

The *C. elegans* model in toxicity testing

Piper Reid Hunt*

ABSTRACT: *Caenorhabditis elegans* is a small nematode that can be maintained at low cost and handled using standard *in vitro* techniques. Unlike toxicity testing using cell cultures, *C. elegans* toxicity assays provide data from a whole animal with intact and metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems. Toxicity ranking screens in *C. elegans* have repeatedly been shown to be as predictive of rat LD₅₀ ranking as mouse LD₅₀ ranking. Additionally, many instances of conservation of mode of toxic action have been noted between *C. elegans* and mammals. These consistent correlations make the case for inclusion of *C. elegans* assays in early safety testing and as one component in tiered or integrated toxicity testing strategies, but do not indicate that nematodes alone can replace data from mammals for hazard evaluation. As with cell cultures, good *C. elegans* culture practice (GCeCP) is essential for reliable results. This article reviews *C. elegans* use in various toxicity assays, the *C. elegans* model's strengths and limitations for use in predictive toxicology, and GCeCP. Published 2016. This article is a U.S. Government work and is in the public domain in the USA. *Journal of Applied Toxicology* published by John Wiley & Sons Ltd.

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Introduction

Much of our knowledge within the field of biology is based on scientific experimentation using *in vivo* and *in vitro* models. Toxicity testing is done with the expectation that information acquired in a particular model will apply to other biological systems, with each model presenting strengths and limitations depending on the information required. Mammalian laboratory animals share similar developmental pathways and most organs with humans, making toxicity testing in mammals the current 'gold standard' in toxicology. However, no model is perfect, and even human trials do not always predict outcomes in the population at large.

Toxicity studies using mammalian models are expensive and time-consuming (Nass and Hamza, 2007; Tralau *et al.*, 2012), and meta-analyses indicate that rodent models predict specific toxic effects in humans only about 50% of the time (Hartung, 2009; Knight *et al.*, 2009; Olson *et al.*, 2000). Using more than one mammalian species can increase predictivity (Olson *et al.*, 2000) but will also increase cost and decrease throughput. Predictive toxicology seeks to use alternative methods to improve prediction of human outcomes while reducing the cost, time and use of mammals in toxicity assessments. Chemicals of concern can be tested far more rapidly and at a much larger range of concentrations if *in vitro* assays are used to assess perturbations in toxicity pathways. While the use of primary human cells does have the potential to more accurately reflect human-specific metabolism and modes of action than testing in lab animals (Li *et al.*, 1999; Miranda *et al.*, 2009; Scott *et al.*, 2013), results cannot be used to predict a response at the organismal level using current techniques. Additionally, testing using immortalized cell lines can have high rates of false positives (Kirkland *et al.*, 2005; Bhattacharya *et al.*, 2011; Pfuhrer *et al.*, 2010) or false-negatives (Knight *et al.*, 2009) depending on the assay type. Thus, the use of *in vitro* tests alone for hazard assessment gives rise to the possibility that compounds that are harmless *in vivo* will be unnecessarily restricted and that harmful compounds will be incorrectly presumed to be safe.

Another option is to utilize a small model organism such as the nematode *Caenorhabditis elegans*, which can be handled using *in vitro* techniques. Unlike *in vitro* testing, *C. elegans* toxicity assays

provide data from a whole animal with intact and metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems (Fig. 1). As government-sponsored efforts to improve toxicity screening and predictive toxicology progress (Tice *et al.*, 2013; Casey *et al.*, 2015), we may eventually find that testing strategies which use multiple types of *in silico*, *in vitro* and non-mammalian small animal model-based assays together has the potential to inform risk evaluation as well or better than *in vivo* toxicity studies using mammals, but much more work is still required (Krewski *et al.*, 2009; Hartung *et al.*, 2013). For now, mammalian models will continue to be used to predict safe human exposure levels because no combination of current alternative assays can replicate the complexity of interacting metabolism, homeostasis and signaling mechanisms that are present in mammals (Tice *et al.*, 2013). However, given the high percentage of commercially available chemicals for which there is little or no toxicity data available (Dix *et al.*, 2007; Judson *et al.*, 2009), toxicity screens that can at least flag for further study those chemicals with the most potential for harm are urgently needed. As an intermediate between *in vitro* and mammalian testing, toxin ranking using various *C. elegans* assays has consistently predicted toxicity ranking in mammals.

Caenorhabditis elegans

C. elegans is a nematode that feeds on fungi and bacteria in soil and rotting fruit. At a bit over 1 mm long, adults are just visible by eye. Since Sydney Brenner's initial characterization of the model in the 1960's, *C. elegans* research has been essential in the

*Correspondence to: Piper Reid Hunt, Division of Applied Toxicology, Mod.1, room 2013, 8301 Muirkirk Road, Laurel, MD 20708-2476, USA.
E-mail: piper.hunt@fda.hhs.gov

Division of Applied Toxicology, Laurel, MD, USA

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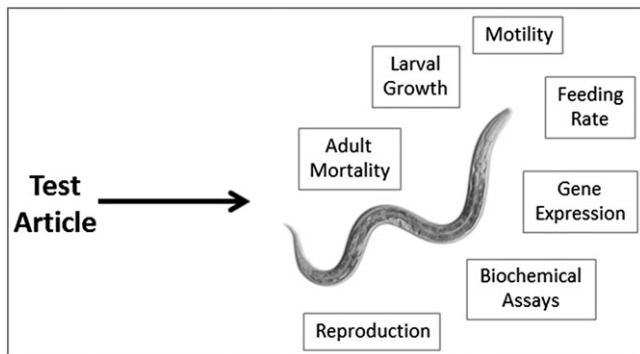


Figure 1. Toxicity testing in *C. elegans* can provide a bridge between *in vitro* and mammalian *in vivo* testing.

elucidation of several basic aspects of biology, including apoptosis, RNA interference, and miRNA function. The majority of these studies were carried out using *E. coli* as a feeder organism. For toxicology purposes however, the use of axenic media is preferred to avoid confounding issues of xenobiotic metabolism. In the lab, *C. elegans* small size means that thousands of animals can be maintained in nutrient media in multi-well plates, so studies assessing multiple compounds or mixtures at a wide range of concentrations can be carried out in a small space. With a reproductive capacity of about 300 progeny per hermaphrodite adult by self-fertilization, and a life cycle of approximately 3 days, millions of animals can be rapidly generated, and most experiments can be completed by one person in a week or less. *C. elegans* has a tough but transparent cuticle, which allows for visualization of internal structures without dissection and facilitates tracking of organellar dyes and structure-specific gene expression in transgenic strains. Importantly, *C. elegans* is non-hazardous to lab workers, and does not reproduce at temperatures above 25°C.

C. elegans somatic cell locations and lineages as well as neural networks have been mapped (White *et al.*, 1986), allowing morphological assessments of toxin induced abnormalities and in depth neurological/behavior correlations. Given that (a) *C. elegans* was the first multicellular organism to have its genome completely sequenced (*C. elegans* Sequencing Consortium, 1998), (b) genes and signaling pathways are well conserved between *C. elegans* and humans (Kaletta and Hengartner, 2006; Leung *et al.*, 2008), (c) studies to understand *C. elegans* genetics have been underway for over 40 years (Brenner, 1974; Corsi *et al.*, 2015; Nass and Hamza, 2007), and (d) mutant and transgenic *C. elegans* strains are readily available for many many genes, this model has great potential for the assessment of human-relevant pathways of toxicity.

Toxicity screening in *C. elegans*

Studies designed to rank toxicity in *C. elegans* have consistently shown good correlation with rodent oral LD₅₀ ranking. In an early ranking study, using *C. elegans* maintained on plates with test articles dissolved in agar, it was found that the toxicity order for eight metal salts based on *C. elegans* adult mortality correlated with rat and mouse oral LD₅₀ ranking at one-tenth the cost of rodent testing (Williams and Dusenbery, 1988). The authors also demonstrated that that LC₅₀ ranking in *C. elegans* was as predictive of acute toxicity in mammals, other than rat and mouse, as LD₅₀ ranking in rat or mouse (Williams and Dusenbery, 1988). In this first assessment comparing LC₅₀ ranking in *C. elegans* to LD₅₀ ranking in mammals, it

was noted that LC₅₀s in *C. elegans* were high when compared to corresponding mammalian LD₅₀s (Williams and Dusenbery, 1988). It was the relative order of toxicity that correlated rather than the concentration, as subsequent studies have also found. This is probably due to robust genetic mechanisms that allow *C. elegans* to exist and reproduce in a wide variety of harsh environments.

The Complex Object Parametric Analyzer and Sorter™ (COPAS; Union Biometrica, Holliston, MA, USA) uses microfluidics and laser-based technologies to automate the analysis of multiple endpoints on hundreds of *C. elegans* per minute. In my lab, using a novel COPAS-based assay to assess the fraction of live *C. elegans* in dosed populations, we also found that LC₅₀ ranking in *C. elegans* matched LD₅₀ ranking in rat for five metal salts (Hunt *et al.*, 2012). These two studies used very different techniques (agar plates vs. axenic liquid media, manual manipulation with microscopy inspection vs. automated assessment), but in both *C. elegans* ranking for lethality predicted the relative rodent toxicity for metal salts.

The COPAS has also been used to assess larval growth and reproductive output, and studies assessing six or seven water-soluble compounds have shown that ranking for these endpoints in *C. elegans* also correlates with rodent LD₅₀ ranking for the same compounds (Boyd *et al.*, 2010; Ferguson *et al.*, 2010). In a much larger, COPAS-based study of hundreds of compounds from the ToxCast™ libraries, *C. elegans* larval growth identified rabbit or rat developmental toxins with a balanced accuracy of 52–53%, whereas the concordance for developmental toxicity between rat and rabbit was 58% (Boyd *et al.*, 2016). These levels of predictivity are consistent with an earlier meta-study which found that a single-species rodent study alone predicted human toxicity less than 50% of the time and that using one rodent and one non-rodent mammalian species together, predictivity increased to 71% (Olson *et al.*, 2000). Unlike a study in mammals, however, a small-scale *C. elegans* larval growth assay can be conducted by a single technician in less than a week with an incubator, a basic microscope and a few simple tools. Interestingly, the sensitivity of the larval growth assay for mammalian developmental toxins was high, but the balanced accuracy was brought down by low specificity (Boyd *et al.*, 2016). In contrast, in a recent study evaluating *C. elegans* egg viability using 72 compounds of known developmental activity in mammals, the specificity of the egg viability test was high whereas the sensitivity was low (Harlow *et al.*, 2016). This begs the question of how the *C. elegans* larval growth and egg viability assays might be used together, or perhaps in conjunction with mammalian cell based *in vitro* assays, to better detect mammalian developmental toxins.

In a screen of 21 chemicals in which *C. elegans* were individually assessed by eye for viability, LC₅₀ ranking in *C. elegans* and LD₅₀ ranking in mouse predicted rat LD₅₀ ranking equally well, but only when the four compounds that reduced pH to below 3.2 were excluded (Li *et al.*, 2013). In another study using motility as an endpoint to assess 15 organophosphate pesticides and herbicides, the ranking of EC₅₀ values in *C. elegans* was statistically consistent with the order of rat LD₅₀s (Cole *et al.*, 2004). However, for the two organophosphates that reduced pH to below 3.5, altered motility tracked more consistently with pH than the test article concentration. In this same study, five out of six organophosphate insecticides tested, which were known to inhibit cholinesterase in mammals, also inhibited cholinesterase activity in *C. elegans*, indicating conservation of the toxicity mechanism (Cole *et al.*, 2004). While factors such as pH and solubility limit *C. elegans* toxicity studies, they are also limitations for *in vitro* and zebrafish embryo testing.

In a study comparing the *C. elegans* endpoints of lethality vs. reproduction for five metabolic poisons, reproduction was suppressed at far lower concentrations than that required for lethality (Middendorf and Dusenbery, 1993). The authors of this study did not compare their data to toxicity in mammals, yet an analysis of the published results relative to rat oral LD_{50s} shows that for the five metabolic poisons tested, *C. elegans* reproductive EC₅₀ ranking differed from rat ranking only in that the toxins ranked two and three are inverted between the two species. In this study (Middendorf and Dusenbery, 1993), *C. elegans* LC₅₀ ranking did not correlate with the mammalian data as well as reproductive EC₅₀ ranking, indicating that some endpoints may detect certain categories of toxins better than others, and that in testing unknowns, the assessment of multiple *C. elegans* endpoints will probably increase the sensitivity of a screen. Blinded validation studies comparing the success of specific *C. elegans* assays at detecting various types of toxins have not yet been conducted, however. It is likely that rather than relying strictly on ranking, combining the results from a few different *C. elegans* assay endpoints into broader categories of nontoxic, mildly toxic, toxic, and very toxic will be more useful for tiered testing and predictive toxicology.

In addition to ranking studies, targeted *C. elegans* screens have also been successful in detecting specific types of toxins. One example is a recently developed elegant assay for the detection of aneugens, toxins that induce alterations in the number of chromosomes per cell. Self-fertilizing *C. elegans* XX hermaphrodites normally make up over 99.8% of the wild-type population, whereas rare XO males arise spontaneously by chromosomal non-disjunction. Using a male-specific promoter to drive green fluorescent protein expression, transgenic *C. elegans* males will fluoresce. When this transgenic strain was exposed to chemicals and assessed for the number of male offspring, seven of eight known mammalian aneugens significantly increased the number of XO male progeny, whereas five known non-aneugens tested negative (Allard *et al.*, 2013). Cadmium is known to induce intestinal pathology in mammals and fish at a low concentration. In a test of four metal chlorides on adult *C. elegans* morphology in my lab, we found that at low concentrations which did not alter viability or other evaluated physical parameters, only cadmium reduced intestinal diameter and opacity, a change that was measurable both by light microscopy and by COPAS (Hunt *et al.*, 2012). This indicates a conserved mode of action for cadmium and the possibility that other intestinal toxins can be identified in *C. elegans* using higher throughput microfluidics techniques.

Predictive toxicology using *C. elegans*: strengths, limitations and GCeCP

Caenorhabditis elegans can be used to predict nanosilver (AgNP) toxicity in other models. In orally exposed *C. elegans* and rats, ionic silver is more toxic and is taken up into tissues more extensively than 10-nm citrate-coated silver nanospheres (Cit-AgNP) (Hunt *et al.*, 2013). Polyvinylpyrrolidone (PVP)-coated AgNP are associated with higher silver uptake and higher toxicity relative to Cit-AgNP in *C. elegans* (Hunt *et al.*, 2014) and zebrafish embryos (Kim *et al.*, 2013), whereas in a subsequent study of orally exposed mice, no nanosilver toxicity was observed at study dosing levels, but there was a trend of greater intestinal uptake for PVP-AgNP over Cit-AgNP (Bergin *et al.*, 2016). In my lab, we have quickly identified toxicity in specific batches of nanomaterials that had been carefully evaluated for quality and consistency by the synthesizing

lab. In one instance, the AgNP producer later identified endotoxin contamination in one of their supplied reagents (Hunt *et al.*, 2013), and in another, a switch from centrifugation-based nanomaterial wash steps to the use of filters for washing led to the retention of unbound, positively charged coat material that should have been removed from the final product (Hunt *et al.*, 2014). Had these batches been tested directly in mammals, toxicity might have been attributed to the nanomaterial itself rather than a contaminant. This underscores both the importance of assessing all component materials plus the soluble fraction as controls in nanomaterial toxicity testing, and the utility of *C. elegans* assays which are rapid and inexpensive enough to be used on each batch of nanomaterial produced.

Given the success of the studies highlighted here, it is interesting to speculate why *C. elegans* is not more widely used in toxicity screening. It may be that the majority of researchers specializing in studying *C. elegans* are more focused on genetics, development and cell signaling than on toxicity screening, whereas most toxicologists and risk assessors are simply unfamiliar with the model. Certainly, many people, including those on scientific funding committees, mistakenly believe that worms are slimy things with no connection to humans. However, the high conservation of genes and signaling pathways between *C. elegans* and mammals (Kaletta and Hengartner, 2006, Leung *et al.*, 2008) indicate that the model is likely to continue to do well when used to predict broad measures of toxicity, if good *C. elegans* culture practice (GCeCP) is followed.

Just as good cell culture practice is essential for reliability and reproducibility in *in vitro* studies (Hartung *et al.*, 2002), *C. elegans* used in toxicity testing must be maintained in a highly consistent manner, with temperature, salt concentration and sufficient nutrient supply held constant to ensure reliable, repeatable results (Table 1). Alterations of even a short duration in any of these variables will lead to altered gene expression and toxin response, potentially for multiple generations. Even the brief contact of a human finger applied to a row of wells for a few seconds by picking up a multi-well plate from the bottom can alter the growth rate of the larvae in those wells.

Males normally make up 0.2% of the *C. elegans* population, although males can be induced by exposure to elevated temperatures or toxins (Corsi *et al.*, 2015). Self-fertilization maintains the population as nearly all hermaphrodites, while mating produces half male and half hermaphrodite progeny. *Caenorhabditis elegans* males are smaller than the hermaphrodites, and mating produces more offspring than self-fertilization. Thus the introduction of males into a culture will skew larval growth and reproductive assays in opposite directions. Additionally, *C. elegans* have an alternative developmental stage called dauer larva that can be induced by stressful conditions such as low nutrient availability and some toxins. *Caenorhabditis elegans* exposed to dauer pheromone have higher levels of stress resistance gene expression and are more likely to become dauers themselves. In the dauer state, the cuticle grows over the buccal and anal openings, making dauers resistant to harsh environments. This makes dauer formation a likely explanation for many published *C. elegans* studies that beautifully demonstrate a mechanism of action or pathway of toxicity, but fail to show a dose response beyond a threshold. Thus, in contrast to some well-established genetics and cell signaling protocols which utilize dauers and males, *C. elegans* cultures containing dauers or males should not be used for standard toxicity assays.

The differences in morphology among hermaphrodites, dauers and males are subtle and likely to go unrecognized without

Table 1. *C. elegans* culture standardization factors for toxicology and GCeCP

Factor	Details
Temperature	Small temperature differences have a large effect on <i>C. elegans</i> growth rate, motility, lifecycle, lifespan, and gene expression. The method and duration of vessel contact with human hands and metal surfaces can significantly affect many endpoints. Handling culture vessels by edges that are not in contact with medium, and a layer of Styrofoam on work surfaces will reduce heat exchange.
Humidity	Low humidity will alter test article and nutrient concentration, the smaller the volume the larger the effect. Unless equipment is enclosed and carefully climate controlled, it is unlikely that the small volumes used in HTS will work well with <i>C. elegans</i> assays. For larger test volumes, incubators can be humidified with an open vessel of water that is cleaned and refilled regularly.
pH	Extreme pH is required to alter adult <i>C. elegans</i> viability, but other endpoints are more sensitive to pH. The appropriate pH range should be determined for each assay, and the pH of test articles in assay medium must be assessed and reported.
Worm Density	<i>C. elegans</i> gene expression and life cycle respond to nutrient availability and secreted hormones. Given a 3-day generation time and ~300 progeny per worm, cultures can easily outstrip nutrients if they are not consistently monitored. Conversely, <i>C. elegans</i> do not grow well if maintained too sparsely. Note that the worms will not necessarily die in these conditions, instead they will adapt epigenetically (Hall <i>et al.</i> , 2010), potentially resulting in altered toxicity test results for several generations.
Cohort Synchronization	A cohort of 1 st larval stage (L1) <i>C. elegans</i> can be isolated by hypochlorite treatment of gravid hermaphrodites (an egg prep) followed by hatching of the released eggs in non-nutrient buffer. In the absence of nutrients, these L1s halt development just after hatching. At 20 °C, about 12 hours are required for all the eggs to hatch. After more than 18 hours in buffer, gene expression is altered resulting in delayed and unsynchronized development, and increased stress resistance (Jobson <i>et al.</i> , 2015, Nass and Hamza, 2007). Some genetics protocols state that hatched L1s can be maintained in non-nutrient buffer and used for a week or more, but this will result in variable toxicity outcomes.
Dauers	The <i>C. elegans</i> dauer larva is a stress resistant, long-lived alternate to the 3 rd larval stage (L3). Dauers must revert to the normal lifecycle in order to grow and reproduce. <i>C. elegans</i> dauers secrete dauer pheromone, which promotes conversion to the dauer state in other larvae and induces increased stress resistance in exposed adults. This will both reduce apparent growth rates as measured by worm length (dauers can remain at the L3 length for months or even years), and increase viability in the presence of many toxins. Dauers are thinner and darker than L3s of similar length, and lack the clearly defined gonadal region and visible intestinal lumen identifiable in developing <i>C. elegans</i> . Liquid cultures are unlikely to produce dauers if they are consistently maintained with adequate nutrient supply and are started from agarose cultures that were well fed for at least 3 generations. Daily media exchange along with a few sequential egg preps as soon as each generation becomes gravid can sometimes free a culture of dauers.
Genetic Drift	Genetics labs often maintain commonly used <i>C. elegans</i> strains at room temperature as dauers. If <i>C. elegans</i> cultures are consistently well fed for optimal toxicity studies, use of frozen stocks must be scheduled in order to prevent genetic drift.
Males	Non-disjunction of the X chromosome results in XO males. This happens rarely in nature, and is induced by toxins and stress. <i>C. elegans</i> males can be identified by their flared tail and single gonad arm. Males are smaller than the XX hermaphrodites, which will result in apparent reduced growth if automated methods are used and technicians are not trained to recognize males. Mating with males more than doubles the progeny per hermaphrodite relative to selfing, so males in a culture will increase reproductive output. Removing males from a culture requires isolating developing hermaphrodites away from the males.
Solid vs. Liquid Medium	When the test article is mixed into molten agar, or spread in solution onto solidified agar, and then the <i>E. coli</i> feeder organism is grown in a lawn on top of the dosed agar, the true exposure will depend on many factors such as humidity, compound solubility, compound-agar interaction, and feeder organism uptake and metabolism. Dosing in liquid medium provides a measureable exposure, but limits the test to water-soluble compounds.
<i>E. coli</i> vs. Axenic Medium	Axenic medium avoids the complicating factor of the metabolic response of the feeder organism, which is especially important for test articles with antibiotic activity. Killed <i>E. coli</i> are sometimes used (usually heat or UV), but the method and exposure time must be carefully controlled to avoid the bacteria producing toxins which will alter assay results.

training. This training is quickly and easily acquired, however. It is not necessary to check every worm in a start culture for phenotype, because if dauers or males are present, they will likely make up a significant portion of the population within a week. A scan of a few microscopy fields of view per flask is sufficient, with the caveat that if males or dauers are identified, assays completed with that culture within the past week are suspect. Once consistent handling and workflow for GCeCP is established, a single *C. elegans* technician, using just a few pieces of equipment, can assess a dozen compounds or mixtures at multiple concentrations in a week. While this type of medium-throughput screening cannot replace a descriptive toxicology study in lab mammals, given the massive backlog of compounds in consumer products and in the environment that have received no toxicological assessment at all (Tice *et al.*, 2013), it makes sense to utilize a small model organism such as *C. elegans* as one component within a battery of tests to help prioritize compounds of unknown toxicity for further assessment.

Some companies and government agencies are beginning to utilize *C. elegans* for rapid toxicity evaluations. For example, in response to the 2014 West Virginia Elk River chemical spill, the National Toxicology Program examined the effects of the major constituents of the spill using several toxicity assessment models. No effects were detected for the 4 most prevalent spill compounds in a panel of 27 different *in vitro* assays (NTP, 2015). Similarly, 12 different compounds and mixtures identified in the spill had no effects on growth, pharyngeal pumping or reproduction in *C. elegans* (NTP, 2015). While mild dermal irritation and sensitization was detected in mice, and prenatal developmental toxicity was detected in rats, doses were high, and rat studies are planned to assess the effects of lower doses that are more relevant to likely human exposures (NTP, 2015). This set of experiments demonstrates some of the strengths and weaknesses of the *C. elegans* model. In *C. elegans*, 12 compounds and mixtures were assessed in a quick study that evaluated three endpoints, whereas a maximum of 3 compounds and mixtures were assessed in the more lengthy and costly mouse and rat assays. The *C. elegans* data were in accord with the *in vitro* assessments indicating no deleterious biological activity at tested concentrations, but did not predict mouse dermal irritation response at high concentration, nor a rat reproductive response to high oral gavage doses that corresponded to over 20 g for a 70-kg person (NTP, 2015). Thus, the *C. elegans* model predicted the absence of general acute toxicity for the spill components at lower doses but failed to detect specific high-dose mammalian toxic outcomes.

In theory, *C. elegans* ease of culture, small size and ability to grow in axenic liquid media indicate that the model would lend itself to the type of high-throughput screening (HTS) now in use by multi-agency efforts to modernize toxicity testing such as Tox21 (Tice *et al.*, 2013). Current HTS equipment may not have the level of temperature, humidity and sterility control that reliable *C. elegans* testing requires, however. In the very small volumes that are used in HTS, small changes in ambient humidity can dramatically alter test article, nutrient and salt concentrations, which will in turn alter assessed endpoints. Additionally, because *C. elegans* are highly responsive to their environment, small temperature differences among metal robotic arms, plastic tubing and mechanical shelving will have a large effect on *C. elegans* metabolism and growth rates. These factors will lead to variable test results that depend on weather (humid or dry), well selection (close or far from mechanical arm grips) and plate location (local air flow, proximity to motors) that are avoided when larger volumes and low-

medium-throughput techniques are used. Consistency in HTS could dramatically improve if factors such as nutrient availability, temperature and humidity are carefully controlled and start cultures are monitored for consistent reproductive capacity and life cycle time, as well as the absence of dauers and males.

Homologous genes and concordant pathways

The first completely sequenced genome of a multicellular organism was that of *C. elegans*, and many genes and signaling pathways are conserved between nematodes and humans (*C. elegans* Sequencing Consortium, 1998). For example, apoptosis, or programmed cell death, an essential process in normal animal development and adult homeostasis, is now known to be a well-conserved process (Vaux *et al.*, 1992), but it was in *C. elegans* that the first genes in the apoptosis pathway were identified (Ellis and Horvitz, 1986). Additionally, many elements of the insulin/IGF-1 signaling (IIS) pathway, which uses both the endocrine system and cellular mechanisms to regulate glucose utilization and metabolism, are shared in *C. elegans* and mammals. DAF-16, the sole *C. elegans* homolog of the mammalian FOXO forkhead transcription factors, was found to be a negative regulator of insulin signaling 5 years before a mouse FOXO was identified as a regulator of the same pathway (Ogg *et al.*, 1997; Nakae *et al.*, 2002). It was first discovered in *C. elegans* that IIS is involved in aging (Kenyon *et al.*, 1993), and later that corresponding genetic variants within this pathway in humans and *C. elegans* have analogous effects on lifespan (Kenyon, 2011). Additionally, dietary restriction-induced increases in healthspan and lifespan are mediated by IIS and other conserved pathways in both nematodes and mammals (Fontana *et al.*, 2010). Drugs such as metformin that are used to alter IIS activity to improve human health can increase the healthspan and lifespan in *C. elegans* (Onken and Driscoll, 2010) and mice (Martin-Montalvo *et al.*, 2013), whereas diets high in glucose are associated with poor health in humans and a decreased lifespan in *C. elegans* (Lee *et al.*, 2009). Thus, a 4-week screening assay in *C. elegans* using lifespan as an endpoint has the potential to identify compounds that can improve or worsen human health.

The majority of familial early-onset Alzheimer's disease (AD) patients have mutations in presenilin-1 (*PSEN1*), a gene required for processing of amyloid precursor protein. Four out of the initial five disease-causing missense mutations found in *PSEN1* alter amino acids that are conserved between *PSEN1* and the *C. elegans* presenilin gene *sel-12* (Levitan and Greenwald, 1995). As with *PSEN1* in humans, mutations in *sel-12* induce altered neuronal morphology and memory defects in *C. elegans* (Levitan and Greenwald, 1995), and the memory defects in *C. elegans sel-12* mutants can be rescued by the normal human *PSEN1* gene (Wittenburg *et al.*, 2000). *Caenorhabditis elegans* also has close homologs in several other genes associated with AD etiology, potentially making the worm a useful model in which to understand and treat AD (Alexander *et al.*, 2014; Lu *et al.*, 2014).

An early example of the mechanism of toxicity identification that was supported by research in *C. elegans* is that of phorbol esters (PE), potent mammalian tumor promoters that induce developmental arrest and altered motility in *C. elegans* (Miwa *et al.*, 1982). In a single assay screening for mutants generated by exposure to ethyl methane sulfonate for reversion to control phenotype in the presence of PE, a *C. elegans* protein kinase C (PKC)

homolog *tpa-1* was identified as required for PE toxicity (Tabuse and Miwa, 1983; Tabuse *et al.*, 1989). In contrast, PKC was identified as the major receptor for PE in mammals in a far more complex and incremental manner involving multiple labs over several years (Blumberg, 1988). Thus, phorbol ester toxicity acts via PKC in mammals and worms, although the endpoints (growth and motility vs. tumorigenesis) differ in the two models. Additionally, PEs induce lysosomal degradation in mammalian cell cultures (Daniels and Amara, 1999), and the major pathway change detected in the analysis of gene expression in PE-exposed *C. elegans* is upregulation of lysosome degradation (Ana DePina and Xiugong Gao, personal communication) indicating at least one conserved mode of toxic action. The somatic cells of *C. elegans* adults are post-mitotic (Gartner *et al.*, 2008), so the species is not used as a direct model for tumorigenesis. However, the example of PE toxicity demonstrates (a) how research using a small organism that lacks many systems and phenotypes present in more complex animals can still provide useful toxicological information, (b) how screening for endpoints without direct correlations to human outcomes such as cancer can still be used to detect conserved perturbations in toxicity pathways, and (c) how a relatively simple mutagenesis experiment in *C. elegans* can be used to identify a conserved mechanism of toxicity. Moreover, given that the majority of cancer genes have a *C. elegans* ortholog (Rubin *et al.*, 2000) and that conserved processes such as apoptosis are required for evading tumorigenesis, it is possible that many human carcinogens could be identified in *C. elegans* by screening for alterations in the activity of genes in related pathways.

Two-thirds of human proteins have *C. elegans* homologs, and nearly 80% of genes for human inborn errors of metabolism have *C. elegans* homologs (Sonnhammer and Durbin, 1997; Kuwabara and O'Neil, 2001;). The *C. elegans* ortholog of TRPML1, the gene for mucopolidosis type IV, is *cup-5* (coelomocyte-uptake defective) (Campbell and Fares, 2010). In humans, mutations in TRPML1 result in psychomotor retardation and ophthalmological abnormalities, whereas *C. elegans* homozygous for mutations in *cup-5* are sterile, yet in both species absence of TRPML1/*cup-5* activity at the cellular level results in inappropriate accumulation of lysosomes. Additionally, the normal human TRPML1 gene rescues *C. elegans cup-5* reproductive and lysosomal abnormalities (Hersh *et al.*, 2002). In a similar way, toxicity endpoints in *C. elegans* such as reduced reproductive capacity, altered motility patterns, or developmental defects may detect toxins that elicit very different organismal endpoints in humans, but subsequent analyses of more subtle effects at the molecular level have the potential indicate shared pathways of toxicity. For example, homologs of genes associated with neural tube defects in humans were recently found to be required for *C. elegans* gastrulation (Sullivan-Brown *et al.*, 2016). Vertebrate neural tube closure and *C. elegans* gastrulation involve many of the same processes such as internalization of surface cells, actomyosin-driven apical constriction and adhesions between specific cells, suggesting that screening in *C. elegans* for early embryonic abnormalities may detect toxins that induce neural tube and other developmental defects.

Caenorhabditis elegans utilize most of the same neurotransmitters and neuronal signaling pathways as vertebrates (Kaletta and Hengartner, 2006; Peterson *et al.*, 2008). From worms to mice, dopamine and serotonin play similar roles in the transition from one form of locomotion to another (Vidal-Gadea *et al.*, 2011). *Caenorhabditis elegans* share several nicotinic acetylcholine receptor-dependent behavioral responses to nicotine with mammals (Feng *et al.*, 2006), and *C. elegans* mutagenesis screens

to detect worms with altered motility has led to the identification of human genes for specific myosins and neurotransmitter transporters (Beron *et al.*, 2015). However, the *C. elegans* genome lacks voltage-gated sodium channels (Bargmann, 1998), so the model is not likely to detect excitotoxins and suppressors that exert their effects via this class of ion channel. Conversely, nematodes and insects have inhibitory glutamate-gated chloride channels that mammals do not, allowing for the selective action of some pesticides (Peterson *et al.*, 2008). These differences underlie the importance of fully understanding any model prior to using it for hazard evaluations.

Promising avenues for toxicity testing in *C. elegans*

Methods to assess lethality in *C. elegans* range from simple and inexpensive for a non-worm lab to set up, to those requiring costly automated motility tracking or microfluidics equipment. Adult *C. elegans* are amazingly hearty, so while more screens for adult mortality have been published showing mammalian-correlated toxicity ranking, screens for larval growth, reproduction and motility have also shown ranking correlations and can be more sensitive by an order of magnitude or more (Anderson *et al.*, 2004; Dhawan *et al.*, 1999; Boyd *et al.*, 2010), allowing for reduced test article concentrations and, therefore, fewer solubility issues. Additionally, assays for motility, enzyme activity, reactive oxygen species, RNAi response and gene expression can provide information on mechanisms of action as well as relative toxicity. Other endpoints to assess chemical toxicity include intestinal morphology (Hunt *et al.*, 2012; Stutz *et al.*, 2015), accumulation of autofluorescence (Gerstbrein *et al.*, 2005), gonadal morphology (Hunt *et al.*, 2012) and internal hatching (Hunt *et al.*, 2013).

The *C. elegans* alimentary system has many facets that are comparable to that of mammals, including an acidified lumen, microvilli that form a brush border, secretion of digestive enzymes, uptake of digested components and peristalsis (Hall and Altun, 2008; Chauhan *et al.*, 2013; Stutz *et al.*, 2015). Mammalian and *C. elegans* intestinal dysmorphology in response to cadmium and toxic lectins is comparable (Hunt *et al.*, 2012; Stutz *et al.*, 2015). In contrast to mammals, however, the damage induced by intestinal toxins can also be visualized in live worms by light microscopy or COPAS detection (Hunt *et al.*, 2012). *Caenorhabditis elegans* feed by pulling liquid phase components from their environment into their pharynx by pumping and peristalsis. Thus, they can be used as an oral toxicity model by adding test articles to their nutrient supply. Additionally, pharyngeal pumping rates, which decrease in the presence of several known mammalian neurotoxins, can be observed by light microscopy in individual worms. A higher throughput method of evaluating the effect of toxins on feeding rates involves exposure to fluorescent microbeads followed by an assessment of internal fluorescence (Boyd *et al.*, 2007).

Over 70% of human lipid genes are conserved in *C. elegans*, and 20% of *C. elegans* lipid genes are orthologs of human metabolic disease genes (Zhang *et al.*, 2013). Distinct long- and short-term fat storage vesicles are easily visualized in *C. elegans* (O'Rourke *et al.*, 2009) allowing for screening of compounds that alter lipid metabolism and storage. Similarly, 90% of human lysosome-associated non-disease genes, as well as 70% of human lysosomal storage disorder genes, have *C. elegans* homologs (de Voer *et al.*, 2008). There are several methods to stain specific subsets of endosome and lysosome-related vesicles in live and fixed worms (Clokey and Jacobson, 1986; Hersh *et al.*, 2002; Chen *et al.*, 2006;

Roh *et al.*, 2012), allowing for rapid evaluation of potential lysosomal toxins.

Adult *C. elegans* somatic cells are post-mitotic, making the model seem at first glance to be a poor one in which to assess carcinogenicity. The cellular machinery for DNA replication and repair is highly conserved between *C. elegans* and mammals (Leung *et al.*, 2008) however, and related pathways that prevent the propagation of carcinogenic mutations, such as apoptosis and cell cycle checkpoints, also have conserved elements (Stergiou and Hengartner, 2004). In addition to the previously discussed assay for the detection of aneuploids (Allard *et al.*, 2013), *C. elegans*-based assays for the detection of DNA damage include DNA sequencing, reporter assays, lethality assays and gene expression assessments (Leung *et al.*, 2008). Specific DNA lesions can also be detected in *C. elegans* by gas chromatography/tandem mass spectrometry (Arczewska *et al.*, 2013; Hunt *et al.*, 2013). In another promising new assay, a green fluorescent protein labeled ZTF-8 altered localization in *C. elegans* specifically in response to agents that induced double-stranded DNA breaks but not other types of DNA damage (Kim and Colaiacovo, 2014).

On a much larger scale, screening of thousands of potential drugs with the COPAS using *C. elegans* pre-infected with human pathogens has been used to identify compounds with antimicrobial activity. This method is a departure from traditional screens for antibiotics, which test compounds for their ability to directly kill microorganisms or inhibit their growth. By using infected *C. elegans*, drug efficacy and host toxicity are simultaneously assessed *in vivo*, while making it possible to identify compounds that require metabolic activation for antimicrobial activity, stimulate innate immune activity, and/or inhibit host-pathogen interactions for the establishment of infection, without harming the host (Moy *et al.*, 2009). This approach was recently used to evaluate 300 000 compounds to identify drug candidates that might improve outcomes for patients infected with multi-drug-resistant *B. pseudomallei* (Lakshmanan *et al.*, 2014). On a much smaller scale, *C. elegans* have also been used as part of a two-tiered approach to identify new non-toxic compounds for the treatment of *B. cenocepacia* K56–2 infections in cystic fibrosis patients (Selin *et al.*, 2015). It is too early to tell if the findings from these studies will result in safe treatments for patients, but the strategy appears promising.

Summary: The *C. elegans* Toxicology Model

Model Strengths

- The first completely sequenced genome of a multicellular organism, plus over 40 years of genetics, neuroscience, and cell signaling research make *C. elegans* a well understood model
- Extensive homology to mammals at the genetic level
- Many key cellular metabolic and signaling pathways are conserved
- Many elements of neuronal function are conserved
- Conserved alimentary features make *C. elegans* a good oral toxicity model
- Unlike cell and tissue cultures, *C. elegans* has neuronal, motor, digestive, and reproductive systems, endocrine signaling, and sensory/behavioral responses to stimuli
- *C. elegans* can be maintained at 15 to 25°C, using relatively simple techniques and without the need for CO₂ incubators
- Inexpensive and compact platform allows multiple concentrations and exposure times to be assessed simultaneously

- Short life cycle allows for low-dose lifespan and multi-generation testing in a couple of weeks rather than years
- Transparent tissues and fully mapped body and neuronal plans allow for rapid morphology and transgene expression assessment at the tissue, cellular and sub-cellular levels
- Multiple types of toxicity ranking screens have demonstrated good correlation of endpoints in *C. elegans* to rat LD₅₀s, with *C. elegans* data and mouse data predicting toxicity ranking in rat equally well

Model Limitations

- *C. elegans* lack many mammalian organs such as eyes, lungs, heart, kidney, and liver
- As with the zebrafish embryo model, *C. elegans* have a functioning innate immune system, but lack adaptive immunity
- In contrast to zebrafish embryos, *C. elegans* is not a good absorption model due to its tough cuticle
- As with cell-based models, pH range is wide but still limited, and liquid culture testing requires soluble test compounds
- Small changes in temperature, nutrient, or salt concentration elicit adaptive responses that can significantly alter assay results, sometimes for multiple generations – this currently limits the number of compounds that can be screened by a single lab, but could change with enclosed, climate-controlled microfluidic HTS equipment
- Incorrect handling of stock cultures can result in altered gene expression patterns and the accumulation of dauers or males – phenotype recognition training and good *C. elegans* culture practice (GCeCP, Table 1) is essential for reliable, repeatable data
- Some *C. elegans* systems act/respond in similar ways as analogous mammalian systems, but lack homology at the genetic level (e.g. the innate immune system) – development of microarray analysis software that is specific for *C. elegans* using data that is already available would fix this problem

Conclusions

Currently accepted standards for hazard assessment using conventional laboratory animals are hampered by duration and cost, as well as species specificity. The need for more affordable, rapid and predictive toxicity testing paradigms has led to several coordinated national and international efforts to test and validate novel assays and toxicity evaluation strategies (Casey *et al.*, 2015). These efforts have focused mainly on high-throughput *in vitro* testing, which can reveal molecular and cellular mechanisms of toxicity, but cannot currently provide information required for hazard assessment, such as the effects of exposure route, metabolism by and transport through multiple tissues, and communication among interacting tissues (Tice *et al.*, 2013). *Caenorhabditis elegans* can provide a bridge between *in vitro* assays and mammalian toxicity testing by combining established *in vitro* handling techniques and cost ratios with oral toxicity test data from an intact organism, although standardized culture practices are required to achieve consistent results. Given that nematodes lack most mammalian organs, it is unrealistic to expect that any combination of *C. elegans* assays alone will replace in-depth descriptive toxicology analyses in mammals. However, although organismal toxicity endpoints often differ, many pathways of toxicity and modes of toxic action are conserved between worms and humans. *Caenorhabditis*

C. elegans screening assays have consistently predicted LD₅₀ ranking in mammals, indicating the utility of the model for screening at an early step in tiered testing strategies. The use of *C. elegans* assays within integrated testing strategies that include other models such as zebrafish or human cell cultures have yet to be evaluated.

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Conflict of interest

The authors do not report any conflict of interest.

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