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Alzheimer's Disease Drug Discovery: *In-vivo* screening using *C. elegans* as a model for β-amyloid peptide-induced toxicity

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Abstract

Alzheimer's disease (AD) is a complex human neurodegenerative disease. Currently the therapeutics for AD only treat the symptoms. While numbers of excellent studies have used mammalian models to discover new compounds, the time and effort involved with screening large numbers of candidates is prohibitive. Cultured mammalian neurons are of often used to perform high through-put screens (HTS) however, cell culture lacks the organismal complexity involved in AD. To address these issues a number of researchers are turning to the round worm, *C. elegans. C. elegans* has numerous models of both Tau and Aβ induced toxicity; the two prime components observed to correlate with AD pathology. These models have lead to the discovery of numerous AD modulating candidates. Further, the ease of performing RNAi for any gene in the *C. elegans* genome allows for identification of proteins involved in the mechanism of drug action. These attributes make *C. elegans* well positioned to aid in the discovery of new AD therapies.

Introduction

Alzheimer's disease (AD) is a chronic, neurodegenerative disorder that is the number one cause of senile dementia. Brains of AD patients contain neurofibrillary tangles of tau (a microtubule protein) and hallmark plaques, constituted primarily of insoluble Aβ [1]. According to the amyloid cascade theory, amyloid precursor protein (APP) is processed to form Aβ, which self-dimerizes into increasingly large oligomers, and is responsible for both sporadic and familial AD. Aβ is predominantly produced as a 40 residue peptide ($A\beta^{1–40}$) and less commonly as a longer 42 residue peptide ($A\beta^{1–42}$) [2]. A large body of evidence suggests that $A\beta^{1–42}$ is more prone to forming oligomers and therefore the more toxic species. The toxic nature of $A\beta^{1–42}$ makes it a marker of AD progression and a target of many screens for new therapeutic treatments [3, 4].

Currently there is a dearth of treatments for AD and the treatments that are available, acetyl cholinesterase inhibitors and NMDA receptor antagonists only treat the symptoms. Much of the research to determine the cause of AD as well as design therapies to treat it has utilized mammalian models. This approach is logical in that AD is a complex neurodegenerative disease, requiring the advanced nature of the rodent brain to see a plausible correlation to a human neurodegenerative disease. Unfortunately, due to the time and the number of animals required, mammals are not amenable to high-throughput screening (HTS). While mammalian neuronal cell culture is often used for (HTS) it lacks the complexity found in a

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living organism or even a whole organ, making this system a somewhat crude predictor of safety and efficacy. To fill the gap between cell culture and rodents, many have turned to a seemingly unlikely model, the round worm, *C. elegans*. An invertebrate with only 302 and neurons, *C. elegans* offers a number of advantages to identify candidate drugs for AD.

Drug Screening in *C. elegans*

Administration of drugs to *C. elegans* is conceptually simple; add varying concentrations of candidate drugs to the medium and add worms. Performing the screen with several concentrations allows for the creation of a classic dose response. The dose response can show the ideal dose, minimally effective dose, and, at higher concentrations, toxicity [5]. Although relatively simple, there is no easy way to know how much of a given drug is being ingested and/or absorbed [6]. Surrounding *C. elegans* is a weakly-permeable membrane known as the cuticle. The cuticle functions as an exoskeleton and as a barrier to environment. Therefore, while potentially functional, a small molecule may fail to produce a phenotype due to reduced bioavailability. Further, the bacteria that serve as food for *C. elegans* could also be metabolizing any drugs administered to the media. To address this issue it is now possible to use a structure based bioaccumulation model (SAM) to identify the candidate molecules most likely to be absorbed while remaining active [6]. While the cuticle may confound administration and dosage of candidates it is not prohibitive. Some drugs are still absorbed through the cuticle while other drugs are likely being ingested with the consumption of bacteria.

The screening of a drug library for modifiers of AD can employ a broad based approach where many molecules are screened. In a screen for enhancers of lifespan, the antidepressant, mianserin was isolated from a library of 88,000 molecules [7]. Mianserin functions as a serotonin receptor antagonist. The large collection of available genetic mutations in *C. elegans* allowed these researchers to identify the specific receptors required for lifespan extension by mianserin (SER-3 and SER-4), and to demonstrate that mianserin is likely to function as a calorie restriction mimetic. This study demonstrates that *C. elegans* is amenable to high-throughput screening and the genetic tools available in this model can be used to determine the mode of action of active compounds identified in an essentially unbiased chemical screen. To date, there have been no large scale screens described using *C. elegans* to identify compound leads for AD drugs. Below we characterize the available AD-relevant *C. elegans* models and suggest how studies in these models might be extended to enable high throughput screening.

Alzheimer's disease modeled in *C. elegans*

The nematode, *C. elegans*, is a completely transparent, approximately 1mm long roundworm. As a hermaphrodite *C. elegans* is self-fertilizing, allowing for rapid production of numerous offspring. With a relatively short lifespan of 23 days, *C. elegans* is ideally suited to study aging and age-related diseases [8]. Although it is unlikely invertebrate models can really capture Alzheimer's disease pathology in total, multiple transgenic *C. elegans* strains have been constructed to model aspects of AD, specifically β-amyloid peptide (Aβ) toxicity (Table 1). The first described model expressed the human $A\beta^{1–42}$ peptide under the control of a constitutive muscle-specific promoter derived from a myosin gene expressed in body wall muscle (*unc-54*) [9]. This transgenic construct employed an N-terminal artificial signal peptide to route the Aβ peptide to the secretory pathway. Mass spectrometry studies have demonstrated the signal peptide is efficiently removed, although this signal peptide cleavage apparently results in the production of $A\beta^{3–42}$ rather than $A\beta^{1–42}$ [10]. Constitutive muscle-specific expression of Aβ leads to the accumulation of intramuscular Aβ deposits (Figure 1, top panel) and the formation of fibrillar amyloid

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detectable with amyloid-specific dyes [11]. This pathology is reminiscent of that observed in Inclusion Body Myositis (IBM), and this transgenic model may be more directly relevant to this disease than AD. The best-characterized strain containing the P*unc-54*::Aβ $^{1–42}$ transgene is CL2006, which shows a progressive, adult onset paralysis.

Strain CL2006 has been used to evaluate the effects of selected compounds on Aβ toxicity and fibrillar amyloid formation. These include natural compounds such gingko biloba extract [12], the soy isoflavone glycitein [13], and epigallocatechin gallate [14]. Drug-related compounds including reserpine [15], fluoxetine [16], and thioflavin T [17], have also been shown to modulate toxicity in this model. Only one study has examined the effects of more than a handful of compounds in the CL2006 model [5]. Thirty drugs that protect against glucose induced toxicity were identified using mammalian primary neurons to screen a library of FDA-approved drugs. These 30 candidates were subsequently screened for both amyloid induced toxicity and lifespan extension in *C. elegans*. From this two-species screen, three compounds, caffeine, tannic acid, and bacitracin, offered protection from Aβ-induced toxicity in the *C. elegans* CL2006 model. These studies indicate that a range of exogenous compounds can modulate Aβ-dependent phenotypes in this model. These compounds could be used as positive controls for higher-throughput assays.

A limitation of the CL2006 constitutive Aβ-expression model for more extensive compound screening may be the age dependence of the paralysis phenotype: it may be difficult to disentangle whether protective compounds are modulating Aβ toxicity or aging physiology (or both). Indeed, all the compounds identified by Lublin et al as protective in CL2006 also increased the lifespan in wild type worms, and genetic backgrounds that lead to increased lifespan also reduce paralysis rates in CL2006 [18]. In addition, the paralysis rate in CL2006 can be variable from experiment to experiment, and some worms never appear to become paralyzed. To circumvent these limitations, transgenic worms have been constructed in which body wall muscle expression of the signal peptide::Aβ $^{1–42}$ minigene can be up-regulated in larval animals by temperature up-shift [19]. (See Figure 1, middle panel). In the best-characterized transgenic strain of this type (CL4176), worms have wild type movement throughout their lifespan when raised at the permissive temperature (16°C). However, up-shift of larval worms to higher temperature (25°C) leads to rapid, reproducible, and 100% penetrant paralysis [20]. CL4176 has been used to demonstrate that coffee extracts [21] and tetracycline and related analogs [22] can be protective against Aβ toxicity.

An obvious limitation of the models described above is that Aβ expression is limited to muscle cells. Transgenic worms with pan-neuronal expression of the signal peptide::Aβ $^{1–42}$ minigene have also been constructed and characterized [23] (see Figure 1, bottom panel). While these worms have more subtle phenotypes than the paralysis that occurs in muscle expression strains, they do show chemotaxis defects and altered movement in liquid. Although suboptimal for high-throughput screens, perhaps the most interesting defect in these transgenic worms is a failure to retain associative memory [24]. A transgenic *C. elegans* model has been recently developed in which the signal peptide::Aβ $^{1–42}$ minigene is expressed solely in 5 glutamatergic neurons, using the promoter from the *eat-4* gene [25]. While this transgenic strain was not reported to have any gross visible phenotypes, visualization of the glutamatergic neurons with a P*eat-4*::GFP construct was used to demonstrate that Aβ expression led to an age-dependent loss of glutamatergic cells. The transparency of *C. elegans* may allow GFP fluorescence-based assays to form the basis of high throughput screens.

Alzheimer's disease is also associated with the deposition of neurofibrillary tangles composed of hyperphosphorylated tau protein. Three transgenic *C. elegans* tauopathy models have been described, two of which expressed human tau using pan-neuronal

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promoters [26]; [27], while one expressed tau only in touch cell neurons [28]. The strains with pan-neuronal tau expression displayed uncoordinated movement and age-dependent neurodegeneration, while tau expression in the touch neurons led to mechanosensory defects. These transgenic models have not yet been used for compound testing. Automated methods have been developed for assaying *C. elegans* movement [29], so the phenotypes of the pan-neuronal expression models may amenable to large scale compound screening.

A major advantage of *C. elegans* models lies in the sensitivity of *C. elegans* to RNA interference (RNAi), which can be very beneficial in identifying molecular mechanisms underlying the action of protective compounds. For example, the protective effects of coffee extracts are completely blocked by RNAi knockdown of *skn-1*, the worm ortholog of the master phase 2 detoxicification transcription factor Nrf2. Similarly, the lifespan extension created by treatment with either caffeine or tannic acid was completely blocked by RNAi of the FOXO transcription factor *daf-16,* suggesting that caffeine and tannic acid require DAF-16 to extend lifespan [5].

Conclusions

In the design of a screen, the short time required to create strains of *C. elegans* offers a clear advantage compared to the time involved in producing murine strains. This advantage is amplified by the ease of acquiring and maintaining previously created strains available from the *C. elegans* Genetics Center (CGC) (http://www.cbs.umn.edu/CGC/strains/), including most of the strains described in Table 1. While the strains with muscle-specific expression of Aβ do not directly capture the neurodegeneration seen in AD, they do provide an easy means to study Aβ-induced toxicity and protein aggregation [11, 19]. Furthermore, these models can be used to study inclusion body myositis, an aging-related disease were Aβ forms aggregates in muscle tissue [30–32]. More recently created strains have expressed $A\beta^{1–42}$ in the neurons of *C. elegans*. These strains produce a less pronounced phenotype then the muscle expressing strains, making them potentially problematic for higher through-put screens, however, they may more accurately represent the amyloid induced toxicity seen in AD [23, 25].

Screening drugs, small molecules, or complex extracts in *C. elegans* is strait forward in design but difficulties in bio-accumulation can create obstacles in identification of promising candidates. To circumvent these issues there are several approaches. Using a large scale HTS reduces the difficulties by screening a large enough number of molecules that one or more candidates will produce a significant effect, as was the case with mianserin [7]. Another approach is to perform a more focused screen on candidates that have an increased probability of functioning in a specific model. The candidates for these screens come from many sources, including specific studies in higher organisms or from a HTS in tissue culture. This approach has lead to the discovery that, in addition to increasing lifespan, caffeine, bacitracin, and tannic acid can reduce the observed proteotoxicity seen in an Aβ expressing *C. elegans* strain (CL2006) [5].

In *C. elegans,* HTS offer the advantage of identifying promising novel, and otherwise unlikely drugs with the caveat that some interesting candidates are likely to be missed. With a more focused screen some of issues with drug administration can be surmounted using a wide range of doses. Furthermore, by using different concentrations a dose response curve can be produced, enabling the identification of the most efficacious dose as well as any doses that produce toxicity.

Once identified, candidates from the screen can be functionally analyzed by utilizing RNAi. Performing RNAi on genes potentially needed for drug activity allows for identification of

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specific proteins and protein pathways involved in the mechanism of its action. Knowing the mechanism responsible for drug action it is possible to investigate other drugs that might more specifically target the same mechanism. Further, combination therapies, similar to those used for the treatment of HIV [33], can be designed in *C. elegans* by utilizing additional drugs or other modulators of the target molecular mechanism.

In closing, with the number of people diagnosed with AD expecting to grow as average age of population increases it is paramount to find new and better ways to treat this growing pandemic [34]. The mouse models will ultimately be needed to demonstrate the efficacy of any therapy prior to clinical trials. However, the spate of candidate AD therapies combined with a large number of failed trails creates a need for a filter to isolate the most promising leads. As *C. elegans* is well suited to screening it serves as a scout, to rapidly identify, efficacy, toxicity, and the molecular mechanisms responsible for any observed benefit. Moving forward, the information gained from research in worms can be applied to the design of mammalian studies. This streamlined approach to the process of drug discovery, may ultimately aid in the production of a valid therapy for AD.

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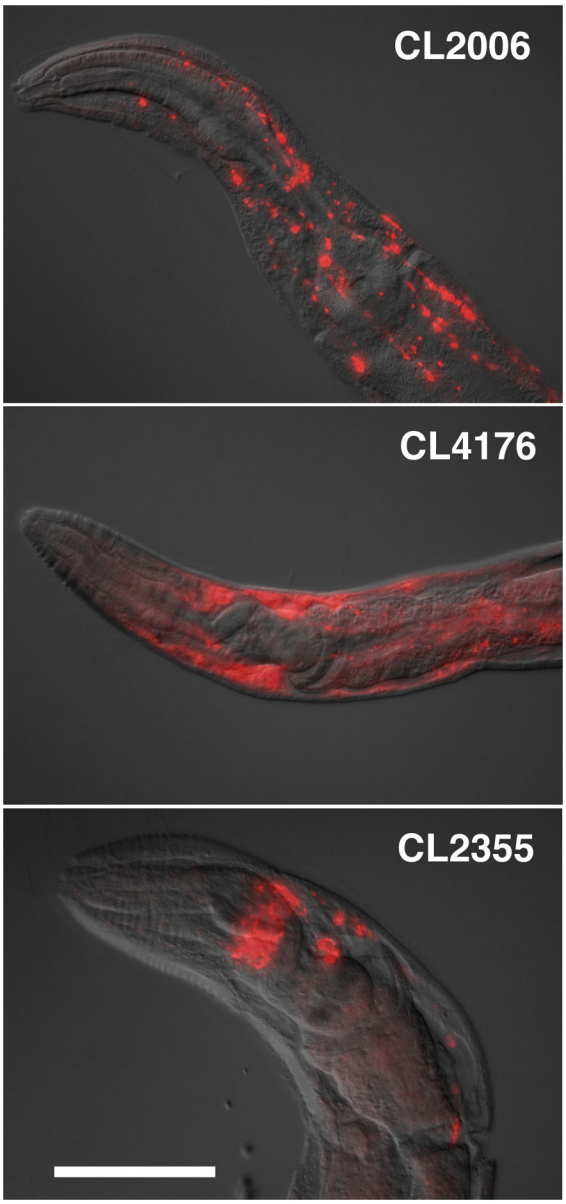

Figure 1.
Anterior regions of transgenic *C. elegans* worms fixed and probed for Aβ. Top panel, CL2006 adult probed with anti-Aβ mAb 4G8. Middle panel, CL4176 3rd stage larvae probed with anti-Aβ mAb 6E10. Bottom panel, CL2355 adult probed with anti-Aβ mAb 6E10. Images are overlays of DIC brightfield image and digitally deconvoluted epifluorescence images. Size bar = 50μM.

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Table 1

C. elegans transgenic strains expressing human Aβ

Strain	Aβ transgene	Aβ Expression	Phenotype
CL2006	pCL12 (P*unc-54*: SP::Aβ $^{1-42}$)	constitutive muscle	adult onset paralysis, β-amyloid formation[9]
CL2122	pCL12 (P*unc-54*: SP::Aβ $^{1-42}$)	constitutive muscle	slow adult movement, β-amyloid formation[11]
CL4176	pAF29 (P*myo-3*::SP::Aβ$^{1-42}$::long 3′ UTR)	inducible muscle	inducible larval paralysis[35]
CL2159	pAF29 (P*myo-3*::SP::Aβ$^{1-42}$::long 3′ UTR)	inducible muscle	inducible larval paralysis[19]
CL2355	pCL45 (P*snb-1*::SP::Aβ$^{1-42}$::long 3′ UTR)	pan-neuronal	memory deficits, abnormal thrashing in liquid, partial sterility[23]
UA166	P*eat-4*::SP::Aβ $^{1-42}$	glutamatergic neurons	age-dependent loss of glutamatergic neurons[25]