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AVALIAÇÃO DO EFEITO DO DISSELENETO DE DIFENILA EM MODELOS DE DOENÇA DE ALZHEIMER UTILIZANDO *Caernorhabditis elegans*

DISSERTAÇÃO DE MESTRADO

Daniele Coradini Zamberlan

Santa Maria, RS, Brasil

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AVALIAÇÃO DO EFEITO DO DISSELENETO DE DIFENILA EM MODELOS DE DOENÇA DE ALZHEIMER UTILIZANDO *Caernorhabditis elegans*

por

Daniele Coradini Zamberlan

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica**

Orientador: Prof. Dr. Félix Alexandre Antunes Soares

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Centro de Ciências Naturais e Exatas
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elaborada por
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como requisito parcial para obtenção do grau de
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"A verdadeira viagem de descobrimento não consiste em procurar
novas paisagens, mas em ter novos olhos".
(Marcel Proust)

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil

AVALIAÇÃO DO EFEITO DO DISSELENETO DE DIFENILA EM MODELO DE DOENÇA DE ALZHEIMER NO NEMATÓDEO *Caernorhabditis elegans*

AUTOR: DANIELE CORADINI ZAMBERLAN

ORIENTADOR: FÉLIX ALEXANDRE ANTUNES SOARES

Local e Data da Defesa: Santa Maria, 21 de fevereiro de 2014.

A Doença de Alzheimer (DA) é uma doença neurodegenerativa evidenciada por distúrbios cognitivos e déficit de atenção e aprendizagem, sendo a principal causa de demência em idosos. A Hipótese Amilóide postula o acúmulo de depósitos extracelulares do peptídio β-amilóide (Aβ) no cérebro como o principal fator da doença. Entretanto, sua etiologia ainda não está completamente elucidada e seu tratamento visa apenas a melhora dos sintomas. Cepas transgênicas do nematódeo *Caernorhabditis elegans* que expressam as espécies tóxicas Aβ, têm sido utilizadas como modelos *in vivo* de DA para elucidar mecanismos e verificar a eficácia de novas moleculas. O disseleneto de difenila ($(PhSe)_2$), composto orgânico de selênio utilizado nesse estudo, tem demonstrado eficácia em melhorar diversos parâmetros em modelos de doenças neurodegenerativas. No presente estudo foram analisados os efeitos do tratamento crônico com $(PhSe)_2$ na toxicidade induzida pela Aβ em *C. elegans*. Os resultados mostraram que a exposição crônica ao $(PhSe)_2$ atenuou o estresse oxidativo induzido pela Aβ, além de recuperar a memória associativa no nematódeo. Além disso, o (PhSe)2 diminuiu a expressão do gene *Aβ*, levando a supressão do peptídio Aβ e reduzindo a expressão do gene *hsp-16.2*, por diminuir a necessidade desta chaperona frente a toxicidade Aβ. Estes dados sugerem que o $(PhSe)_2$ desempenha um importante papel na proteção contra a toxicidade induzida por estresse oxidativo, além de representar um promissor agente farmacológico por atenuar a expressão do Aβ.

Palavras-chaves: compostos de selênio, Alzheimer, *C. elegans*, β-amilóide, doenças neurodegenerativas.

ABSTRACT

Master Dissertation
Graduation Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EVALUATION OF DIPHENYL DISSELENIDE EFFECT IN THE NEMATODE *Caernorhabditis elegans* ALZHEIMER DISEASE MODEL

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ADVISOR: FÉLIX ALEXANDRE ANTUNES SOARES
Date and Place of the Defense: Santa Maria, 21 de fevereiro de 2014.

Alzheimer's (DA) is a neurodegenerative disease evidenced by cognitive disorders and attention deficit and learning, and is the main cause of dementia in the elderly. The amyloid hypothesis posits that extracellular amyloid-β (Aβ) deposits are the fundamental etiological factor of the disease. However, the AD etiology has yet to be fully understood and common treatments remain largely non-efficacious. *Caernorhabditis elegans* transgenic strains expressing toxic Aβ has been employed as AD in vivo model in order to elucidate mechanisms and verifying the effectiveness of pharmacological compounds. The organoselenium compound tested in this study, Diphenyl-diselenide $(PhSe)_2$, has shown efficacy in ameliorate several parametres in neurodegenerative disease models. In the present study, we analyzed the effects of $(PhSe)_2$ chronic treatment on Aβ peptide-induced toxicity in *C. elegans*. This data shows that chronic exposure to $(PhSe)_2$ attenuated oxidative stress induced by Aβ with concomitant recovery of associative learning memory in worms. In addition, $(PhSe)_2$ decreased Aβ transgene expression, suppressing the Aβ peptide and down-regulating *hsp-16.2* by reducing the need of this chaperone under Aβ toxicity. This observations suggest that $(PhSe)_2$ plays an important role in protection against oxidative stress-induced toxicity, this representing a promising potential pharmaceutical modality by attenuating Aβ expression.

Key words: selenium compounds, Alzheimer, *C. elegans*, amyloid-β, neurodegenerative diseases.

SUMÁRIO

APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrito uma sucinta revisão bibliográfica sobre os temas trabalhados nesta dissertação.

O **DESENVOLVIMENTO** da dissertação está apresentado sob a forma de um artigo o qual se encontra alocado no item **ARTIGO CIENTÍFICO.** As seções Materiais e Métodos, Resultados, Discussão dos Resultados, Conclusão e Referências Bibliográficas, encontram-se no próprio artigo e representam a íntegra deste estudo.

Os itens **CONCLUSÕES e PERSPECTIVAS** são encontrados no final desta dissertação e apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** uma vez que o artigo científico contém as suas próprias referências.

1. INTRODUÇÃO

Nas últimas décadas, a expectativa de vida na população tem aumentado significativamente. Sendo a idade o principal fator de risco para o desenvolvimento de doenças neurodegenerativas, estas têm se tornado cada vez mais comuns, sendo a Doença de Alzheimer (DA) a mais comum, com 20 milhões de casos no mundo [1]. Estima-se que na DA a neurodegeneração comece cerca de 20 a 30 anos antes do aparecimento dos sintomas clínicos [1], os quais incluem principalmente distúrbios cognitivos, perda de memória e disfunção das funções mentais, podendo evoluir para demência e até a morte em seu estágio final [2].

A DA é caracterizada principalmente pela presença de placas senis e emaranhados neurofibrilares no cérebro, os quais causam morte neuronal e, consequentemente, na redução da neurotransmissão [3]. A formação dos emaranhados neurofibrilares está associada a mutações e a hiperfosforilação da proteína tau, presente no citoesqueleto dos neurônios [1]. As placas senis são formadas por agregados extracelulares do peptídio tóxico beta-amilóide (Aβ), derivado da clivagem da proteína precursora amilóide (APP) pelas enzimas β-secretase e γ-secretase [4]. Aβ apresenta-se predominantemente na forma de um peptídio de 40 resíduos de aminoácidos ($A\beta_{1-40}$) e menos comumente, porém mais predisposta a formar oligômeros tóxicos, com 42 resíduos ($A\beta_{1–42}$) [5]. A APP é produzida normalmente por diferentes células e a presença de mutações genéticas em formas familiares da DA, acarretam em um aumento da produção da Aβ ou da proporção da forma $A\beta_{1-42}$ [6], evidenciando a hipótese da cascata amilóide nesta patologia [7] (Fig. 1). A natureza tóxica do $A\beta_{1–42}$ o torna um marcador da progressão da DA, bem como um alvo de novos tratamentos terapêuticos [8].

Estas mudanças neuropatológicas iniciam-se no hipocampo, posteriormente espalhando-se pelo córtex temporal, parietal e frontal [9]. As primeiras lesões características da DA aparecem em neurônios pouco mielinizados, em áreas relacionadas a memória e aprendizagem, como o hipocampo Neurônios altamente mielinizados, em áreas relacionadas ao humor, sentidos, entre outras, são afetados apenas nas fases finais da doença [10]..

Vários estudos demonstraram que esses sintomas da DA estão relacionados a redução nas taxas de acetilcolina (ACh) e outros neurotransmissores como noradrenalina, dopamina, serotonina, glutamato e substância P. A deficiência de

ACh ocorre por atrofia dos núcleos basais de Meynert, estruturas responsáveis pela produção da enzima colina acetiltransferase (CAT) que, por sua vez, é responsável pela catálise da síntese de ACh [11]. Como consequência desta atrofia, há uma diminuição na produção de CAT e, portanto, uma diminuição na geração de ACh, afetando a sinalização colinérgica e evidenciando a hipótese colinérgica da DA [12].

Desta forma, o mecanismo pelo qual a proteína tau e o peptídio Aβ atuam como agentes tóxicos na DA ainda não está claro. Além disso, os tratamentos disponíveis, como inibidores da Ache, visam apenas melhorar a cognição, retardar a evolução da doença e atenuar os sintomas e alterações comportamentais, sem, contudo, oferecer a cura efetiva. Desta forma, numerosos estudos em mamíferos visando elucidar causas e tratamentos vem sendo realizados. Porém, tendo em vista o tempo e o grande número de animais requeridos nestes estudos, modelos invertebrados como o nematódeo *Caernorhabditis elegans* proporcionam uma ponte entre estudos em culturas celulares e mamíferos na descoberta de novas drogas para a DA.

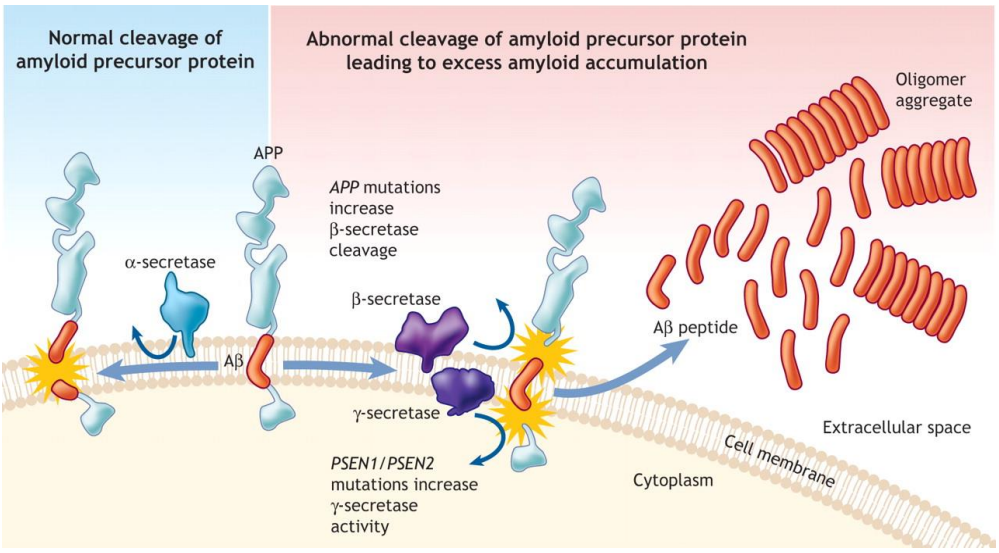

Figura 1. Clivagem da APP, adaptada de Patterson et al. 2008 [13].

O C. elegans é um organismo multicelular utilizado como sistema modelo para estudos do funcionamento do sistema biológico [14]. Este nematódeo de vida livre foi introduzido em 1965 por Sydney Brenner com a finalidade de oferecer um grande potencial principalmente para análises genéticas [15]. Seu pequeno tamanho (aproximadamente 1 mm), ciclo de vida rápido (aproximadamente 3,5 dias – Fig. 2), grande número de progênie (300-1000 animais de um único hermafrodita), curto

tempo de vida (cerca de 20 dias a 20°C) e fácil manutenção [15], o tornam um vantajoso modelo experimental.

Este nematódeo é um bom modelo animal para pesquisas relacionadas à toxicologia, farmacologia, biologia molecular e ao envelhecimento. Ele permite abordagens simples na determinação da toxicidade e da ativação de genes responsíveis ao estresse, e ainda, efeitos tóxicos podem ser facilmente detectados através da análise do comportamento do animal [14]. Seu genoma totalmente sequenciado [16] e suas vias metabólicas e biossintéticas são altamente conservados nos mamíferos (aproximadamente 60% de semelhança), incluindo vias envolvidas no desenvolvimento celular, na manutenção do sistema nervoso e na apoptose [14, 17]. O *C. elegans* possui diferentes sistemas de neurotransmissores que coordenam seu comportamento, incluindo o sistema dopaminérgico, colinérgico, serotoninérgico, glutamatérgico e gabaérgico [14]. Além disso, como os vermes são transparentes, fusões em 'genes repórteres' permitem a visualização direta da morfologia celular e dos padrões de expressão proteica [18, 19]. Ainda, mutantes genéticos e vermes knockouts podem ser facilmente gerados via RNA de interferência [18, 19], havendo atualmente milhares de mutantes já produzidos disponíveis.

Desta forma, o *C. elegans* representa um importante sistema modelo *in vivo* para o estudo de mecanismos patológicos em diversas doenças neurodegenerativas. Morte de células neuronais e inclusões proteicas podem ser facilmente detectadas e quantificadas utilizando técnicas ópticas. Além disso, este nematódeo possui um sistema nervoso simples, composto de 302 neurônios em um hermafrodita adulto [20] e a maioria do sistema neurotransmissor conservado [21]**.**

O *C. elegans* possui um gene relacionado ao que codifica a APP em mamíferos, o apl-1. Entretanto, este não contempla a região codificante do peptídeo tóxico $A\beta_{1-42}$ [22]. Desta forma, foram criadas cepas transgênicas que expressam as espécies tóxicas Aβ, as quais têm sido utilizadas como modelos de DA [23-25]. Estes modelos são capazes de reproduzir os processos celulares que fundamentam a DA, apesar de não serem capazes de retratar a complexidade neuronal cognitiva de mamíferos [26]. Estas cepas apresentam características específicas em decorrência do acúmulo do peptídeo $A\beta_{1-42}$ no nematódeo. Além do aumento da produção das espécies reativas de oxigênio, a expressão muscular do peptidio $A\beta_{1-42}$ (CL2006 e CL4176) ocasiona uma diminuição dos movimentos e paralisia

progressiva [27]. Cepas com expressão neuronal deste peptídeo (CL2355) apresenta baixo número de progênie, parcial esterilidade, diminuição do tempo de vida e da memória associativa [28].

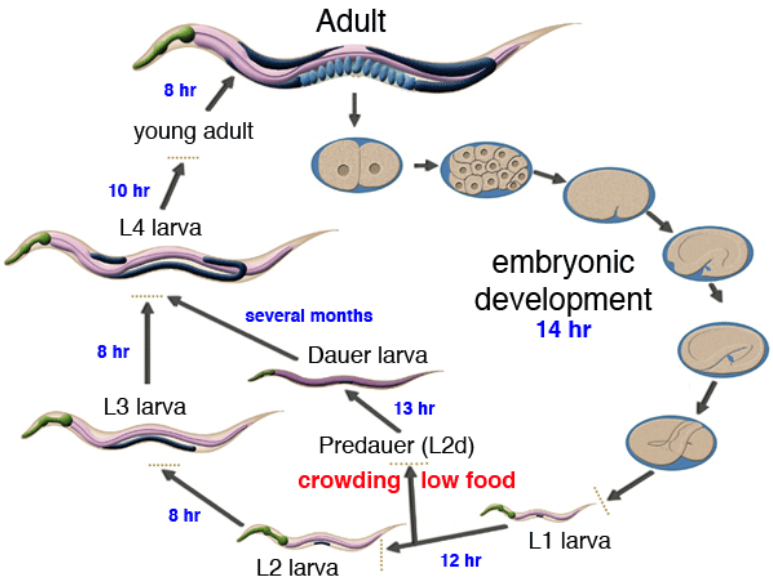

Figura 2. Ciclo de vida de *C. elegans* a 22°C [29]

Vários estudos têm utilizado este nematódeo para verificar a eficácia de compostos com potencial farmacológico. O Selênio (Se) é um micronutriente essencial para o organismo, sendo absorvido pelo sistema nervoso central [30] e requerido para a função muscular [31]. Estudos demonstraram que o Se está envolvido na maioria das vias moleculares implicadas na progressão da DA [32]. Pacientes com DA apresentam níveis plasmáticos reduzidos de Se quando comparados a pacientes saudáveis [33], fator que deve contribuir para o declínio das funções neurofisiológicas observado em idosos [34]. Além disso, em cultura de células, o Se está associado a redução da produção do βA e consequentemente da morte neuronal [35].

O tratamento com Se tem demonstrado melhorar diversos parâmetros em modelos de doenças neurodegenerativas, como redução de danos oxidativos, declínio cognitivo e neurodegeneração em ratos modelos de DA [36, 37] e retardo da progressão da neurodegeneração em parksonismo [38]. Em contrapartida, a exposição a altas doses de Se (Na_4SE 5mM) induziu perda neuronal e alteração da sinalização colinérgica em *C. elegans* [39].

Existem evidências de que compostos orgânicos de Se possuem uma maior biodisponibilidade e atividade biológica do que os inorgânicos. Desta forma, estes composto orgânicos de selênio vem sendo cada vez mais estudados, dentre eles, o disseleneto de difenila $(PhSe)_2$ (Fig. 3). O $(PhSe)_2$ é caracterizado pelo seu potencial antioxidante [40], além de suas propriedades anti-inflamatórias [41], hepato [42] e neuroprotetoras [43]. Recentemente, as atividades biológicas do $(PhSe)_2$ têm sido amplamente estudadas. Ratos tratados com (PhSe)2 demonstraram uma melhora na memória de curto e longo prazo comprometida por scopalamina, sugerindo que este composto possui um significativo valor terapêutico no tratamento da disfunção cognitiva, a qual é característica da DA [44]. Contudo, a complexidade de modelos mamíferos dificulta a determinação das vias moleculares e proteínas específicas moduladas em resposta ao tratamento com esses compostos. *C. elegans* expostos ao $(PhSe)_2$ não apresentaram aumento da mortalidade, mesmo em doses maiores que 100 µM. Além disso, foi evidenciado que o pré-tratamento com este composto aumentou o tempo de vida em vermes expostos a doses tóxicas de Manganês [45].

Considerando o efeito neuroprotetor do composto $(PhSe)_2$ e a efetividade do *C. elegans* como modelo alternativo para o estudo de doenças neurodegenerativas, o presente estudo foi delineado a fim de elucidar os efeitos e possíveis mecanismos do composto $(PhSe)_2$ em um modelo experimental de DA, utilizando o *C. elegans* como organismo vivo.

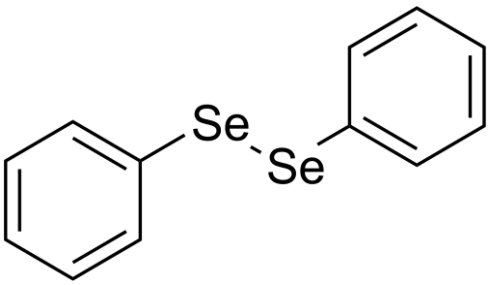

Figura 3. Estrutura química do disseleneto de difenila.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar os efeitos do tratamento crônico com $(PhSe)_2$ *in vivo* em modelos de Doença de Alzheimer no nematódeo *Caernorhabditis elegans*.

2.2. Objetivos específicos

- Analisar o efeito do $(PhSe)_2$ sobre a toxicidade mediada pelo peptidio Aβ em *C. elegans,* como produção de ROS, déficits comportamentais, neurotoxicidade;
- Determinar o efeito do $(PhSe)_2$ sobre os parâmetros comportamentais ensaio de paralisia, movimento, quimiotaxia e memória em *C. elegans*;
- Investigar a capacidade do $(PhSe)_2$ em modular a expressão do RNAm e do peptídio Aβ em *C. elegans*;
- Investigar a capacidade do $(PhSe)_2$ em modular a sinalização colinérgica e a expressão da enzima Acetilcolinesterase em *C. elegans*.

3. DESENVOLVIMENTO

O desenvolvimento que faz parte desta dissertação está apresentado sob a forma de artigo científico, o qual encontra-se na formatação para publicação da revista científica *Neuroscience.* Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprio artigo.

3.1 Artigo Científico

Disseleneto de difenila suprime o peptídeo β-Amilóide em *Caernorhabditis elegans* modelo de Doença de Alzheimer

Diphenyl-diselenide suppresses amyloid-β peptide expression and toxicity in *Caenorhabditis elegans* model of Alzheimer's disease

Daniele Coradini Zamberlan, Leticia Priscilla Arantes, Ronaldo Golombieski, Cintia Leticia Tassi, Marina Lopes Machado, Bruna Puntel, Félix Alexandre Antunes Soares

Diphenyl-diselenide suppresses amyloid-β peptide in *Caenorhabditis elegans* model of Alzheimer's disease

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Diphenyl-diselenide suppresses Amyloid-β Peptide in *Caenorhabditis elegans* Alzheimer's disease model

Abstract

Alzheimer's disease (AD) is the most common and devastating neurodegenerative disease. The etiology of AD has yet to be fully understood, and common treatments remain largely non-efficacious. The amyloid hypothesis posits that extracellular amyloid-β (Aβ) deposits are the fundamental etiological factor of the disease. The present study tested the organoselenium compound diphenyl-diselenide $(PhSe)_2$, which is characterized by its antioxidant and antiinflammatory properties and has shown efficacy in several neurodegenerative disease models. We employed a transgenic *C. elegans* AD model to analyze the effects of $(PhSe)_2$ treatment on Aβ peptide-induced toxicity. Chronic exposure to $(PhSe)_2$ attenuated oxidative stress induced by $A\beta_{1-42}$, with concomitant recovery of associative learning memory in *C. elegans*. Additionally, $(PhSe)_2$ decreased $A\beta_{1-42}$ transgene expression, suppressed $A\beta_{1-42}$ peptide, and downregulated *hsp-16.2* by reducing the need for this chaperone under $A\beta_{1-42}$-induced toxicity. These observations suggest that $(PhSe)_2$ plays an important role in protecting against oxidative stress-induced toxicity, thus representing a promising pharmaceutical modality that attenuates $A\beta_{1-42}$ expression.

Keywords: selenium compounds, Alzheimer's disease, *C. elegans*, amyloid-β, neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases have become increasingly prevalent. However, their etiology has yet to be fully deciphered, and treatment modalities are largely ineffective (Dimitriadi and Hart 2010). Alzheimer's disease (AD) is the most common and devastating neurodegenerative disease, predominantly characterized by the presence of senile plaques (SPs) in the brains of affected individuals (Goedert and Spillantini 2006). The major constituents of SPs are toxic species of amyloid-β (Aβ) peptide, mainly $A\beta_{1-42}$ (Goedert and Spillantini 2006), which is produced by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase, respectively. $A\beta_{1-42}$ peptide is considered to cause AD pathology and induce the formation of neurofibrillary tangles, cell loss, vascular damage, and dementia (Hardy and Higgins 1992). To date, there is no cure for AD, and the available treatments are largely designed to only alleviate the symptoms of the disease (Klafki et al. 2006). Therefore, the discovery of efficient and novel pharmacological agents that may attenuate disease progression and its symptoms is needed.

Selenium (Se) is an essential nutrient for organism function. It has been shown to have pharmacotherapeutic efficacy in numerous experimental models of brain disease. Se effectively attenuates oxidative damage, improves cognitive decline, and reduces neurodegeneration in rat models of AD (Ishrat et al. 2009). It has also been shown to exert a protective effect in a rat model of Parkinson's disease (PD; (Zafar et al. 2003).

Evidence indicates that organoselenium compounds have higher bioavailability and biological activity than inorganic compounds and are promising pharmacological agents. $(PhSe)_2$ is characterized by its antioxidant (Bruning et al. 2012), antiinflammatory (Jesse et al. 2009), hepatic (da Rosa et al. 2012), and neuroprotective (Nogueira et al. 2004; Luchese et al. 2007) potential. In *Caenorhabditis elegans*, $(PhSe)_2$ did not increase mortality, even at exceedingly high doses (Avila et al. 2012). Moreover, pretreatment with $(PhSe)_2$ attenuated manganese-induced toxicity, restoring the nematodes' longevity (Avila et al. 2012).

In vivo models, such as the nematode *C. elegans*, offer alternative and complementary systems for deciphering neurodegenerative etiologies and aiding drug discovery (Wu and Luo 2005); (McColl et al. 2012). *C. elegans* transgenic strains that express Aβ have been established, allowing mechanistically driven studies of AD (Link 1995; Link et al. 2003; Wu

et al. 2006; Dosanjh et al. 2010). These models are able to recapitulate several of the cellular processes that underlie the etiology of AD (Wu and Luo 2005).

Considering the protective properties of $(PhSe)_2$ and the lack of an effective pharmacological treatment for AD, we investigated the efficacy of $(PhSe)_2$ in attenuating Aβ toxicity in a transgenic *C. elegans* model of AD. We tested the hypothesis that $(PhSe)_2$ reduces Aβ peptide expression and associated reactive oxygen species (ROS) generation in the nematode.

2. Experimental Procedures

2.1 Chemicals and Reagents

$(PhSe)_2$, dimethyl sulfoxide (DMSO), 5-hydroxy-1,4-naphthoquinone (Juglone), 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), aldicarb, butanone, ethanol and thioflavin T (ThT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Caenorhabditis elegans Strain, Maintenance and Treatment

The wild-type *C. elegans* strain N2 (Bristol) and transgenic worms, CL2006 [pCL12(*unc-54*/Aβ_{1-42})+pRF4], CL4176 [*smg*-1(*myo-3*/Aβ_{1-42}/letUTR)+pRF4] and its control strain CL802 (*smg*-1ts), CL2355 [*smg-1ts* (*snb-1*/Aβ_{1-42}/long 3'-UTR) and its control strain CL2122 (unc-54 vector + mtl-2::GFP) were obtained from the *Caenorhabditis elegans* Genetics Center (University of Minnesota, Minneapolis, MN).

The CL2006 strain constitutively expresses a body-wall muscle-specific Aβ (Link 1995), whereas the expression of muscles specific Aβ in CL4176 depends on increasing the ambient temperature from 16 to 23°C (Link et al. 2003). The CL2355 strain employs the promoter of the *C. elegans* synaptobrevin ortholog (*snb-1*) to drive panneuronal expression of Aβ, which is also inducible by temperature upshift (Dosanjh et al. 2010).

For all worms, age-synchronized eggs were obtained by isolating embryos from gravid hermaphrodites using bleaching solution (1% NaOCl, 0.25M NaOH) (Lewis and Fleming 1995). The L1 population were transferred to 10 mL NGM (nematode growth medium) plates seeded with *E. coli* OP50 as a food source and 100 µL of $(PhSe)_2$ at 10, 25 and 50 µM or vehicle (DMSO 1%) and allowed to develop until 1-day adult. The wild-type N2 and the transgenic CL2006 were maintained at 20°C. CL4176, CL2355 and their respective controls were grown at 16ºC until L3 stage followed by a temperature upshifting to 23°C to induced Aβ expression until adulthood.

2.3 Survival assay in a juglone induced stress model

Synchronized N2 L1-larva were transferred to treatment plates containing $(PhSe)_2$ (10-50 μM) or DMSO (control) and allowed to develop at 20°C up to adultohood (approximately 2 days). The pretreated worms were collected, washed three times with M9 buffer and transferred into Eppendorf tubes containing 100 μM juglone for one hour. The number of living worms was observed in each of the treatments, and expressed as percent of control.

2.4 Measurement of reactive oxygen species (ROS)

Intraworm ROS generation was measured in treated and control *C. elegans* wild-type (N2) and transgenic (CL4176) strains using CM-H_2DCFDA, following a previously described method (Sakaue et al. 2010) with minor modifications. Briefly, L1 age-synchronized worms were transferred onto culture plates containing either a vehicle or $(PhSe)_2$. Wild-type worms were maintained at 20°C until adulthood (48 hours). CL4176 were maintened at 16°C for 24 hours followed by a temperature upshift to 23°C during 24 hours to induced Aβ expression. The worms were collected, washed with fresh M9 buffer three times and transferred to Eppendorf tubes containing 20μM CM-H_2DCFDA (final concentration) and incubated for 2 hours. The worms were washed and the fluorescence intensity was measured every hour over a 5 hour interval with a plate reader (Excitation: 488nm; Emission: 510nm).

2.5 Chemiotaxis assay associated with learning

The odorant preference assay was performed according to a previously described method (Dosanjh et al. 2010) with minor modifications. Briefly, age-synchronized transgenic *C. elegans* (CL2355) and vector control (CL2122) were transferred onto culture plates containing either a vehicle or $(PhSe)_2$ and cultured for 24 hours at 16◦C, and then the temperature was upshifted to 23◦C for additional 36 hours to induce neuronal expression of Aβ. Well-feed worms were collected, washed 3 times (without centrifugation) and transferred to NGM plates out of food for conditioning. To evaluate the effect of pairing an attractant with starvation, we selected the volatile chemical compound butanone, that is naturally preferred by worms (Bargmann et al. 1993). To condition the worms, 2 μL of the odorant was placed on the lid of the plates and plates were inverted for 2 h. Conditioned worms were maintained starved at 20°C for 2 hours in the presence of butanone. Naive worms were maintained simultaneously starved in the absence of the odorant. At the end of the starvation period, both groups were subjected to a basic chemotaxis to performed an associative learning

assay (Bargmann et al. 1993) One μl butanone 0.01% (in 95% ethanol) was placed 1 inch above the center of the plate. On the opposite side (at equi-distance) of the plate, 1 μl drop of 95% ethanol was added as vehicle control. Approximately 50 worms were immediately transferred to the center of the plate. After the transfer was complete, an additional 1 μl of each odorant and 1 μl of 1M sodium azide to paralyze the worms on contact were added to the preexisting spots placed prior to the transfer of the worms. Chemotactic index (CI) was scored (number of worms at the attractant- number of worms at control spot/total number of worms) (Bargmann et al. 1993).

2.6 Paralysis Assay

Synchronous L1 population of transgenic worms (CL4176) and their control (CL802) were allowed to develop at 16°C until the third larval stage (L3). The worms were then upshifted to 23°C and scored for paralysis every hour, starting 24 h after upshift until all worms became paralyzed. Animals were scored as being paralyzed when they did not move even after repeated prodding, or if they were associated with a "halo" of ingested bacterial lawn, indicative of an inability to move to access food (Link et al. 2003).

2.7 Body Bends Frequency

Well-fed, 1-day adult worms were transferred to food-free NGM plate and allowed to freely move. After 3 minutes of adaptation, they were scored for the number of body bends generated in a 20 s time interval. A body bend was defined as a change in the direction of propagation of the part of the worm corresponding to the posterior bulb of the pharynx along the *y* axis, assuming the worm was travelling along the *x* axis (Tsalik and Hobert 2003).

2.8 Aldicarb-induced paralysis

The resistance to aldicarb assay was performed according to a previously described method (Nonet et al. 1998) with some modifications. Age-synchronized 1-day adult N2 worms pre-treated with $(PhSe)_2$ or vehicle, were placed on plates containing a final concentration of 0.5 mM Aldicarb (Nonet et al. 1998) and assayed for paralysis every 30 min for a total of 2 hours.

2.9 Acetylcholinesterase Activity assay

Acetylcholinesterase (AChE) activity in adult wild-type and transgenic worms was analyzed with a colorimetric assay (Ellman et al. 1961) with adaptations (Cole et al. 2004).

Following the $(PhSe)_2$ exposure period, 1-day adult worms were washed three times in M9 buffer and transferred to microcentrifuge tubes. The samples were frozen three times in liquid nitrogen prior to sonification 5x 15 s with 10s breaks on ice at 30% amplitude, centrifuged for 30 min at 15,000 x g, and the supernatants (lysates) were collected. The protein content was determined as described previously (Bradford 1976) with bovine serum albumin (BSA) as standard. A 160 µl portion of the sample was mixed with 1,200 µl of 0.25 mM 5,5 Dithiobis (2-nitrobenzoic acid) (DTNB), and 40 µl of 156 mM acetylthiocholine iodide (ASChI) and incubated at 30°C for 5 min. The rate of change in absorbance was measured at 405 nm at 30s intervals for 4 min by spectrophotometry. Kinetic measurements were recorded and converted to total cholinesterase activity using the extinction coefficient for the colored product, 5-thio-2-nitro-benzoic acid (II) (DTNB) (Ellman et al. 1961).

2.10 Quantitative real-time PCR

Aβ mRNA and the small heat shock proteins (sHSP), *hsp-16.1* and *hsp-16.2* mRNA was quantified in the CL2006 strain treated with $(PhSe)_2$ for 7 days after hatching. We used a transgenic strain, which expresses Aβ constitutively, in order to exclude a possible effect of the compound at the promoter gene expression. Next, the animals were washed with M9 buffer into Eppendorf tubes and placed on ice. The worm pellet was resuspended in Trizol® reagent (Invitrogen®) followed by chloroform extraction and isopropanol precipitation. Isolation of total RNA was performed accordingly to the manufacturer's suggested protocol.

Total RNA samples were treated with DNase I (Invitrogen®) to remove genomic DNA contamination in the presence of RNase inhibitor. Reverse transcription (RT) of approximately 2 µg total RNA was performed with random primer, RNase inhibitor, dNTPs and M-MLV reverse transcriptase enzyme (Invitrogen®), according to the manufacturer's suggested protocol. RT products (cDNAs) were maintained at -20°C. Quantitative real-time PCR was performed with gene-specific primers (Table 1) in 20 µl PCR mixture containing 1 µl RT product (cDNAs) as template, 1x PCR Buffer, 5 mM dNTPs, 0.2 µM of each primer, 50 mM $MgCl_2$, 0.1x SYBR Green I (Molecular Probes®) and 1U Taq DNA Polymerase (Invitrogen®). PCR mixtures were subjected to PCR at 95°C for 5 min followed by 45 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 72°C for extension in a Thermocycler StepOne Plus (Applied Biosystems, Foster City, CA, USA). All samples were analyzed in triplicates with a non-template control also included. SYBR Green fluorescence was analyzed by StepOne Plus Software version 2.0 (Applied Biosystems, Foster City, CA, USA) and Cq value (ΔCq) for each sample was calculated and reported using the ΔΔCq method (Livak and Schmittgen

2001). Briefly, for each well, a ΔCq value was obtained by the difference in Cq values (ΔCq) between the target gene and the reference gene. The ΔCq mean value obtained from the control group of each gene was used to calculate the ΔΔCq of the respective gene ($2^{-\Delta\Delta Cq}$).

2.11 Aβ analysis with thioflavin-T

The abundance of Aβ was quantified with the thioflavin T dye according to a previously described method (Xin et al. 2013) with minor modifications. Twenty-seven hours post temperature shift from 16°C to 23°C, the CL4176 worms were collected, washed 3 times with M9 and transferred to a microcentrifuge tube. The worms were frozen three times in liquid nitrogen and sonicated 5x for 15 s (with 10s breaks) on ice at 30% amplitude, centrifuged for 30 min at 15,000 x g, and the supernatants (lysate) collected. The protein content was determined as described previously (Bradford 1976) with bovine serum albumin (BSA) as standard. Equal amount of total protein from every sample was used in each independent experiment. Each tube was mixed with 10 µl M9 and 2 µl 1mM thioflavin-T (final concentration 20mM) in a final volume of 100 µl. Fluorescence resulting from Aβ stained by thiflavin-T was measured by a 96-well microplate reader (Excitation: 440 nm; Emission: 482 nm).

2.12 Statistical analyses

Statistical analysis was performed using *GraphPad* (Version 5.0 for Macintosh OSX, *GraphPad* Software, San Diego, CA). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's Test, or two-way ANOVA, followed by Bonferroni Test for *post hoc* comparison. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1. $(PhSe)_2$ protects against oxidative stress

We first investigated whether $(PhSe)_2$ is able to protect wildtype worms from oxidative stress. Juglone is a redox-active quinone that is very toxic to *C. elegans* and at high concentrations leads to premature death (Blum and Fridovich 1983). *C. elegans* is more susceptible to juglone than other agents and exhibits reductase activity that is able to recruit NADH and NADPH to reduce juglone (Blum and Fridovich 1983). To analyze the protective effect of $(PhSe)_2$ against juglone-induced toxicity, wildtype worms were treated with $(PhSe)_2$. The percentage of worms that survived juglone exposure was determined. The LD_{50} with 1 h

of juglone exposure was approximately 100 μM (data not shown). Wildtype worms that were treated with $(PhSe)_2$ exhibited a significant reduction of juglone-induced mortality (39% and 36.5% reductions by 25 and 50 μM $(PhSe)_2$, respectively) compared with untreated worms (71.5%) but did not attain mortality levels that were similar to controls (5%; Fig. 1A).

Oxidative stress is associated with AD and Aβ toxicity, and we tested the efficacy of $(PhSe)_2$ in attenuating Aβ-induced ROS generation. Oxidative stress in transgenic worms was evaluated using CM-H2DCFDA, a fluorescent probe that is sensitive to the formation of various types of ROS. As shown in Fig. 1B, untreated transgenic worms that expressed Aβ (CL4176) exhibited a prominent increase in DCF fluorescence, whereas Aβ non-expressing worms (CL802) exhibited basal fluorescence levels that corresponded to the physiological endogenous production of ROS. Treatment with 25 and 50 μM $(PhSe)_2$ significantly attenuated DCF fluorescence in the CL4176 strain compared with untreated worms.

3.2. $(PhSe)_2$ treatment recovers associative learning memory in C. elegans

We used the transgenic strain CL2355 to investigate whether $(PhSe)_2$ is able to restore the impairment in associative learning memory induced by Aβ. After pairing butanone with food deprivation, CI was significantly decreased in CL2122 control conditioned worms (Fig. 2). The CL2355 strain displayed an initial decrease in chemotaxis (non-conditioned). After conditioning, the worms persisted in approaching the odorant, indicating a failure to associate the deprived environment with the odorant. CL2355 worms that were treated with 50 μM $(PhSe)_2$ continued to exhibit a decrease in chemotaxis but showed recovery of associative learning that was similar to the control strain (Fig. 2). No difference was observed in the worms treated with 10 μM (data not shown) and 25 μM $(PhSe)_2$. These data indicate that the overall levels of response to butanone decreased in the worms treated with $(PhSe)_2$, suggesting that the treatment may have affected the chemotaxic response (Fig. 2).

3.3. $(PhSe)_2$ reduces muscle function, leading to an increase in paralysis

A strain of *C. elegans* that inherently expresses human Aβ peptide in muscle cells and exhibits a paralysis phenotype (Link et al. 2003) was used to determine whether $(PhSe)_2$ protects against Aβ-induced toxicity. A significant acceleration of paralysis was observed in transgenic worms that were fed $(PhSe)_2$ compared with untreated controls (Fig. 3A). Twenty-seven hours after the increase in temperature, 20% of the transgenic $A\beta_{1-42}$-expressing worms were paralyzed, compared with 31.5%, 37.5%, and 50% of the worms treated with 10, 25, and 50 μM, $(PhSe)_2$, respectively. Paralysis was observed in 100% of the worms in the absence of

$(PhSe)_2$ at 33 h. In worms fed 25 and 50 μM $(PhSe)_2$, full paralysis was observed at 32 and 31 h, respectively, demonstrating significant acceleration of the onset of paralysis by $(PhSe)_2$ in worms that expressed $A\beta_{1-42}$.

To investigate whether the accelerated rates of paralysis that resulted from $(PhSe)_2$ exposure were attributable to an increase in toxicity induced by Aβ or resulted from a general effect on movement or muscle function in the worms, we assessed the effects of $(PhSe)_2$ on locomotor behavior in the wildtype N2 strain. The results showed a decrease in the number of body bends in worms treated with 25 and 50 μM $(PhSe)_2$ (48/min and 47/min, respectively) compared with control worms (56/min; Fig. 3B).

3.4. $(PhSe)_2$ increases acetylcholinesterase activity

The acetylcholinesterase (AChE) inhibitor Aldicarb produces an accumulation of acetylcholine in the synaptic cleft, leading to paralysis in worms. To investigate possible alterations in cholinergic signaling, worms that were treated with $(PhSe)_2$ were tested for resistance to paralysis induced by Aldicarb. As shown in Fig. 4A, wildtype worms that were treated with $(PhSe)_2$ became paralyzed at a slower rate than untreated worms when exposed to Aldicarb ($p < 0.001$). These data suggested a reduction of acetylcholine levels at the neuromuscular synapse. To investigate this possibility, we measured AchE activity in wildtype (N2) and transgenic (CL4176) worms. Acetylcholinesterase activity was significantly higher in $(PhSe)_2$-treated worms than in controls, showing a dose-dependent effect ($r^2 = 0.9703$; Fig. 4B).

3.5. Aβ and hsp-16.2 expression in transgenic C. elegans

Transgenic worms with constitutive muscle-specific Aβ expression (Link 1995) were used to examine the effect of $(PhSe)_2$ on the expression of the *Aβ* and HSP genes (*hsp-16.1* and *hsp-16.2)*. Real-time quantitative PCR revealed that chronic treatment with 25 and 50 μM $(PhSe)_2$ significantly decreased the levels of *Aβ* (Fig. 5A) and *hsp-16.2* (Fig. 5B) mRNA compared with controls but not *hsp-16.1* mRNA levels (data not shown).

Fig. 5C shows that 27 h after Aβ-induction, when approximately 20% of the untreated transgenic worms (CL4176) were paralyzed (Fig. 3A), the levels of the soluble Aβ peptide were significantly increased compared with the control strain (CL802). Treatment with 25 and 50 μM $(PhSe)_2$ significantly decreased Aβ peptide levels in the worms compared with the control group. No significant differences were observed between treated worms and the control strain (Fig. 5C).

4. Discussion

The efficacy of organoselenium compounds, such as $(PhSe)_2$, in attenuating disease has been extensively studied in recent years (Nogueira et al. 2004; Jesse et al. 2009; da Silva et al. 2012; Dias et al. 2012; Costa et al. 2013; de Oliveira et al. 2013; Glaser et al. 2013; Menezes et al. 2013). In the present study, we investigated the protective properties of $(PhSe)_2$ in a *C. elegans* model of Aβ-induced toxicity. The simple *C. elegans* model described in this study does not replicate many aspects of human AD. The rationale for using this model is that it can recapitulate some of the disease-relevant biology that underlies AD, such as the mechanisms by which Aβ perturbs cell function and causes toxicity. *C. elegans* that were exposed to $(PhSe)_2$ did not exhibit an increase in mortality, even at doses greater than 100 μM (Avila et al. 2012). Moreover, $(PhSe)_2$ pretreatment prolonged the lifespan in a model of manganese-induced toxicity (Avila et al. 2012). Corroborating its antioxidant properties (da Silva et al. 2012), the present findings indicate that $(PhSe)_2$ protected worms from oxidative damage, decreasing both ROS production and mortality (Fig. 1). Additionally, $(PhSe)_2$ treatment restored associative learning memory in *C. elegans* that pan-neuronally expressed Aβ (Fig. 2) and decreased soluble Aβ peptide levels in muscles in Aβ-expressing worms (Fig. 5).

In vivo Aβ-induced toxicity in *C. elegans* has been used as a model of AD (Link et al. 2003; Wu et al. 2006; Abbas and Wink 2010). These worms constitutively and upon increases in temperature express Aβ in the muscles of the body wall, leading to increased ROS levels (Wu et al. 2006) and progressive paralysis (Link et al. 2003). However, previous studies demonstrated that paralysis is not associated with H_2O_2 levels (Wu et al. 2006). Corroborating the propensity of this strain to express altered redox status, we showed that $(PhSe)_2$ significantly reduced Aβ-induced ROS production (Fig. 1B), despite the increase in paralysis (Fig. 3A). These observations suggest that Aβ peptide-induced toxicity is multifaceted and includes both oxidative stress and other toxic mechanisms.

An acceleration of the rate of paralysis was observed in CL4176 worms that were treated with 25 and 50 μM $(PhSe)_2$ (Fig. 3A). Moreover, $(PhSe)_2$ treatment decreased the overall levels of response to butanone, despite the recovery of associative learning (Fig. 2). $(PhSe)_2$ also decreased locomotion in wildtype worms (Fig. 3B). These data suggest that the $(PhSe)_2$-induced increase in paralysis occurred independently of Aβ toxicity and is potentially mediated by alterations in muscle signaling or function. To test this hypothesis, we used the pharmacological agent Aldicarb, a cholinesterase inhibitor that prevents the breakdown of

Ach, thus inducing paralysis in the worms. The observed resistance to Aldicarb-induced paralysis in $(PhSe)_2$-treated worms (Fig. 4A) indicates that changes in Ach levels may contribute to the $(PhSe)_2$-induced decrease in movement and acceleration of paralysis. This is supported by earlier studies, in which high concentrations of Se reduced cholinergic signaling, causing paralysis in *C. elegans* (Estevez et al. 2012).

To confirm the changes in cholinergic signaling, AChE activity was assayed in adult wildtype and transgenic worms. The increase in AChE activity observed in $(PhSe)_2$-treated worms (Fig. 4B) offers a plausible mechanism for the decrease in worm motility observed in the present study, in which the AChE breakdown of acetylcholine at the synaptic cleft reduced the concentration of this neurotransmitter at the neuromuscular junction. The decrease in Ach levels is a concern in AD, but our data do not exclude the possibility of using $(PhSe)_2$ in AD research. Mammalian studies demonstrated that this compound inhibited cerebral AchE activity (Luchese et al. 2007). Moreover, $(PhSe)_2$ prevented the stimulation of AchE activity caused by ovariectomy and ameliorated cognitive deficits (da Rocha et al. 2012). Additionally, another organoselenium compound, p,p-methoxyl-diphenyl diselenide $(MeOPhSe)_2$, was neuroprotective in a mouse model of sporadic dementia of the Alzheimer's type, improving performance in cognitive tasks and protecting against the increase in cerebral AChE activity (Pinton et al. 2010). These studies showed an opposite effect of $(PhSe)_2$ treatment in mammals and nematodes with regard to AChE activity. The inhibition of AchE activity may contribute to the beneficial effects of $(PhSe)_2$ in AD, although this was not demonstrated in *C. elegans*.

C. elegans that express Aβ peptide in neurons are also used as a tool to understand the mechanisms involved in Aβ-induced toxicity (Dosanjh et al. 2010). The neuronal expression of Aβ affects behavioral phenotypes in worms, leading to a decrease in lifespan and impaired associative learning (Dosanjh et al. 2010). In the present study, we evaluated whether $(PhSe)_2$ treatment can restore some of these effects. After pairing butanone with food deprivation, CI was significantly decreased in the control transgenic strains (CL2120), whereas transgenic worms that expressed Aβ (CL2355) exhibited a decrease in chemotaxis. After conditioning, they still consistently pursued the odorant (Fig. 2), indicating a failure to associate the deprived environment with the odorant. Our data indicate that $(PhSe)_2$-treated worms continued to exhibit a decrease in chemotaxis but exhibited a recovery of associative memory, in which they avoided the odorant after pairing it with the absence of food. Acute $(PhSe)_2$ administration in mice has been shown to improve cognitive performance in an object recognition task (Rosa et al. 2003). Additionally, rats that were sub-chronically exposed to

$(PhSe)_2$ exhibited cognitive improvements in the Morris water maze (Stangherlin et al. 2008). The decrease in chemotaxis is consistent with the decrease in movement and increase in paralysis, thus contributing to the evidence of reduced cholinergic signaling induced by $(PhSe)_2$.

Given that soluble Aβ oligomers but not aggregates are toxic species of Aβ (Hardy and Selkoe 2002), we used CL4176 worms to quantify Aβ peptide levels using fluorescent thioflavin-T staining, which specifically detects non-aggregated proteins (Hatters and Griffin 2011). Prefibrillar assemblies might also be neurotoxic *in vivo*, which seems plausible when considering the synaptic, electrophysiological, and behavioral changes observed in young APP transgenic mice before plaque formation (Mucke et al. 2000; Walsh et al. 2002). Additionally, microinjection of a culture medium that contained naturally secreted human Aβ into living rats revealed that Aβ oligomers (in the absence of monomers and amyloid fibrils) can inhibit long-term potentiation in the hippocampus, which is required for memory formation (Walsh et al. 2002). In *C. elegans*, the increase in amyloid deposits triggered by copper and the associated decrease in some oligomeric Aβ species are related to improvements in Aβ-induced pathological traits (Rebolledo et al. 2011). CL4176 worms express Aβ mostly in a soluble form and do not form Aβ plaques in muscle cells (Dostal and Link 2010). Our data demonstrated that Aβ peptide levels were significantly lower in $(PhSe)_2$-treated worms (Fig. 5C), consistent with the ability of this compound to modulate Aβ transgene expression (Fig. 5A). This downregulation of Aβ and consequent decrease in Aβ levels may explain the reduction of ROS levels observed in $(PhSe)_2$-treated transgenic worms (Fig. 1B), given that the overexpression of this peptide in CL4176 worms induced the overproduction of ROS. Similarly, the recovery of the deficit in associative learning induced by $(PhSe)_2$ was also associated with the downregulation of Aβ transgene expression.

Previous studies showed that the overexpression of HSP-16.2, a *C. elegans* chaperone protein homologous to B crystallin, can suppress toxicity associated with human Aβ in a *C. elegans* model of AD (Fonte et al. 2008). Heat Shock Proteins (HSPs) are a group of low-molecular-weight polypeptides that are expressed under stress conditions (de Jong et al. 1998). The HSP-16.2 precursor gene is upregulated by the induction of Aβ expression (Link et al. 2003), and HSP-16 protein directly interacts with Aβ (Fonte et al. 2002). Our data demonstrated that $(PhSe)_2$ treatment decreased *hsp-16.2* expression, likely by reducing the need for this chaperone upon $A\beta_{1-42}$-induced toxicity because Aβ levels were also concomitantly decreased.

5. Conclusion

Altogether, the present study indicated that $(PhSe)_2$ downregulated Aβ transgene expression in a *C. elegans* model of AD, decreasing the levels of Aβ peptide and consequently affording protection to the worms. This protection was reflected by a decrease in ROS generation, the restoration of associative memory, and a reduction of *hsp-16.2* mRNA expression but not paralysis rates. This apparent discrepancy may be explained by the propensity of $(PhSe)_2$ to increase AChE activity and the consequent decrease in movement (Scheme 1).

Furthermore, the findings indicate that $(PhSe)_2$ effectively protects *C. elegans* from Aβ-induced toxicity, not just by attenuating ROS generation and restoring redox status but also by its ability to thwart *Aβ* transgene expression and decrease Aβ levels. These findings, together with earlier studies, suggest that $(PhSe)_2$ is a potential promising pharmacological modality for the treatment of AD and possibly other diseases.

Conflict of interest

The authors declare no conflict of interest.

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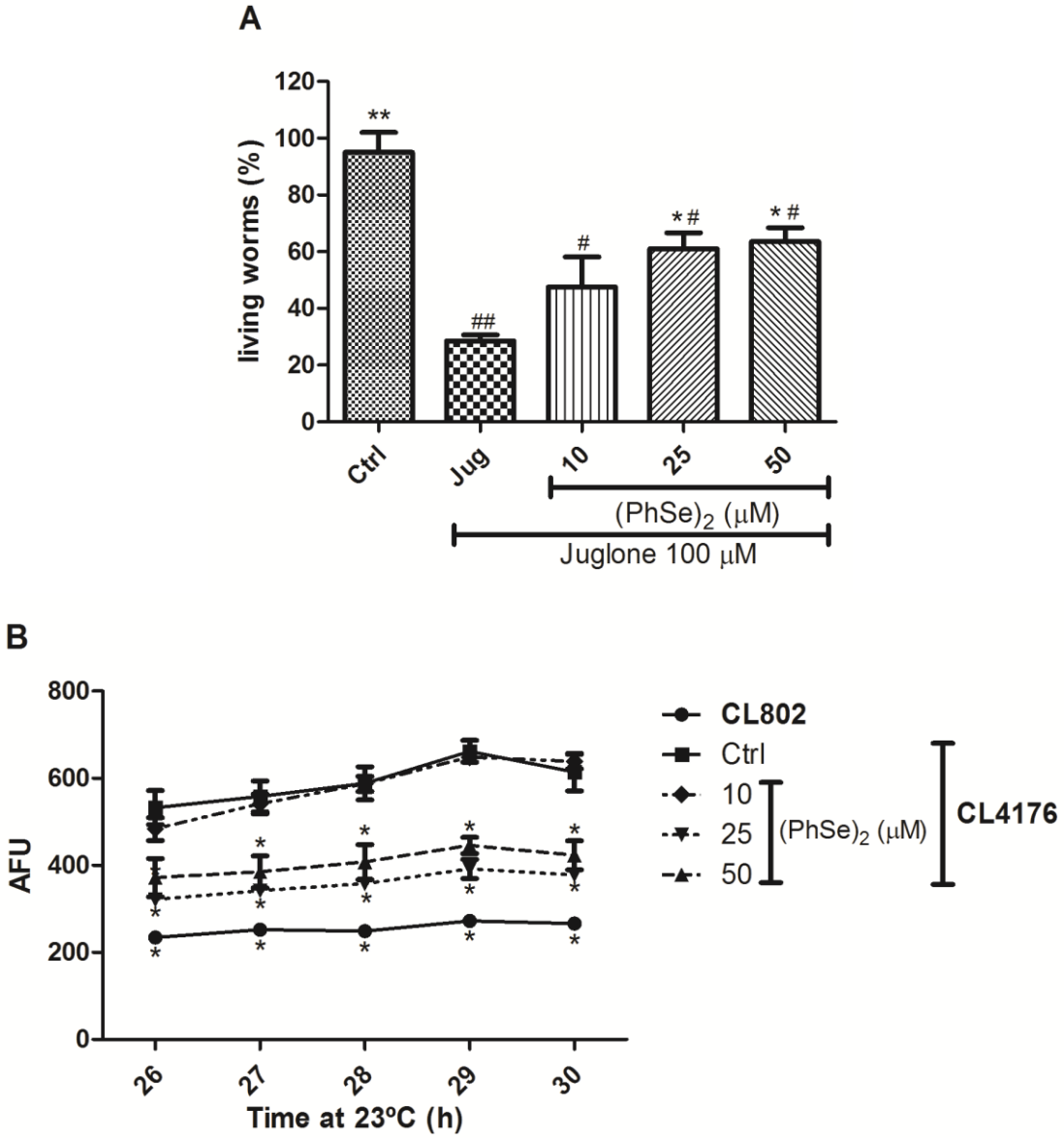


Figure 1. Protective effect of $(PhSe)_2$ on oxidative stress. (A) Survival to 100 µM/1h juglone-exposition in wild-type (N2) worms. Data are expressed as percentage of living worms from four independent assays of 100 worms in each experiment (N = 400). Errors bars represent the SEM. *p<0.01, **p<0.001 versus Jug, #p<0.05, ##p<0.01 versus Ctrl (one-way ANOVA). (B) Levels of ROS production induced by the peptide Aβ in transgenic strain CL4176 expressed in Arbitrary Fluorescence Unity (AFU). Time represent the hours after Aβ expression was induced by temperature upshift. Data are expressed as CM-H2DCF fluorescence in four independent assays (N = 4). Error bars represent the SEM. p<0.001 versus Ctrl (two-way ANOVA).

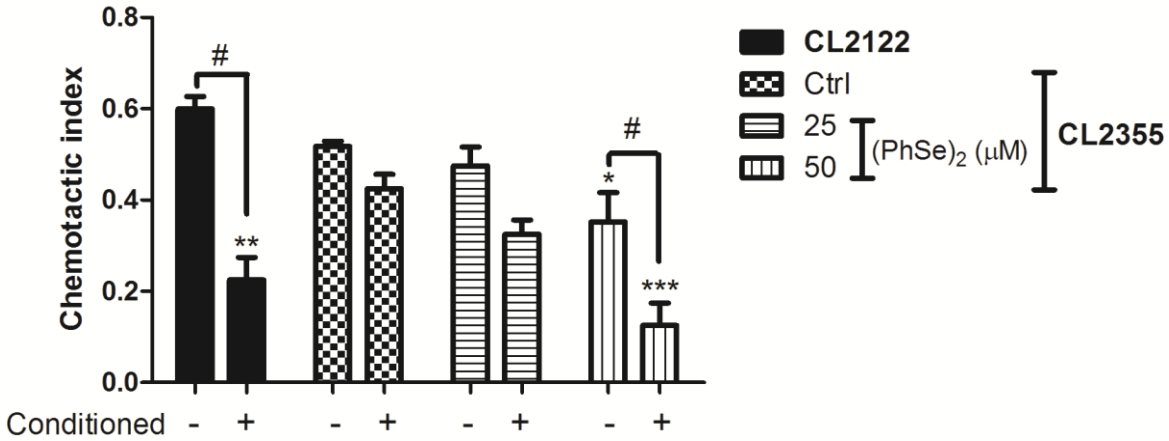


Figure 2. $(PhSe)_2$ effect in associative learning assay. Transgenic worms were starved for 2 h either in the presence (+) or absence (-) of the odorant butanone (0.01%) and subsequently subjected to a chemotaxis assay for 1h. Data are expressed in four independent assays with 50 worms each (N = 200). Errors bars represent the SEM. *p<0.05, **p<0.01, ***p<0.001, versus Ctrl. #p<0.01 + versus – (Two-way ANOVA).

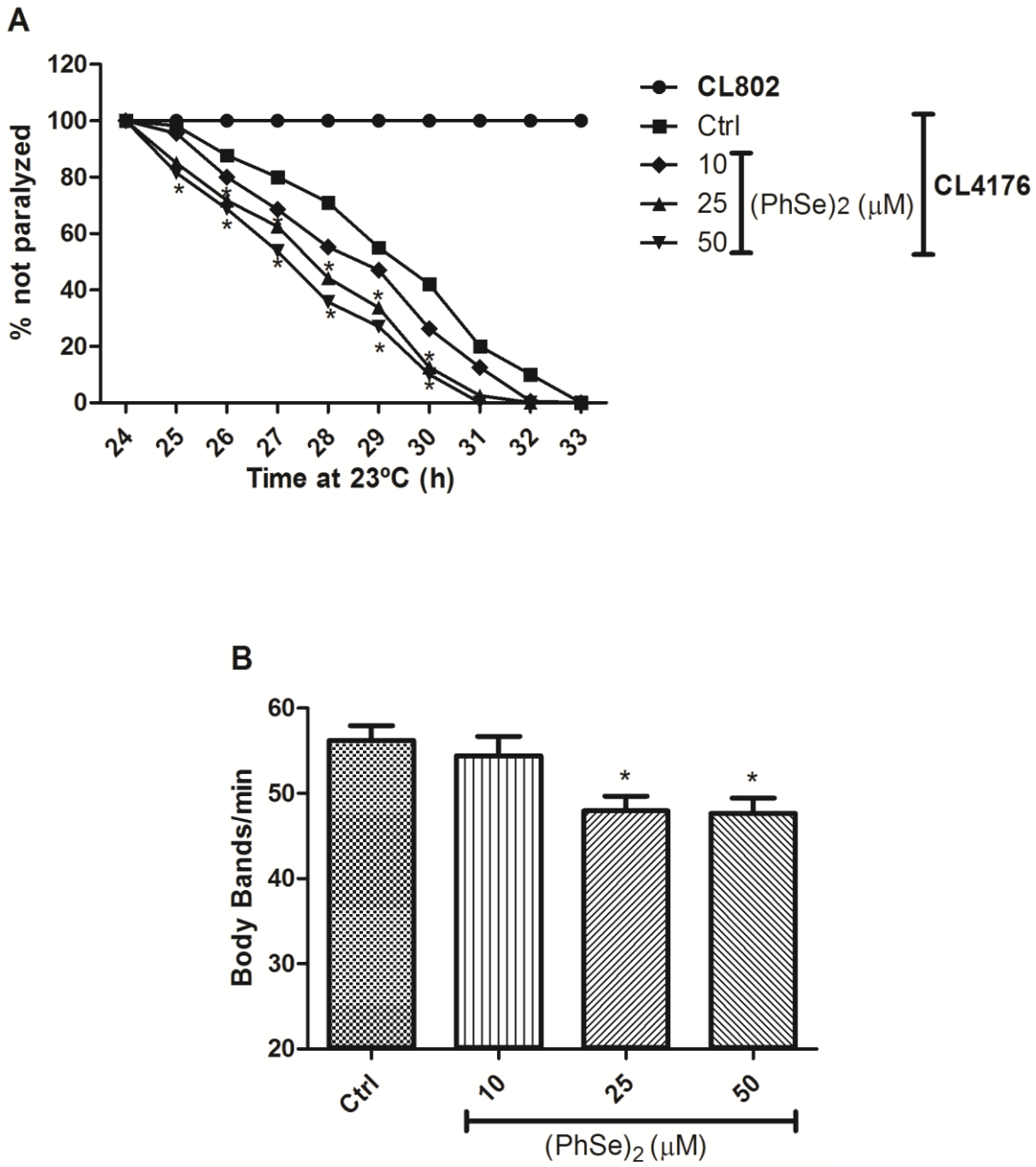


Figure 3. Effect of $(PhSe)_2$ on movement in *C. elegans*. (A) Aβ-induced paralysis in the transgenic strain Aβ muscles expressing (CL4176) and control strain (CL802). Time represent the hours after Aβ expression was induced by temperature upshift. Data are expressed as percentage of no paralyzed worms from three independent assays with 100 worms in each group (N = 300). Error bars indicate the SEM. $p<0.001$ (two-way ANOVA). (B) Locomotor activity assay in wild-type (N2) worms. Data are expressed as number of body bends performed by the worms. Error bars indicate the SEM. *$p<0.05$ versus Ctrl (one-way ANOVA).

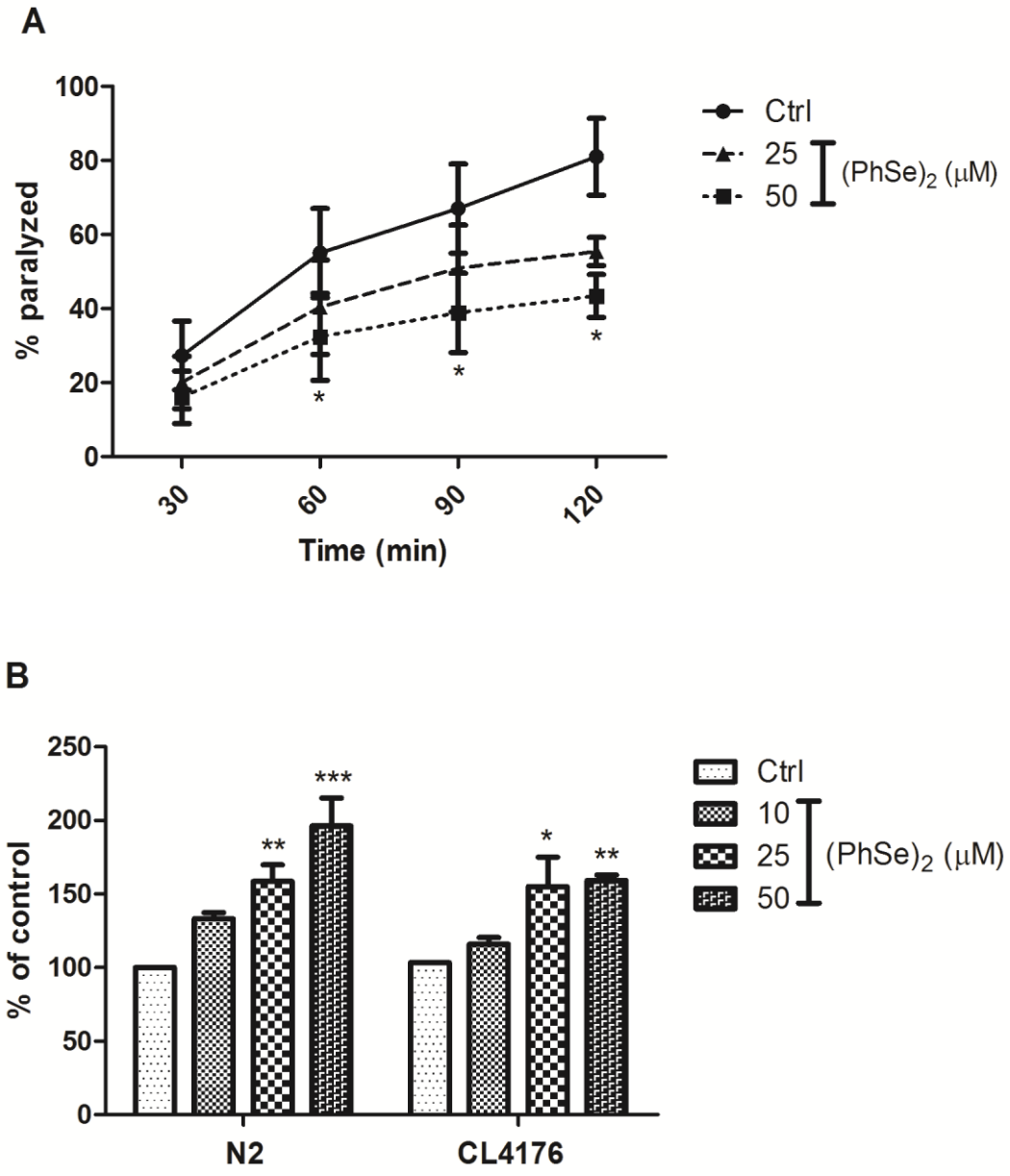
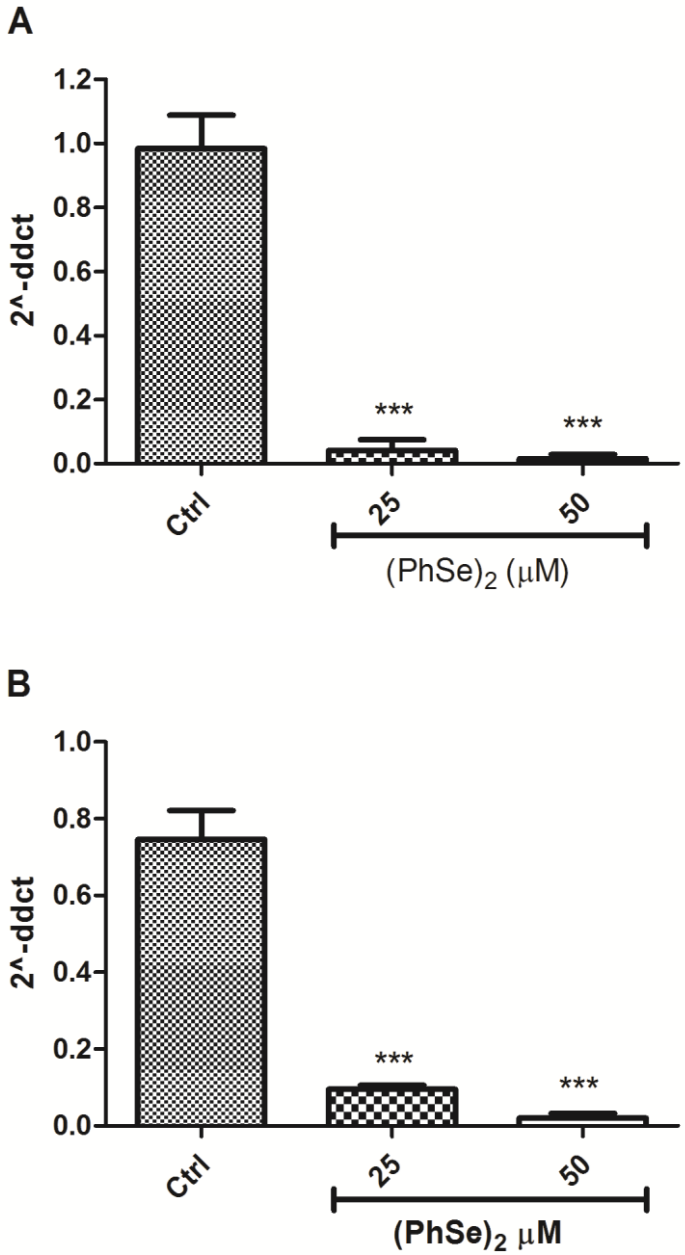


Figure 4. **Effect of $(PhSe)_2$ on cholinergic signaling.** (A) Aldicarb-induced paralysis in *C. elegans* wild-type strain (N2). Worms were placed on plates containing 0.5 mM aldicarb and assayed for forward movement every 30 min for a total of 2 h. Data represent the percentage of paralyzed animals from three independent assays with 50 worms in each group (N = 150). *p<0.001, 25 and 50 μM $(PhSe)_2$ versus Ctrl. (B) Ache activity in wild-type strain (N2) and transgenic Aβ muscle expressing strain (CL4176). Data are expressed as percentage of control from four independent assay (N = 4). *p<0.01, **p<0.001, #p<0.05, ##p<0.01, versus its respective Ctrl (Two-way ANOVA). Errors bars represent the SEM.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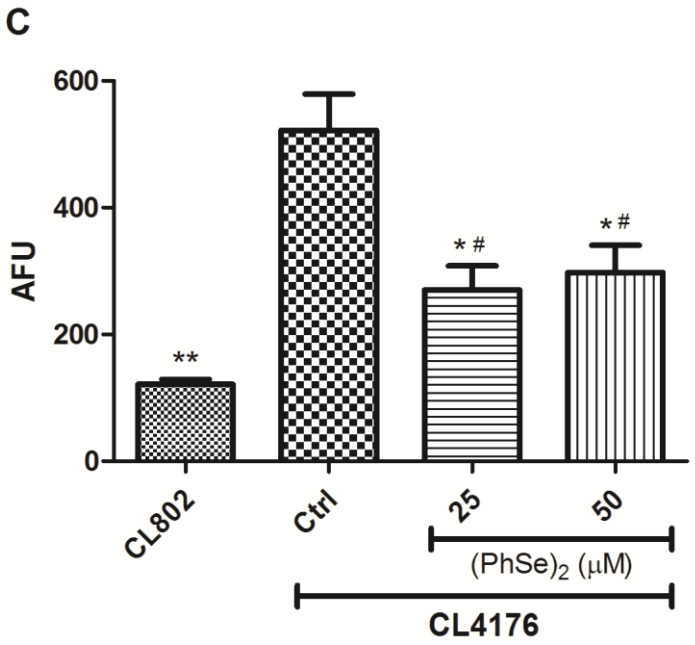
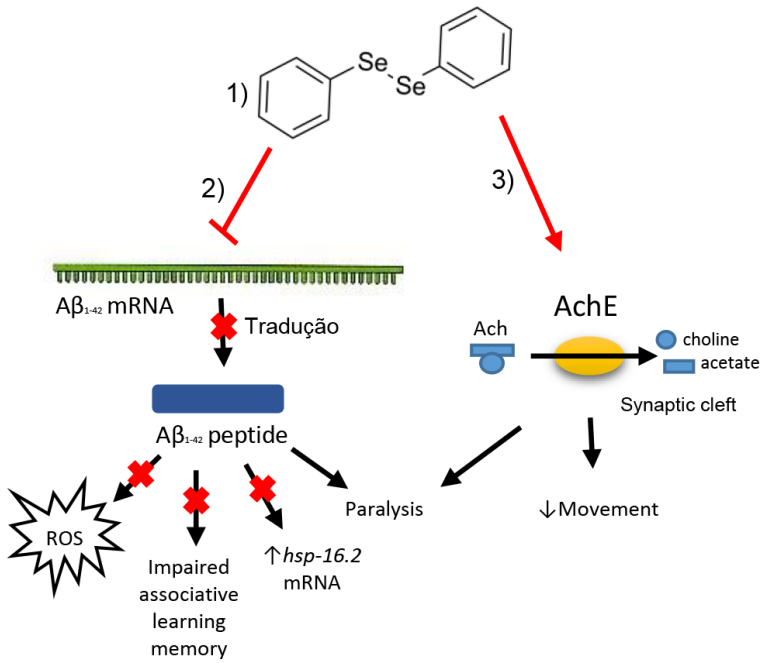


Figure 5. **Effect of $(PhSe)_2$ on *Aβ* and *hsp-16.2* transgene expression and Aβ accumulate on in transgenic Aβ strain.** Data represent the quantitative RT-PCR measurements for (A) *Aβ* mRNA levels and (B) *hsp-16.2* mRNA levels using a ΔΔCq method in transgenic Aβ strain (CL2006) from three independent assays (N =3). (C) Quantification staining of Aβ peptide in the transgenic Aβ strain (CL4176) 27 h after induction using thioflavin-T dye (N = 4). Errors bars represent the SEM.

#p<0.05 versus CL802, *p<0.01, **p<0.001 versus Ctrl (one-way ANOVA).



Scheme 1. Summary. $(PhSe)_2$ effect in *C. elegans* AD models. 1) Chemical structure of $(PhSe)_2$. 2) $(PhSe)_2$ down-regulation of Aβ peptide mRNA confers a reduction in the levels of the peptide $A\beta_{1-42}$ in the worms. By consequence, it prevents the overproduction of the ROS and the oxidative damage, besides the impaired associative learning memory and the up-regulation of the *hsp-16.2*. 2) By another way, $(PhSe)_2$ induce an increase in AchE activity in the worms, reducing the Ach neurotransmitter at the synaptic cleft and taking to an decreased motility and to the increased paralysis.

4. CONCLUSÕES

4.1 Conclusão geral:

Ainda há um conhecimento limitado a respeito do mecanismo da DA e o papel do peptidio Aβ nesta patologia. Além disso, os tratamentos disponíveis apenas visam apenas atenuar os sintomas da doença. Desta forma, há um grande interesse na descoberta de novos compostos efetivos em prevenir o desencadeamento da doença, bem como retardar a sua progressão. Neste contexto, os resultados obtidos neste trabalho, apesar de preliminares, permitem concluir que o $(PhSe)_2$ mostrou-se um composto eficaz contra a toxicidade mediada pelo peptidio Aβ no nematódeo *C. elegans.*

Considerando que a agregação do peptídio tóxico Aβ é um evento crucial para o desenvolvimento da DA, sugerimos que o $(PhSe)_2$ é um composto promissor a ser estudado como possível agente farmacológico, uma vez que mostrou eficiência na prevenção do acúmulo deste peptídio e dos seus consequentes danos. Entretanto, mais estudos são necessários a fim de comprovar sua eficácia, bem como elucidar os mecanismos de atuação do $(PhSe)_2$.

4.2 Conclusões específicas:

De acordo com os resultados apresentados nesta dissertação podemos concluir que o $(PhSe)_2$, no nematódeo *C. elegans*:

- Possui atividade antioxidante, protegendo contra a toxicidade induzida pelo agente estressor Juglone, reduzindo a mortalidade;
- Diminui a produção de espécies reativas de oxigênio induzida pelo peptídio Aβ;
- Restaura a memória associativa em modelo de expressão neuronal do peptídio Aβ;
- Exacerba a paralisia induzida pela expressão muscular do peptídio Aβ;
- Reduz a expressão do mRNA do gene *hsp-16.2*, diminuindo sua necessidade frente a toxicidade induzida pela Aβ;
- Diminui a expressão do mRNA do gene *Aβ* e consequentemente o acúmulo da proteína Aβ;

5. PERSPECTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores em *C. elegans* modelos de DA são:

- Determinar vias de atuação do $(PhSe)_2$;
- Elucidar outros mecanismos de ação do $(PhSe)_2$;
- Investigar o aumento da paralisia induzida pelo $(PhSe)_2$;
- Investigar os efeitos do $(PhSe)_2$ em modelos de DA em mamíferos.

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