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List of Abbreviations

A β	Amyloid β
AD	Alzheimer disease
APP	Amyloid Precursor Protein
AQ	Anthraquinone
AC	Anthracene
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CGC	<i>Caenorhabditis</i> Genetic Stock Centre
CNS	Central Nervous System
Cyt C	Cytochrome C
DAT	Dopamine Transporter
<i>D. melanogaster</i>	<i>Drosophila melanogaster</i>
FUdR	5-Fluoro-2'-deoxyuridine
GFP	Green Fluorescent Protein
ILS	Insulin growthfactor-1 like signaling
LTP	Long Term Potentiation
nACh	Nicotinic Acetylcholine Receptor
NGM	Nematode Growth Media
NMDA	N-methyl-D-aspartate
NT	Neurotransmitter
6-OHDA	6-Hydroxydopamine
PD	Parkinson Disease
RNAi	RNA interference
SNP	Single Nucleotide Polymorphism
YFP	Yellow Fluorescent Protein

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1. Introduction

1.1 Introduction

The human brain contains approximately 100 billion neurons which are expressed by more than 60% of known genes and it is extremely plastic during the whole human life: In neonate, adult and elderly brains vary in morphology and phenotype. Neurotransmitters and receptors which are present during development disappear in the adult brain. Also diseased brain can be different from a healthy brain because receptors change in number and function due to the kind of disease or trauma. Mechanisms to sustain brain homeostasis are negatively impacted by aging, due to an accumulation of environmental insults. The function of the brain can be influenced by hormones and by peripheral system function (e.g. cardiovascular/vascular influences) (Williams, 2001). This complexity makes it difficult to understand the complete neuronal network.

But for the design of new drugs against neuronal disorders with less side effects and better efficiency it is important to understand this complexity and the targets which should be addressed by a screening method. A promising approach for this purpose is the screening of chemical libraries on whole animals. The mouse *Mus musculus* is one of the most common animal model but its costs and generation time reduces its use in high throughput screenings (West et al. 2000). Small and simple animals like *C. elegans* or *D. melanogaster* could solve that problem (Giacomoto and Segalat, 2010). The characterization of *C. elegans* has prompted a shift from the reductionist approach (molecular function at the synaptic level) to a renewed appreciation of the hierarchical complexity (gene, synapse, pathway, phenotype) of the nervous system. With its simplified neuronal network we are now able to integrate the structure with its function at both the tissue and the whole animal level. Further we can integrate animal studies at the systems and compound levels. In this way a number of highly effective CNS drugs have been discovered in the last 40 years, the majority of which act synaptically, either mimicking (agonists), facilitating (transmitter uptake blockers, allosteric modulators) or antagonizing the effects of endogenous neurotransmitters and neuromodulators (Williams, 2001).

Similarities in neurodegenerative diseases

The hallmarks of neurodegenerative diseases are progressive and gradual loss of neurons in affected areas of the brain. Neurodegenerative disorders include Alzheimer disease, Parkinson disease, Huntington disease, amyotrophic lateral sclerosis and others. Despite differing clinical phenotypes all seem to have a pathogenic mechanism that involves the aggregation and deposition of abnormally folded proteins either extracellularly or intraneuronally. The pathogenic mechanisms are still poorly understood but currently it is assumed that toxic intermediates like the oligomeric conformation of β -amyloid, tau and α -synuclein lead to the neuronal degeneration (Arya et al., 2010).

1.2 Alzheimer's Disease

With nearly 8 million patients in the United States, France, Germany, Italy, Spain, the United Kingdom and Japan Alzheimer's disease remains the most prevalent neurodegenerative disorder. In accordance with the world's leading research and advisory companies for pharmaceuticals and healthcare issues "Decision Resource" the market will nearly triple over the next ten years increasing from \$5.4 billion in 2010 to \$14.3 billion in 2020 (www.DecisionResourcesGroup.com).

Dementia is generally defined as "a state of serious emotional and intellectual disorientation" and marks deficits in memory, language, visuospatial skills and cognition. In post-mortem brains pathological changes include β -amyloid plaques, neurofibrillar tangles composed of tau, inflammation, neuron loss and synapse loss and the loss of synapses correlates with memory impairments (Snyder et al., 2005).

In 90% of all cases it affects the elderly population with an age over 65 (Lockhart and Lestage, 2003). The early-onset familial form is linked to mutations in presenilin genes and amyloid precursor protein gene or a trisomy in chromosome 21. Another risk factor is apolipoprotein E, which carries cholesterol in the blood but can bind to amyloid β too and is so involved in aggregation (Poirier, 2005). Furthermore, high blood pressure, insulin resistance, hypercholesterolemia and cerebrovascular disease are able to increase the risk to develop AD with age (Poirier, 2005).

Molecular studies identified additionally to tau protein identified amyloid peptides as the major components of the plaques. Amyloid 1-40 and 1-42 peptides are the dominant forms derived from proteolytic cleavage of amyloid precursor protein (APP), a trans- membrane protein, by enzymatic complexes known as β - and γ -secretase

(Yoshiike et al., 2003). Amyloid 1-42 peptide is highly hydrophobic and accumulates both extracellular and intracellular (Snyder et al., 2005).

It is a fact that the formation of misfolded β -amyloid leads to its deposition and consequently to the degeneration of the neurons. But the question whether the early soluble or the mature fibrillar species of the amyloid- β peptide is the toxic species is discussed controversially. Based on their strong genetic and pathologic association with disease it is not surprising that in the past there has been a focus towards developing therapies against the β -amyloid plaques. But until now no strong correlation between plaque deposition and the degree of cognitive decline has been established and therefore it is now in question if β -amyloid plaque is responsible for the damage during the process of neurodegeneration or not (Mondragón-Rodríguez et al., 2012). A more relevant hypothesis is based on the view that β -amyloid reduces synaptic plasticity. This is the process by which synapses modulate their connections with other neurons and an insult of β -amyloid results in the impairment of Long Term Potentiation (LTP) and the facilitation of Long Term Depression (LTD) (Mondragón-Rodríguez et al., 2012). But the mechanisms are still unclear. It is known is that N-methyl-D-aspartate (NMDA) receptors are involved in synaptic plasticity and that their overactivation leads to neuronal cell death (Jin H. et al., 2007). Tanzi claimed 2005 in the “synaptotoxic β -amyloid hypothesis” that persistent binding of the soluble species of β -amyloid to nACh receptor leads to reduced NMDA receptor density at synapses, thereby decreasing glutamatergic transmission and impairing synaptic plasticity (Figure 1a).

In contrast, Hoe et al. (2009) found that β -amyloid enhances NMDA receptor function by direct interaction (Figure 1b).

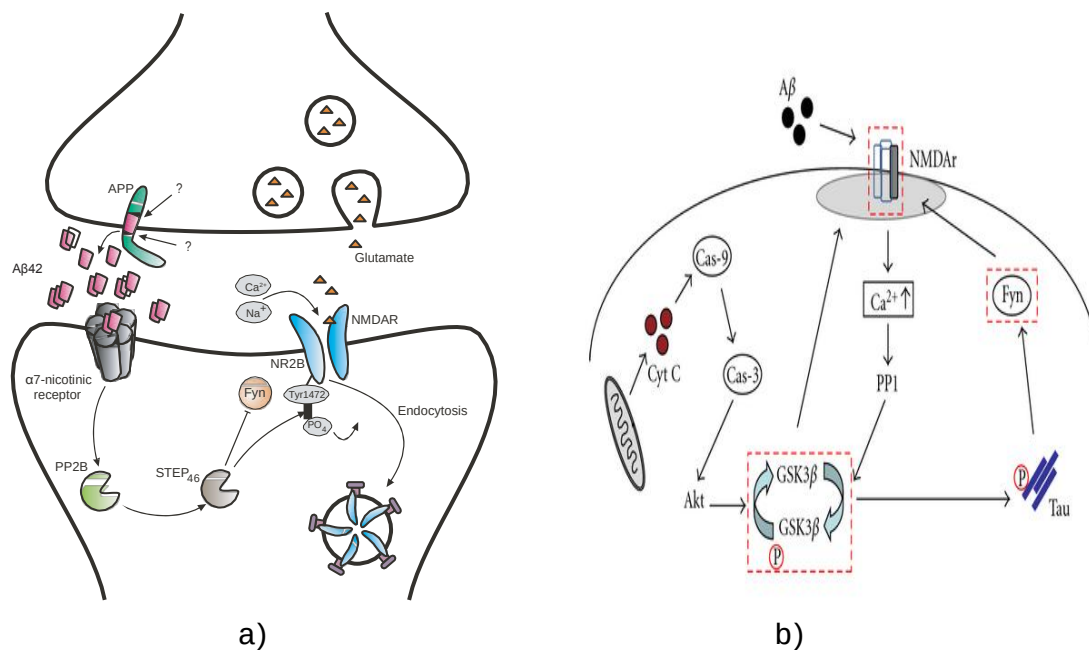


Figure 1: The two synaptic amyloid hypotheses:

a) Aβ42 influences NMDA receptor at the synapse indirectly. β- and γ-secretase cleave β-amyloid from its precursor protein APP. This leads to interaction of β-amyloid with the α7-nicotinic receptor and finally to decreased NMDA receptor density and impairment of synaptic plasticity (Tanzi, 2005).

b) β-amyloid interacts with NMDA receptor directly and leads to impairment of LTP (Mondragón-Rodríguez et al., 2012).

In this context, synaptic plasticity modulators could be an interesting target. The most important aspect of this approach is that synaptic plasticity links β-amyloid and tau to the synaptic terminal. In this regard, some molecules have already been tested. Memantine, for example, is a partial antagonist of NMDA receptor function. But Memantine in its current therapeutic form only slows down the neuronal degeneration process and does not improve cognitive function (Weiner et al., 2011). The most serious problem today in the treatment of Alzheimer's disease is that till now only symptoms but not the cause can be treated. Beside the treatment with NMDA receptor antagonists, the decreased synthesis of acetylcholine is compensated with acetylcholinesterase inhibitors like donepezil, galantamine or rivastigmine.

Other strategies to reduce the pathogenicity of β -amyloid peptide are:

1. To decrease amyloid production by inhibiting the activity of β -secretase and up-regulation of γ -secretase activity (Golde and Younkin, 2001; Postina et al., 2004).
2. To reduce amyloid aggregation curcuminol or curcumin are currently in phase II and III clinical trials (Ringman and Cummings, 2006).
3. To enhance amyloid clearance by increasing amyloid degrading enzymes activity (Saido and Iwata, 2006).
4. Immunotherapy: Active and passive immunization showed reduced amyloid deposits and cognitive and behavioural improvements with no adverse events (Morgan and Gitter, 2004) and has proceeded late-stage clinical trials. Most promising are the anti-beta-amyloid (anti-A β) monoclonal antibodies (MABs) solanezumab and bapineuzumab.

Sealife's novel compounds mainly belong to the class of quinones and here specifically to naphthoquinones and anthraquinones. In this work we wanted to test Sealife's marine compound library towards their ability to protect transgenic *C. elegans* from Alzheimer's phenotype. Quinones act as inhibitors of different metabolic pathways in the cell and they have anti-bacterial, anti-viral and anti-cancer activities (Convertino et al., 2009). In the work of Jin et al. (2006) was found that tetraamine derivatives of anthraquinone and anthracene are potent NMDA antagonists. Another study has shown that anthraquinones can serve as aggregation inhibitors because of the dipole interaction of the quinonic ring with aromatic recognition sites within the amyloidogenic proteins (Figure 2).

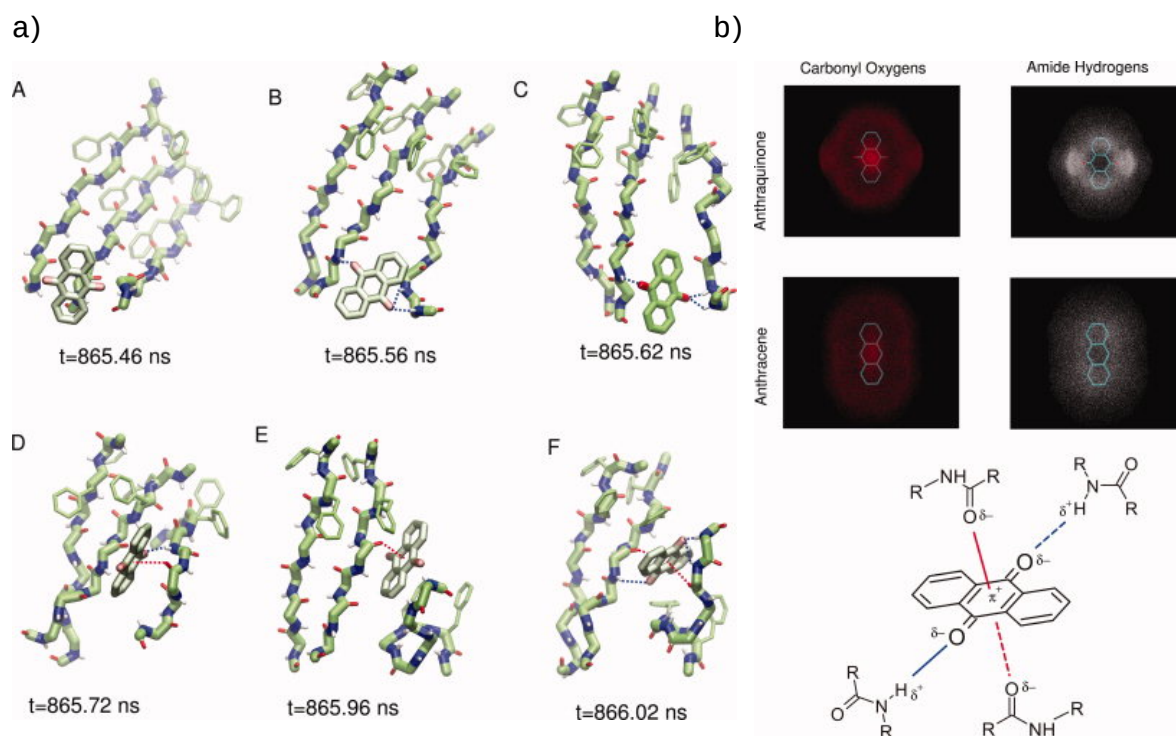


Figure 2: a) Anthraquinones (AQ) disrupt β -amyloid. This happens in a kind of “butter-knife” mechanism. An Anthraquinone draws near the oligomeric β -amyloid (A), interacts (B and C, blue dashed lines), intercalates into (D–F, red dashed line) and breaks the ordered peptide via hydrogen bonds and $\pi^+\delta^-$ interactions (F) (Convertino, 2009).

b) Interactions between backbone polar groups and anthraquinones or anthracenes (AC) (Convertino et al., 2009).

In 2010 Scherzer-Attali et al. demonstrated that 1,4-naphthoquinon-2-yl-L-tryptophan completely inhibited β -amyloid oligomerization and fibrillization, as well as the cytotoxic effect of β -amyloid oligomers towards a cultured neuronal cell line. Further when fed to a transgenic *Drosophila* model of Alzheimer’s disease it prolonged the life span and completely abolished defective locomotion. Immuno-staining of the brains showed a significant reduction in β -amyloid accumulation. In a NMR and CD spectroscopic approach they provided evidence for the compound’s activity which is likely mediated by clamping of the aromatic recognition interface in the central segment of β -amyloid. This provided proof of concept for a new class of drugs modifying Alzheimer’s disease (Scherzer-Attali et al., 2010).

1.3 Parkinson's disease

Parkinson's disease affects more than four million people worldwide and is the second most prevalent neurodegenerative disorder after Alzheimer disease. As described by the English physician James Parkinson (1755–1824) in "*An Essay on the Shaking Palsy*" Parkinsonism is clinically characterized by the triad of tremor, rigidity and bradykinesia (Parkinson, 2002). However, non-motor manifestations, including autonomic and cognitive dysfunction based on abnormal levels of the neurotransmitters serotonin, noradrenalin and acetylcholine are increasingly recognized as being part of the wider clinical syndrome. The histopathology of PD is characterized by a loss of dopaminergic neurons in the *Substantia Nigra* that project to the striatum.

The main risk factors for developing such a disease are inheritance and age (Semchuk et al., 1993). A single mutation in one of the genes Park1 – Park13 has been discovered to cause PD. But this monogenetic cause does not explain the great majority of 90% sporadic cases. Pesticides and insecticides like rotenone and paraquat or heavy metals like manganese are toxic to dopaminergic neurones by inhibiting the mitochondrial complex I (Stredrick et al., 2004; Chesselet and Fernagut, 2008).

Therefore current therapies are aiming to substitute the missing dopamine. **Levodopa**, the immediate precursor of dopamine, has been used for more than forty years. It is widely available at a low price. Within five years of treatment with Levodopa about 60% of patients develop complications of disabling response fluctuations ("wearing-off" effect) and dyskinesia (abnormal involuntary movements). Patients at an age of less than 60 years) are particularly prone to develop these problems faster. Therefore younger patients start with **D1 and D2 agonists** like apomorphine or bromocriptine although they are less effective (Fahn, 2008). Further strategies in treatment are the more potent **monoamine oxidase B inhibitors** such as safinamide. A new kind of cell therapy based on spheramine should stop the progression of PD but bears a high risk of adverse effects. In treating both symptomatic phenotypes, motor and non-motor **non-dopaminergic agents** are an option. **Anti-muscarinic drugs** including amantadine have been widely used since the 1950s. Amantadine increases synaptic dopamine release, inhibits the reuptake of dopamine and increases the expression of D2 receptor. It further blocks NMDA receptors but its effects decrease with time (Fahn, 2008). They can reduce the severity of tremor. Because of the risk of memory impairment and hallucinations in the elderly population, anti-muscarinic drugs should be avoided in patients over the age of 70 years. **Coenzyme Q10** is currently tested in a controlled clinical trial to

determine if it has protective effects as well as **creatine** to see if it reduces the progress in clinical worsening in patients who are already on a stable dose of levodopa.

But so far it has not been possible to stop the progression of the disease (Artal-Sanz et al., 2006). The main reason for the lack of an efficient therapy is that the molecular mechanisms are still unclear, making it difficult to identify a target for treatments. The recent detection of the five genes (LRRK2, α -Synuclein, Parkin, PINK, DJ1) in the pathogenesis of PD stimulates a new direction of research (Testa, 2007). Those genes affect the formation of Lewy bodies composed of α -synuclein.

Based on the finding that a point mutation in the gene encoding α -synuclein (α -Syn) protein causes a rare familial form of PD, it is now confirmed that α -Syn is a major component of Lewy bodies in patients with sporadic PD, too. Therefore abnormal accumulation of α -Syn protein is considered a neurotoxic event in the development of both familial and sporadic PD (Figure 3) (Yasuda 2010). The function of α -synuclein is largely unknown. It is suggested that this protein is needed for synaptic vesicle docking, dopamine biosynthesis, DAT function and neuroprotection (Fortin et al. 2008).

Recent research focuses on compounds which interact with α -synuclein as target in PD treatment. Until now the anti-tuberculosis drug rifampicin and baicalein from the Chinese herbal drug *Scutellaria baicalensis* have been analyzed and shown to inhibit α -synuclein fibrillation (Li et al., 2004; Hong et al., 2008). Xiaoyun et al. (2010) have demonstrated in fibrillation kinetic assays that flavonoids significantly affect α -synuclein propensity to aggregate in vitro by binding and stabilizing oligomeric and monomeric protein conformations or by disaggregation of fibrils. Since it is suggested that oligomers are more toxic than fibrils the question raises if oligomers induced by flavonoids show this high toxicity as well. To test this hypothesis, much effort had been made to investigate the flavonoid-synuclein complexes and benign effects on cell permeability (Hong et al., 2008) or cytotoxicity (Masuda et al. 2006). Neuron degeneration in worms and mice has been shown. Furthermore, this complex did not induce cell death in human dopaminergic neuroblastoma cells as native α -synuclein oligomers did (Masuda et al., 2006).

These findings supported our hypothesis that phenolics such as flavones, flavanones or anthraquinones might provide a new class of drugs in treatment of amyloidosis by decelerating the progression of neuronal degeneration.

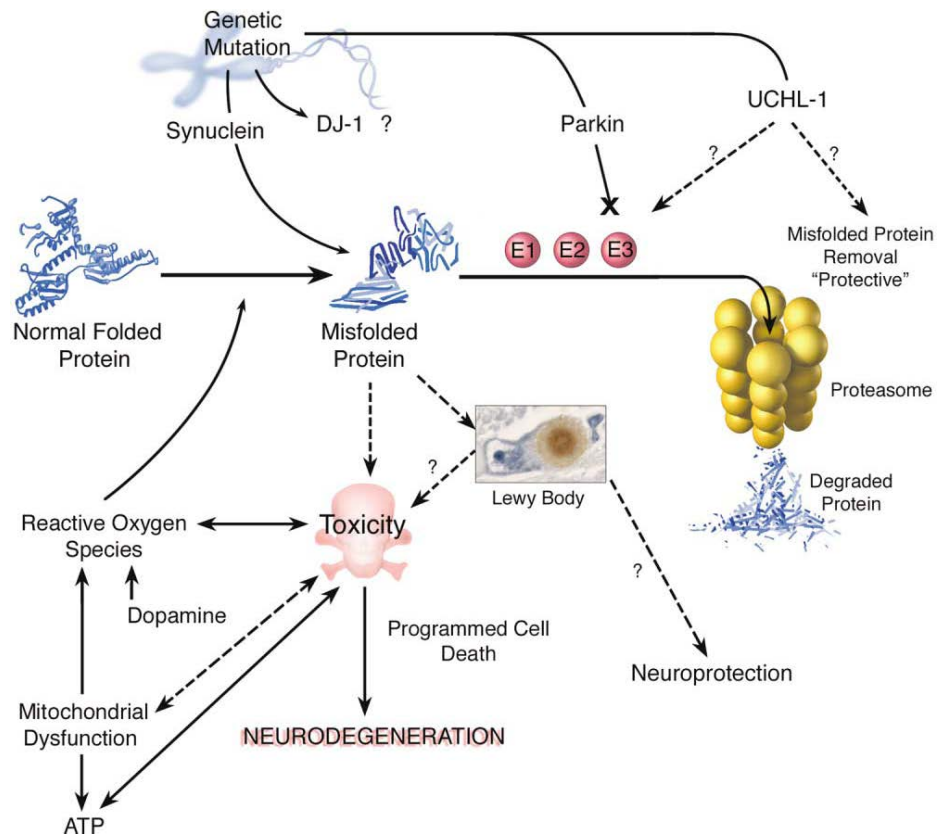


Figure 3: Mechanisms of Neurodegeneration in Parkinson's Disease
(Dauer and Przedborski, 2003)

An invertebrate model like *C. elegans* facilitated the identification of genes and molecular pathways which are involved in PD and this information is essential for the development of therapeutic targets and chemical leads. Researchers used transgenic *C. elegans* models which show α -synuclein misfolding to screen for the genetic background of Parkinson's etiology. Hamamichi et al. (2008) created a transgenic *C. elegans* strain that expressed human α -synuclein fused to GFP in the body wall muscles which led to the accumulation of this complex in the cytosol. The accumulation increased with aging. Using the RNA interference method to knock down gene expression about 900 target genes were screened which could be involved in PD. With this transgenic worm model 20 genes were identified which could increase α -synuclein misfolding when they have been knocked down. Among those were six which had been previously identified in a genome wide polymorphism screen for candidate single nucleotide polymorphisms (SNPs) in PD patients (Fung et al., 2006). Further Hamamichi et al. (2008) created transgenic *C. elegans* strains in which candidate genes were overexpressed together with α -synuclein in dopaminergic

neurones. This model had more direct implications to Parkinson's disease and results showed that five of seven tested genes protected worm's dopaminergic neurones against α -synuclein induced neurodegeneration. Atg-7 has been validated in a mouse model (Komatsu et al., 2006). Knockdown of atg-7 led to neuronal degeneration and overexpression of vps-41 in a mammalian cell line and it protected against PD related neurotoxins like 6-OHDA and rotenone (Ruan et al., 2009). VanHam et al. (2008) created a transgene *C.elegans* model which expressed human α -synuclein fused to yellow fluorescent protein (YFP) in the body wall muscles to find out which mechanisms led to age-dependent α -synuclein aggregation. α -synuclein expression led to cytosolic inclusions which increased with aging of the worm (Figure 4). Using the RNAi method 80 suppressors of inclusion formation were found and many of those genes had a human counterpart.

In our study we will use this transgenic worm model. In accordance to the work of Lakso et al. (2003) we want to establish a high-throughput drug screening method where positive drugs should be able to hinder or reduce α -synuclein formation with aging.

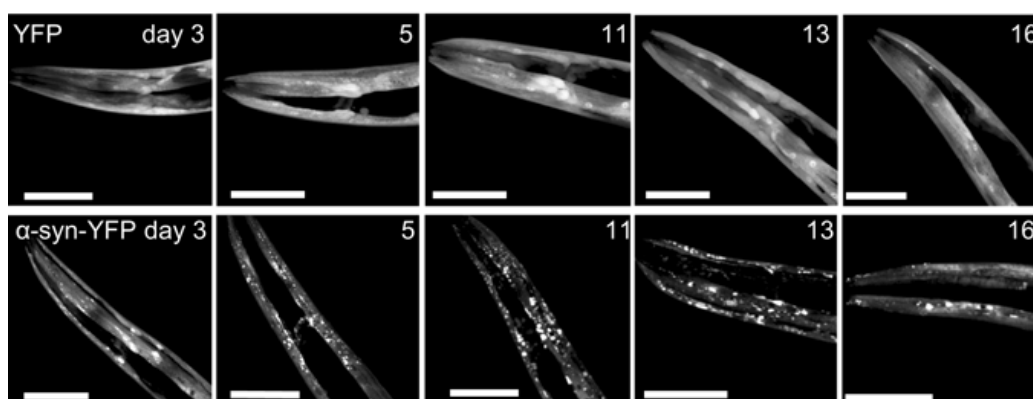


Figure 4: Top: In transgenic animals YFP is expressed alone whereas in the bottom pictures worms which express the α -synuclein-YFP complex are shown. Expression of the complex leads to formation of inclusions in the head region of strain NL5901 which increase during aging. (VanHam et al., 2008)

1.4 Modeling disease in an invertebrate

1.4.1 *Caenorhabditis elegans* versus *Drosophila melanogaster*

There are a lot of scientific approaches to study neurodegenerative diseases and this work deals with a relatively recent area of human disease biology, the modeling of a disease in an invertebrate organism.

The nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* are both inexpensive to culture and have short life spans. The rapid development of these models is particularly advantageous for the study of neurodegenerative disorders as nervous system maturation and subsequent neuronal degeneration occur over few days (Penny and McCabe, 2008). Both models have the advantage of fully sequenced genomes, which have revealed remarkable genetic conservation to humans. Molecular and genetic techniques have been developed which allow rapid, cell-specific labeling, gene knockdown and gene over-expression. The combination of these techniques with the short life spans of these models enables an extremely fast high-throughput screening.

However, there are four main reasons for using *C. elegans* in this work. First *C. elegans* offers a more rapid system for *in vivo* screening and high-throughput drug discovery than *Drosophila* (Jorgensen and Mango, 2002). Second, *C. elegans* are hermaphrodites and in a mutagenesis screen each mutant animal will produce its own homozygous line without mating, which increases the speed of the high throughput experiments. Finally, the anatomy of the worm is less complex than that of the fly and every individual neuron has been characterized, including both their connections and lineage (Wood, 1988). Therefore, the study how genes and cells affect circuitry and how circuitry controls behavior is possible.

Invertebrate models may bridge the gap between traditional high-throughput screenings and the validation in mammalian models. Moreover, the *C. elegans* model will considerably reduce the cost of drug screening and time consumption as compared to animal models. *C. elegans* is far superior to the *in vitro* mammalian cell culture models which are generally used for primary screening, because it is a multicellular organism with defined systems which provides physiological relevance (Arya et al., 2010).

1.4.2 *Caenorhabditis elegans*

This 1mm long, not segmented, soil dwelling nematode has been used as a model organism for the last four decades (Brenner, 1974).

The life span of *C. elegans* is 3 weeks and the life cycle consists of an embryonic phase followed by four larval stages, in the last stage the animals are sexually mature (Wood, 1988). The majority of *C. elegans* are hermaphrodites. In the wild, males are rare accounting for only 0,05% of the population. The generation time of *C. elegans* takes 3 days and each hermaphrodite can bear several hundred progeny and large numbers of animals can be rapidly produced (Figure 5) (Wood, 1988).

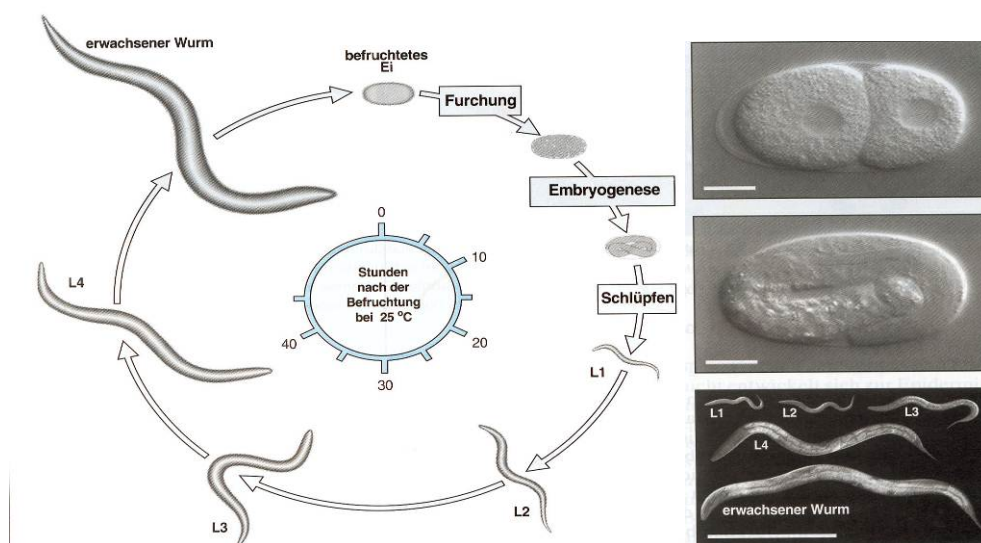


Figure 5: “Development of larvae needs 2 – 3 days, depending on the growth temperature. Adults have a lifespan of 2 – 3 weeks. When food source is depleted L1 or L2 are able to enter in a permanent “dauer” stage. In this stage they are able to survive for up to 4 weeks and do not develop until feeding.”
(<http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm>)

C. elegans is the only multicellular organism in which routine cryo-conservation has been established. They are permeable to aqueous and organic molecules and since they have no blood-brain barrier compounds diffuse into the nervous system. Test compounds can be dissolved in 1-2% DMSO since this is the nontoxic concentration (Nass and Hamza, 2007). In addition, worms carrying mutations in a variety of highly conserved biochemical pathways are displaying different phenotypes that can easily be scored. This makes *C. elegans* especially attractive for investigating the biological effects of drugs and for identifying their molecular targets. The small size, the rapid

life-cycle, and the high fecundity of *C. elegans* permit screening of thousands of animals over multiple generations in micro titer plates. Furthermore, the transparency of the worm allows appropriate fluorescent markers to be readily followed *in vivo*. There is a high degree of differentiation; *C. elegans* is composed of 959 somatic cells including muscles cells (111), neurons (302), intestine (34 cells), epidermis (213 cells), which are not able to regenerate (Harris et al., 2004). Although specific organs like the eye, ear and bone and cardiovascular system are absent, brain-related neurodegenerative diseases can be modeled in *C. elegans* (Kaletta, 2006). The worm does not have a distinct, confined central nervous system (CNS) but a central nerve ring, where processes from many neurons are connected (Figure 6). The 302 neurons of the hermaphrodite make 10,000 connections (Wood, 1988).

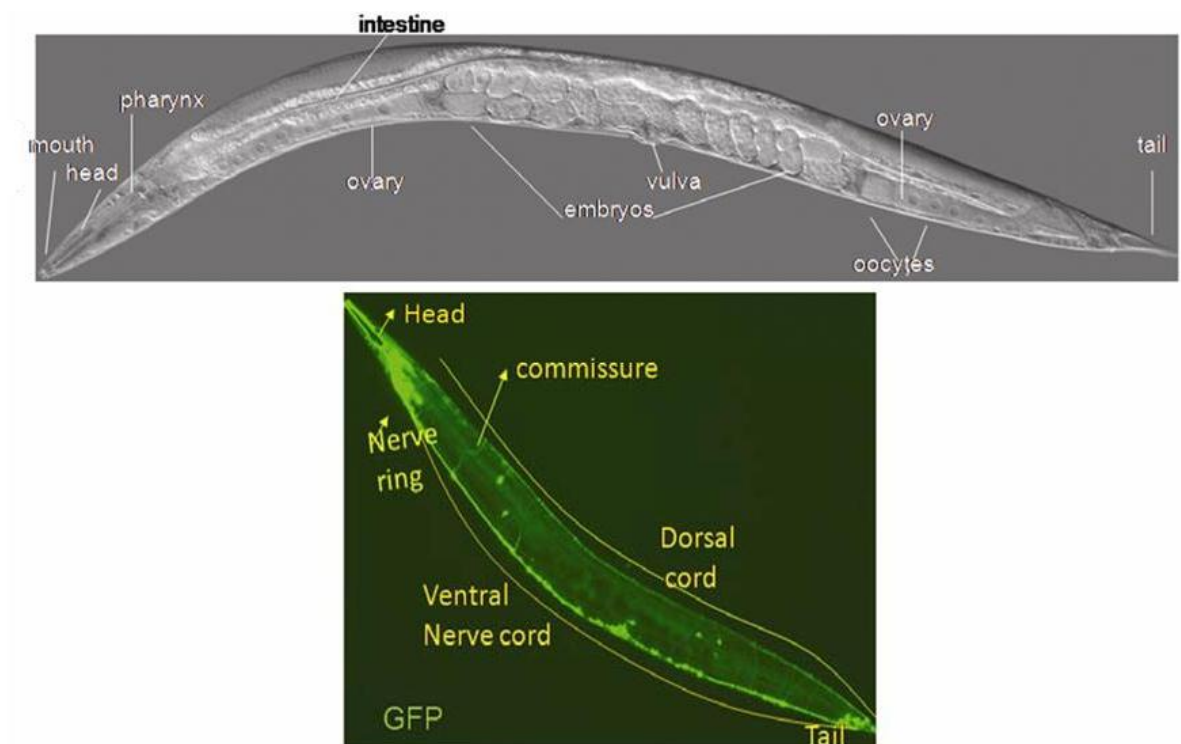


Figure 6: *C. elegans* digestive, reproductive (top) and nervous system (bottom) marked by GFP (Arya, 2010).

Although the neuroanatomy of invertebrates seems very different to that of mammals, there are many similarities at the molecular and cellular levels between vertebrate and invertebrate neurons (Adams, 2000). For example, ion channels, receptors, neurotransmitters (NT), and the machinery of NT release are similar in both structure and function (Bargmann, 1998; Yoshihara *et al.*, 2001). Body wall muscles are

attractive to study misfolding of proteins as it occurs in diseases like Alzheimer (β -amyloid peptide) or Parkinson (α -synuclein). In this way several modifiers of proteotoxicity have been identified (Harrington et al., 2010). Therefore the worm's nervous system provides a unique setting for studying the action of many drugs that act on the central nervous system (CNS) in humans.

The use of invertebrate model organisms for the study of human diseases has increased significantly since Sydney Brenner got the Nobel Prize for highlighting the strong genetic similarities in organ development and programmed cell death between nematodes and humans. In 2006 the work of Andrew Fire which confirmed these results was also awarded with the Nobel Prize. This is a reverse genetic approach to knock down the expression of any gene product, allowing for phenotypic analysis in whole-genome screens (Nass et al., 2008). The third Nobel Prize for studies on *C. elegans* received Martin Chalfie in 2008 for his work on the fusion of GFP with proteins in *C. elegans* for the first time.

The availability of a fully sequenced worm genome, in which 60–80% of the genes have a human counterpart (Harris et al., 2004), the access to an exhaustive database (<http://www.wormbase.org/>; ref. (Chen et al., 2005) containing data on gene structures, mutant and RNAi phenotypes, microarray data and protein–protein interactions, and the free availability of more than 8000 mutant strains at the *C. elegans* Genetic Stock Centre (<http://elegans.swmed.edu>), have led to the rapid adoption of this worm as a popular platform to study the mode of action of many drugs as well as for the discovery of new bioactive compounds (Artal – Sanz et al., 2006).

The urgent needs of 21st century medicine for an improvement in drug discovery and target validation may finally be implemented by this small, ancient nematode.

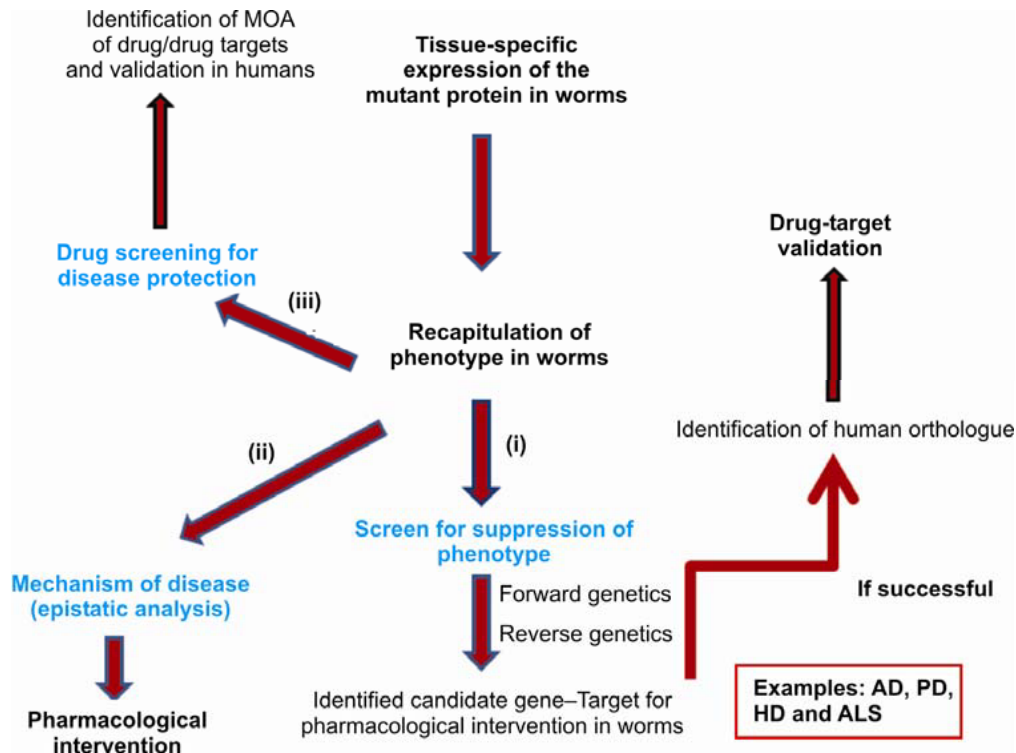


Figure 7: “An invertebrate disease model can be used (i) to identify suppressors of disease phenotype; (ii) to understand disease mechanism and (iii) for drug screening. MOA...Mode of action, AD...Alzheimer’s disease, PD...Parkinson’s disease, HD...Huntington disease, ALS...amyloid lateral sclerosis” (Arya, 2010).

High-throughput Drug-Screening in *C. elegans*

Current high-throughput screening methods for drug discovery are based on the existence of targets. Most of the hits generated during screenings turn out to be invalid when further tested in animal models. Therefore efforts are made to screen chemical libraries on whole animals like *C. elegans* or *Drosophila melanogaster* (Giacomotto and Segalat, 2010).

C. elegans models of neurodegenerative diseases have been extensively subjected to HTS (Figure 7). Its use is not dependent on the prior identification of a target and permits the selection of compounds with an improved safety profile. For example the pharmaceutical company NemaRx Pharmaceuticals has used the worm model to test drugs against important neurodegenerative diseases such as Alzheimer's disease (Arya et al., 2010). High Throughput Screening with whole animals can be separated into two distinct approaches (Figure 8). On the one hand we can screen compounds if they reverse a detectable phenotype like growth, behavior in a wild-type strain. Or on the other hand we can screen compounds if they are able to reverse an induced disease phenotype to the wild-type phenotype (Giacomotto and Segalat, 2010) as we will do in our work.

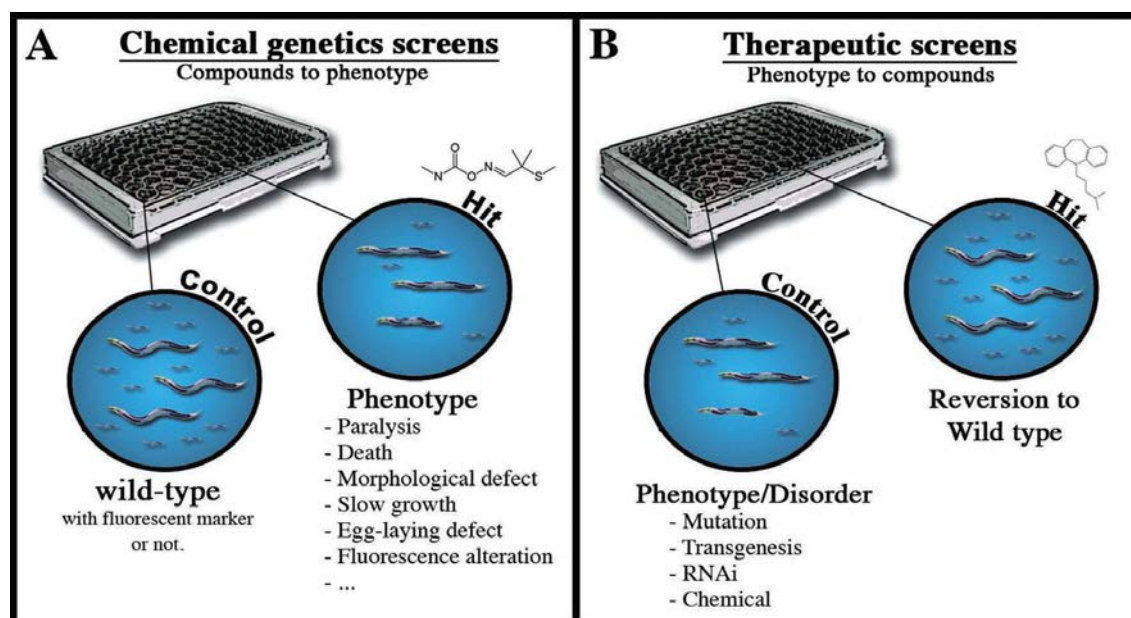


Figure 8: “Two distinct approaches in High-throughput screening systems with animals. **(A)** A substance can turn a wild-type into a phenotype or can retract an abnormal phenotype in the wild-type” **(B)** (Giacomotto and Segalat, 2010).

1.5 Marine compounds as novel candidates for drug development

Today the search for novel drug candidates especially in chronically diseases like Alzheimer or Parkinson is an important task in pharmacy and medicine. Beside the molecular biology approach and the chemical synthesis, natural compounds get back into the line of interest and here especially the marine area with its large number of unknown and unexplored organisms. Approximately half of the total biodiversity on earth are comprised by marine organisms which are recognized as a big resource of novel metabolites against human diseases. There are many reports about the discovery and development of marine compounds originating from marine microbes, cyanobacteria, micro- and macroalgae, soft corals, sponges, echinoderms, ascidians or fishes. Many of those compounds showed promising antimicrobial, antiviral and anticancer activities. Other compounds showed activity against malaria, osteoporosis, Alzheimer`s disease, neuronal disorders and inflammation (Blunt et al., 2006).

Recently scientists from the James Cook University (School of Pharmacy & Molecular Sciences) have discovered some striking similarities between the genetics of corals and humans. Corals, normally very simple animals, have as many genes as humans (20000-25000 genes in corals compared to human 20000-23000) and many of the proteins in the nervous systems are similar to higher animals (<http://www.sydneybashi-bangla.com>).

This suggests that the possibility to find novel marine neuroprotective compounds in the future is high. As an example Noscira, a Spanish biopharmaceutical company, has two marine derived compounds (NP-12 and NP-61) against Alzheimer`s disease in its pipeline. The company develops drugs for the treatment and prevention of diseases of the nervous system using a primary screening platform for marine samples. The most promising compound is the GSK-3 inhibitor NP-12 which is currently undergoing phase II clinical trials in the EU. NP-12 has proven to be capable of acting on all of the histopathological lesions associated with the disease in experimental models: it reduces phosphorylation of the tau protein and hippocampal and entorhinal cortex neuron loss, improves spatial memory deficits and significantly reduces the accumulation of amyloid plaques in the brain. NP-12 also provides neuroprotection in vivo and has a potent anti-inflammatory effect in a range of animal models.

1.6 Aim of the study

1.6.1 Establishment of screening methods with different transgenic *C. elegans* models for Alzheimer`s and Parkinson`s disease

The screening of marine extracts and compounds in this project focuses on the identification of novel pharmacologically active compounds against β -amyloid and α -synuclein toxicity in a high throughput screening. For this purpose we will use *C. elegans* as an innovative animal model for Alzheimer`s and Parkinson`s disease.

First, cultivation and propagation of *C. elegans* in both solid and liquid media, its freezing and recovery and the propagation of its food source, an *E. coli* strain, have to be established. Second, different transgenic strains expressing β -amyloid or α -synuclein have to be tested.

Strain CL4176 shows temperature inducible muscle expression of a human β -amyloid transgene, resulting in a reproducible paralysis phenotype by temperature upshift. Compounds can be tested against the acute toxicity of soluble oligomeric β -amyloid whereas in the second model (strain CL2006) we will screen the ability of compounds to dissolve amyloid deposits or to hinder β -amyloid to aggregate.

Strain CL2006 shows constitutive expression of amyloid β and so the deposits can be stained with thioflavin dye and then be detected by fluorescence microscopy.

For two other strains (CL2120 and NL5901) no high throughput screening protocol exists and therefore we want to set it up during this work. In the strain CL2120, which accumulates β -amyloid fibrils in the muscles, GFP is expressed in the intestine. Our hypothesis states that toxicity of β -amyloid kills enterocytes and the degree of the decreasing GFP signal correlates with β -amyloid expression. Active compounds should be able to protect intestinal cells from degeneration. In contrast, the Parkinson model with the second strain NL5901 expresses α -synuclein in muscle cells. Coupling of α -synuclein to YFP permits direct detection of α -synuclein expression. Positive compounds should be able to reduce the YFP signal which should be detected in the multi-well plate reader. Using this platform, different doses of the compounds which have been evaluated regarding their toxicity in prior human cell culture tests (not published) are fed to worms and the rate of paralysis or fluorescence is measured and compared to different controls. To examine if the screening system is suitable to respond to drugs affecting β -amyloid or α -synuclein peptide, we will test positive controls as protective against β -amyloid or α -synuclein toxicity in both other test systems and in nematodes. Coffee extract (Dostal et al., 2010) thioflavine (Alavez,

2011) or reserpine (Arya et al., 2009) are known to protect *C. elegans* from β -amyloid peptide toxicity. Furthermore Pooja et al. showed 2011, that an aqueous extract of *Bacopa monnieri* (L.) Pernel has an anti-Parkinson effect in *C. elegans* models.

All the compounds or extracts will be included as positive control in the planned study.

1.6.2 Screening of SeaLifePharma Lead Compounds for their neuroprotective potential

SeaLife Pharma as an innovative marine biotech-company has a focus on the discovery and development of microbial pharmaceutical lead structures. In the last three years the company isolated and characterized a lot of novel compounds. One group are anthraquinones, discovered originally in the endophytic fungi *Stemphyllium botryosum*, isolated from an saltwater mangrove.

Originally, this group of anthraquinones was characterized by Stoessl et al. in 1968 from *Alternaria solani* and the first compound was named *altersolanol A* (Figure 9)

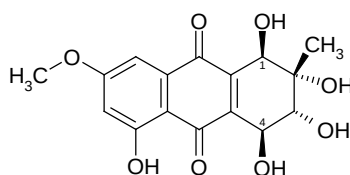


Figure 9

In the last year the company synthesized in its chemical platform more than 70 different derivatives of that compound group. Beside the monomeric compounds dimeric anthraquinones called alterporriols exists in endophytic fungi (Figure 10).

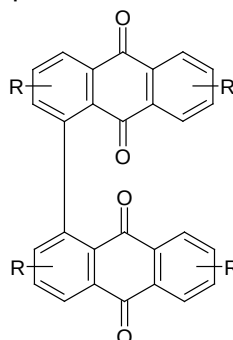


Figure 10

Convertino et al. discovered 2009 that anthraquinones from marine sources could be interesting for an application in Alzheimer treatment.

2. Material and Methods

2.1 Material

2.1.1 Bacteria

The uracil auxotroph *Escherichia coli* strain OP50 has been provided from CGC (*Caenorhabditis* Genetic Stock Center), University of Minnesota, St. Paul, U.S.A.

2.1.2 Different transgenic strains of *C. elegans*

All strains used in this work are provided from CGC (*Caenorhabditis* Genetic Stock Centre), University of Minnesota, St. Paul, U.S.A. Following strains have been used for different assays:

CL2006: *dvls*(pCL12(*unc-54*/human A β peptide 1-42 minigene)+pRF4) For the construction of the first *C. elegans* for Alzheimer's disease model Link et al. (2003) chose the promoters from the *C. elegans* gene *unc-54* to drive the expression of the A β 1-42 minigene (fig.11). *Unc-54* encodes the major body wall muscle myosins (Link et al. 2006). The A β transgenes were co-injected with pRF4, which encodes a mutant collagen gene whose expression leads to an easily recognized "Roller" phenotype (Mello et al., 1991). This strain shows adult onset of paralysis and they are egg-laying deficient. β -amyloid is expressed constitutively and staining with amyloid specific dyes like thioflavine S showed the formation of deposits. Further immune-electronmicroscopical studies (Link et al., 2001) demonstrated that β -amyloid was located in the cytoplasm. The construction of this strain with both a detectable phenotype and amyloid deposits allows the investigation of toxicity of the amyloid fibrils formation (Link et al., 2006). This strain is used to investigate the influence of test compounds on fibrillation of β -amyloid by staining with thioflavine S after incubation with substances.

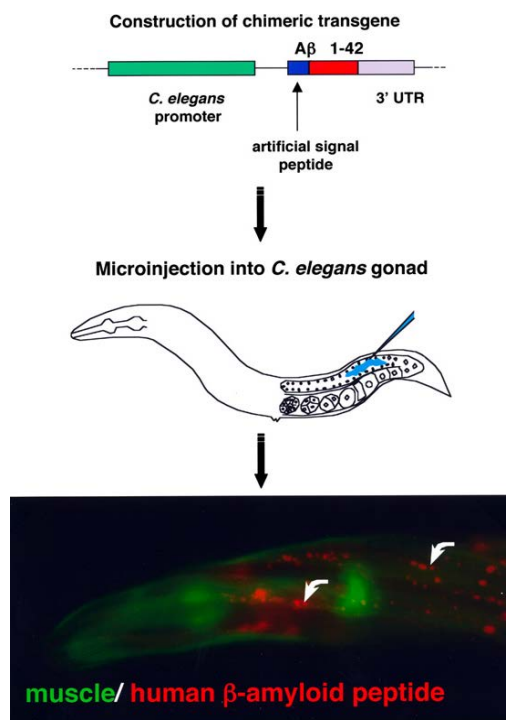


Figure 11: “Transgenic *C. elegans* Alzheimer disease model. GFP is fused to β -amyloid. Immunohistochemical β -amyloid specific antibody staining (mAb 4G8) shows β -amyloid deposits (arrows)” (Link et al. 2006).

CL4176: This strain has been engineered by Link et al. (2003) to inducibly express A β upon temperature upshift in muscle cells (Link et al., 2003). Muscles are the postsynaptic target for neurotransmitters and the neuromuscular junction is the equivalent of synapse. A correlation between neurotransmission increase and paralysis progression has been reported previously (Garcia et al., 2007). This has been achieved by using transgene constructs with abnormally long 3' un-translated regions, resulting in transgenic transcripts that are subject to degradation by the mRNA surveillance system. Introduction of these transgenes, containing a temperature-sensitive mutation in a gene essential for mRNA surveillance (*smg-1*), resulted in strains that had a ~5-fold increase in transgenic transcripts when shifted from permissive (16°C) to non-permissive (23°C) conditions (Steinkraus et al., 2008). This strain has wild type movement at the permissive temperature but becomes paralyzed upon temperature upshift within approximately 24 hours. Treatments that counter A β toxicity in this model (e.g. exposure to *Ginkgo biloba* extracts (Wu et al., 2006) alter the rate of paralysis in these worms. There is a direct correlation between β -amyloid expression and the rate of paralysis. In accordance to the work of Dostal et al. (2010) we used this screening protocol for measuring the rate of paralysis. The inducible A β expression does not lead to amyloid deposits and the paralysis phenotype appears

independent of amyloid deposition (Drake et al., 2003). Therefore the acute toxicity of induced A β expression resulting from the accumulation of soluble oligomeric A β can be measured.

CL2120: *dvIs14 [unc-54/beta 1-42(pCL12) + mtl-2::GFP (pCL26)]*. Mtl-2::GFP produces strong constitutive intestinal expression of GFP fused to metallothionein. Metallothioneins are small, metal-binding proteins. Mtl-2 expression is induced in larval and adult intestinal cells following exposure to cadmium or heat shock (Hall, 2012). MTL-2 intestinal expression is dependent upon ELT-2, an intestine-specific GATA-type transcription factor and it is downregulated by DAF-16 in *daf-2* mutants (www.wormbase.org/species/c_elegans/gene).

Further CL2120 expresses human β -amyloid peptide and accumulates β -amyloid fibrils. This strain has been constructed for a study of the influence of different amino acids substitutions on the in vivo production of amyloid deposits (Fay et al., 1998).

In our study this strain is used to establish an innovative high throughput screening model for analyzing the mode of action of substances which have been positive in the other screenings.

CL2122: *dvIs14 [mtl-2::GFP (pCL26)]* In this strain metallothionein is marked by GFP. In contrast to CL2120, this strain does not express amyloid β and serves as control strain for CL2120.

NL5901: (*pkIs2386 [unc54::a-synuclein::YFP unc-119(+)*). VanHam et al. (2008) expressed human α -synuclein fused to yellow fluorescent protein in the body wall muscle of *C. elegans*, where it accumulates into “Lewy bodies” with increasing age. The *unc-54* promoter is powerful and muscle cells are large allowing for visual detection of α -synuclein expression and its subcellular localization. This model was applied in a genome-wide RNAi screen to identify genes and cellular processes involved in age-related α -synuclein accumulation in inclusions (VanHam et al., 2008). In our study this strain is used to establish an innovative high throughput screening model against synucleopathies.

2.1.3 Equipment

Fluorescence microscope (Carl Zeiss, Austria)

Pipetboy (LLG Labware by ISOLAB Laborgeräte GmbH, Germany)

Flameboy (Integra, Germany)

Bacterial Loop (VWR, Austria)

Incubator (Lucky Reptile, Austria)

Bigger Bill Digital Orbital Shaker - Barnstead Thermolyne M73735 (Clarkson laboratory & supply inc., USA)

Freezer 4°C (Liebherr ProfiLine, Germany)

Freezer 16°C (Öko-Santo Superelectronic, AEG, Austria)

Vortexer (Janke & Kunkel IKA-Labortechnik, Germany)

Jouan CR3-22 Centrifuge (ABI, France)

Phase Contrast Microscope (Nikon TMS, Germany)

2.1.4 Consumable materials

Petri dishes, 35 x 10mm (VWR, Austria)

96-well microtiter plates (TPP, Austria)

24 well plates (TPP, Austria)

2 liter autoclavable bottles (containing a stir bar) with caps (VWR, Austria)

90-mm plastic bacterial petri dishes (VWR, Austria)

24 well plates (TPP, Austria)

15ml and 50ml conical tubes, sterile (VWR, Austria)

PP tubes, 15ml and 50ml (Greiner bio-one, Germany)

Eppendorf tubes (VWR, Austria)

Cryo tubes (VWR, Austria)

Cryo box (VWR, Austria)

Serological pipets (VWR, Austria)

2.1.5 Buffer, solutions, media

For the following solutions deionized water has been used. After addition of water, solutions were autoclaved for 20 min at 121 °C and 3,0 bar. The substances were analytical grade and obtained from Sigma (Germany), Merck (Germany) or Applichem (USA).

2.1.5.1 For manipulation of bacteria

LB-Agar:

5,0g NaCl (Sigma)

2,6g yeast extract (Sigma)

5g peptone (Sigma)

7,6g agar (Merck)

Add H₂O to 1 liter, pH 7,5

Autoclave for 35 min

Dispense the solution into petri dishes. Fill plates 2/3 full of agar.

L Broth:

5,0g NaCl (Sigma)

2,6g yeast extract (Sigma)

5,0g peptone (Sigma)

Add H₂O to 1 liter, pH 7,5

Autoclave for 35 min

2.1.5.2. For manipulation of *C. elegans*

1 M Potassium phosphate buffer pH 6

108,3g KH₂PO₄ (Merck)

35,6g K₂HPO₄ (Merck)

Add H₂O to 1 liter

Measure pH value with pH meter

Swirl and autoclave for 20 min

NGM Agar

3g NaCl (Sigma)

17g agar (Applichem)
2,5g peptone (Sigma)
Add H₂O to 1 liter
Cool down for 15 min in a 55°C water bath
0,5ml 1M CaCl₂ (Merck)
5mg cholesterol in 1ml ethanol (Sigma)
1ml 1 M MgSO₄ (Merck)
25ml 1 M Potassium phosphate buffer, pH 6 (Sigma)
200µl amphotericin B (250µg/ml) (Applichem)
Dispense the solution into petri dishes. Fill plates 2/3 full of agar.

S Basal Solution

5,8g NaCl (Sigma)
1g K₂HPO₄ (Merk)
6g KH₂PO₄ (Merk)
Add H₂O to 1 liter
Autoclave for 20 min at 122°C
Add 5mg cholesterol in 1ml ethanol

1M Potassium citrate buffer pH 6, 0

20g citric acid monohydrate (Merck)
293,5g tri-potassium citrate monohydrate (Merck)
Add H₂O to 1 liter
Autoclave for 20 min at 122°C

Trace metal solution

1,86g disodium EDTA (Sigma)
0,96g FeSO₄*7 H₂O (Sigma)
0,2g MnCl₂*4 H₂O (Sigma)
0,29g ZnSO₄*7H₂O (Sigma)
0,025g CuSO₄*5 H₂O (Sigma)
Add H₂O to 1 liter
Autoclave for 20 min at 122 °C

1M CaCl₂ Solution

111g CaCl₂ in 1 liter H₂O (Sigma)
Autoclave for 20 min at 122 °C

1M MgSO₄ Solution

120g MgSO₄ in 1 liter H₂O (Sigma)

Autoclave for 20 min at 122 °C

5M NaOH

200g NaOH in 1 liter H₂O (Merck)

Autoclave for 20 min at 122 °C

0.05M KH₂PO₄ solution

6,8g in 1 liter H₂O

Autoclave for 20 min at 122 °C

0.05M K₂HPO₄ solution

8,7g in 1 liter H₂O

Autoclave for 20 min at 122°C

S Medium

1l S Basal

10ml M potassium citrate pH 6

10ml trace metal solutio

3ml 1 M CaCl₂

3ml 1 M MgSO₄

200µl Amphotericin B (250µg/ml) (Applichem)

Autoclave for 20 min at 122 °C

Bleaching solution

5M NaOH (Sigma)

5% NaCl (Sigma)

Mix in the ratio 1:1

M9 buffer

3g KH₂PO₄ (Merk)

6g Na₂HPO₄ (Sigma)

5g NaCl (Sigma)

1ml MgSO₄ (Sigma)

Add H₂O to 1 liter

Autoclave for 20min at 122°C

S buffer

129ml 0,05M K₂HPO₄ (Merck)

871ml 0,05M KH₂PO₄ (Merck)

5,9g NaCl₂ (Sigma)

S Buffer with 30% Glycerol

70ml S Buffer (Sigma)

30ml glycerol (Sigma)

Permeabilization solution

125mM Tris, pH 7,4

1% Triton X-100,

5% Mercaptoethanol (Sigma)

0,125% Thioflavin T (Sigma)

PBS-T

PBS (Applichem) plus 0,1% Triton X-100 (Sigma)

4% Formaldehyde in PBS (Sigma)

6,27mM Caffeine in S-Medium (Sigma)

60µM Reserpine in acetic acid (Sigma)

Add 0,15g to 5ml acetic acid and dilute 5µl from this solution in 198µl M9 buffer

500µM Thioflavin S (ThS) in S-Medium 10X (Sigma)

60mM 5-fluoro-2'-deoxyuridine (FUdR) 100x (Sigma)

Dissolve 10mg/ml in S-Medium and store at -20° C in the freezer.

Amphotericin B solution (Applichem)

250µl in 1ml H₂O

Extracts

1mg of each extract was dissolved in 200µl DMSO (Sigma) and diluted with 800µl Cell Culture Grade Water (PAA, Austria)

Talaromyces extract (Patent 3)

Ethyl acetate extract of the fungi *Talaromyces cerevisiae* which has been isolated from the terrestrial plant *Aloe vera*.

Herba Bacopa monnieri

Ethanol extract of the aerial parts of the plant which is used in the traditional Chinese medicine (TCM).

Huang Chi (*Radix Astragali membranaci*)

Water extract of the roots from *Astragalus membranacus*, which are used in the TCM.

SeaLifePharma´s lead compounds from the class of anthraquinones

BK110/RP

BK112/RP

BK118/K1

2.2 Methods

2.2.1 Maintenance of *C.elegans*

We maintained *C. elegans* according to the protocol from the CGC (*Caenorhabditis* Genetics Center), University of Minnesota, Minneapolis, MN 55455 USA. Requested strains were obtained from the CGC in NGM petri plates with the worms still feeding on *E. coli* OP50. All worms were kept at room temperature were maintained at 20° C. The permissive temperature for CL4176 was 16° C. All work was done under the laminar hood or using sterile procedures. To avoid contamination the cover of the plates was removed for a minimum time, when pouring or seeding plates or when transferring worms. The petri plates always were labeled in the same way: NGM – date of preparation – OP50 – date of seeding – name of the worm strain – date of transfer.

2.2.1.1 Preparation of bacteria as food source on NGM plates

C. elegans grows monoxenically using the *E. coli* strain OP50 as food source (Brenner, 1974). We used a starter culture from CGC to isolate single colonies on a streak plate with LB agar. After 24 hours we took a single colony to inoculate L broth. After growing for 24 hours at 37° C on the shaker this OP50 culture was ready for seeding onto NGM plates. The streak plate and the liquid culture could be stored at 4° C for several months.

Seeding of NGM plates

0,1ml liquid culture of *E. coli* OP50 was pipetted on NGM plates. With a trigalski spatula the drop could be spread so that there is a larger lawn. This is useful when visualizing worms with the microscope. Worms spent all time in the bacterial lawn. Bacteria were left to grow overnight at room temperature before transferring worms on it. When plates were seeded they could be stored in an air-tight bag for 2-3 weeks.

2.2.1.2 Preparation of bacterial food source for liquid *C. elegans* culture

E. coli OP50 was cultured overnight in liquid broth at 37° C on the shaker. Next day the culture was pelleted by a centrifugation step for 15min at 3500rpm. We determined the weight of the pellet and the falcon could be stored for several weeks at

4°C. For further standard use we adjusted the concentration to 0,1g/ml by re-suspending the pellet in S Medium.

With 200µl of this suspension we inoculated 40 ml liquid culture with worms from 1 petri dish which were growing for 4 - 5 days.

2.2.1.3 Culturing *C. elegans* on petri plates

For an ideal development and rapid growth worms were cultured on large NGM plates seeded with OP50 as food source. To prevent plates from drying out they were wrapped with a strip of parafilm and put without stacking into the incubator at 20° C or as otherwise indicated.

2.2.1.4 Transferring worms

The frequency of transfer depended on the genotype of the worm strain, temperature and the experimental setting. For harvest of every stage of development they should be transferred every 1 – 2 days. If permanent larvae were necessary they were not transferred until the food source was exhausted. Several methods exist to transfer *C. elegans* from one petri plate to another. We used this two:

Transferring by chunking

For this method a chunk of agar was cut with a sterilized scalpel from an old plate with a lot of worms and transferred to a new one inoculated with OP50 bacteria. Within a few minutes worms crawl into the bacterial lawn.

Transferring by picking

A worm picker was made from a platinum wire which has been flattened at the end and mounted into a Pasteur pipette. With this instrument single worms could be transferred. For easier use we dipped the picker into an OP50 suspension to create a sticky end. To put a picked worm on the fresh plate we touched the surface of the agar and waited till the worm crawled from the picker.

2.2.1.5 Culturing of *C. elegans* in liquid medium

For culturing *C. elegans* in liquid medium S Medium has been used. The advantage of this culturing method was that food could be added as needed, every time when media appeared clear. Further substances could be added at each time point too, e.g. after contamination with a fungus amphotericin B was added. This method enabled

testing of compounds in 96 well micro titer plates and highlighted the use of *C. elegans* in a high-throughput screening system.

Normally we used 40ml S Medium which was inoculated with worms from 1 large plate just cleared of bacteria which had been grown for 4 – 5 days. We washed the plate 3 times with 3ml S Medium and added *C. elegans* to the flask. The flask was put on the shaker so that the culture was well oxygenated. If not otherwise indicated the cultures were growing at 20° C. Every day they were monitored by checking a 10µl drop of the culture under the microscope. This way we controlled food supply, contamination, condition of the worms or stage of development. Usually on the 4th or 5th day the culture consisted of many gravid adults. At this time point the culture was ready for harvesting. For this we pelleted the culture in a 50ml falcon for 5 min at 2500 rpm.

2.2.1.6 Freezing protocol

The first or second larval stage survived freezing best. We used one plate with freshly starved L1 or L2 worms. 0,6ml S buffer for each vial were used to wash the worms from the plate. An equal volume S Buffer with 30% glycerol (final concentration: 15 %) was added and the solution was transferred to a 50ml falcon. After shaking, aliquots of 1ml in 1,8ml cryo tubes were packed into the cryo conservation device allowing gradually reduction of temperature for 1°C/min. This was placed for at least 12 hours into a –80° C freezer. Next day the survival of worms in the freezing procedure was checked in one tube.

2.2.1.7 Thawing protocol

The tube was thawed at room temperature. Then the whole content was poured on a NGM plate seeded with OP50. After few minutes motion of the worms was examined. After 3 days they were transferred to a new plate by chunking.

2.2.2 Different Screening procedures in *C. elegans*

For each assay we used an age-synchronized worm population. For this purpose we used the “egg prep method” (Lewis and Fleming, 1995)

For the preparation of eggs a mixture of 1ml household bleach with 0,5ml 5M NaOH for every 3,5ml worm suspension was freshly prepared. A NGM plate full with gravid adults (3 – 4 days old) was washed 3 times with sterile water and the liquid transferred into a 15ml falcon. We mixed it with the synchronisation solution and vortexed it for few seconds. Then we pipetted 10µl of this suspension on a slide to monitor the egg release under a light microscope. Accordingly, the process was stopped at the accurate time, normally after 5 – 7min. We vortexed the suspension every minute and stopped the worm lyses by adding an equivalent volume M9 Buffer. The eggs were pelleted for 5min at 3000rpm and the liquid was discarded. The pellet was resuspended in 5ml sterile water and pelleted again. This washing step was repeated two times. We discarded the supernatant, added 10ml S Medium, transferred the solution to a sterile Erlenmeyer flask and added 30ml S Medium to a final volume of 40ml. The flask was shaken overnight at 16° C on a shaker. The first larval stage was 250µm long and arrests in this stage if no food is applied. Therefore no food was provided until all worms have hatched to guarantee a synchronous population.

2.2.2.1 Screening against β -amyloid toxicity with CL4176 – Paralysis Assay

This assay was performed according to Dostal et al., 2010

Preparation of NGM plates for paralysis assay

We prepared 24 well plates 4 – 7 days before the paralysis assay started and only plates from the same batch were used in one experiment. After inoculation of the plates with OP50 they were kept overnight for growth to an optical density of 0,4 – 0,6. For testing for the effect of compounds we diluted them in S-Medium and distributed 50 µl of the compound over the surface of each well. In each well we had 1ml agar which resulted in a dilution of 1:20. Three different concentrations per compound were tested. Those concentrations had been determined in earlier toxicity tests in human cell cultures. We allowed compounds to dry over night at room temperature. A solution of 6,27mM caffeine served as positive control. The next day 10µl OP50 of an overnight culture were added and incubated for 2 hours at 37° C. After this worms which had been synchronized 48 hours before were added. As negative control we used 50µl of the OP50 suspension. For statistical analysis minimum 3 wells/ concentration were analyzed.

Seeding plates with L1 larvae

We determined the concentration of worms in the medium by counting the number of worms in 10 μ l drops using the light microscope. For statistical significance we counted at least ten drops for each sample and calculated the mean value. We re-suspended worms at a concentration of 1000 – 2000 worms/ml in S-Medium and pipetted 10 μ l in each well of the 6 well plate so that there were 10 – 20 worms per well. We wrapped the plates with a strip of parafilm and let them grow to L3 larvae in about 40 hours at 16° C.

Determining the stage of development

For the success of the assay it was essential to determine the stage of development because the myo-3 promoter of myo-3/A β transgene worms is regulated developmentally in strain CL4176. For this purpose we determined the length of the worms under the fluorescence microscope and evaluated every stage of development. L1 is 250 μ m long, L2 360 – 380 μ m, L3 490 – 510 μ m, L4 620 – 650 μ m, a young adult 900 – 940 μ m and the adult is 1110 – 1150 μ m long.

Transgene induction and scoring for paralysis phenotype

When worms reached the length of 400 +/- 10 μ m (usually after 40 hrs) their growth temperature was upshifted from 16° C to 25° C so that expression of the β -amyloid peptide started. 22 hours after temperature upshift we started scoring the paralysis of worms. At this time point all worms should have reached the fourth larval stage. For the paralysis curve we continued scoring in 2 hours intervals until all worms in each well were paralyzed. Paralyzed worms were recognized when forming halos at the head region because head is the last part of the worm to cease moving thereby clearing the bacterial lawn around it. If there was no halo although the worm did not move we used the worm picker to prod it. A prodded worm should undergo a full body wave propagation to be not counted as paralyzed. To avoid rescore of a paralyzed worm these worms were picked with the worm picker and flamed.

Paralysis curve

At each time point the amount of not paralyzed worms on each plate was determined and the average “unparalyzed” percentage was plotted against time from initiation of upshift. This curve is analogous to a survival curve.

2.2.2.2 Assaying β -amyloid toxicity with strain CL2120

The strain CL2120 expresses human β -amyloid and forms oligomers without building deposits like CL4176. Further the transgene Mtl-2::GFP produces strong constitutive intestinal expression of GFP. We used this strain to establish an innovative high throughput screening model for analyzing the mode of action of substances which have been positive in the other screenings. We used this strain for testing compounds regarding their ability to increase metallothionein expression. We performed this assay in a 96-well format allowing for detection of fluorescence by the multi well plate reader. The 96 well formats allowed testing of many compounds at different concentrations. In the first row of the plate we pipetted 100 μ l of the starting concentration. In the other wells we filled 90 μ l S-Medium. For the dilution step we transferred 10 μ l from the first well into the second, mixed it and transferred 10 μ l from the second into the third well. After this plates were seeded with 10 μ l of the worm/bacteria suspension, using synchronized L1 worms with a concentration of about 2000 worms/ml. We incubated at 16° C for 48 hours and added 1 μ l 60mM FUdR for sterilization of the worms after this time. The addition of FUdR marks time point "0 days". We measured fluorescence intensity at two different time points: after 3 days and after 5 days.

2.2.2.3 Assaying α -synuclein toxicity with strain NL5901

The strain NL5901 expresses human α -synuclein in all muscle cells. The protein is marked with YFP which allows detecting the increasing protein expression directly in the multi well plate reader. The setting was the same as in the assay with strain CL2120. Positive compounds in this assay are hypothesized to inhibit α -synuclein accumulation.

2.2.2.4 Screening compounds against β – amyloid accumulation in CL2006

Strain CL2006 expresses β -amyloid constitutively and develops β -amyloid deposits in muscles with aging. Compounds which were able to inhibit β -amyloid deposition were tested. Synchronized CL2006 larvae were transferred to a Petri dish containing NGM agar inoculated with OP50 bacteria. We let them grow for four days at 16° C until they reached the fourth larval stage. Then they were transferred to a 96 well plate with S

Medium containing 60µM FUdR for sterilization, OP50 bacteria as food source and the different test compounds. As positive control served 6,27mM caffeine dissolved in OP50 suspension whereas as negative control we used OP50 suspension alone. The worms were incubated for 14 days at 23° C to build β -amyloid deposits. During this time they were monitored every day and food source was added immediately after depletion.

Staining of β -amyloid deposits with thioflavin T

Thioflavin staining was performed as described previously (Fay et al., 1998).

After 2 weeks we pipetted 50µl of worm suspension into an Eppendorf tube containing 950µl 4% formaldehyde in PBS for fixation and incubated at 4°C for 24 hours. After this we pelleted worms and discarded the supernatant. For permeabilisation we added 1ml solution containing 125mM tris buffer (pH = 7,4), 1% Triton X-100 and 5% mercaptoethanol. Worms were incubated at 37° C for 24 hours. Then worms were pelleted again and washed 3 times in PBS with 0,1% Triton X-100. Staining with 0,125% thioflavin T in 50% ethanol for 2 minutes and de-staining in 50% ethanol for further 2 minutes followed. The stained samples were resuspended in PBS and visualized with the fluorescence microscope by an excitation of 360nm and an emission of 465nm.

2. Results

The aim of the master thesis was the establishment of different screening models and the investigation of different SeaLifePharma lead compounds. Therefore the following results were achieved on the route of the master thesis:

2.2. Synchronization of a worm population with different developmental stages using egg prep method

Eggs could be prepared by adding hypochlorite and NaOH to the worm suspension leading to the lysis of the gravid hermaphrodites and to the release of their eggs. The reaction had to be stopped at about 10min by adding M9 buffer because otherwise the protective eggshell would have been lysed too. After three washing steps and an incubation time of 48 hours at 16° C all larvae were hatched and were in the same developmental stage because of absence of food.



Figure 12: Gravid adult hermaphrodite. Eggs can be seen within the first cleavage (thick arrow) in the translucent worms. This is the right time for harvesting eggs to synchronize the worm population. The small arrow shows a L3 larva. (Magnification 20x)

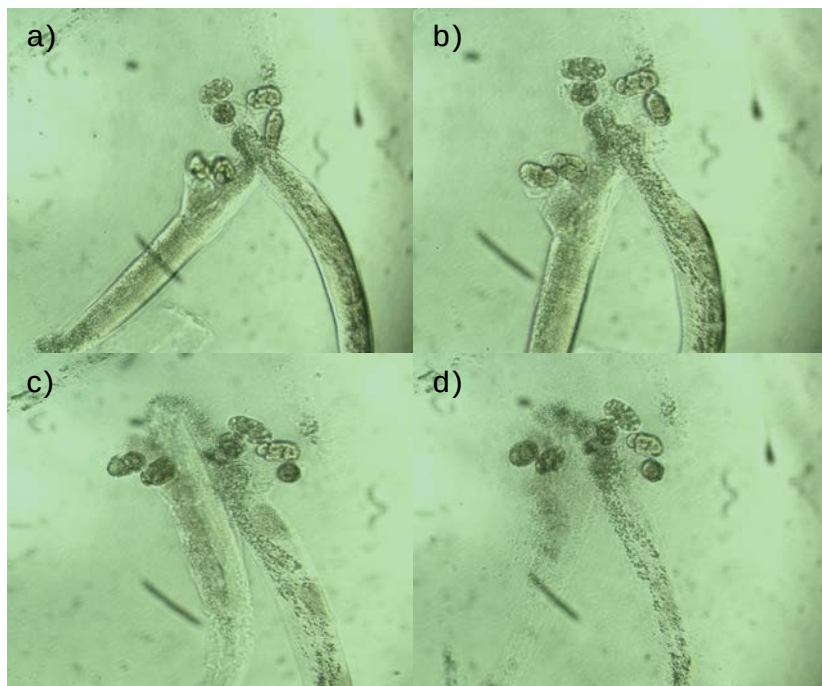


Figure 13: Egg Prep Method. To induce egg release gravid worms were treated with hypochlorite solution. Pictures a-d show egg release and lyses of the adult within 10 minutes. (Magnification 20x)

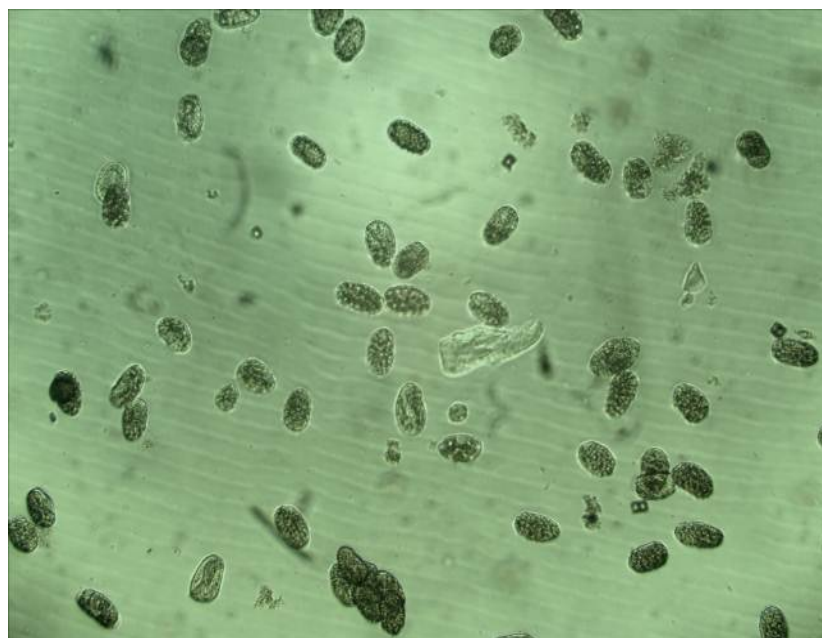


Figure 14: Harvested eggs after Egg Prep Method. The eggs were kept overnight in media without any food source for L1 arrests. (Magnification 20x)

2.3. Determining the right stage of development of CL4176 to induce β - amyloid expression

24 hours after egg hatch at 16° C worms were 250 μ m long in the first larval stage. Upshifting temperature from 16° C to 25° C resulted in the paralysis phenotype of 100% worms after 40 hours. 48 hours after egg hatch at 16°C worms were 390 μ m (L2) long and developed paralysis phenotype too. 72 hours after egg hatch they reached a length of 540 μ m (L3) and upshifting temperature at this time point did not result in paralysis but in development to adults. For every paralysis assay we elevated temperature when the worms reached the second larval stage after 48 hours on NGM agar containing a test compound. This way sufficient time for intake of the compound was guaranteed.

2.4. Screening for compounds with anti-proteotoxic effects

Several drugs with known activity against β -amyloid toxicity like thioflavin t, caffeine and reserpine have been tested. Additionally the activity of an extract of *Bacopa monnieri* which acts against α -synuclein toxicity and of the extract from *Talaromyces cervisiae* and SeaLifePharma`s lead compounds which belong to the class of anthraquinones were evaluated. The water extract of the TCM drug Huang Qi was tested as well.

2.4.1. Assaying β -amyloid toxicity using strain CL4176

In our study we were able to establish an assay for the evaluation of the β -amyloid toxicity by using the strain CL4176. The extent of β -amyloid expression correlated with the paralyzed phenotype. Worms in this assay were counted as paralyzed when they formed a halo around the head region as seen in Figure 15 (arrow). The formation of the halo is the result of the fact that the head of a worm is the last part which paralyzes. Therefore bacteria around them are cleared in this region.

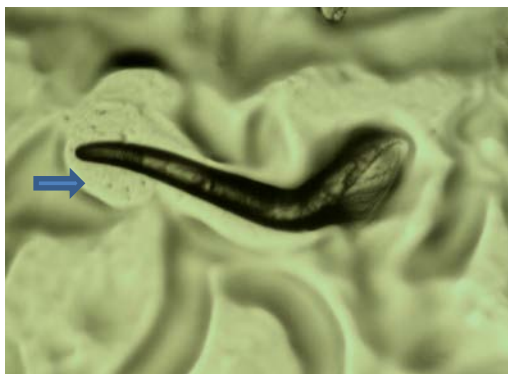


Figure 15: Paralyzed worm with halo. Bacteria cleared around the head region. (Magnification 20x)

2.4.2. Metallothionein expression with strain CL2120

We were able to establish the worms from the strain CL2120 expressing metallothionein and β -amyloid. Metallothionein marked with GFP is expressed in the intestine whereas β -amyloid is expressed in the body wall.

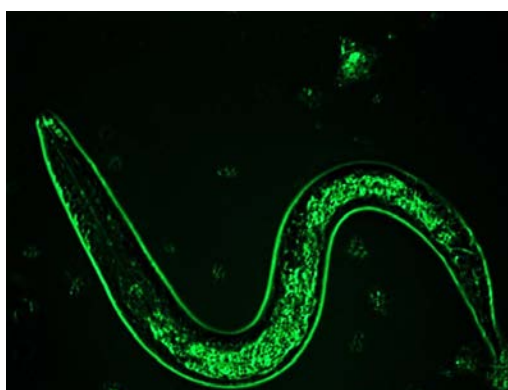


Figure 16: Worms from the strain CL2120 expressing metallothionein and β -amyloid. (Magnification 20x)

For the analysis of the ability of substances to increase metallothionein expression and protect in this way worms from toxic insults of β -amyloid we detected GFP at different time points, on the first, the third, the fifth and the seventh day. In this assay caffeine reduced fluorescence to the half of the untreated control whereas reserpine increased it twofold.

3.3.3 Assaying α -synuclein toxicity using strain NL5901

In strain NL5901 the peptide α -synuclein is marked by YFP. α -synuclein expression leads to its cytoplasmic accumulation in this strain. The extent of accumulation can be measured using the Tecan fluorescence reader or by fluorescence microscopy as seen in this picture.

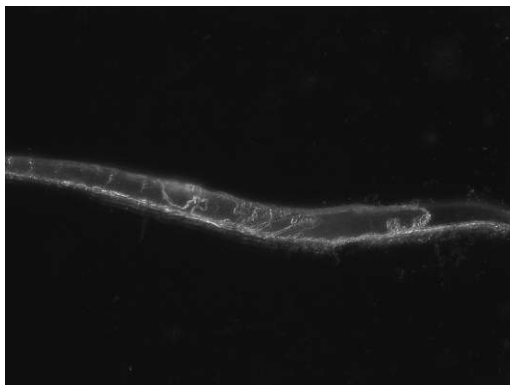


Figure 17: Detection of α -synuclein marked with GFP in strain NL5901. (Magnification 20x)

3.4 Detailed results from the screening activities

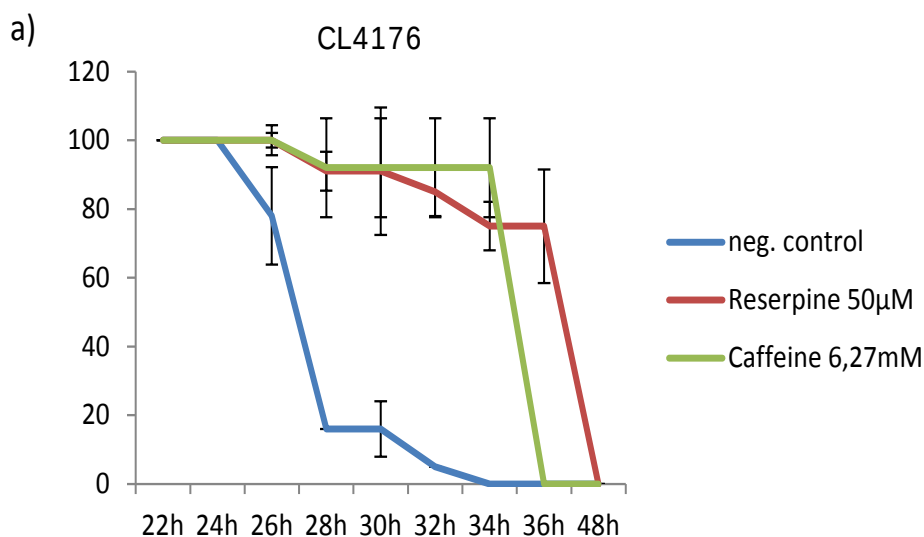
The results of all screenings are compiled graphically in the following chapters.

3.4.1. Caffeine and reserpine

To investigate the applicability of our screening systems we tested the compounds caffeine and reserpine because of their known activity against β -amyloid toxicity in *C. elegans*. We used 6,27mM caffeine (Dostal et al., 2010) and 50 μ M reserpine (Arya et al., 2009).

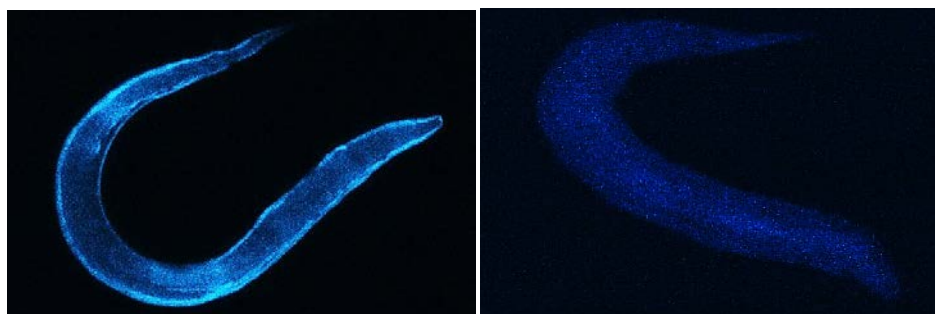
3.4.1.1. Effects of caffeine and reserpine on β -amyloid and α -synuclein toxicity

Figure 18:

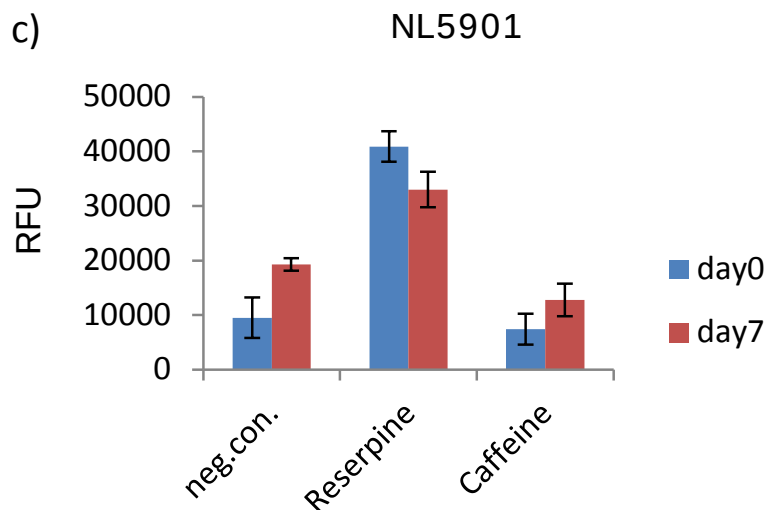


- a) Paralysis curve for strain CL4176 untreated or exposed to 6,27mM caffeine and 50 μ M reserpine resulted in a delay in paralysis of approximately 5 hours, which we interpreted as indicative of suppression of β -amyloid toxicity in this model. Time point 22h marks initiation of temperature upshift.

b) CL2006

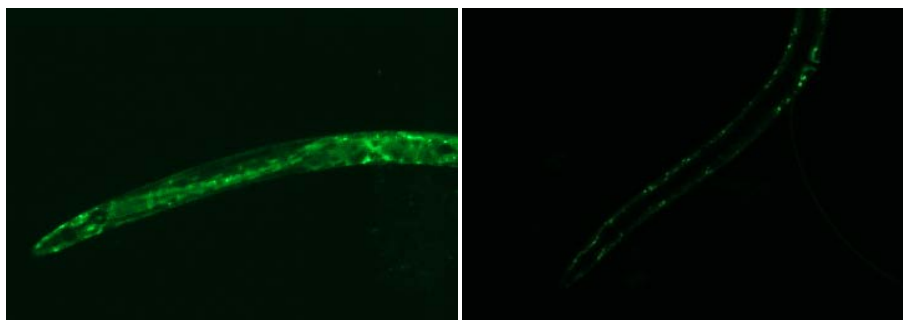


- b) Strain CL2006 after staining β -amyloid deposits with thioflavin T dye. Constitutive expression of β -amyloid in untreated worms led to visible deposits (left picture). In the right picture a worm exposed to 6,27mM caffeine can be seen. Treatment resulted in no detectable β -amyloid deposition. Worms were growing on NGM plates with 0,6mM FUDR for 14 days. (Magnification 20x)

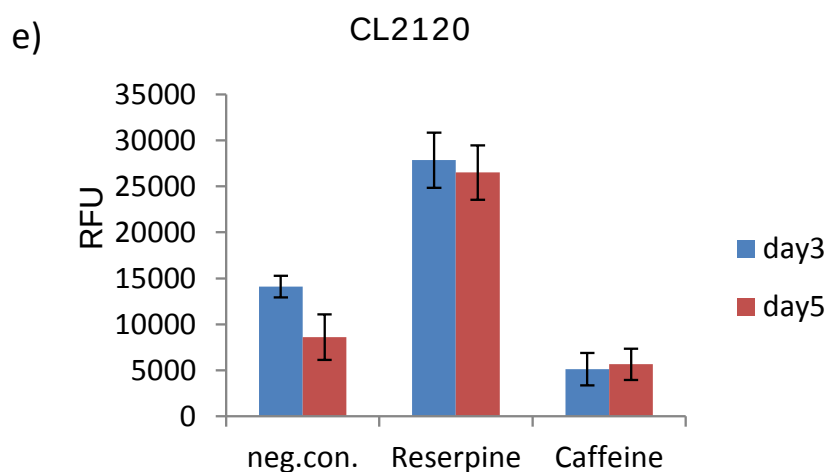


- c) The bar graph shows fluorescence of α -synuclein coupled to GFP in strain NL5901 at day 0 and after 7 days of adolescence. Worms were either left untreated or exposed to 6,27mM caffeine or 50 μ M reserpine. Only caffeine decreased α -synuclein fluorescence whereas reserpine even increased fluorescence.

d) NL5901



- d) Fluorescence pictures of NL5901 worms after 7 days with or without treatment. In the picture left fluorescence of α -synuclein is visible whereas in the right picture fluorescence is weakened after exposition to 6,27mM caffeine. (Magnification 20x)



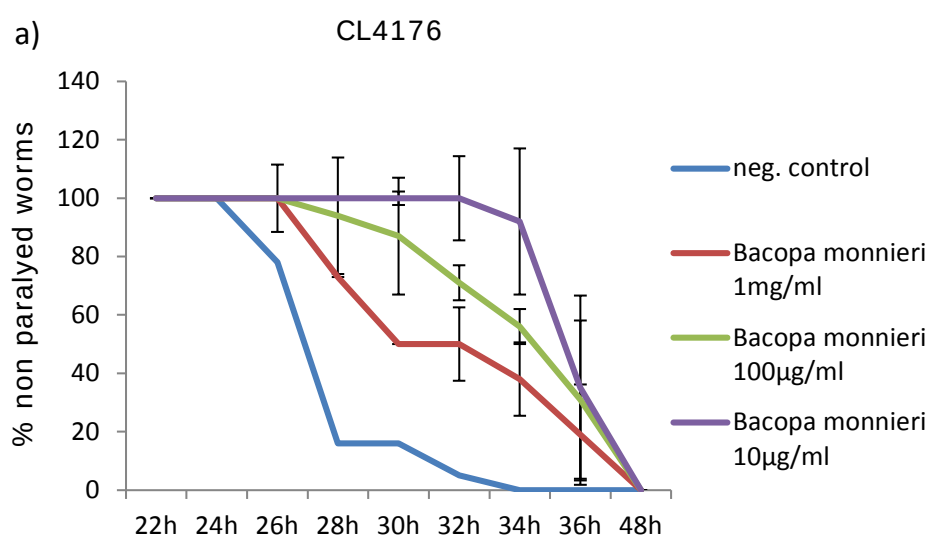
- e) In strain CL2120 fluorescence of metallothionein was measured on day 3 and day 5 of adolescence. In the untreated worms β -amyloid expression led to an increased metallothionein expression after 3 days but to a decreased after 5 days. In worms exposed to reserpine the level of metallothionein is increased after 3 and 5 days whereas β -amyloid expression in worms exposed to caffeine did not influence metallothionein expression.

3.4.2. *Bacopa monnieri*

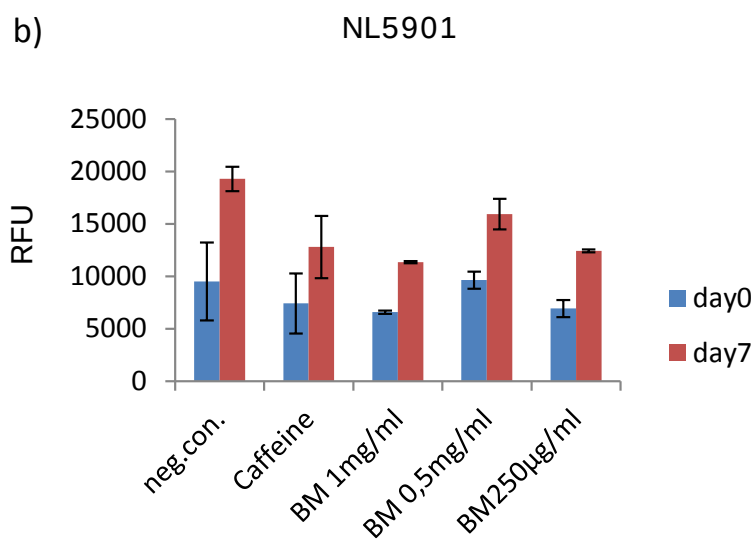
The Ayurvedic medicinal plant *Bacopa monnieri* has antioxidant, antidepressant, anti-inflammatory and antimicrobial properties and is commonly used to enhance memory (Pooja et al., 2011). Pooja et al. (2011) observed a marked and significant reduction in the aggregation of α -synuclein in strain NL5901 after exposition to concentrated mother tincture of the herb *Bacopa monnieri*. In our study we wanted to evaluate if the ethanol extract of *Bacopa monnieri* protects against Alzheimer's disease too and to replicate the results of Pooja et al. (2011) in strain NL5901.

3.4.2.1. Protection from β -amyloid and α -synuclein toxicity by an ethanolic extract of *Bacopa monnieri*

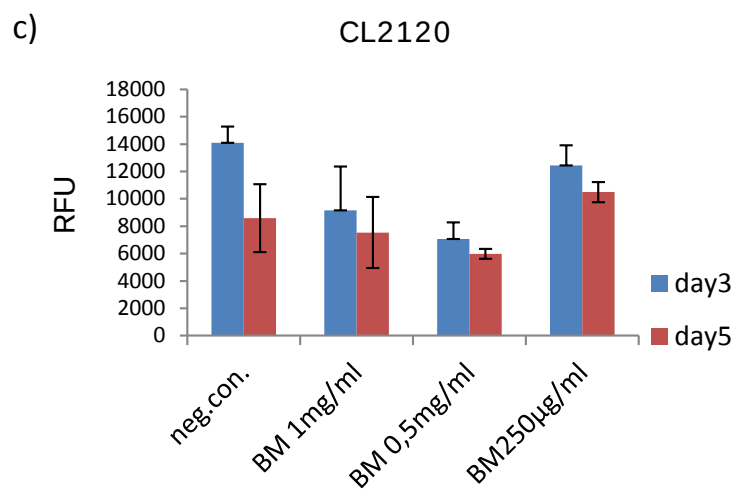
Figure 19:



- a) Paralysis curve. CL4176 worms were exposed to different concentration of *Bacopa monnieri* extract or left untreated (neg. control). Time until paralysis is dependent on concentration. Best results were achieved with the lowest dose.



- b) NL5901 worms treated with different concentrations of *Bacopa monnieri* showed reduced fluorescence after 7 days when compared to the negative control. Fluorescence intensity was nearly the same as in worms exposed to 6,27mM caffeine.



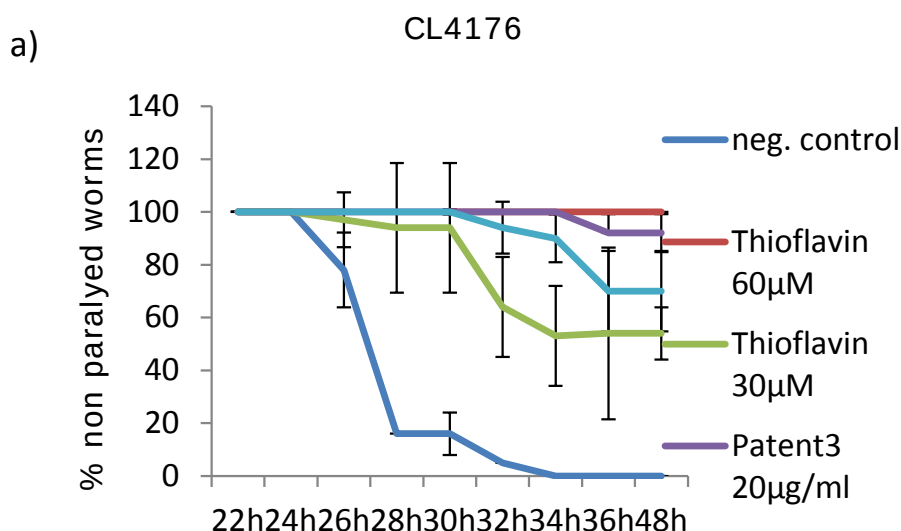
- c) Compared to the negative control metallothionein expression is increased in CL2120 worms when treated with 250µg/ml of *Bacopa monnieri* extract but decreased when exposed to higher concentrations.

3.4.3. Thioflavin T

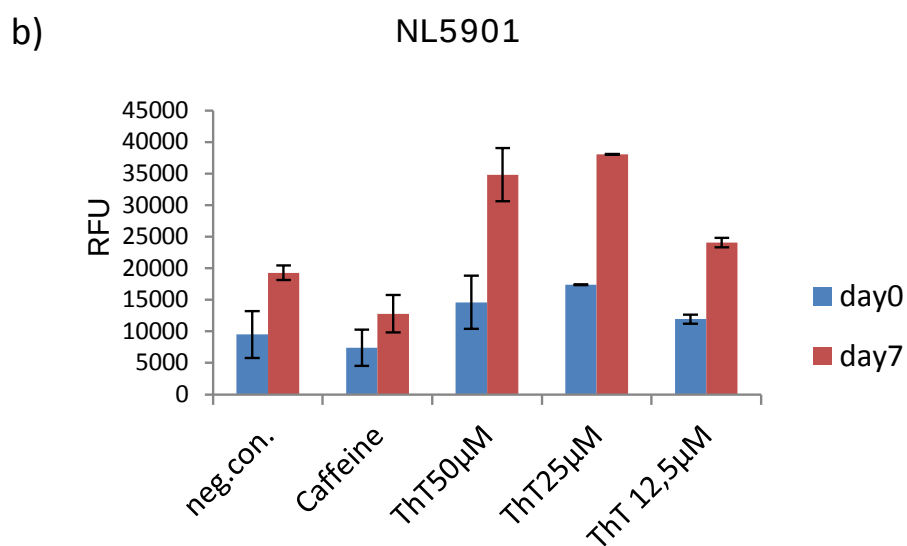
Thioflavin T (4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride) is a protein-fibril- and protein-aggregate-binding flavonoid commonly used to stain β -amyloid deposits. Alavez et al. (2011) showed that exposition of CL4176 worms to 50 – 100 μ M thioflavin t suppressed paralysis. It reduced the aggregation affinity and the level of the soluble oligomeric β -amyloid. This was achieved by activating stress-response pathways. Beside the positive controls caffeine, reserpine and Bacopa monnieri extract we used thioflavin T to evaluate the applicability of our test sytems.

3.4.3.1. Effects of Thioflavin T on β -amyloid and α -synuclein toxicity

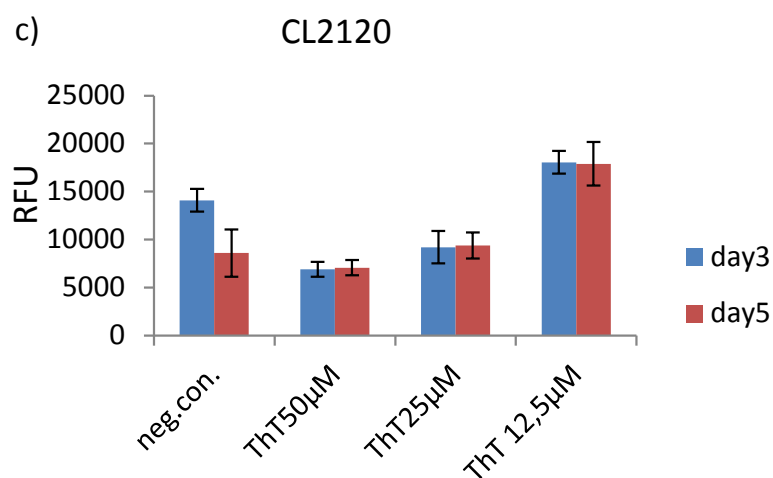
Figure 20:



- a) Dose-response paralysis curve for CL4176 worms untreated (neg. control) or exposed to 20 to 60 μ M thioflavin T. Most untreated worms were paralyzed at about 28 hours after temperature upshift. In contrast with 60 μ M thioflavin T 100% of worms did not paralyze even after 48 hours. Approximately 50% of worms exposed to 30 μ M thioflavin T showed paralysis after 48 hours. Thus a dose dependent effect was shown.



b) Compared to the negative and the positive control caffeine thioflavin T increased α -synuclein fluorescence in strain NL5901.

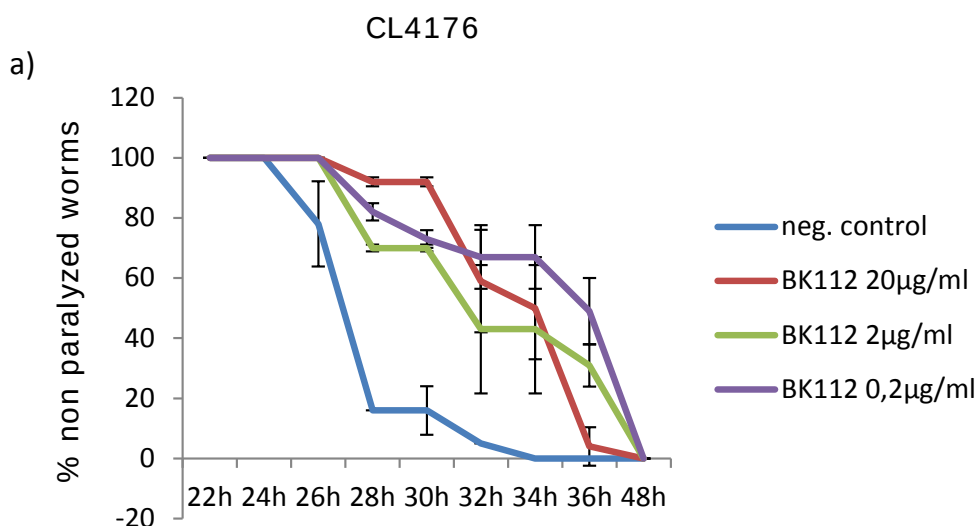


c) The lowest concentration of thioflavin T increased metallothionein expression in transgenic worms CL2120 but this did not lead to protection against β -amyloid toxicity in the paralysis assay (Fig. 20a)

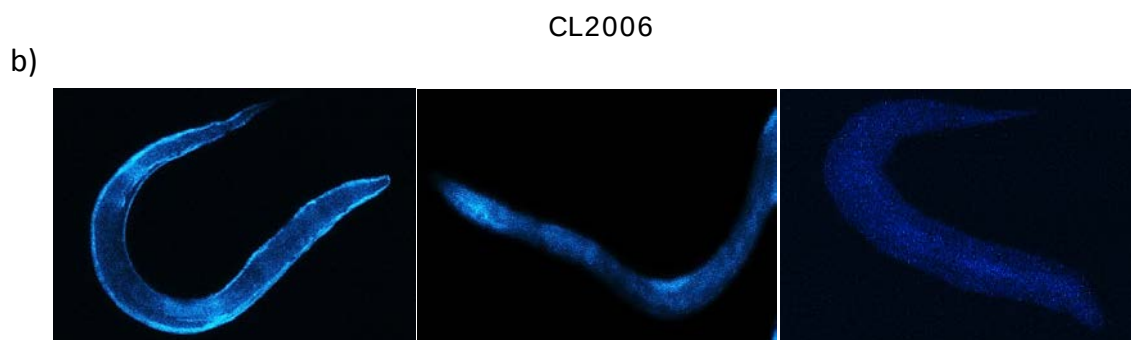
In the following chapters results achieved with anthraquinone-type compounds from marine organisms are compiled. Due to pending patent applications the structures of these substances cannot be presented.

3.4.4. Effect of compound BK112/RP on β -amyloid toxicity by increased metallothionein expression and on α -synuclein accumulation

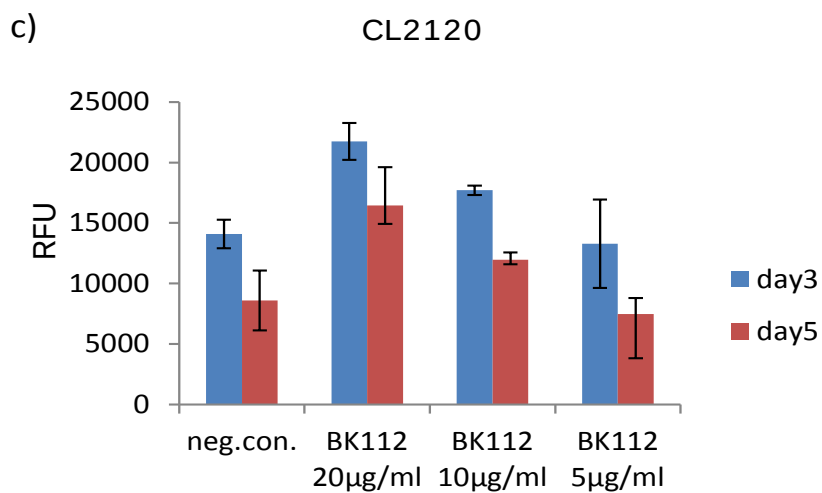
Figure 21:



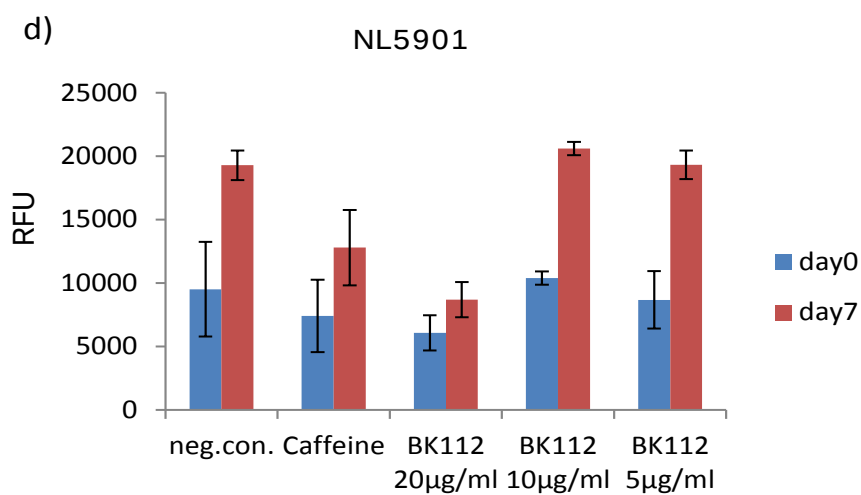
- a) Dose-response paralysis curve of CL4176 worms untreated (neg. control) or exposed to 0,2-20µg/ml BK112/RP. With all three concentrations of BK112/RP a delay in paralysis phenotype of approximately 6 hours was observed.



- b) β -amyloid deposits in strain CL2006 were stained with thioflavin t and visualized with fluorescence microscopy at 360nm extinction and 590nm emission. Plaques were decreased after administration of 10µg/ml BK112/ (middle). The inhibition of plaque formation was less than after 6,27mM caffeine (right). When compared to the untreated worm (left) deposits appeared different. Magnification 20x



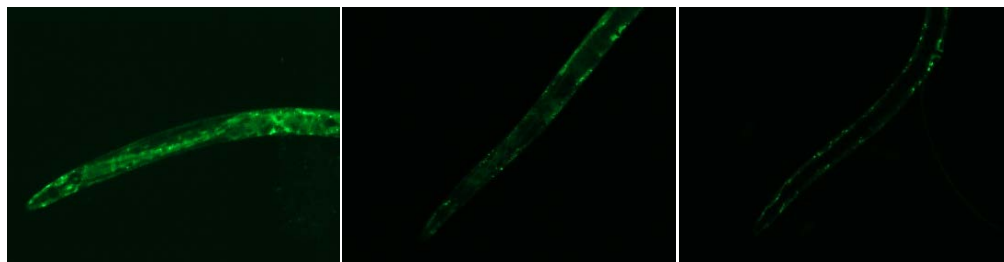
- c) In assaying β -amyloid toxicity 20µg/ml BK112/RP led to a dose dependent increase of metallothionein expression in CL2120 worms suggesting a protection of β -amyloid toxicity via this mechanism.



- d) The effect of 20µg/ml BK112/RP against α -synuclein accumulation was stronger than the one of 6,27mM caffeine.

e)

NL5901

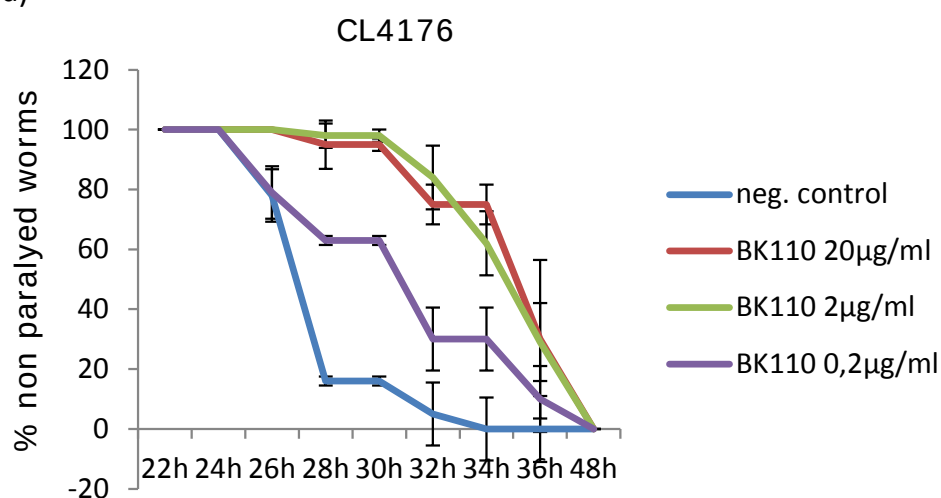


- e) Detection of α -synuclein in NL5901 worms treated with 10 μ g/ml BK112/RP (middle). There are fewer deposits than in the unexposed worm (left). The fluorescence is comparable to the worms treated with 6,27mM caffeine (right). Magnification 20x

3.4.5. Effects of BK110 /RP on β -amyloid and α -synuclein toxicity

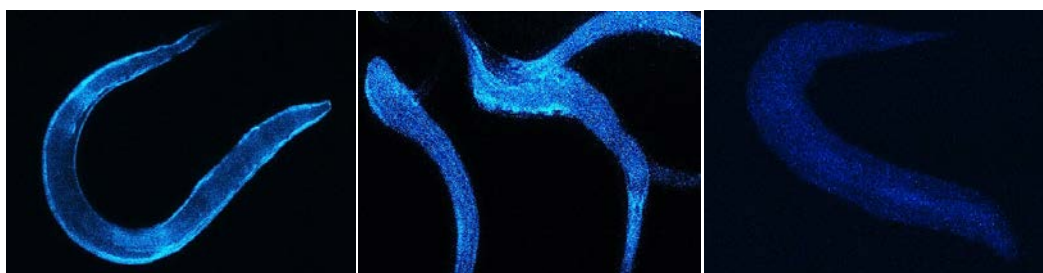
Figure 22:

a)

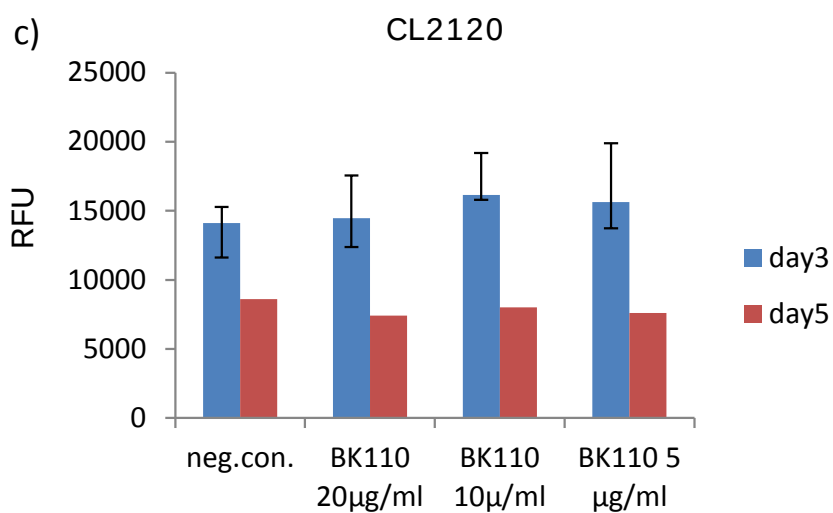


- a) Dose-response paralysis curve for CL4176. A delay in paralysis of CL4176 treated with BK110/RP at higher concentrations of BK110/RP was observed.

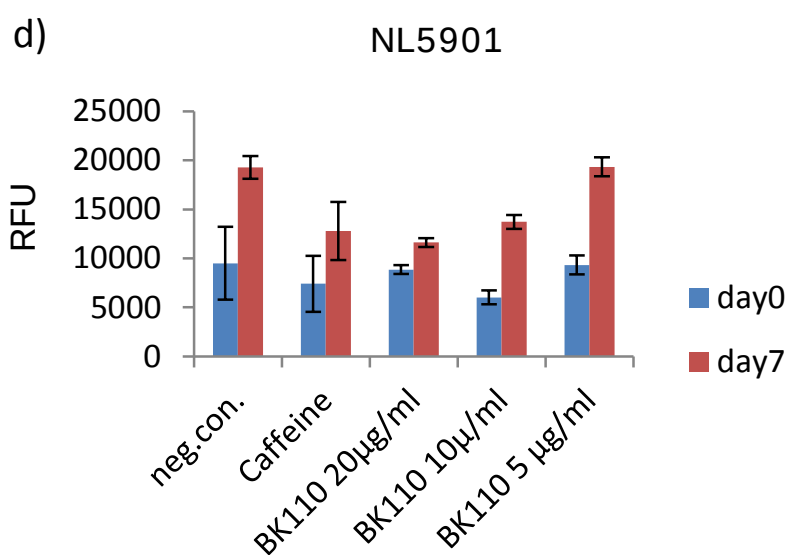
b) CL2006



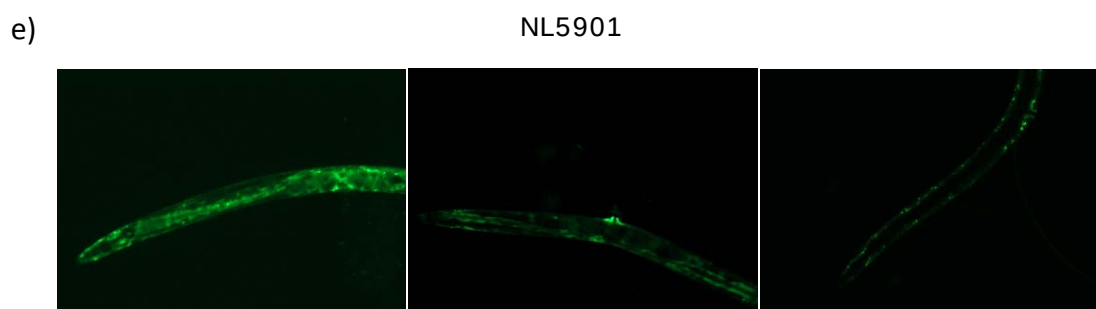
b) After thioflavin T staining of β -amyloid deposits no significant decrease after treatment with 10 μ g/ml BK110/RP was detected as compared to 6,27mM caffeine in the right picture. Magnification 20x



c) Metallothionein expression was not influenced with this compound.



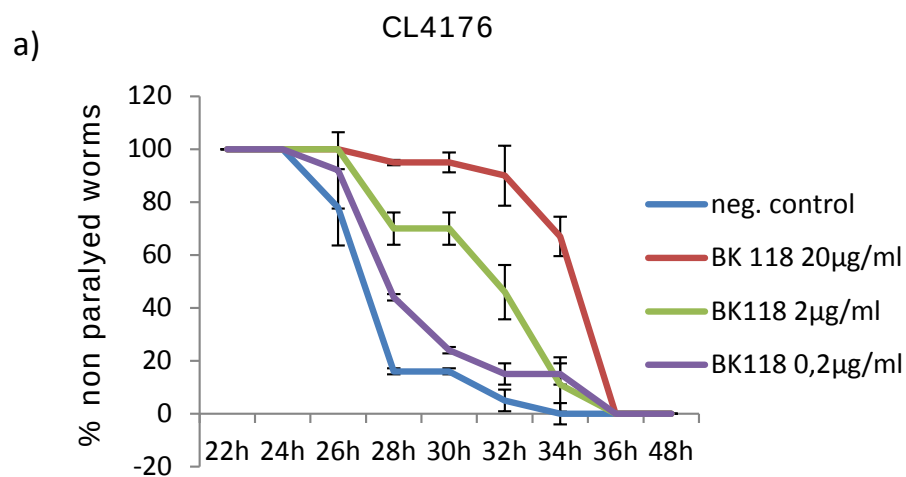
d) α -synuclein aggregation could be reduced with 10 and 20 µg/ml BK110.



e) Detection of α -synuclein in NL5901 worms treated with 10 µg/ml BK110/RP (middle). There are less deposits than in the unexposed worm (left) but more than in the caffeine treated one (right). Magnification 20x

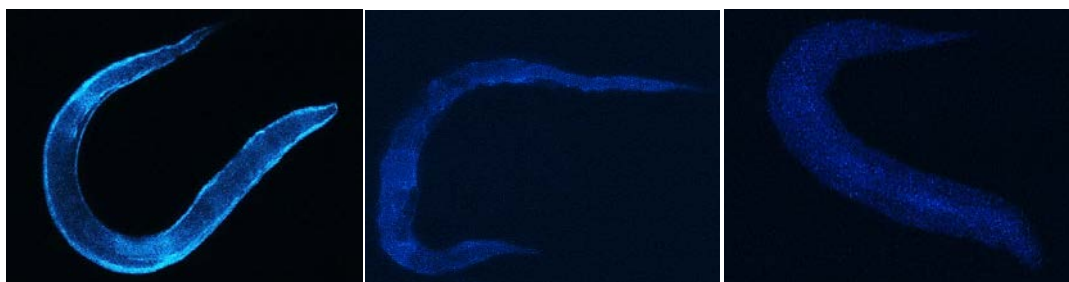
3.4.6. Effects of BK118 /K1 on β -amyloid and α -synuclein toxicity

Figure 23:

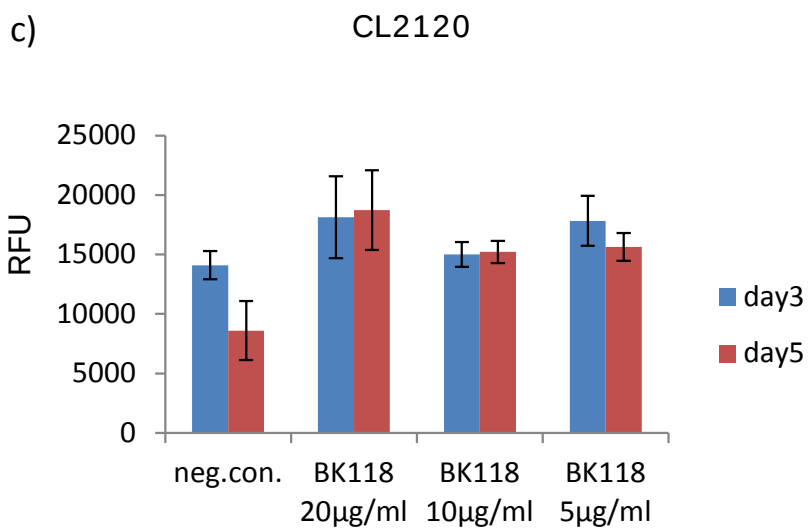


- a) Dose-response paralysis curve for CL4176. The delay in paralysis was dependent on the concentration of BK118/K1. The higher concentration (20µg) resulted in a delay of approximately 5 hours.

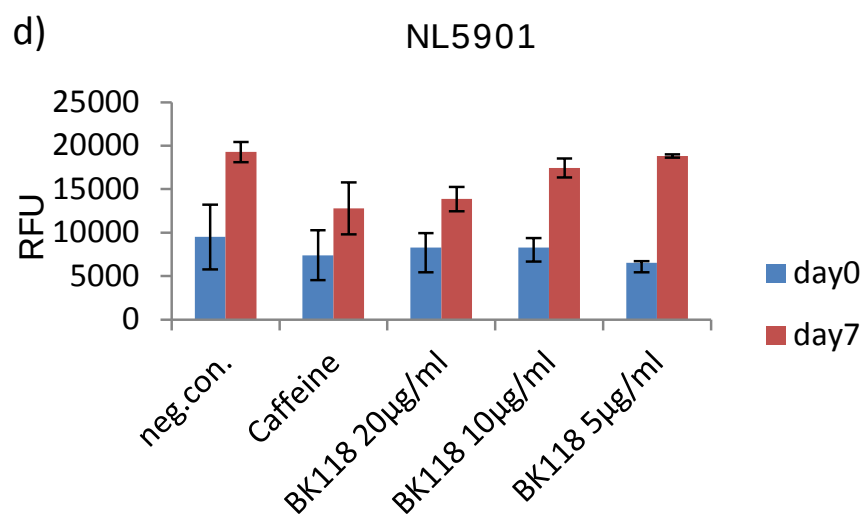
b) CL2006



- b) When stained with thioflavin T CL2006 worms treated with BK118/K1 (middle) showed less deposits as compared to the negative control (left) but approximately the same as the caffeine treated one (right). Magnification 20x



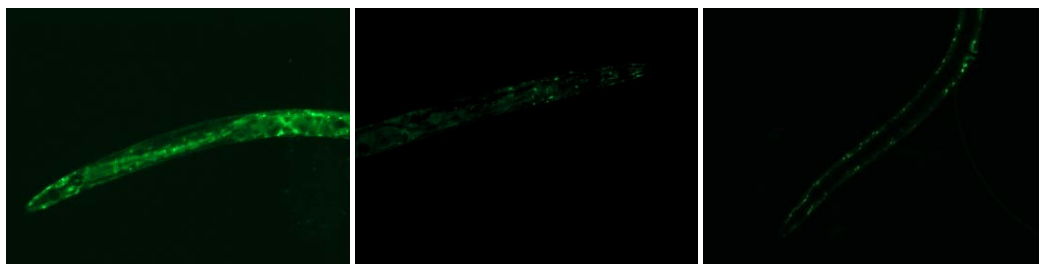
- c) Assaying β -amyloid toxicity. In this assay treatment with 20 – 5µg/ml anthraquinone BK118/K1 led to an increased signal even after 5 days. The level of metallothionein expression is increased during the whole observation period after treatment with this compound.



- d) α -synuclein accumulation was not influenced with the anthraquinone BK118/K1.

e)

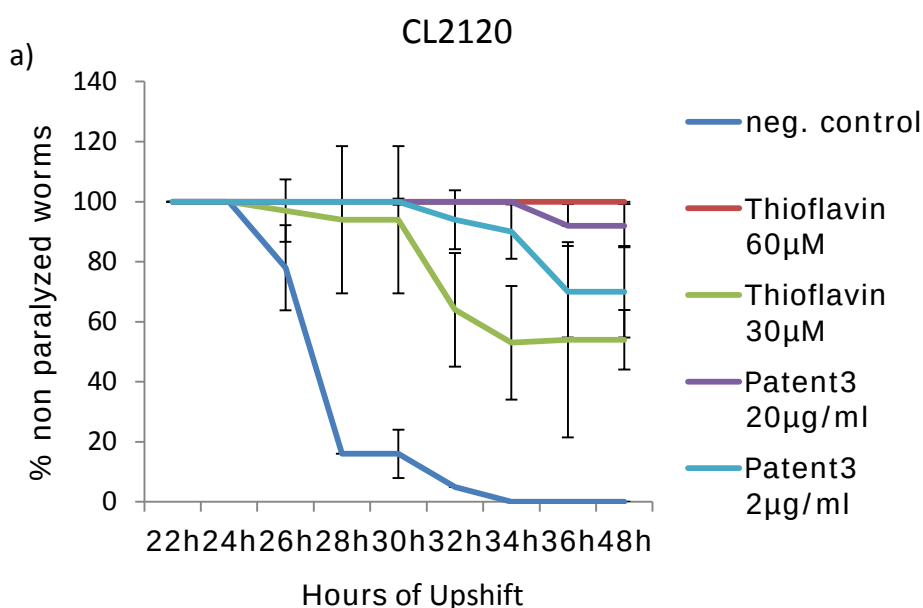
NL5901



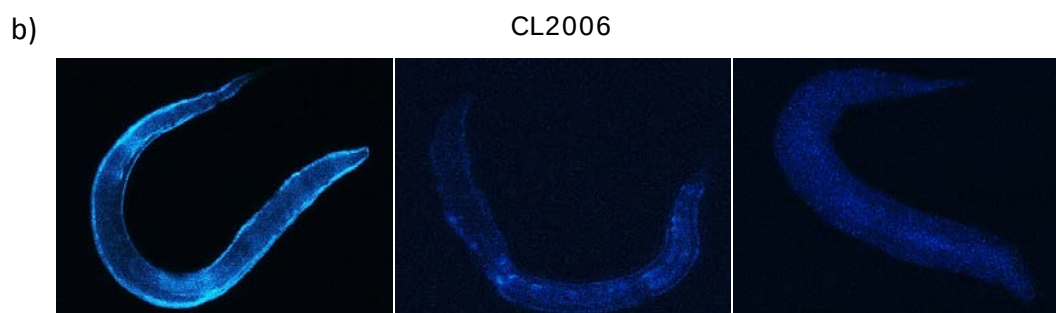
e) Fluorescence of NL5901 worms treated with 10 μ g/ml BK118/K1 showed more deposits when compared to caffeine (6,27mM) treated ones. Magnification 20x

3.4.7. Effects of *Talaromyces* extract ("Patent 3") on β -amyloid toxicity through early protective mechanisms

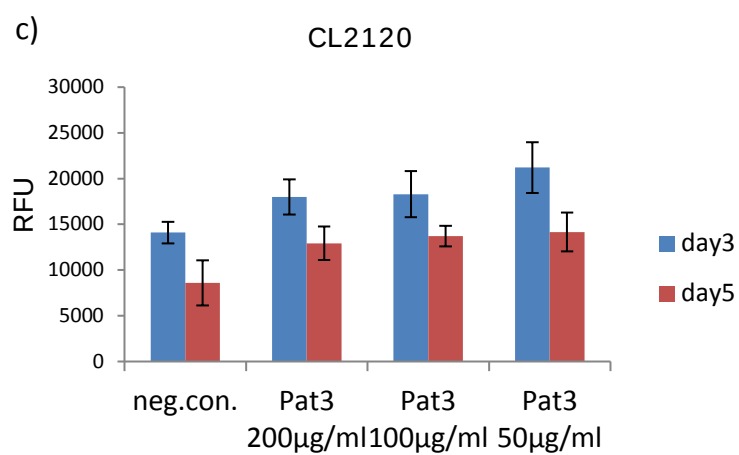
Figure 24



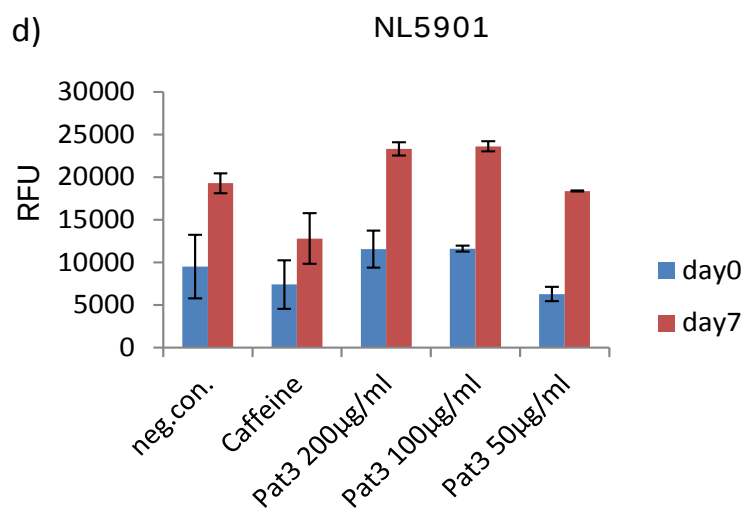
a) Dose-response paralysis curve. Unexposed CL4176 worms (negative control) were paralyzed at about 28 hours after temperature upshift. As positive control serve the worms treated with 60 μ M thioflavin T which did not paralyze even after 48 hours. 70% of worms exposed to 2 μ g/ml Patent3 and 90% of worms exposed to 20 μ g/ml Patent 3 showed no paralysis phenotype after 48 hours. After 60 hours the worms laid eggs whereas worms in thioflavin T remained sterile.



- b) When stained with thioflavin T CL2006 worms treated with *Talaromyces* extract (middle) showed less deposits as compared to the negative control (left) but approximately the same as the caffeine treated one (right). Magnification 20x

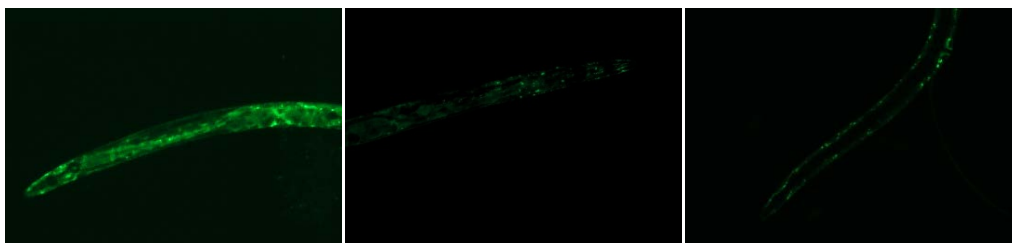


- c) Minor influence on methallothionein expression was achieved with CL2120 worms exposed to the *Talaromyces* extract (Pat.3).



- d) The *Talaromyces* extract (Pat.3) did not reduce α -synuclein fluorescence in strain NL5901 after 7 days of exposition.

e) NL5901



- e) When visualized under fluorescence microscope NL5901 worms treated with 1µg/ml *Talaromyces* extract showed reduced α -synuclein deposition (middle) as compared to the negative control (left) and to 6,27mM caffeine (right). Magnification 20x

3.4.8. Effect of *Radix Astragalii* (“Huang Qui”) on β -amyloid toxicity

The Chinese drug *Radix Astragalii* is traditionally used to boost the immune system. To date this plant is not used to treat neurological disorders. Because every tested substance delayed time until paralysis, we wanted to evaluate if exposition of worms to any substance resulted in this effect. Therefore we tested the water extract of this root.

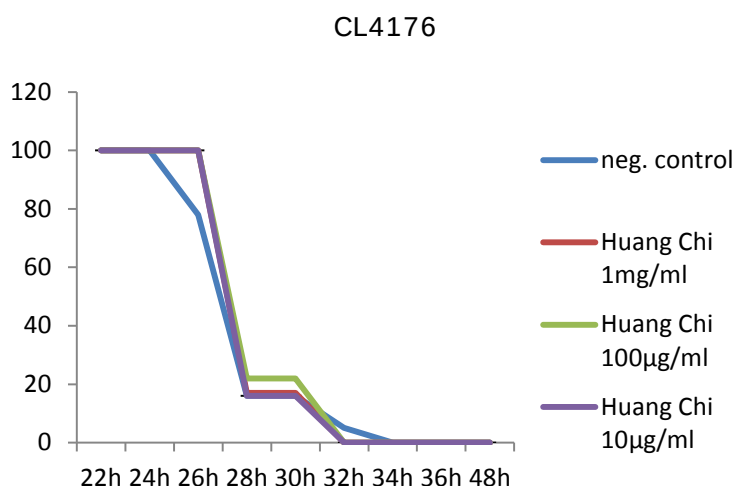


Figure 25: After treatment with an extract of Huang Qui there was no delay in paralysis. Thus, Huang Qui was not able to protect from β -amyloid toxicity.

3.5. Influence of amyloid β expression on the level of metallothionein

In strain CL2120 metallothionein is coupled to GFP. Further β -amyloid is expressed in this strain. After three days of adolescence worms had the strongest fluorescence signal which gets weaker after five days. To investigate if the fluorescence intensity is weaker in worms without β -amyloid expression we compared strain CL2120 with CL2122 which did not express β -amyloid at the third day of adolescence.

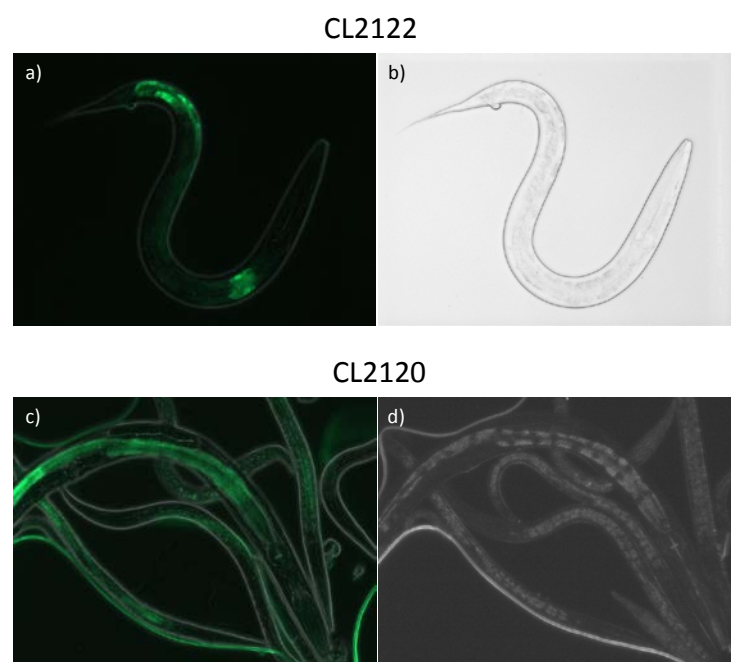


Figure 26: In worms of strain CL2122 at the third day of adolescence metallothionein marked by GFP is visible behind the pharynx and in the tail region. a) GFP b) Light microscopy. In CL2120 fluorescence was increased in the complete intestine. c) GFP d) Light microscopy. (Magnification 20x)

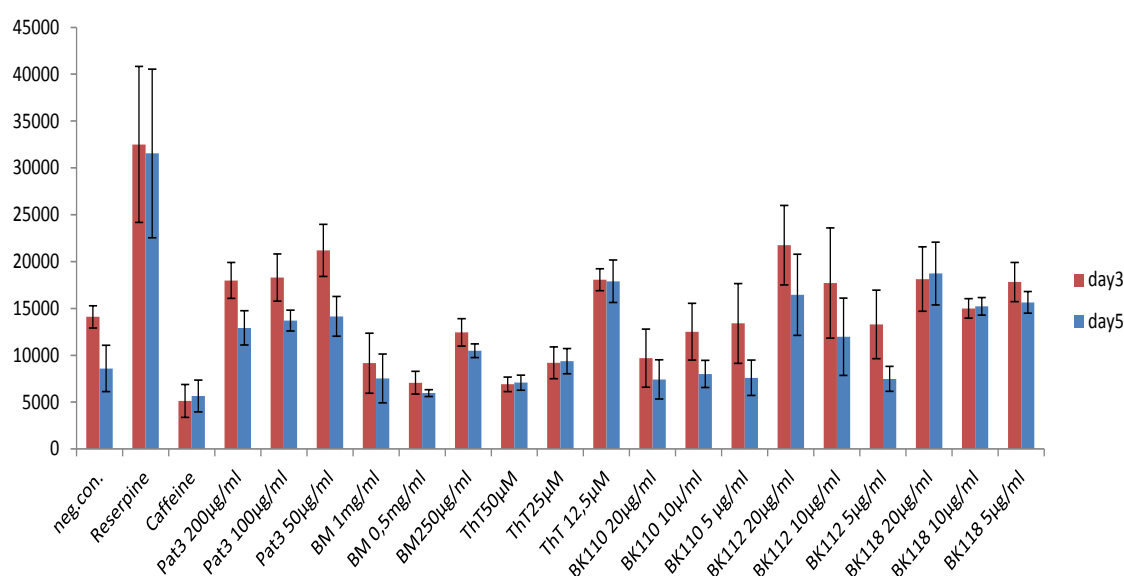


Figure 27: General graph of fluorescence intensity in CL2120 worms exposed to different substances. With 6,27mM caffeine, 1mg - 0,5mg *Bacopa monnieri* extract and 25µM-50µM thioflavin T fluorescence intensity was the half of the negative control. With 50µM reserpine fluorescence intensity doubled. With all three concentrations of *Talaromyces* extract, with 10µg/ml - 20µg/ml BK112 and 5µg/ml - 20µg/ml BK118 fluorescence increased too whereas BK110 shows the same intensity as the negative control.

4. Discussion

4.1. Finding drug targets in neurodegenerative diseases

The pathological hallmark of dementia in Alzheimer`s and Parkinson`s disease is the neurodegeneration. The molecular mechanisms of developing such a disease are still unclear but it is suggested that many different factors are involved in aetiology. Both neuronal disorders exhibit a failure in the folding of proteins which leads to their accumulation and deposition. Namely β -amyloid and the tau protein in Alzheimer`s disease and  $\alpha$ -synuclein in the Parkinson`s disease. It is actually under discussion whether the misfolding is the cause of neurotoxicity or a mechanism to protect neurones from degeneration. Further the question if the polymeric or the soluble oligomeric conformations of the proteins are the toxic species is discussed intensely. It has been proved that no correlation between the degree of neurodegeneration and the amount of plaques can be deduced (Drouet et al., 2000, Mondragón-Rodríguez et al., 2012). Further a study in transgenic mice has shown that the process of neurodegeneration starts before accumulation of peptides occurs (Hsia et al., 1999, Mucke et al., 2000) assigning toxicity to the soluble oligomeric conformation of β -amyloid.

The toxic insults correlated positively with the increase of the soluble β -amyloid form which means that the soluble oligomers inhibit LTP (long-term-potentialiation), a process which enables recognition (Walsh et al., 2002). During the last ten years new synaptotoxic theories shifted away from the hypothesis to eliminate the plaques. The research of Tanzi et al. (2005) and Hoe et al. (2012) showed the involvement of NMDA receptors in the neurodegenerative process. Both groups claim that soluble β -amyloid binding modulates NMDA receptors which leads to an increased Ca influx and consequently to apoptotic cell death. This leads to the hypothesis that an ideal Alzheimer drug should inhibit the oligomeric conformation at their very early stage (Convertino et al., 2009), because already β -amyloid dimers are reported to be synaptotoxic (Mc Donald et al., 2010). The inhibition of the protein`s misfolding effects the receptor binding affinity. In the case of  $\beta$ -amyloid misfolding, drugs are named  $\beta$ -sheet breakers. There is evidence that neither extracellular  $\beta$ -amyloid peptides nor intracellular neurofibrillar tangles alone result in Alzheimer`s symptoms but both are always involved in aetiology (Eriksen et al., 2003). Thus, an ideal anti-Alzheimer drug should inhibit both the accumulation of the extracellular and of the

intracellular proteins. Although many studies dealt with the mechanism of tau aggregation the question remains which conformation is responsible for toxicity of this protein (Götz et al., 2004). Research on other neurodegenerative diseases like Parkinson provided insights. In Parkinson's disease the accumulation and cellular stress induced by intracellular aggregated proteins namely α -synuclein may underlie the neurodegenerative mechanisms (Caughey and Lansbury, 2003 and Harrington et al., 2010). Since the degree of misfolding and aggregation of the natively unfolded and soluble tau or α -synuclein protein correlates with the progression of the diseases it is suggested that inhibition or reversion of protein aggregation can further protect affected cells (Pickhardt et al., 2005). For those intracellular localized proteins prospective targets in drug development should influence intracellular mechanisms (Harrington et al., 2010).

4.2. An animal screening model to bypass the target problem

Since these intracellular drug targets are still unclear and Alzheimer's and Parkinson's disease's aetiology are multifactorial an animal screening model was used. *C. elegans* obviously has more advantages as compared to *Drosophila melanogaster*. The main one is, that *C. elegans* are hermaphrodites and the genetic transmission should be stable (Wood, 1988). Further *C. elegans* seemed to be ideal for developing a high throughput drug screening method because it can be cultured in liquid medium with an *E. coli* strain as food source and grows in 96 well plates. The possibility of freezing and thawing equates *C. elegans* handling to cell culture techniques (Wood et al., 1988).

4.3. Different strains of *C. elegans* for different assays

The first assay was performed in accordance with the protocol from Dostal et al. (2010) with the transgenic strain CL4176 in which β -amyloid expression can be induced by an upshift of the temperature from 16°C to 23°C. The degree of expression correlates with the occurrence of paralysis by which β -amyloid induced toxicity is detectable. The assay was slightly modified by use of the hypochlorite method (Lewis and Fleming, 1995) for a faster synchronization procedure and performed in a 24-well plate format instead of single petri dishes. The resulting paralysis curve for 6,27mM caffeine was consistent with published results. For the estimation if test compounds are able to inhibit β -amyloid aggregation an assay under use of the transgenic *C.*

C. elegans strain CL2006, which expresses β -amyloid constitutively and accumulates β -amyloid fibrils, was established. For detection of the deposits staining with the fluorescent dye thioflavin T was applied (Fay et al., 1998). To estimate activity towards intracellular accumulated proteins we used strain NL5901 in which the human α -synuclein is fused with GFP. Our hypothesis was that the fluorescence should be decreased by an active compound as compared to untreated control. Jadiya et al. (2011) analyzed α -synuclein protein aggregation after incubation of L1 larvae for 48hrs on 10cm NGM plates seeded with the compound in OP50 and measured GFP fluorescence by monitoring under a confocal microscope and fluorescence intensity by image J software. We used a set-up of the assay in a 96 well format to detect differences in fluorescence in a multiwell plate reader. Synchronized worms were suspended in medium with *E. coli* OP50 and pipetted into a 96 well microtiter plate containing different compounds in different concentrations. Since the degree of α -synuclein aggregation correlates with time fluorescence was detected in late age after 7 days in adulthood. To prevent eggs from hatching FUdR was added on day 0 when worms reached the fourth larval stage, according to Solis and Petraschek (2011) who used a protocol to measure the influence of compounds on the life span of wild type worms using 96 well microtiter plates. Fluorescence was measured on day 0 and day 7. When comparing those two time points in the untreated control fluorescence increased whereas the positive control caffeine was able to inhibit α -synuclein aggregation (Fig. 18c). Jadiya et al. (2011) have shown that the mother tincture of the ayurvedic medicinal *plant Bacopa monnieri*, which is commonly used as an antioxidant, reduced α -synuclein aggregation. To verify the applicability of the established assays an ethanol extract of the leafes *Bacopa monnieri* was tested (Fig. 19a-c) and additionally the compounds thioflavine T (Alavez et al., 2011) and reserpine (Arya et al., 2009) which have been active in the paralysis assay with strain CL4176. Our results show that both delayed paralysis in CL4176 (Fig. 18a and 20a) but both increased α -synuclein aggregation (Fig. 18c and 20b). Additionally *Bacopa monnieri* is able to delay paralysis (Fig. 19a). After reproducing the results from Jadiya et al. (2011), Alavez et al. (2011) and Arya et al. (2009) we started testing of SeaLifePharma`s anthraquinones BK110/RP, BK112/RP, BK118/K1 and of the *Talaromyces* extract "patent 3". In the strain NL5901 BK110/RP, BK112/RP and BK118/K1 were able to decrease α -synuclein aggregation at the highest concentration (Fig. 21d, 22d and 23d) whereas the *Talaromyces* extract and BK118/K1 did not show activity in this assay (Fig. 24d). In the paralysis assay all tested substances were able to delay the onset of paralysis after elevating temperature (Fig.21a-24a). The comparison of paralyzed worms after 32 hours resulted in about 95% paralyzed

worms in the negative control and 0% in the positive controls caffeine, reserpine, thioflavin T and the *Bacopa monnieri* extract and in the test compound *Talaromyces* extract (Fig.18a-20a and 24a). After treatment with 20µg/ml BK118/K1 approximately 5 % of worms (Fig. 23a) and with 20µg/ml BK110/RP or BK112/RP about 20% were paralyzed after 32 hours of temperature upshift (Fig. 21a and 22a). To exclude the probability that addition of any substance prolongs time till paralysis we further tested *Radix Astragali* ("Huang Qi") a TCM drug traditionally used to boost the immune system. Worms treated with Huang Qi were paralyzed within 32 hours (Fig.25) in the same way as the negative control. The achieved results showed that anthraquinones could be used as a new class of substances in drug research against neurodegenerative diseases. To further investigate these compounds we tested them in strain CL2006, the worm model which expresses and accumulates β -amyloid deposits.

4.4. Do the tested compounds prevent plaque building?

The strain CL2006 forms deposits that can be stained with fluorescent dye. Contrary to CL4176 in which β -amyloid expression can be induced CL2006 constitutively expresses the human transgene β -amyloid. The strains differ in the kind of β -amyloid conformation as well. CL4176 does not exhibit deposits and is a preferable test system when assaying toxicity of oligomeric β -amyloid. CL2006 can serve as a model to screen the ability of compounds to prevent accumulation or dissolve already accumulated β -amyloid polypeptides. Thioflavin T is a fluorescent dye with protein aggregate binding properties. We used it to stain the β -amyloid deposits of treated or untreated worms from the strain CL2006 according to the protocol from Fay et al. (1998). After staining and visualizing worms under the fluorescence microscope, a clear distinction was possible between untreated worms and worms treated with 6,27mM caffeine. Whereas the untreated control exhibited deposits throughout the wall muscles, caffeine treated positive control remained without deposits (Fig. 18b). When investigating the effect of our test substances in this strain worms showed diffuse fluorescence throughout the whole body which differed from the negative control (Fig. 21b-23b). Only the *Talaromyces* extract exhibited distinct deposits like the negative control (Fig.24b). This led us to the conclusion that anthraquinones are able to protect strain CL4176 from β -amyloid toxicity and strain NL5901 from α -synuclein toxicity to a lesser extent. The compounds' anti-aggregating properties led to visible differences in the strain CL2006. Previously Yoshiike et al. reported that metals force amyloid to form aggregates but are apparently not characterized by β -

sheet fibrils. It seems that they prevent individual amyloid molecules from aggregating in a single direction, thus preventing fibrillogenesis (Yoshiike, 2003), which might be the reason for the diffuse fluorescence in CL2006.

4.5. Hypothesis on the mode of action

Caffeine hinders aggregation of β -amyloid peptide and delays paralysis in CL4176 (Dostal et al., 2010). The genetic mechanisms underlying these protective mechanisms were studied by Dostal et al. (2010) examining if caffeine acts via an induction of a stress response pathway. For this hypothesis several stress response pathways have been tested using transgenic *C. elegans*. A significant induction of glutathione-S-transferase was shown. Glutathione-S-transferase is controlled by genes *skn-1* or *nrf2* in vertebrates. *Skn-1* controls phase II detoxification response genes (Kahn et al., 2008) and is involved in cellular response to oxidative stress (Osburn and Kensler, 2008). Dostal et al. (2010) elucidated that the *skn-1* pathway is needed for the protection of β -amyloid induced toxicity by caffeine when tested in a strain where the gene *skn-1* is knocked out and where β -amyloid is expressed. For thioflavin T and other amyloid-binding compounds it is proposed that protection against β -amyloid toxicity is achieved by mimicking stress response and activating stress-response pathways (Alavez et al., 2011).

Anthraquinones are known to hinder β -amyloid aggregation by destabilizing the interstrand hydrogen bonds through polar interactions with the backbone of the peptide (Fig.2) (Convertino et al., 2009). Previously has been shown that aromatic interactions contribute to the inhibition of fibril formation as well as to the reduction of amyloid β toxicity (Porat et al., 2004). It was suggested that such interactions are not sufficient to significantly perturb the β -sheet formation (Convertino et al., 2009). In contrast was reported that even small perturbations can dramatically influence the kinetics of the aggregation process (Pellarin and Caflish, 2006). A promising group of compounds that interrupt β -amyloid aggregation and thus protect against toxicity are metals. Although it has been assumed that zinc, copper, aluminium or iron promote β -amyloid aggregation now there is evidence that they disrupt amyloid β aggregation and improve cell viability (Yoshiike et al., 2001). These controversially discussed properties of metals led to the suggestion that metals are toxic in “healthy organisms” but protective when β -amyloid is present. It seems that the metal-amyloid complex reduces toxicity of both metals and β -amyloid. As already mentioned metals force β -amyloid to form aggregates which are apparently not characterized by β -sheet fibrils. It seems that metals prevent individual amyloid molecules from aggregating in a

single direction, thus preventing fibrillogenesis (Yoshiike et al., 2003). From these data was deduced that metallothionein activation is an important step in cell protection from β -amyloid toxicity by anthraquinones. Helmcke (2012) showed that metallothionein expression is increased after exposure to metals as well as after non-metals. Metallothionein is a small molecular weight protein that binds the probably toxic metal and acts as an antioxidant to repair the damage caused by the reactive oxygen species (ROS) (Swindell, 2011). To find out if anthraquinones are able to act in this way we established an additional screening model.

4.5.1. Metallothionein activation as the protective mechanism

For this purpose we used the strain CL2120 which expresses β -amyloid in body wall muscle cells and forms deposits. Metallothionein which is expressed in the intestine under the *mtl-2* promotor is marked by GFP and metallothionein overexpression can be measured in the multiwell plate reader or be visualized by fluorescence microscopy. To screen the compounds the synchronized worm population was added to a 96 well microtitre plate containing medium, OP 50 and a test substance. Each compound was tested in triplicates. 0,6mM FUdR was added to adult worms to prevent eggs from hatching at day 0. We measured the difference of fluorescence at day3 and day5. To be positive in this screening assay with CL2120 the test compound should prevent the decrease of metallothionein fluorescence between the third and the fifth day (Fig.27). Fluorescence intensity decreased in the negative control and in the worms treated with BK110/RP and 5 μ g/ml BK112/RP (Fig. 21c, 22c). It was half of the negative control in the worms treated with caffeine (Fig. 18c), thioflavin T (Fig. 20c) or *Bacopa monnieri* (Fig. 19c). Exposition to reserpine increased fluorescence intensity twofold after five days (Fig. 18c). All three tested concentrations of the *Talaromyces* extract “patent 3” increased the metallothionein level (27) as did the highest concentration BK112/RP. From the results can be deduced that caffeine, *Bacopa monnieri*, and thioflavin T act in a different manner than the other test substances.

After these results the question raised to what extent metallothionein is expressed when β -amyloid transgene is absent. To investigate this task we used strain CL2122 in which GFP is fused with metallothionein and β -amyloid is not expressed. By comparison of fluorescence intensity between 3 day old adults of strains CL2120 and CL2122 we observed that in CL2122 worms GFP is expressed only in two regions, behind the pharynx and in the tail region (Fig. 26a). In contrast, in CL2120 the

complete intestine showed fluorescence which means that β -amyloid expression leads to an increased mtl-2 expression in early adolescence (Fig. 26b).

For the two anthraquinones BK118/K1 and BK112/RP (Fig. 21c, 23c) similar activity as for reserpine was shown (Fig. 18e). Arya et al. (2009) reported previously that reserpine increases the lifespan with a high quality of life and ameliorates β -amyloid toxicity in *C. elegans*. Because reserpine does not act via pathways known to extend lifespan or via inhibition of its known target, the vesicular monoamine transporter, it is still in question which pathway is activated by this compound (Saharia et al., 2012). Recently was reported that acetylcholine is playing an important role in reserpine mediated lifespan extension (Saharia et al., 2012).

To sum up, it seems that BK118/K1, BK112/RP and BK110/RP force amyloid to form aggregates that are apparently not characterized by β -sheet fibrils in the same manner like metals (Yoshiike et al., 2001). These compounds prevent individual amyloid molecules from aggregating in a single direction, thus preventing fibrillogenesis. This hypothesis could explain the different fluorescence patterns of CL2006 after treatment with anthraquinones (Fig. 21b, 22b, 23b) compared to worms treated with the *Talaromyces* extract (pat.3) (Fig.24b) which exhibits distinct deposits comparable to the negative control (Fig. 18b). Further is suggested that this complex hinders β -amyloid to interact with nACh receptors thus avoiding overactivation of the NMDA receptor protecting cells from cell death.

Although the anthraquinones were toxic in our cell culture tests no toxicity in *C. elegans* was observed. It is assumed that this toxicity of anthraquinones abolishes β -amyloid induced toxicity and in turn β -amyloid neutralizes anthraquinone toxicity by its binding affinities. Cells are indeed able to protect themselves from β -amyloid toxicity by mtl-2 activation but for unknown reasons its expression decreases with aging of the worms. Maybe this is an age dependent phenomenon and since Alzheimer`s and Parkinson`s disease are both age-dependent this could be the important step in drug research of age-dependent diseases. The *Talaromyces* extract (pat. 3), BK118/K1, BK112/RP and reserpine are able to increase the mtl-2 activation and to hold the metallothionein level with aging (Fig. 21c, 23c and 24c). In a review from 2011 Swindell discussed that metallothionein obviously is relevant to basic aging mechanisms. It is one of a few proteins that, when overexpressed, has been demonstrated to increase mouse lifespan. Cohen et al. published 2006 in Science that aggregation-mediated amyloid β toxicity was reduced in *Caenorhabditis elegans* when aging was slowed by decreased insulin/insulin growth factor-1 like signaling (ILS). Since ILS pathway is central to the regulation of longevity in worms, flies and mammals, they propose a mechanistic link between the aging process and

aggregation-mediated proteotoxicity (Cohen et al., 2006), thereby strengthening our theory of age-dependent metallothionein detoxification of β -amyloid.

5. References

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6. Appendix

Kurzzusammenfassung

Alzheimer und Parkinson sind die am häufigsten auftretenden neurodegenerativen Erkrankungen, wobei hauptsächlich ältere Menschen davon betroffen sind. Zu beiden gehören pathologische Veränderungen wie die Aggregation fehlgefalteter Proteine, welche sich in Folge extrazellulär oder intraneuronal ablagern. Die Erkrankungsursachen sind noch unklar, was eine Wirkstofffindung sehr erschwert. Ohne definierten Angriffspunkt erscheint die Wirkstoffsuche in einem lebenden Organismus, wie zu Beispiel in *C. elegans*, eine vielversprechende Alternative zu sein. Der hermaphrodite Wurm produziert homozygote Nachkommen, ohne auf eine Paarung angewiesen zu sein. Das und die kurze Lebensdauer dieser Nematoden ermöglichen die Wirkstoffsuche im Hoch-Durchsatzverfahren. In dieser Arbeit wurden verschiedene Substanzen gegen die β -amyloid- und α -synuclein-Toxizität getestet. Dazu verwendeten wir verschiedene transgene Wurmstämme, welche das humane Peptid gemeinsam mit GFP exprimieren. Als positive Kontrolle wurden aktive Substanzen wie Koffein, Thioflavin, Reserpin und ein Extrakt der Pflanze *Bacopa monnieri* verwendet. Diese sind hinsichtlich ihrer protektiven Eigenschaften gut untersucht. Um die Wirkstoffe genauer zu charakterisieren, verwendeten wir 2 zusätzliche transgene Stämme. Einen Stamm, um zu überprüfen, ob die Plaquebildung beeinflusst wird, und einen weiteren, ob der Wirkmechanismus der getesteten Substanzen auf einen erhöhten Metallothioneingehalt zurückzuführen ist.

Schlagworte:

Alzheimer, Parkinson, *C. elegans*, Hoch-Durchsatzverfahren, Ablagerung, Plaquebildung, Metallothionein

Abstract

Alzheimer and Parkinson are the most prevalent neurodegenerative diseases affecting mainly the elderly population. The pathogenic mechanism involves the aggregation and deposition of abnormally folded proteins, either extra-cellular or intra-neuronal. Until now the cause of developing such a disease is unclear. But without a target a screening method can hardly be set up and therefore an animal screening model like *C. elegans* seems a promising tool. This hermaphrodite produces its own homozygous line without mating. This advantage together with the short life span enables a high-throughput screening. To screen SeaLifePharma`s compounds against β -amyloid and α -synuclein toxicity we used transgenic strains expressing the human peptide together with GFP. As positive controls served caffeine, thioflavine, reserpine and an extract of *Bacopa monnieri* which are known to be protective against this toxicity. To further evaluate compounds mode of action we tested if they are able to inhibit plaque formation and if they increase the metallothionein level after exposition.

Keywords:

Alzheimer, Parkinson, *C. elegans*, high-throughput screening, plaque formation, metallothionein

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