside molecular mechanisms to unravel pathological features and to develop effective strategies to treat these diseases.

## 3.2 | *C. elegans* as model of parasite nematodes

Parasitic nematodes infect a wide range of species, including humans, companion animals, livestock, and crops producing a devastating impact on human life quality and economy. The World Health Organization (WHO) claims that helminth infections are the most common neglected tropical diseases and estimates that 30% of the human world population is infected with at least one parasite (https://www.who.int/neglected_diseases/en/). This prevalence could be even higher in rural areas and in low-income countries. Although helminth infections are, in general, not lethal, human helminthiases are associated with morbidity. The consequences are particularly serious in children, impairing growth, nutrition, cognition, and school performance.

Several issues are of concern in relation to helminthiasis management and treatment, namely (i) the loss of effectiveness caused by parasite resistance, (ii) the environmental impact of drugs used for crop protection, and (iii) the lack of interest in this field by the pharmaceutical industry.

The development of novel pharmacological agents for helminthiasis treatment has been delayed for decades and the repertoire of available anthelmintics is limited.[77,207,208] Since drug-resistant parasitic nematodes have been reported for all classes of currently used anthelmintics,[209–213] there is an urgent need to advance in pharmacological research to develop new antiparasitic drugs.

Due to their complex life cycles, growing and maintaining parasitic nematodes under standard laboratory conditions are both challenging. The limited molecular genetic tools available for these nematodes hampers the study of molecular mechanisms.[214] It has been shown that compounds that induce C. elegans death likely also kill parasitic nematodes,[68] highlighting the potential of this worm as an anthelmintic screening platform (Table 1).[39,215,216]

## 3.2.1 | Current use of C. elegans in anthelmintic drug discovery

In the past, C. elegans was used to dissect the target pathways and molecular mechanisms of known anthelmintics, such as levamisole, ivermectin, benzimidazoles, nitazoxanide, and amino-acetonitrile derivatives.[75,77,78,108,217–219] Nowadays, it is a recognized platform to screen new compounds and drug repurposing[220] that must be subsequently tested in parasites. Many of the currently used anthelmintics are imidazole derivatives.[221] In general, the mechanisms underlying the anthelmintic effect can induce worm death or generate paralysis to facilitate parasite expulsion. The anthelmintic mechanisms of imidazole-derivatives are diverse. For example, levamisole causes nematode spastic paralysis through the potent activation of a muscle nicotinic receptor (AChR)[218,222–225] whereas the anthelmintic action of benzimidazoles (e.g., albendazole and mebendazole) arises from their capacity to block tubulin polymerization in nematode cells.[78] Thus, while the presence of the imidazole ring appears to be important for bioactivity, it does not restrict the molecular targets where imidazole-containing anthelmintics can act. Recently, taking advantage of C. elegans as an established model for parasitic nematodes, we screened the nematicidal potential of novel imidazolium and imidazole derivatives.[66] We identified a new compound, diisopropylphenyl-imidazole (DII), that is lethal to C. elegans through a novel mode of action that includes differential targeting in larvae and adult nematodes. This lethal effect appears to be specific for nematodes because at DII concentrations, proven to be toxic to C. elegans, no significant lethality on bacteria, Drosophila melanogaster and HEK-293 cells has been detected. Using C. elegans mutant and transgenic strains, we found that DII effects on adult nematodes rely on a previously unidentified UNC-29-containing muscle AChR, different from the classical Levamisole-sensitive AChR. Interestingly, DII targets appear to be different between larvae and adults as unc-29 null mutant

larvae are sensitive to the drug.[66] Summing up, using the model C. elegans we demonstrated that DII fulfills the major criteria necessary for the development of a novel anthelmintic, namely phylogenetic specificity and a novel biochemical mode of action. The next step will be to expose parasite worms to this already characterized drug.

Other researchers performed a screen of a small-molecule library using C. elegans to test the anthelmintic activity of FDA-approved drugs with the aim of repurposing their clinical activity.[226] The availability of data on their toxicity and pharmacokinetic characteristics will expedite its potential use for new therapeutic indications. From this screen, they found that the neuromodulatory drugs sertraline, paroxetine, and chlorpromazine kill C. elegans at multiple life stages and inhibit worm feeding. C. elegans mutants with resistance to known anthelmintic drugs are as sensitive as wild-type worms to these three drugs, suggesting that they may act through novel targets. They also demonstrated that these drugs affect divergent parasitic helminth species, such as Trichuris muris and Ancylostoma caninum and the trematode Schistosoma mansoni. These researchers therefore conclude that these drugs may represent a new class of anthelmintic drugs that could be used in combination with classic anthelmintics to boost effectiveness as well as to avoid parasite drug resistance.

As a result of the limited repertoire of available anthelmintics and the alarming emergence of anthelmintic resistance, there is an urgent need to develop novel anthelmintics. C. elegans becomes a useful platform to accelerate the screening process for anthelmintic drug discovery. The use of C. elegans can permit the development of new parasite control strategies by identifying new drugs with better and broad-spectrum, repurpose already approved drugs, and combine different therapeutic options.

## 4 | CONCLUSIONS

Traditional preclinical drug discovery is a complex process that takes more than 10 years. Today, innovative strategies are required to satisfy both the increasing demand for better and more efficient therapies and the need to save resources and time for drug development. Under this scenario, the use of invertebrate animals, such as the nematode C. elegans, during the early phases of drug development becomes a very convenient strategy to achieve these two aims. Since the introduction of C. elegans into research studies, this nematode has been extensively used to understand fundamental biological processes. During the last years, C. elegans has also become an invaluable tool for the screening of compounds with potential therapeutic uses. This nematode is probably the best cost-effective choice for early target validation scheme among animal models. Primary hits could be identified in C elegans as an initial filter. The efficacy and safety information gathered by the use of this nematode, significantly reduce costs, time, and the number of vertebrate animals required.

In this review, we have outlined several attributes that highlight the strength of C. elegans as a model for drug screening, drug repurposing, drug target identification, drug combination,

and molecular drug mechanism unraveling. The advances in massive and automated high-throughput drug discovery assays for *C. elegans*, the amenability of genetic manipulation, and the extended information available on gene structures, mutant and RNAi phenotypes, microarray data and protein–protein interactions (Wormbase) should be further exploited to facilitate the development of new drugs. The variety of *C. elegans* disease models now available in the drug discovery pipeline does secure a significant boost to our understanding of human diseases and accelerates the reach of effective disease treatments.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

## AUTHOR CONTRIBUTIONS

All authors were involved in manuscript preparation for this project.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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