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Targeting abnormal metal homeostasis to combat neurodegenerative disorders by natural products

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Abstract

Today, the search for new drugs against neurodegenerative diseases (NDs) is facing an innovation deficit. A promising tool in the drug discovery process can be provided by whole animal screenings, e.g., based on the nematode *Caaenorhabditis elegans*.

To generate extracts for the discovery of bioactive multicomponent mixtures we combined the knowledge from traditional medicine and natural product chemistry. We established a miniaturized multi-content screening method based on *C. elegans* to investigate extracts for their ability to increase the nematodes' survival time and to suppress fat accumulation as hallmarks of "healthy aging" (paper I).

Age-dependent metal dyshomeostasis has been recognized as a causative factor involved in protein aggregation in NDs. We could show that metallothionein (MT), an endogenous metal detoxifying protein, is increased in young amyloid ß (Aß) expressing *C. elegans*, whereas it is not in wild type strains. Further MT induction collapsed aged Aß expressing worms, indicating the age dependency of disease outbreak. A medium throughput screening assay has been established to identify compounds increasing the MT level as a putative novel therapeutic target. Thereby we could show that compounds prolonging the time of MT induction were active in both models of Alzheimer (AD) and Parkinson´s disease (PD) (paper II).

Besides other extracts the alkaloid fraction of *Psychotria nemorosa* was able to significantly reduce proteotoxicity in our AD and PD models of *C. elegans* and induced MT in aged nematodes. Therefore, the two main alkaloids, nemorosine A and fargesine, have been further evaluated for their molecular mechanism (paper III).

Based on current data, it can be hypothesized that MT promotion can be further taken into account for the treatment of Huntington´s disease (HD), amyotrophic lateral sclerosis, prion disease and many other disorders of the central nervous system (paper IV).

Zusammenfassung

Heutzutage sieht sich die Medikamentenentwicklung gegen neurodgenerative Erkrankungen (NDs) mit einem Innovationsdefizit konfrontiert. Ein vielversprechendes Werkzeug in der Wirkstoffsuche könnten Screenings in ganzen Organismen wie z.B im Fadenwurm *Caenorhabditis elegans* bereitstellen.

Für die Erforschung von Mehrstoffkomponenten (Extrakten) und deren wirksamen Inhaltsstoffe, kombinierten wir das Wissen um die traditionelle Medizin mit der Naturstoffchemie. Wir etablierten eine miniaturisierte Screeningmethode um Mehrstoffgemische hinsichtlich ihrer Aktivität auf eine Lebensverlängerung und reduzierte Fettakkumulation als Merkmale gesunden Alterns im Wurmmodell zu untersuchen (Paper I).

Eine altersabhängige Metalldishomöostase ist Mitverursacher der Proteinaggregation in ND. Wir konnten zeigen, dass Metallothionein (MT), ein endogenes Metall-entgiftendes Protein, im Amyloid ß (Aß) exprimierenden Wurmmodell im Gegensatz zum Wildtyp anfangs erhöht ist. Bei fortschreitendener Alterung konnten wir weiters feststellen, dass die MT Induktion zusammenbricht, was die Altersabhängigkeit aufzeigt. Deshalb wurde eine Screeningmethode entwickelt, um Verbindungen zu identifizieren, die die MT Induktion selbst in gealterten Nematoden ermöglichen. Die Induktion von MT stellt ein potentiel neues Target für die Arzneimittelentwicklung dar. Die auf diese Weise ermittelten bioaktiven Verbindungen waren zusätzlich im *C. elegans* Alzheimer- und Parkinsonmodell aktiv (Paper II).

Neben weieren Extrakten war besonders die Alkaloidfraktion von *Psychotria nemorosa* Gardner fähig, MT in gealterten Nematoden zu induzieren. Sowohl diese Fraktion als auch deren zwei Hauptverbindungen, Nemorosin A und Fargesin, erwiesen sich im *C. elegans* Modell für Alzheimer (AD) - und Parkinson (PD) Erkrankung als vielversprechende Naturstoffe. *In silico* Studien dienten der Identifizierung der molekularen Mechanismen dieser zwei Hauptalkaloide (Paper III).

Schließlich wurde erläutert, dass eine MT Induktion auch in anderen Erkrankungen, wie Huntington Krankheit (HD), amyotrophe Lateralsklerose oder der Prionenerkrankung als ein neues Target in Betracht gezogen werden sollte (Paper IV).

List of scientific papers

I. Zwirchmayr J, Kirchweger B, Lehner T, Tahir A, Pretsch D, Rollinger JM. A robust and miniaturized screening platform to study natural products affecting metabolism and survival in *Caenorhabditis elegans*. Sci Rep. 2020 Jul 23; 10(1):12323. doi: 10.1038/s41598-020-69186-6.

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III. Klein-Junior LC, Pretsch D, Kirchweger B, Chen Y, Cretton S, Gasper AL, Heyden YV, Christen P, Kirchmair J, Henriques AT, Rollinger JM. Effects of Azepine-Indole Alkaloids from *Psychotria nemorosa* on Neurodegenerative Disorders Evaluated in Transgenic *C. elegans*. Submitted on June 11, 2021.

IV. Pretsch D. Abnormal metal homeostasis as a common drug target to combat neurodegenerative diseases. Neural Regen Res. 2021 Dec; 16(12):2388-2389. doi: 10.4103/1673-5374.313039.

Additional publications (not included in the thesis)

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CONTENTS

List of Abbreviations

ND	Neurodegenerative disease
AD	Alzheimer´s disease
PD	Parkinson´s disease
NMDA	N-methyl-D-aspartate
MT	Metallothionein
Aß	Amyloid ß
HD	Huntington´s disease
MPACs	Metal-protein attenuating compounds
C. elegans	*Caenorhabdits elegans*
RNAi	RNA interference
TTM	Tibetian traditional medicine
ITM	Iranian traditional medicine
FDA	Food and drug administration
AchEIs	Acetylcholinesterase inhibitors
CNS	Central nervous system
CQ	Clioquinol
GABA	γ-aminobuturyl acid
CGC	*Caenorhabditis* Genetic Stock Center
NGM	Nematode growth medium
FUdR	5-fluorodeoxyuridine
DMSO	Dimethyl sulfoxide
ROS	Reactive oxygen species
GFP	Green fluorescence protein
YFP	Yellow fluorescent protein
DT	Death time
AICAR	5-aminoimidazole-4-carboxamide-ribonucleoside
OD	Optical density
DCM	Dichlormethane
M	Methanol
BuChE	Butyrylcholinesterase
MAO-A	Monoamine oxidase A
IDO	Indolamin-2,3-dioxygenase
5-HTR	5-hydroxytryptamine receptors
1-(PARP1)	Poly (ADP-ribose) polymerase

1. Introduction

1.1. Neurodegenerative diseases

As life expectancy increases, the prevalence of age-related diseases will also increase due to fact that the aging process leads to a progressive loss of function and a decline in resilience. There has been an estimation that 50 million individuals are living with dementia in 2019. This number will increase to 75 million in 2030 and 131.5 million by 2050.[1] As the aging process in general, neurodegenerative diseases (ND) such as Alzheimer´s disease (AD), Parkinson´s disease (PD), Huntington disease (HD) and many others have multifactorial etiologies.[2] The predominant symptoms of AD are impairment in cognition and profound memory loss. Neuronal loss occurs in the temporal and parietal lobe and in parts of the frontal cortex and cingulate gyrus. The characteristic pathological signs are senile plaques, formed from fibrillary deposits of ß-amyloid peptides (Aß) and neurofibrillary tangles which are mainly composed of the tau protein.[3] To date, the only drugs approved by the Food and Drugs Administration (FDA) for the treatment of AD patients are the acetylcholinesterase inhibitors (AChEIs) tacrine, donepezil, galantamine and rivastigmine, and the non-competitive N-methyl-D-aspartate (NMDA)-receptor antagonist memantine. The AChEIs exert their affect by preventing the enzymatic degradation of the neurotransmitter acetylcholine (ACh), resulting in increased ACh concentrations in the synaptic cleft and enhanced cholinergic transmission.[4] Memantine, however, protects neurons against NMDA receptor activation-mediated glutamate excitotoxicity and also inhibits τ-hyperphosphorylation and aggregation.[5,6] A new therapeutic approach, using a combination therapy of donepezil and memantine, has been reported to have significant beneficial effects on cognitive function, activities of daily living and behaviour.[7] Meanwhile, potent and more selective AChEIs (e.g. Huperzine) and NMDA-receptor antagonists (Dimebon) are being assessed. However, all current approaches for the treatment of AD provide only temporary symptomatic relief and do not inhibit or reverse the underlying disease mechanisms. This stresses the urgent need for disease-modifying drugs for AD – small, easily administered, well-tolerated, bioavailable compounds that cross the blood-brain barrier and have little or no adverse effects and contraindications. There are currently more than 50 compounds in various stages of clinical investigation for the treatment of AD (www.alzforum.org) including: statins[8], peroxisome proliferator-activated receptor-γ agonists[9] non-steroidal anti-inflammatory drugs[10] neurotrophic molecules and even metabolic or nutritional drinks e.g. Ketasyn, (Accera, Broomfield, CO, USA) or; Souvenaid (Danone Research-Centre for Specialized Nutrition, Palaiseau, France). In addition, there are many more candidate molecules at pre-clinical stages of development. Most of these pharmacological agents have been designed and/or developed based upon a notion that has been

dominating the AD field for the past two decades – **`the amyloid cascade hypothesis'**. This theory claims that the metabolism of the Aβ (both generation and clearance) is the main initiator of AD, which together with the downstream formation of the τ-protein aggregates, leads to neuronal and synaptic dysfunction and loss, microglial activation and neuronal death.[11, 12] Thus, most of the pharmacological agents under development target one or both of the principal cerebral proteins implicated in the pathogenesis of AD: τ and Aβ. However, pharmacological strategies directed at these targets in human studies have not yet proven to modify the disease. In particular, several investigational drugs that target Aβ have failed to show any correlation between a reduction in amyloid burden and improvement of cognitive functions in large-scale clinical trials. Such data might indicate that the `amyloid cascade hypothesis' of AD is not necessarily the correct one.[13]

This could be a hint that in the search of new drugs against ND, other considerations must be made. The problem is that we are used to think that NDs like AD, PD or HD are different clinical entities which target different brain regions and displaying separate pathology and symptoms. But on the genetic, the molecular and the cellular levels, we should realize that many players and patterns appear consistently in all NDs: an early vascular dysfunction, an accumulation of misfolded proteins, a selective sensitivity of specific neurons, and an activation of immune responses, to name but a few.[14] Accumulation of misfolded proteins in the brain is one of the most prominent pathological manifestations occurring in all NDs: extracellular oligomers of the peptide Aβ form the notorious plaques found in AD; the protein α-synuclein accumulates within dopaminergic neurons in PD and the misfolded huntingtin protein oligomerizes in HD. Further, there has been shown that the interaction of each of these proteins with metal ions like copper or iron is the initial step of oligomerization.[15]

1.1.1. The`metal hypothesis'

Bush and Tanzi supposed that age-related stochastic changes play essential roles in the biochemical events and reactions that cause Aß to accumulate in specific brain regions affected in AD. They first discovered in 1994 that Aß becomes amyloidogenic in reaction to stoichiometric amounts of Zn^{2+} and Cu^{2+}. In the subsequent years it has become clear that Aß is a metalloprotein.[16] Metals are implicated in interactions with the major protein components of NDs. But this is not merely due to increased (e.g., toxicological) levels of exposure, but rather due to a breakdown in the homeostatic mechanisms that compartmentalize and regulate these metals.[17] The equilibrium (concentrations, distribution, stability and bio-availability) of metal ions is a critical factor for the CNS, where metals are essential for development and maintenance of enzymatic activities,

mitochondrial function[18], myelination[19], neurotransmission[20], learning and memory.[21, 22] Due to their importance, cells have evolved complex machinery for controlling metal-ion homeostasis.

In the `metal hypothesis of AD´ it is assumed that age-related endogenous metal dyshomeostasis in the brain causes binding of redox-active metal ions (Cu^{2+} and Fe^{3+}) to Aβ.[17] This can lead to neurotoxicity as Cu^{2+} stabilizes the neurotoxic, oligomeric Aβ species[23, 24, 25] and induces the covalent di-tyrosine crosslink of Aβ.[26] The production of H_2O_2 by Aβ is dependent on the presence of those redox active metal ions. The generated peroxide may degrade forming highly reactive hydroxyl radicals (ROS) via Fenton chemistry or a Haber–Weiss reaction.[27] This induces oxidative damage of lipids, proteins and DNA, ultimately leading to synaptic and neuronal loss.[28]

PD is characterized by the accumulation of α-synuclein and other proteins into Lewy bodies, proteinaceous aggregates within dopaminergic neurons in the substantia nigra where has been shown that ionic levels are increased. Iron and copper can cause oxidative damage and play a role in α-synuclein oligomerization. Iron levels are currently used to monitor disease progression.[29, 30]

Based on the metal hypothesis, pharmacotherapeutics, which restore metal homeostasis, inhibit protein (Aß, α-synuclein, huntingtin)-metal interactions and/or inhibit metallated protein (Aß, α-synuclein, huntingtin)-catalysed oxidation should be developed.

Many preclinical and clinical studies showed that metal modifying complexes and chelators have therapeutic potential by reducing neurodegeneration and improving clinical symptoms.[31]

1.1.2. Metal chelators

Based on the metal hypothesis, the belief that chelation (the removal of metal ions from tissue) is the obvious intervention is a common misconception. Although there are several medical chelators, their approved use is confined to genuine situations of metal overexposure (e.g. Wilson's disease or lead toxicity) or rheumatoid arthritis. The risk of chelation therapy is that the removal of essential metal ions will lead to serious adverse effects (e.g. iron-deficiency anemia). Further, chelators cannot cross the blood brain barrier due to their hydrophilic nature. Thus, there is a need for the development of small molecules with more sophisticated properties, e.g. metal-protein attenuation compounds (MPACs) that serve as metal exchangers and ionophores.[17]

1.1.3. Metal-protein attenuating compounds (MPACs)

Small molecule MPACs target Aß oligomerization and Aß related generation of free radicals to employ MPACs that can prevent reactive metals from participating in potentially harmful redox chemistry.[17] MPACs have weak, reversible affinity towards metals, which enables them to compete with endogenous ligands for metal ions, target the harmful 'up stream' metal-protein reactions and restore normal metal levels in specific cellular compartments.[32] The first-generation of MPACs was based on clioquinol (CQ; 5-chloro-7-iodo-8-hydroxyquinoline). CQ is highly lipophilic, absorbed quickly and is able to cross the BBB.[33] CQ was initially shown to dissolve synthetic Aβ-Cu^{2+}/Zn^{2+} aggregates and amyloid deposits from post-mortem AD brain.[34] This finding prompted a study of the oral administration of CQ to Tg2576 mice over 9 weeks, which resulted in the normalization of cerebral ionic Cu^{2+} and Zn^{2+} levels, a reduction in H_2O_2 synthesis, and a significant decrease in cortical amyloid deposition by ∼49%, compared to control . Subsequently, CQ was shown to reverse Cu-suppressed, but not Zn-suppressed Aβ fibril formation.[35] However, other studies have suggested that CQ increases oxidative neurotoxicity.[17]

1.1.4. Metallothioneins (MTs)

MTs provide another possibility for the chelation of metal ions. MTs consist of a diverse superfamily of endogenous multipurpose proteins. With their heavy metal-binding capacity they are involved in the transport, homeostasis, and detoxification of heavy metals. While there is considerable variation in MTs within the animal kingdom, all MTs share similarities of being cysteine-rich, and showing a regulatory response to essential and non-essential metal exposure. In mammals there can be found 4 isoforms. MT1 and MT2 are found in all tissues of the body, throughout the brain and spinal cord, predominantly in astrocytes. But they can also be found in endothelial cells of blood vessels, meningeal cells, ependymal cells, epithelial cells and in neurons. MT3 is mainly expressed in neurons of the cerebellum, hippocampus and amygdala.[36] The primary mode of action for MTs is formation of metal-thiolate bonds and subsequent removal of the metal from the cytoplasm. Redox control of the metabolic influence of Zn has been proposed as core function of MT.[37, 38] The importance of MT in maintaining metal homeostasis is further demonstrated in studies involving exposure to heavy metals in MT1/2 knock out mice, which leads to metal toxicity, while MT1/2 overexpressing mice are relatively protected from heavy metal toxicity.[39] Under physiological conditions MTs are expressed at relatively low levels. But they can be induced by many types of stress, including heavy metal toxicity, alkylating agents, drug toxicity, bacterial endotoxin, viral infection, endoplasmic reticulum stress and oxidative stress.[38] Aging is another important inducer of

MTs as Miyazaki et al. showed that MT3 expression was increased in aged rat brain, but after treatments with lipopolysaccharids inducibility of MT3 as seen in the young brain, was lost in the aged adult brain. Finally, MT3 was shown to be markedly diminished in brains in AD, amyotrophic lateral sclerosis, PD, prion disease, brain trauma, brain ischemia, and psychiatric diseases.[40] Transgenic MT overexpression reduced proteotoxicity in different mouse models of PD[41] and overexpression of MT3 in HeLa cells decreased the polyQ toxicity which is associated with HD.[42] In AD research the metal-exchange process between Zn7MT3 and Aβ1-40 Cu^{2+} was recently elucidated, by which the production of ROS or the related cellular toxicity was quenched.[43] Many experiments showed that some drugs/compounds (e.g. apomorphin) exert neuron protective effects mainly via up-regulation of MT.[44, 45] Mice exhibited a 500-fold increase in MT1 and MT2 expression after zinc injections.[45] Zinc supplementation has been shown to promote healthy aging and improves lifespan in mice.[46] Other interventions shown to increase the lifespan affected the MT abundance too. In rodents for example, treatments with resveratrol increased the lifespan and MT protein levels.[47] Polyphenol compounds, including quercetin and tannic acid, have been identified as inducers of MT, both of which increased *C. elegans* lifespan.[48] With these studies highlighting the function of MT in neuroprotection and recovery of ND, it is evident that MT activation is a therapeutic target for the therapy of several neurodegenerative diseases.[49]

1.2. Screening for drugs restoring metal imbalance during ageing

In the search for new drugs affecting multiple targets in NDs, three main variables must be considered if we want to conduct a single definitive screening assay which is based on the metal hypothesis: first, the complex interplay between metals; second, the diversity of metal:protein interactions and third, the change in metal abundance with ageing and during the course of disease.[50] These requirements can be achieved by using a whole animal screening model. The most commonly used animal model in drug discovery is the mouse *Mus musculus* due to its genetic, physiological and anatomical similarities to the human system.[51] However, its use is limited by its costs in large scale therapeutic screenings. With the development of robotic and automated imaging small animal models like *C. elegans*, *D. melanogaster* and *D. rerio* became popular screening tools for drug discovery.[52] Genetic amenability, low cost and easy handling are combined by these organisms and their small size makes them perfect for high-throuput screenings. Further they can be used as models for drug screening even if the target is unknown. Human diseases can be artificially engineered by expressing the human disease gene in such models. With their use in drug screenings compounds can be identified which are able to suppress the disease phenotype. New molecular mechanisms and targets could be found

when hits are further investigated in models where single genes are knocked down using RNAi. Therefore, whole animal screenings with small organisms can bridge the gap between hit identification in cell based assays and the validation in mammalian models.[53] Regarding metal hypothesis *C. elegans* is widely used in studies of metal homeostasis, aging and ND. Results from age-related analyses of the metallome even indicated that aging of *C. elegans* is associated with the accumulation of iron, copper and manganese.[54]

1.2.1. C. elegans

„*To understand development, one would need the simplest differentiated organism, small enough to be handled in large numbers, easy to cultivate, with a short life cycle and amenable to genetic analysis*" reasoned Sydney Brenner when introducing the soil nematode *C. elegans* as a model organism in the 1960s. The adult nematode is about 1mm long. Their diet consists primarily of bacteria (*Escherichia coli*) OP50 and under optimal laboratory conditions the nematode has a 3-day growth-reproduction cycle. *C. elegans* has two sexes: hermaphrodite and male. An adult hermaphrodite produces close to 300 progenies by self-fertilization. Besides the ability to grow a homogenous population, the worm is small enough to be handled in large numbers and easily cultivated in the laboratory. Its optical transparency allows for in vivo detection of morphology, pathological changes and fluorescent or luminescent reporters.[55] Although there is a large evolutionary distance between *C. elegans* and humans, 41% of the *C. elegans* genes have human orthologs[56] and up to 75% of disease-related genes in humans have *C. elegans* orthologs.[57] There has been shown that *C. elegans* is an excellent model to evaluate toxicity. The ranking of compounds for toxicity in *C. elegans* closely matches that investigated in mammalian models.[58] Therefore, a major advantage of phenotypic drug screening in *C. elegans* is that toxic hits can be eliminated early on in the drug developmental stages. Another aspect is that besides the simple structure of the nervous system, consisting of only 302 neurons in an adult nematode there are remarkable similarities at the molecular and the cellular levels between nematodes and vertebrate neurons. For example, ion channels, receptors classic neurotransmitters (acetylcholine, glutamate, GABA, serotonin and dopamin), vesicular transporters and the neurotransmitter release machinery are similar in both structure and function.[59] This aspect and the ease of genetic manipulations, evident in the availability of mutants and by application of RNA interference (RNAi) knockdown, makes *C. elegans* valuable for screening drugs against age-associated ND, such as Alzheimer's disease[60,61], Huntington's disease[62], amyotrophic lateral sclerosis, Duchenne muscular dystrophy[63,64] and hereditary transthyretin amyloidosis.[65] These disease models can furhter be easily ordered at the Caenorhabditis Genetic Stock Center CGC (http://cbs.umn.edu/cgc/home). Many examples illustrate the power of *C. elegans* in screening for new drugs

against ND. Almost 70% of drugs listed in the DrugAge database have been tested in *C. elegans* (https://genomics.senescence.info/drugs/stats.php) compared to just 10% in mice. Some lead molecules originated from worm-based screening assays are in advanced stages of drug discovery.[66,67,68,69] This indicates the popularity of this model organism in neuroprotective drug screening.

1.2.2. Screening with *C. elegans*

Whole animal screening based on *C. elegans* can be treated as an innovative tool in the drug discovery process. For screenings with *C. elegans* two approaches can be used: First we can screen extracts/compounds for their ability to produce a particular phenotype like slow growth, lethality or lifespan extension to name just a few. With the second apporach we can screen compounds/extracts towards their ability to reverse an abnormal phenotype or a disease model to a wild type phenotype. A huge library of mutant strains which mimic a human disease is already available at the CGC. Further, when screening with *C. elegans*, there exist several screening protocols for culturing and dispensing fluorescent[70] and phenotypic[71] strains of *C. elegans* to search for extracts or constituents thereof, which are able to express a specific gene, turn a wild type into a phenotype or retract an abnormal phenotype into the wild type.[72] However, these screening models can be viewed as a complementary alternative to the cellular or in vitro screening assays. The real added advantage of this model can be found in their ability to reveal targets and pathways. The flexibility of *C. elegans* is a powerful tool for evaluating the mechanisms of action at very low cost.[73] With RNAi screens we are able to rapidly conduct the identification of potential targets. Identified targets may in turn feed the traditional HTS assays based on target binding function.[72]

1.2.3. Culturing of *C. elegans*

C. elegans was maintained with little alterations according to the protocol of Stiernagle.[74] Requested strains were obtained from the CGC (Caenorhabditis Gentic Stock Center). All worms were held at 16° C. They were growing on plates containing nematode growth medium (NGM) seeded with *Escherichia coli strain* OP50 (CGC) as food source. For a synchronized population eggs were isolated from gravid worms by a bleaching solution (12% NaClO and 10% 1 M NaOH), transferred to liquid media without bacteria and then incubated at 20°C overnight to obtain newly hatched animals or L1 larvae. To get L3 or L4 larvae, synchronized L1 larvae were transferred onto NGM plates containing *E. coli* OP50 and incubated for 48h at 16°C instead of 24h.[75]

For conducting a screenig assay we found out that it is best to use L3/L4 larvae. They have been added to 96-well plates in defined densities containing the compounds. In vivo fluorescence intensity was measured with a fluorescence

microplate reader - phenotypical changes with a stereo light microscope. For maintaining the synchronized population we exposed L4 larvae to 0,6mM FUdR.[76]

1.2.4. Lifespan extension in *C. elegans* wild type strain

Aging is the main risk factor for developing NDs.[77] Lifespan extension is a result of changes in signaling pathways and transcriptions factors of the aging process. A lot of these pathways are evolutionary conserved.[78] *C. elegans* shows many age-related changes similar to mammals like less motility, reduced feeding and accumulaton of insoluble proteins to name but a few.[79,80] Measurement of lifespan extension is the main experiment in the search for drugs against age-related disorders in humans.[81,82] To find out if an agent is able to prolong the lifespan measurements of survival during aging of a nematode in a population have been done.

The screening procedure was carried out as previously described with some alterations.[83] We incubated the assay plates at 25°C without any addition of antibiotics or antimycotics and all extracts/ compounds have been dissolved in 1 % DMSO.

In short, 10–20, age-synchronized L4 larvae of wild-type *C. elegans* (N2) were incubated in S-complete media with 0,6mM FUdR in wells of 96-well plates containing *E. coli* OP50 as feeding bacteria. The concentration of added test compounds/ extracts varied between 100µg/ml and 10µg/ml or 100µM and 10µM. Each agent was investigated in triplicates. Controls contained 1% DMSO. Living animals were scored by eye using a dissecting microscope at different time points. For easier scoring we induced movement by gentle vortexing or by light to the investigated well. For statistical analysis we converted the amount of living worms into a percentage using an excel file. Significance was evaluated using student´s t-test.

1.2.5. *C. elegans* models of human NDs

A diverse set of *C. elegans* models has been developed and characterized of different human NDs like AD[84], PD[85] or HD[86]. They have been developed by over-expressing human ND associated genes or by altering the expression of the orthologous worm genes. There has been shown a genotype to phenotype correlation between human NDs and the phenotypes of transgenic worm models.[87] Nevertheless, there are aspects of ND pathophysiology that cannot be modelled in worms. Brain inflammation and microglial activation play an important role in NDs, but there is no microglial equivalent among the 56 glia

cells of *C. elegans*.[88] However, *C. elegans* has proven to be a useful pharmacological model for screening potential neuroprotective compounds.

1.2.5.1. *C. elegans* models of AD

A diverse range of compounds have been shown to be neuroprotective when screened in strain CL2006, which constitutively expresses human Aß3-42. These include natrual products such as ginkolides[89] soya isoflavone glyctein[90], the green tea component epigallocatechin gallate[91,92] and coffee extract.[93] FDA-approved drugs such as tannic acid, bacitracin, rifampicin[94], thioflavin-T[95], reserpine[96] and fluoxetine. Further polyphenolic compounds like curcumin and ferulic acid .[97,98] Treatments with this compounds/extracts prolonged the lifespan and conferred cellular stress tolerance as a consequence of suppressing the Aß3-42 induced increase in toxic ROS levels and inhibiting Aß3-42 oligomerization and deposition.[99] Because strain CL2006 shows an age-dependent paralysis phenotype investigators introduced strain CL4176 where paralysis phenotype can be observed within 48h after temperature upshift because this strain expresses Aß1-43. The rapid paralysis from Aß1-42 is well suited for the assessment of drug effects.[100] This strain CL4176 has been engineered by Link et al. (2003) to inducibly express Aβ upon temperature upshift in muscle cells.[101] 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.[102] 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 increased the transcription of their transgene when shifted from the permissive temperature of 16°C to the non-permissive of 25°C.[103] As a consequence wild type movement is shown at the permissive temperature but paralyzation is induced within 48 hours after elevating the temperature. Treatments against Aβ toxicity in this worm model (e.g. exposure to Ginkgo biloba extracts[104] are able to change the rate of paralysis. There has been already shown a direct correlation between ß-amyloid expression and the rate of paralysis. When compared to the strain CL2006 and CL2120 the inducible Aβ expression does not lead to amyloid deposits and the paralysis phenotype is independent of amyloid deposition in strain CL4176.[105] So it is possible to measure the acute toxicity of induced Aβ expression resulting from the accumulation of soluble oligomeric Aβ. This is an advantage because several studies have shown that soluble oligomers are likely to be the toxic form of Aß.[106] Recently there has been reported that clioquinol and PBT2 rescued Aß1-42 toxicity in *C. elegans* body wall muscle cells and

glutamatergic neurons. PBT2 further improved cognition and reduced Aß in cerebrospinal fluid in a small Phase 2A trial in AD patients.[107]

With some alterations we used the screening protocol of Dostal *et al.* (2010)[94] for measuring the rate of paralysis in CL2659 which is the same strain as CL4176 but with the advantage that MT is GFP tagged. In contrast to Dostal *et al.* we performed this assay in 96 well plates instead on NGM agar plates and did not repeat the counting step all 2 hours but three times altogether to screen extracts/ compounds in a medium throughput manner.

Briefly, age-synchronized animals were grown on NGM plates with OP50 as food source at 16°C. After reaching the third larval stage worms were transferred to a 96-well plate containing OP50 and test compounds in triplicates. Aβ transgene expression in muscle cells was induced by temperature upshift from 16°C to 25°C and lasted until the end of the paralysis assay. At three time points (0, 24 and 48 hours) after temperature upshifts the number of paralyzed worms was scored under the microscope and recorded in an excel file to determine the percentage of non-paralyzed worms after 24 hours or 48 hours.

1.2.5.2. *C. elegans* models for PD

α-synuclein is a small, highly soluble, predominantly presynaptic cytoplasmic protein that has been implicated in PD and other synucleinopathies.[108] The normal physiological structure and function of α-synuclein is still not completely explored but it is assumed that it assists in compartmentalization, storage, and recycling of neurotransmitters.[109] Further, α-synuclein is able to regulate a variety of enzymes, probably increases the number of dopamine transporters, and has shown molecular chaperone activity, which is linked to neurotransmitter release.[110] A mutation in the a-synuclein gene SNCA was the first gene to be linked to the disease.[111] *C. elegans* do not have an α-synuclein homolog. To model α-synuclein aggregation and accumulation in vivo, researchers have generated transgenic *C. elegans* strains that express the human α-synuclein gene in body wall muscle cells and in neurons. In these models, increased or decreased fluorescence intensity associated with YFP linked to α-synuclein can be quantified to determine the levels of protein expression.[112] There has been reported that copper/iron ions accelerate intracellular α-synuclein aggregation and the release of mature fibrils to the extracellular space to induce further propagation. These aggregates were cytotoxic, causing increased ROS production, cell apoptosis, and shortened the lifespan of a *C. elegans* PD model overexpressing human α-synuclein. The study futher showed that the deleterious effects were ameliorated by chelators like triethylenetetramine and deferiprone which are also used in the clinic. Therefore, investigators assumed a new role for heavy metal ions, e.g. copper and iron, in the pathogenesis of PD through

accelerating prion-like propagation of α-synuclein fibrils.[113] A popular PD *C. elegans* strain modeling α-synuclein aggregation is NL5901 (unc-54p::human α-synuclein::YFP+unc-119; yellow fluorescent protein expression in the muscles). In these strain, the human α-synuclein gene is tagged to yellow fluorescent protein (YFP), which drives the expression of α-synuclein in the body wall muscle cells under the control of the unc-54 promoter.[114] The protein is marked with YFP allowing in vivo detection of compounds ability to reduce its accumulation by the multi well plate reader.

We established an assay procedure first.[115] Assays have been done in 96 well formats allowing testing of many compounds at different concentrations. Age synchronized L4 larvae of the strain NL5901 were transferred to 96 well plates containing media with *E. coli* food source, FudR for sterilization and test compounds in different concentrations in triplicates. Changes in intensity of α-synuclein coupled GFP fluorescence has been measured by a multiwell plate reader daily until the 6th day to identify hits that are able to reduce fluorescence which in turn can be interpreted as reduced α -synuclein accumulation.[53]

1.2.5.3. Manipulation of MT in *C. elegans*

C. elegans remains a widely used animal model to identify genes that modify neurodegeneration in vivo. This can be shown by genetic screens performed on worm models which have identified a wide variety of conserved genes that can suppress or increase disease progression and are thus potential therapeutic drug targets.[116] Human MT3 is a regulator of metal homeostasis and has been shown to be downregulated at least 30% in AD brain. Treatment of mice with Zn_7MT3 has been shown to significantly ameliorate cognitive deficits, regulate metal homeostasis, abolish Aß plaque load and reduce oxidative stress.117 Miyazaki and colleagues showed that the expression of MT3 and its mRNA was up-regulated in the healthy aged rat brain. Lipopolysaccharide treatment induced expression of MT3 and its mRNA only in young but not in aged rat brain regions. Therefore investigators assumed that the reduced inducibility of MT3 against oxidative stress with aging is responsible for the vulnerability and neurodegeneration of aged brain tissue.[40] Activation of failed MT induction in age-dependent NDs like AD or PD could be a promising novel therapeutic target.[118,119] This has been shown in studies where treatments with compounds known to induce MT release were active in models of AD and PD; e.g. progesterone[120], quercetin[121], dexamethasone[122] and apomorphine[123]. In *C. elegans*, there are two genes, mtl-1 and mtl-2, which encodes the MTs of *C. elegans*.[124] They have been shown to be responsive to metals, and they function like their mammalian counterparts.[125] In our study we used two different transgenic *C. elegans* models of AD where changes of GFP tagged MT2 can be monitored over time.

CL2659: dvIs770 [myo-3::Abeta 1-42 wt::3' UTR(long) + mtl-2::GFP]. This strain is equivalent to CL4176 but with the advantage that MT2 is GFP tagged and can be monitored by the fluorescent multiwell plate reader during the paralysis assay which has been described above (2.5.1).

CL2120: dvIs14 [(pCL12) unc-54::beta 1-42 + (pCL26) mtl-2::GFP]. Contrary to strain CL2659 where the expression of $A\beta_{1-43}$ can be induced resulting in soluble Aß oligomers, constitutive expression of $A\beta_{3-43}$ in strain CL2120 leads into plaque formation during ageing which can be stained with thioflavin. In both strains MT is gfp tagged so that compounds influence on MT induction can be monitored.[100]

The MT assay has been established in this thesis. Assays have been done in 96 well formats allowing testing of compounds in a medium throughput manner.

Age synchronized worms in the L4 stage have been incubated with *E. coli* food source, FudR for sterilization and test compounds in triplicates at 25°C for 9 days. Fluorescence intensity has been measured by a multiwell plate reader during ageing but predominantly at d0, d3 and d9 to determine the breakdown of MT induction.[53] Depletion of food source has been monitored by measuring the OD_{600}. Statistical significance has been assessed by student´s t-test.

1.2.5.4. Suppression of MT using RNAi

Since its discovery, RNA interference (RNAi) has become an important tool for *C. elegans* research and the scientific community.[126] RNAi is a form of gene silencing induced by double stranded RNA (dsRNA) that is processed into short interfering RNAs (siRNAs). RNAi refers to silencing genes by the use of dsRNA at transcriptional and post-transcriptional levels in a sequence-specific manner.[127] RNAi is systemic in worms and plants, but not in other organisms. Notable, RNAi is heritable in *C. elegans* RNAi because the effect can be maintained for three or more generations.[128,129] To date, this phenomenon has not been noticed in other organisms.[127] In *C. elegans*, RNAi can be generated by applying dsRNA by microinjection, feeding, and soaking. In the microinjection and soaking protocols, in vitro formulations of dsRNA are delivered mechanically with a needle to inject dsRNA directly into the body or passively by soaking worms in dsRNA solution.[130] The protocol of the feeding procedure is simple: *E. coli* that had been engineered to produce dsRNA of interest is fed to the worms of any stage. Different from other dsRNA delivery protocols, here L1s can be used instead of L4s. With this method a large number of animals can be treated at once on agar plates or in liquid culture. A research group cloned about 18,000 genes covering over 85% of the *C. elegans* genome into a dsRNA producing vector, and generated a feeding RNAi library of bacterial strains which fortunately is now commercially available.[131]

For the RNA interference by feeding we used the protocol of Conte *et al*. (2015).[126] Clones carried in *E. coli* HT115(DE3) were purchased from GE Dharmacon (www.horizondiscovery.com). Clone Id for MT 1 is: K11G9.6 ORF and clone Id for MT 2 is T08G5.10 ORF. Synchronized L1 larvae of the strain CL2659 were transferred to agar plates inoculated with the *E. coli* strain carrying the double stranded RNA. L3 larvae were harvested and paralysis assay was performed as usual (2.5.1).

1.3. Traditional and Complementary Medicine

Today, drug discovery is facing many challenges because a lot of drugs are failing due to toxicity and safety reasons. Currently, the search for new drugs is limited by an innovation deficit. Therefore traditional knowledge becomes a valuable resource for new drug leads. Traditional and complementary medical systems have described thousands of drugs and formulations.[132]

1.3.1. Traditional Tibetan Medicine (TTM)

TTM is one of the oldest known medical systems with nearly 2000-year-old legacy of holistic and naturopathic approaches. The Tibetan system of medicine postulates that the cause of all human sufferings are three mental poisons: attachment, aversion and ignorance. The aim of TTM is to treat the root cause of the disease instead of symptomatic relief. All life forms consist of the five basic cosmic elements, namely earth, water, fire, air and space. Diseases develop because of a manifestation of disequilibrium of these elements.[133] All the elements are inherent in each cell and tissue of our body. The elements are dynamic forces characterized by their inherent energetic functions rather than their actual state. The three principle energies – wind (rlung), phlegm (badkan) and bile (mkhrispa) – are directly linked with the five cosmic elements.[134]

With this holiststic point of view it is clear that the concept in Tibetan medicine is the multitarget drug design combined with a network–dependent approach to combat multifactorial diseases like cancer or ND. To control complex disease systems simultaneous disruption of multiple targets located in distant cellular networks should be considered.[135]

The main herbs in Tibetan medicines which support the detoxification of the body are *Terminalia chebula* Retz. (Aru ra), *Terminalia belerica* Gaertn (Baru ra) and *Phyllantus emblica* Kurz (Kyuru ra). The combination of these three Myrobalans, well known as Triphala churna, is a constituent of about 1500 Ayurveda formulations. The importance of these plants is shown by the prominent Medicinal Buddha who is an enlightened being and protects humans from sickness and assists to eliminate all three mental poisons. He holds a blooming Myrobalan twig in his left hand which shows a healing touch.[133] In the Tibetan pharmacopoeia Myrobalan is the only herbal remedy which is able to heal all three mental poisons-related diseases, especially those of metabolism, cancer and degenerative changes. Triphala combats degenerative and metabolic disorders possibly through lipid peroxide inhibition and free radical scavenging. Some studies have already shown its beneficial effect to suppress neurodegeneration in fly models of HD and AD.[136]

1.3.2. Iranian Traditional Medicine (ITM)

Many cognition enhancing drugs are used in the Iranian Traditional Medicine too. Iran is among those countries with a long and rich history in traditional medicine, as shown by 'The Canon of Medicine' of Avicenna or 'The Continents' by Rhazes.[137] There are several authoritative written documents of ITM such as Al-Qanun fi-Tibb, Al-Hawi fi-Tibb, Tuhfat al-Mu'minin, Makhzan al-adviyah, in which a number of chapters is related to cognitive disorders. In the 10th century 34 antidementia drugs have been described whereas this digit reached 130 at the end of the 18th century (440% growth).[137] Here we can see the dynamic feature of ITM in drug discovery. The most prominent formulation is a herbal drug mixture named Anaqardia. Anarqadia has already been used in the Hippocratic era to increase intelligence because former physicians did not classify dementia as a disease. They thought that the loss of intelligence is just a normal consequence of aging.[138] The name Anaqardia has been assigned to the main drug of the formulation, namely *Semecarpus anacardium* L.f., because of the similarity of its fruits to the heart (*cardia*). The other components are: *Terminalia chebula, T. belerica, Emblica officinalis* Kurz, *Nigella sativa* (L.), *Bambusa bambos* Voss, *Elettaria cardamomum* Marton, *Cyperus rotundus* (L.), *Piper nigrum* L., *P. longum* (L.), *Zingiber officinale* Roscoe, and *Pimpinella anisetuum* (L.). For the formulation the herbs have to be powdered and mixed with honey. Searching the novel pharmacological activities of Anaqardia constituents presented amazing results: eight herbs of 12 have shown an acetylcholinesterase inhibitory effect; six have been reported with anti-inflammatory activities; and five with antioxidant activities; one showed an N-methyl-D-aspartate (NMDA) antagonistic effect, and one an anti-Aß production activity. In sum, this formulation seems to be able to attack multiple pathways involved in AD underpinning the importance of the multitarget approach.[138]

1.3.3. Impact of natural products on ND

Recently, a great number of medicinal plants have been tested for their therapeutic properties, and promising constituents for the treatment of ND were isolated from the respective crude extracts. These compounds belong to the groups of polyphenols, alkaloids and terpenes, among others, that are produced by plants as secondary metabolites. The neuroprotective effects of polyphenols have been described in several models of ND, and involve mainly signaling pathways mediators, modulation of neurotoxicity via ionotropic glutamate receptors, anti-amyloidogenic and anti-inflammatory effects. E.g., Acticoa, a cocoa bean-derived polyphenol extract improved cognitive performance, increased life expectancy and preserved free dopamine levels in urine of Wistar rats after daily administration.[139] Vincamine, a monoterpenoid indole alkaloid purified from *Vinca minor* L., has shown antioxidant activity, increase of cerebral

blood flow, utilization of glucose and oxygen in neural tissue and the rise of dopamine, serotonin, and noradrenalin levels. Further, it reduced about 50% of the brain iron.[140] Cryptotanshinone is an active component of *Salvia miltiorrhiza* Bunge with anti-inflammatory, antioxidant, and anti-apoptotic properties. This compound crossed the blood brain barrier and decreased cognitive deficits in male IRC mice with scopolamine-induced cognitive impairments. It reduced Aß aggregation in brain tissue and improved spatial learning and memory in APP/PS1 transgenic mice by promoting APP metabolism via a-secretase pathway.[141] Silymarin also showed anti-amyloid properties in vitro which is attributable to the blockade of its aggregation.[142] An extract from *Centella asiatica* (L.) Urban decreased amyloid deposition and plaque formation in a dementia mouse model.[143] Nobiletin, a flavonoid purified from *Citrus depressa* Hayata, prevented memory loss in APP695 mice and Aß treated rats.[144] Other compounds that can prevent Aß aggregation by inhibition of the metabolic pathway that generate Aß plaques are berberine, palmatine, jateorrhizine, alkaloids isolated from *Coptis chinensis* Franch rhizome. These compounds also exhibit AchE inhibiting properties and antidepressant effects and enhance cognitive impairments. Coptisine and groenlandicine present moderate total ROS inhibitory activities.[145] An extract from *Bacopa monnieri* (L.) Pennell prevented neuronal death by the inhibition of AChE activity in primary cortical culture pretreated with Aß.[146] Furthermore, volunteers presented enhanced memory. The use of *Panax ginseng* C. A. Mey. was evaluated in AD patients thereby showing a significant improvement in the AD assessment scale and the clinical dementia rating scale compared to control patients. [147,148]

1.3.4. Extract preparation

A detailed extraction procedure is provided in Paper 1. Small-scale dichloromethane (CH_2Cl_2) and methanol (MeOH) extracts have been generated.

We grounded and defatted dried natural starting materials two times with n-hexane before extracting with CH_2Cl_2 and MeOH. Both extracts were combined again. To deplete tannins we performed a solid phase extraction. These small-scale extracts were added to Eppendorf tubes. After a drying step in a desiccator aliquots of the prepared extract were dissolved in DMSO to a concentration of 10 mg/mL and stored at −20 °C.

2. Aims

The overall objective of this thesis was to identify extracts derived from traditionally used herbal drugs and if applicable also their constituents, which are able to prolong lifespan and target metal dishomeostasis in *C. elegans* models of NDs.

Specific aims of the individual studies

Paper 1: Establishment of a phenotypic medium-throughput lifespan and obesity assay with *C. elegans* and screening of extracts traditionally used in medicine against age related and metabolic disorders. To integrate the extracts to our liquid-based *C. elegans* screening platform, we aimed to establish a standardized small-scale extraction procedure with an enriched yield of drug-like secondary metabolites and depleted metabolites prone to assay-interferences.

Paper 2: The first aim evaluated the MT induction for its potential use as a novel drug target in *C. elegans* models of NDs. Secondly, we aimed to establish a novel medium-throughput screening assay to search for extracts and compounds increasing MT levels and reducing proteotoxicity of the major proteins correlated with the specific disease in *C. elegans* models.

Unpublished data: Screening of extracts traditionally used in the Iranian and Tibetian medicine in *C. elegans* models of AD and PD and evaluation of their effect on MT induction.

Paper 3: Investigation of the main azepine-indole alkaloids of *Psychotria nemorosa*, namely nemorosine A and fargesine isolated from the *P. nemorosa* alkaloid fraction in the *C. elegans* based AD, PD and MT assay. Further, exploration of their pharmacological profile and their putative mechanisms with respect to NDs by implementation of a similarity screening of the ChEMBL database has been described.

Paper 4: An outlook for future research involving MT induction as a novel target for further NDs is given in this paper.

3. Results and Discussion

3.1. Paper 1: A robust and miniaturized screening platform to study natural products affecting metabolism and survival in *Caenorhabditis elegans*

C. elegans has been proven to be a perfect model in several fields of biology and pharmaceutical screenings because important pathways are highly conserved between those nematodes and humans. With such a simplified model it is possible to enlighten complex interplays of different diseases where the causes are unknown like metabolic or neurodegenerative disorders. Therefore it would be a good idea to screen compounds against those disorders in a phenotypic model because hits can be further investigated in cell based assays to identify the corresponding pathway. In the first paper we describe the establishment of a robust phenotypic assay based on *C. elegans* to screen small amounts of complex natural products against fat-accumulation and age-related disorders. Evaluation of fat accumulation has been done by Nile red staining and pathways of age related disorders can be matched by searching for extracts which are able to prolong survival time in the lifespan assay. With both assays we can detect extracts which promote “healthy aging”. For the lifespan assay we observed single worms of a population throughout the whole lifespan. We used th wild type strain N2 and incubated them at 25°C in liquid media containing the compounds an the bacterial food source. We counted living worms three times a week until 50% of the worms were dead. By this way we introduced the term DT50 which means the time span by which 50% of worms of a population has been died.

To conduct the fat accumulation assay in a medium through put manner we added the dye Nile red to the liquid medium where worms have been cultivated. Worms were able to ingest the dye together with food and test compounds/extracts which accumulated in lysome related organelles and fluorescence has been detected because the signal correlated with the lipid content. SS104 served as an appropriate strain for this assay because it is sterile when temperature reaches up to 25°C.

To avoid the well-known low solubility of natural products we used DMSO as a solvent. It is known that DMSO affects several pathways of *C. elegans.* Therefore we evaluated different DMSO concentrations in both assays next and found out that 1% DMSO can be used without an effect on the outcome.

1% DMSO served as vehicle control. To find an appropriate positive control we screened several drugs which are known to prolong survival time or to reduce Nile red fluorescence. The results showed that reserpine can be used for lifespan extension and all three test compounds namely olanzapine, fluoxetine and 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) can be used for the Nile red assays.

The next step was to screen 24 extracts in the established assays. The starting material has been chosen according their traditional use in medicine. For testing small amounts of extracts without fearing to get false-positive or false-negative results we introduced a standardized small scale extraction procedure.
As a result 9 of the 24 investigated extracts were positive in the lifespan assay and 9 were active in the Nile red assay. Further 2 were active in both the lifespan and the Nile red assay, namely Chaga and *G. jasminoides* fruits.

My contribution in this paper: I did all preliminary tests for this study and optimized the liquid based cultivation techniques. Regarding the survival assays I evaluated the impact of different DMSO concentrations in the samples and tested appropriate positive controls.

3.2. Paper 2: Prolongation of Metallothionein Induction combats Aß and α-synuclein toxicity in aged transgenic *Caenorhabditis elegans*

With this paper which has been published in Scientific Reports we first gave a discussion regarding the similarities of the different NDs. We mentioned that the amyloid cascade hypothesis failed to generate effective therapies which could be able to ameliorate the cause of the diseases. We further hypothesized that the right target for several NDs could be the restoration of the metal dyshomeostasis which occurred in NDs. A very interesting fact is that the breakdown of the homeostasis goes along with the aging process because NDs are age dependent disorders. Although chelation of metals in transgenic *C. elegans* reduced the proteotoxic burden of Aß, there has been shown that chelation is not the optimal solution because metals fulfill important functions and so there could be the problem of inducing a deficit. Therefore we hypothesized that it would be better to harmonize the metal imbalance by an indirect exertion of influence. We studied the endogenous pathway of detoxification which is performed mainly by multipurpose proteins named MTs. The metal hypothesis which has been stated by Bush and Tanzi in 2008 revealed that metals react with Aß and other proteins of NDs to form the toxic oligomers. The protein MT could disturb this process but many studies have shown that MTs are reduced in several NDs. In another study from Miyazaki there has been further shown that MTs are induced in healthy aged brain but when LPS has been administered, which means that there is a high level of ROS, MT induction has been disturbed in the aged brain tissue. To find out if this is also the fact in *C. elegans*, we followed MT induction in nematodes during ageing too. We could show that in the healthy worms there is a slightly increase in MT with age whereas in Aß expressing worms there is an intense induction first followed by a breakdown after a few days. Therefore we assumed that induction of MT could be an innovative therapeutic target in the treatment of NDs. Based on these outcomes we established a robust medium throughput assay based on the *C. elegans* strain CL2120, to identify natural

products which are able to prolong MT induction. In this strain MT is GFP tagged and can be detected by the fluorescence multiwall plate reader or by fluorescence microscopy. For a strong signal worm concentration should be between 30 and 50 worms per well. Measurement have been done when starting the assay, when highest level of MT has been achieved and after breakdown of MT induction. To guarantee optimal feeding of the worms throughout the whole experiment we measured optical density at 600 nm.

For the assay evaluation procedure, we hypothesized that the efficacy of compounds in prolonging the time of MT induction in strains CL2120 or CL2659 will correlate with a reduction in Aß and α-synuclein toxicity burden. Therefor we screened compounds known do induce MT which are ZnSO4, apomorphin, dexamethasone and quercetin. We could show that all compounds were able to prolong MT induction, extended the lifespan and reduced proteotoxicity in a AD and PD assays.

After assay evaluation, we investigated if novel neuroactive lead compounds like thioflavin T, clioquinol, sesamin and emodin decrease proteotoxicity of Aß and α-synuclein and extend the lifespan by prolonging MT induction in *C. elegans*. We found out that all compounds but sesamin induced MT and were active in the AD and PD assay. All but clioquinol and sesamin further prolonged the lifespan.

For target evaluation we knocked down MT1 and MT2 in strain CL2659. Bioactivity of emodin in the paralysis assay has been lost when MT 2 has been knocked down with RNA interference and has been reduced when MT1 has been knocked down. However, bioactivity of quercetin in the paralysis assay with CL2659 has not been lost because we assume that MT induction is not the solely mode of action of quercetin in AD and PD transgenic nematodes.

We studied the gene expression of metallothionein-1 (mt-1) and metallothionein -2 (mt-2) in emodin and clioquinol treated CL2120 strain and found out that the increasing MT2 level in CL2120 strain must be a result of post-transcriptional regulation.

This study shows that MT has beneficial effects on aging and in NDs. It underpins the hypothesis that the age dependent brake down of metal homeostasis can be balanced by prolonging time of MT induction. The establishment of a novel screening assay provides a tool to examine extracts and compounds towards this activity.

My contribution in this paper: In this paper I planned, performed and analysed all experiments but the RT-PCR studies and wrote the main part of the manuscript.

3.3. Unpublished data: Screening of neuroprotective extracts traditionally used in the Iranian and the Tibetan medicine

We screened dichloromethane (DCM), methanol (M) and mixed DCM/M extracts of the Iranian Traditional Medicine (ITM) (Tab.1) and the traditional Tibetan Medicine (TTM) (Tab.2) supplied by the Department of Pharmaceutical Sciences, Division of Pharmacognosy, University of Vienna, Austria, in collaboration with ao Prof. Dr. Liselotte Krenn and Hamid – Reza Adhami from the Department of Pharmacognosy, Teheran University of Medical Sciences for testing in *C. elegans* strains NL5901, CL2120 and CL2659.

Tab.1: Extracts of the ITM were screened in *C. elegans* based assays using transgenic strains CL2120, CL2659 and NL5901

ITM Extracts

Int. No.	Conc.	Scientific name	Part used	Voucher number	CL2120		CL2659_MT		CL2659_Paralysis		NL5901	
	µg/ml				DCM extract	MeOH extract	DCM extract	MeOH extract	DCM extract	MeOH extract	DCM extract	MeOH extract
2	100	*Acorus calamus L. (Acoraceae)*	Rhizome	PMP-201	+	+	+	-	+	-	+	-
2	50	*Acorus calamus L. (Acoraceae)*	Rhizome	PMP-205	+	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
3	100	*Terminalia chebula Retz. (Combretaceae)*	Fruit (unmatured)	PMP-612	+	+	-	-	-	-	-	-
3	50	*Terminalia chebula Retz. (Combretaceae)*	Fruit (unmatured)	PMP-616	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
5	100	*Terminalia chebula Retz. (Combrectaceae)*	Fruit (matured)	PMP-613	+	-	+	-	+	+	-	-
5	50	*Terminalia chebula Retz. (Combrectaceae)*	Fruit (matured)	PMP-617	-		n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
9	100	*Terminalia bellirica (Gaertn.) Roxbb. (Combretaceae)*	Fruit	PMP-611	+	-	+	-	++	-	-	+
9	50	*Terminalia bellirica (Gaertn.) Roxbb. (Combretaceae)*	Fruit	PMP-615	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
10	100	*Piper cubeba L. (Piperaceae)*	Fruit	PMP-607	+	+	+	+	+	+	-	+
10	50	*Piper cubeba L. (Piperaceae)*	Fruit	PMP-611	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
6	100	*Piper longum L. (Piperaceae)*	Fruit	PMP608	n.t.	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
6	50	*Piper longum L. (Piperaceae)*	Fruit	PMP613	-	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
11	100	*Bunium persicum (Boiss.) B. Fedtsch (Apiaceae)*	Fruit	PMP-602	+	+	+	+	-	-	-	-
11	50	*Bunium persicum (Boiss.) B. Fedtsch (Apiaceae)*	Fruit	PMP-603	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
13	100	*Ferula Assa foetida L. (Apiaceae)*	Oleo gum resin	PMP-806	n.t.	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
13	50	*Ferula Assa foetida L. (Apiaceae)*	Oleo gum resin	PMP-811	n.t.	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
14	100	*Nardostachys jatamansi DC. (Valerianaceae)*	Hypocotyl	PMP-606	+	-	+	-	+	+	+	-
14	50	*Nardostachys jatamansi DC. (Valerianaceae)*	Hypocotyl	PMP-609	+	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
15	100	*Cinnamomum zeylanicumstem Nees (Lauraceae)*	Stem bark	PMP-901	+	-	+	-	-	+	-	-
15	50	*Cinnamomum zeylanicumstem Nees (Lauraceae)*	Stem bark	PMP-902	+	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
16	100	*Emblica officinalis Gaertn.) (Euphorbiaceae)*	Fruit	PMP-606	+	-	-	-	-	+	-	-
16	50	*Emblica officinalis Gaertn.) (Euphorbiaceae)*	Fruit	PMP-607	+	-	-	-	-	+	-	-
18	100	*Iris germanica L. (Iridaceae)*	Rhizome	PMP-204	-	-	-	-	-	-	-	-
18	50	*Iris germanica L. (Iridaceae)*	Rhizome	PMP-205	-	-	-	-	-	-	-	-
19	100	*Zingiber officinalis R*	Rhizome	PMP-206	n.t.	-	n.t.	-	n.t.	+	n.t.	+
19	50	*Zingiber officinalis R*	Rhizome	PMP-207	n.t.	-	n.t.	-		-	n.t.	+
20	100	*Asarum europaeum L. (Aristolochiaceae)*	Rhizome	PMP-202	+	n.t.	-	n.t.	+	n.t.	+	n.t.
20	50	*Asarum europaeum L. (Aristolochiaceae)*	Rhizome	PMP-203	+	n.t.	-	n.t.	+		+	n.t.
22	100	*Piper nigrum L. (Piperaceae)*	Seed (peeled)	PMP-704	+	-	-	-	n.t.	-	-	-
22	50	*Piper nigrum L. (Piperaceae)*	Seed (peeled)	PMP-705	+	-	+	-	n.t.	-	-	-
23	100	*Eugenia caryophyllata Thunb. (Myrtaceae)*	Bud	PMP-501	+	-	-	-	+	n.t.	-	-
23	50	*Eugenia caryophyllata Thunb. (Myrtaceae)*	Bud	PMP-502	+	+	-	-	+	n.t.	+	-
24	100	*Nigells sativa L. (Ranunuculaceae)*	Seed	PMP-702	n.t.	-	n.t.	-	n.t.	-	n.t.	-
24	50	*Nigells sativa L. (Ranunuculaceae)*	Seed	PMP-703	n.t.	-	n.t.	-	n.t.	-	n.t.	-
27	100	*Semercarpus anacardium L. (Anacardiaceae)*	Fruit	PMP-609	-	-	-	-	n.t.	-	-	+
27	50	*Semercarpus anacardium L. (Anacardiaceae)*	Fruit	PMP-610	-	-	-	-	n.t.	+	-	+
28	100	*Semercarpus anacardium L. (Anacardiaceae)*	Fruit resin	PMP-610	-	-	-	n.t.	-	-	+	n.t.
28	50	*Semercarpus anacardium L. (Anacardiaceae)*	Fruit resin	PMP-611	-	-	-	n.t.	+	n.t.	+	n.t.
29	100	*Pistacia lentiscus (Anacardiaceae)*	Oleo gum-resin	PMP-808	-	-	-	-	-	-	-	-
29	50	*Pistacia lentiscus (Anacardiaceae)*	Oleo gum-resin	PMP-809	-	-	-	-	-	-	-	-

+….p ≤ 0,05; -….p ≥ 0,05 not active (student´s t-test), n.t.….not tested

Tab. 2: Extracts of the TTM were screened in *C. elegans* based assays using transgenic strains CL2120, CL2659 and NL5901.

TTM-Extract								
Int. No.	**Conc. (µg/ml)**	**Scientific name**	**Part used**	**Voucher number**	**CL2120**	**CL2659_ MT**	**CL2659_ Paralysis**	**NL5901**
1	100	*Eriobotrya japonica* (Thumb.) Lindl	Leaf	ErijapFDMp	-	-	-	+
1	50	*Eriobotrya japonica* (Thumb.) Lindl	Leaf	ErijapFDMp	-	-	-	-
2	100	*Sida cordifolia* L.	Herb	SidcorHDMp	+	-	+	-
2	50	*Sida cordifolia* L.	Herb	SidcorHDMp	+	-	+	+
3	100	*Gloeophyllum odoratum* (Wulfen) Imazeki	Seed	GloodoOE	-	+	-	-
3	50	*Gloeophyllum odoratum* (Wulfen) Imazeki	Seed	GloodoOE	-	+	-	-
4	100	*Potentilla aurea* L.	Herb	PotaurHDMp	+	-	+	-
4	50	*Potentilla aurea* L.	Herb	PotaurHDMp	+	+	-	-
5	100	*Cannabis sativa* L.	Flower	CansatBCO2	n.t.	n.t.	n.t.	n.t.
5	50	*Cannabis sativa* L.	Flower	CansatBCO3	n.t.	n.t.	n.t.	n.t.
6	100	*Humulus lupulus* L.	Flower	HumlupFD	+	-	+	+
6	50	*Humulus lupulus* L.	Flower	HumlupFD	-	-	+	+
7	100	*Euphrasia officinalis* L.	Herb	EupoffHDMp	+	-	+	-
7	50	*Euphrasia officinalis* L.	Herb	EupoffHDMp	+	+	+	-
8	100	*Azadirachta indica* A. Juss	Fruit	AzaindODMp	+	-	+	+
8	50	*Azadirachta indica* A. Juss	Fruit	AzaindODMp	-	+	-	-
9	100	*Polygonum aviculare* L.	Herb	PolaviHDMp	+	-	-	-
9	50	*Polygonum aviculare* L.	Herb	PolaviHDMp	-	-	-	-
10	100	*Plantago lanceolata* L.	Herb	PlalanHDMp	+	-	+	+
10	50	*Plantago lanceolata* L.	Herb	PlalanHDMp	+	-	+	-
11	100	*Aquilegia vulgaris* L.	Herb	AquvulHDMp	+	-	+	-
11	50	*Aquilegia vulgaris* L.	Herb	AquvulHDMp	+	+	+	-
12	100	*Lactuca sativa* L.	Leaf	LacsatFDMp	+	-	+	-
12	50	*Lactuca sativa* L.	Leaf	LacsatFDMp	+	-	+	-
13	100	*Saussurea costus* (Falc.) Lipsch	Rhizome	SaucosRDMp	+	-	+	-
13	50	*Saussurea costus* (Falc.) Lipsch	Rhizome	SaucosRDMp	+	-	+	-
14	100	*Pimenta dioica* (L.) Merr	Fruit	PimdioODMp	-	-	-	-
14	50	*Pimenta dioica* (L.) Merr	Fruit	PimdioODMp	+	-	+	-
15	100	*Pterocarpus santalinus* L.F.	Wood	PtesanXDMp	-	-	+	-
15	50	*Pterocarpus santalinus* L.F.	Wood	PtesanXDMp	-	-	+	-
16	100	*Piptoporus betulinus* (Bull.) P. Karst.	Fruititing body	PipbetODM	-	-	-	-
16	50	*Piptoporus betulinus* (Bull.) P. Karst.	Fruititing body	PipbetODM	-	-	-	-
17	100	*Fomitopsis pinicola* (Sw. : Fr.) P. Karst.	Fruititing body	FompinODM	+	-	-	-
17	50	*Fomitopsis pinicola* (Sw. : Fr.) P. Karst.	Fruititing body	FompinODM	+	-	+	-
18	100	*Inonotus obliquus* (Ach. ex Persoon:Fr.) Pilát	Fruititing body	InooblODM	-	-	+	-
18	50	*Inonotus obliquus* (Ach. ex Persoon:Fr.) Pilát	Fruititing body	InooblODM	-	-	+	-
19	100	*Terminalia chebulia* Retz	Fruit	TercheODMp	-	n.t.	n.t.	-
19	50	*Terminalia chebulia* Retz	Fruit	TercheODMp	-	n.t.	n.t.	-
20	100	*Ganoderma lucidum* (Curtis) P. Karst	Fruititing body	GanlucDMp	+	-	-	-
20	50	*Ganoderma lucidum* (Curtis) P. Karst	Fruititing body	GanlucDMp	+	+	-	-
21	100	*Calendula officinalis* L.	Flower	CaloffBDMp	+	-	-	-
21	50	*Calendula officinalis* L.	Flower	CaloffBDMp	-	+	+	+
22	100	*Valeriana officinalis* L.	Root	ValoffRDMp	+	-	+	-
22	50	*Valeriana officinalis* L.	Root	ValoffRDMp	+	+	+	-
23	100	*Lichen islandicus* (L.) Ach.	Lichen	LicislODMp	+	-	-	-
23	50	*Lichen islandicus* (L.) Ach.	Lichen	LicislODMp	+	-	-	-
24	100	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Flower	SyzaroODMp	+	+	+	+
24	50	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Flower	SyzaroODMp	+	+	-	+
25	100	*Drynaria fortunei* (Kunze ex Mett.) J.Sm	Rhizome	DryforRDMp	-	-	+	-
25	50	*Drynaria fortunei* (Kunze ex Mett.) J.Sm	Rhizome	DryforRDMp	-	-	+	-
26	100	*Cynanchum paniculatum* (Bunge) Kitag. Ex H. Hara	Rhizome	CynpanRDMp	-	-	-	+
26	50	*Cynanchum paniculatum* (Bunge) Kitag. Ex H. Hara	Rhizome	CynpanRDMp	-	-	-	-
27	100	*Cynanchum stautonii* (Decne.) Schltr. ex H.Lév	Rhizome	CynstaRDMp	-	-	-	-
27	50	*Cynanchum stautonii* (Decne.) Schltr. ex H.Lév	Rhizome	CynstaRDMp	-	+	-	-
28	100	*Andrographis paniculata* (Burm.f.) Nees	Herb	AndpanHDMp	-	-	-	-
28	50	*Andrographis paniculata* (Burm.f.) Nees	Herb	AndpanHDMp	-	-	-	-
29	100	*Gardenia jasminoides* J.Ellis	Fruit	GarjasODMp	-	-	-	+
29	50	*Gardenia jasminoides* J.Ellis	Fruit	GarjasODMp	-	-	-	-
30	100	*Psychotira nemorosa* Gardner *(alkaloid fraction)*	Leaf	PNAF	+	n.t.	-	-
30	10	*Psychotira nemorosa* Gardner *(alkaloid fraction)*	Leaf	PNAF	+	n.t.	+	+

+….p ≤ 0,05; -….p ≥ 0,05 not active (student´s t-test), n.t….not tested

Based on the extract screening performed at a concentration of 100µg/ml and 50µg/ml, most promising extracts were selected supposed to have a neuroprotective potential, because of a significant MT induction:

- *Acorus calamus* (roots, DCM)
- *Piper cubeba* (fruits, DCM)
- *Nardostachys jatamansi* (hypocotyl, DCM)
- *Asarum europaeum* (rhizom, DCM)
- *Syzygium aromaticum* (*Eugenia cariophylata)* (flower, DCM)
- *Sida cordifolia* (herb, DCM/M)
- *Humulus lupulus* (flower, DCM/M)
- *Azadirachta indica* (fruit, DCM/M)
- *Plataginis lanceolate* (herb, DCM/M)
- *Calendula officinalis* (flower, DCM/M)
- *Syzgium aromaticum* (flower, DCM/M)
- *Psychotria nemorosa* (leaf, alkaloid fraction)

The selection of an extract for further phytochemical investigations was done primarily based on the results from the extract screening performed in two concentrations and the knowledge from previous studies accessed via an intense literature search. Two different extracts from clove, the buds of *Syzygium aromaticum* (i.e. *Eugenia cariophylata)*, were screened in the four different assays and resulted in comparable results. It is well reported that the major constituent from the essential oil is eugenol (up to 90%), which has previously been shown to be neuroprotective against aluminium induced toxicity in rat brains.[149] Further constituents are caryophyllene, caryophyllene oxide, tannins and humulene. The *Syzgium aromaticum* extract is also rich in flavonoids mainly of quercetin and ß-sitosterol. Amber et al. assumed that the expression of the antioxidant enzymes may possibly be due to the metal chelating abilities of quercetin.[150] Quercetin was also used as positive control in our MT and AD assay. Extracts rich in quercetin and eugenol are therefore not regarded as promising starting materials for our further study. However, it would be worth to further scrutinize the molecular mechanism of eugenol as pure constituent.

Recently, it has been reported that *Calendula officinalis* decreased cognitive impairment in diabetic rats.[151] Although quercetin is also a reported ingredient in *Calendulae flos*, it might be of interest to closer look at the other constituents potentially contributing to the observed effect in *C. elegans*. Interestingly, the extract of this medicinal plant showed a better effect at lower concentration, which might indicate a reverse dose-dependent effect.

A neuroprotective ingredient of *Humulus lupulus* is reported to be xanthohumol, because treatment of Neuro2a/APP_{swe}, a cell model of AD, inhibited Aβ accumulation and APP processing and ameliorated tau hyperphosphorylation.[152]

Recently, the preventive role of *Piper nigrum* in animal models of AD has been elucidated.[153] As an active constituent cubebene has been identified. It is able to induce autophagy via PI3K-AKT-mTOR pathway to protect primary neurons against amyloid beta in Alzheimer's disease.[154] Cubebene is a main constituent in *Piper cubeba* too but not in *Piper longum*. In our study *Piper cubeba* is active whereas *Piper longum* is not active in the AD and PD assay. Therefore, we assume that the activity of *Piper cubeba* is due to cubebene.

The traditional use of *Acorus calamus* in Indian Ayurvedic system is widely accepted. The pharmacological studies have established numerous beneficial properties including anti-oxidant, anti-inflammatory, anti-cancer, anti-ulcer, anti-fungal, anti-allergic, anti-diabetic, anti-microbial, wound healing, neuroprotective, radioprotective, pesticidal, insecticidal and cardioprotective effects and others. *Acorus calamus* consists of various phytoconstituents namely glycosides (xanthone), volatile oil, sesquiterpenes, monoterpenes, flavonoids, steroids, saponins, lignin, tannins, mucilage, alkaloid and polyphenolic compounds. One of the known neuroprotective constituents in *Acorus calamus* are alpha- and beta- asarone and ß-sitosterol.[155]

Asarum europaeum was used as a treatment for rhinitis, pneumonia, angina pectoris, migraine, liver diseases and jaundice in the past. It was also used as a remedy for dehydration and to induce menstruation and abortion. However, the plant also includes some harmful constituents, such as β-asarone and aristolochic acid, and has therefore been banned for medical application in several countries. The rhizome of this plant contains resin, starch, tannins, flavonoids, aristolochic acid and up to 4% essential oil. The essential oil contains a variety of different substances, mostly asarone (30%), methyleugenol (20%) and bornyl acetate (15%). Therefore, we assume that asarone is the active constituent in *Asarum europaeum* too.[156]

Azadirachta indica is effective in reversing the neurobehavioral changes, attenuating the cognitive deficits and decreasing the oxidative stress in experimental AD rat models.[157] In another study treatment with *Azadirachta indica* improved functional recovery in the 6-hydroxydopamine induced rat Parkinson's disease (PD) model. By suppression of PD-induced catalase, glutathione-peroxidase, iNOS activity and iNOS protein expression, inflammatory factors, acetylcholinesterase activity and cyclo-oxygenase-2 protein expression levels.[158] Further, it has anti-diabetic and anti-cancer properties. *Azadirachta indica* has various constituents including nimbin, nimbidin, nimbolide, and limonoids. Although quercetin and ß-sitosterol, which was reported to have neuroprotective activity too [159], were the first polyphenolic

flavonoids purified from fresh leaves[159],it would be worth to investigate the constituents of the fruit extract used in our screening for the main active constituents.

Significant nootropic effect of aqueous and hydroethanolic extracts of *Sida cordifolia* was investigated in mice.[160] Another study evaluated the aqueous extract of *Sida cordifolia* (AESC), and its different fractions; hexane (HFSC), chloroform (CFSC) and aqueous (AFSC), against rotenone induced biochemical, neurochemical, histopathological and behavioural alterations in a rat model of PD. Its results indicate the possible therapeutic potential of most polar fraction of AESC, i.e. AFSC, in PD by virtue of its antioxidant actions.[160] Further it has anti-diabetic and anti-cancer properties. The aerial parts of *Sida cordifolia* contain palmitic, stearic, heacosanoic acid and ß-sitosterol and 0,31% alkaloids.[160]

An extract of *Nardostachys jatamansi* exhibited memory retention and learning enhancing abilities in aged and young mice and reversed scopolamine and diazepam induced amnesia.[161] Further it has shown an anti-parkinson and an anti-diabetic activity. It contains mainly the sesquiterpenes valeranone and alpha-patchoulense and ß-sitosterol.[162]

Nothing is known in literature about cognitive enhancing activities of *Plantaginis lanceolota*. This famous European medicinal plant shares its medicinal effects with its close relative, *Plantago major*. The leaves of both plants play an important role in the management of certain ailments and diseases such as ulcers, bacterial and viral infections, diarrhoea, pain, inflammation and cancer. *P. lanceolata* has been shown to contain several classes of biologically active compounds; flavonoids, alkaloids, iridoid glycoside, fatty acids, vitamins, phenolic compounds (caffeic acid) and terpenoids. This field still needs more study to determine the exact mechanisms and the main bioactive constituents responsible for treating certain diseases.[163]

A decoction of the leafs of *Psychotria viridis* and *Banisteriopsis caapi* is named Ayahuasca and has been long used by indigenous people for the treatment of many diseases, including neurological illness. Active compounds belong to the indole alkaloids.[164] Besides this prominent beverage other *Psychotria* species has been investigated towards its pharmacological activities too. In a study there has been shown that the alkaloid fraction of *P. nemorosa* (PNAF) and it´s main isolated indole alkaloids nemorosine A and fargesine are able to inhibit butyrylcholinesterase (BuChE) and monoamine oxidase A (MAO-A).[165]

3.4. Paper 3: Effects of Azepine-Indole Alkaloids from *Psychotria nemorosa* on Neurodegenerative Disorders Evaluated in Transgenic *C. elegans*

In this paper we described different approaches to evaluate the activity of *P. nemorosa* and its alkaloids nemorosine A (1) and fargesine (2) against NDs. First an in-silico study has been done to find out the different targets. Compounds with chemical similarity are supposed to have similar activities. The results showed that 14 of the 20 top targets in NDs are stimulated by 1 and 2. Regarding the prediction important targets of 1 and 2 are for example indolamin-2,3-dioxygenase (IDO), poly[ADP-ribose]polymerase-1 (PARP1) and 5-hydroxytryptamine receptors (5-HTR). Most interesting is the 5-HTR target because the serotoninergic signaling system plays an important role in AD and is highly conserved between *C. elegans* and humans. In a next step we found out that there are the activities of 1 and 2 on the human 5-HT_{2A} receptor using a cellular assay. Because of the promising predicted and experimentally investigated activities of PNAF, 1 and 2 we were interested in the outcome of *C. elegans* based screenings using transgenic nematodes of AD and PD. We examined if the alkaloids and PNAF prolong time until paralysis in strain CL2659 and if test samples reduce the fluorescence of gfp coupled α-synuclein in strain NL5901. Both readouts correlate with a reduction of proteotoxicity. Further we investigated if test compounds are able to induce MT during aging in strain CL2120. The treatment with PNAF, **1** and **2** significantly reduced Aβ and α-synuclein proteotoxicity and prolonged MT induction time in *C. elegans* similar to results obtained previously for quercetin, apomorphine and other known MT-inducers in paper 2.

In this study with our established *C. elegans* models and the target based assays we could show the multifunctional profile of azepine-indol alkaloids both in AD and PD.

My contribution in this paper: In this study I planned, performed and analyzed all *C. elegans* based experiments. I screened the alkaloid fraction PNAF and the two main isolates nemorosine A and fargesine in the AD and PD assay and evaluated the mode of action towards MT induction.

3.5. Paper 4: Abnormal metal homeostasis as a common drug target to combat neurodegenerative diseases

With this perspective article I summarized our findings regarding metal hypothesis in NDs and demonstrate its potential for future applications. To sum up targeting the failed MT induction in aged nematodes with an AD or PD phenotype reduced the proteotoxicity by presumably restoring the metal homeostasis. In my opinion, it is worth to have this target in mind when

searching for new therapeutics against NDs, and here I am not only referring to AD and PD. MT induction may be be further a promising target in the treatment of HD, amyotrophic lateral sclerosis, prion disease, brain trauma, brain ischemia, and psychiatric diseases, because there has been shown that MT is reduced in those disorders too. Therefore, our newly established MT assay could provide a first stage selection of compounds which should be further investigated in the adequate disease models.

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A robust and miniaturized screening platform to study natural products affecting metabolism and survival in *Caenorhabditis elegans*

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In this study a robust, whole organism screening based on *Caenorhabditis elegans* is presented for the discovery of natural products (NP) with beneficial effects against obesity and age-related diseases. Several parameters of the elaborated workflow were optimized to be adapted for probing multicomponent mixtures combining knowledge from traditional medicine and NP chemistry by generating optimized small-scale extracts considering scarcity of the natural source, solubility issues, and potential assay interferences. The established miniaturized assay protocol allows for in vivo probing of small amounts of even complex samples (~ 1 mg) to test their ability to increase the nematodes' survival time and the suppression of fat accumulation assessed by Nile red staining as hall marks of "healthy aging". The workflow was applied on 24 herbal and fungal materials traditionally used against symptoms of the metabolic syndrome and revealed promising results for the extracts of *Gardenia jasminoides* fruits and the sclerotia from *Inonotus obliquus*. Tested at 100 µg/mL they were able to significantly reduce the Nile red fluorescence and extend the 50% survival rate (DT_{50}) compared to the control groups. This phenotype-directed in vivo approach opens up new horizons for the selection of natural starting materials and the investigation of their active principles as fast drug discovery tool with predictive value for human diseases.

Caenorhabditis elegans (Maupas, 1900), a 1 mm sized roundworm, is a popular model organism in almost all areas of modern biology. It can be maintained at low cost, has a short reproductive cycle of three days with a large brood size of 300 progenies per hermaphrodite worm and a transparent body comprising exactly 959 somatic cells[1]. In recent years it has been increasingly used as a model organism for drug screenings[2–7]. The fundamental idea behind is that basic molecular processes which are causal for the development of diseases including aging processes are conserved in the animal kingdom. Indeed *C. elegans* shares many similarities with humans such as autophagy, mitochondrial regulation, apoptosis, proteostasis, energy control, fat-storage, stress response systems and neuronal regeneration[8–16]. A recent meta-analysis estimated that 41.7% of the protein-coding genes in *C. elegans* have orthologs in humans[17]. In this light, screening for substances beneficial to a disease phenotype in *C. elegans* can have important predictive value also for human diseases[18,19]. The simplicity and tractability of the worm compared to classical mammal models represents a large advantage. Its small size makes it amenable to whole organism screening in microtiter plates for medium/high-throughput screening with little consumption of materials and sample[20]. This possibility is particularly helpful for drug discovery from natural sources, which is often impeded by scarcity of natural starting materials, and even more relevant for their isolates which require tedious isolation or synthesizing efforts[21–24]. There is an increasing interest in these approaches as shown by a growing number of isolated NP and extract screens[25–30]. However, extracts are complex mixtures consisting of a broad range of metabolites with different chemical properties which are not necessarily drug-like. Some are prone to give false-positive or false-negative results due to insolubility or interferences with optical readouts[31–34]. Therefore, in this study special attention was given to an extract preparation which allows the enrichment of

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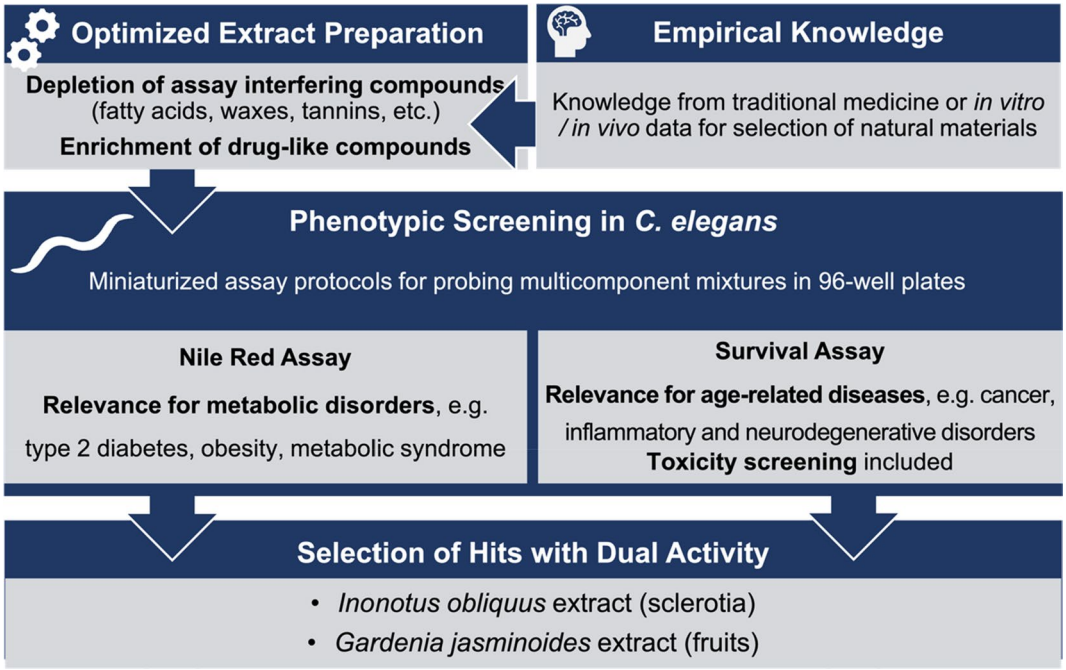

Figure 1. Workflow of the presented approach.

lead-like constituents and meets the requirements for a reliable readout in the phenotypic *C. elegans* assays. By means of these optimized extracts a screening protocol was established with two robust and miniaturized *C. elegans*-based assays for performing survival and Nile red fluorescence experiments capable of testing multicomponent mixtures and their isolates (Fig. 1). Natural products exerting a significant reduction in the *C. elegans*-based Nile red assay avoid of any lifespan shortening or even additionally causing an increase in lifespan have been identified. They are considered as promising candidates in the search for NP able to overcome metabolic diseases as well as age-related diseases with a prescreened low risk of toxicity.

Results and discussion

Selection of natural raw materials. Selection of starting materials was guided by literature on NPs with potential effects on human health. The following criteria for the selection of the starting material were considered:

(i) a species (and its corresponding organ) traditionally used in the field of metabolic disorders, enhancement of the immune system and promotion of health and longevity, and/or
(ii) in vitro data from extracts derived from enzyme or cell-based assays which lack more in-depth analysis in an in vivo model organism, and/or
(iii) pharmacological in vivo data of NPs with effects related to metabolic syndrome and/or anti-aging effects.

The selection presented in this study comprises 24 materials from plant and fungal species belonging to 20 different families, either used in Traditional Chinese, European, African or American medicines, but also in different medicinal systems such as e.g. Ayurveda, Unani or Siddha systems. The literature results for the selected 24 species is given in Table S1.

Survival assay. An increase in lifespan is considered to be a result of interventions in pathways of aging. The measurement of lifespan is therefore a key experiment leveraged to seek for lifespan delaying agents endowed with the potential to treat age-related diseases in vertebrates/humans[35,36]. This investigation requires analysis of survival over time during the aging process of an individual in a population. Survival assays in *C. elegans* have been performed in both, solid and liquid culture at temperatures ranging from 15 °C to 25 °C[36]. The use of liquid culture offers substantial benefits over solid culture: (1) nematodes encounter less mechanical stress when assayed in liquid culture[37], since no prodding or transferring of worms with a platinum wire is necessary, (2) there is no need of censoring worms that are buried into the solid media, (3) it is adaptable to large-scale screenings[38], and (4) less sample amount is needed. In this study, survival analyses were performed at 25 °C with N2 wild-type worms and the commonly used liquid medium S-complete supplemented with OP50 bacteria. The survival of worms at 25 °C was recorded three times a week until only 50% of the worms were alive (death time 50%; DT_{50}). DT_{50} for survival measurement was chosen because it enables a shorter observation time, compared to standard protocols with the necessity of survival monitoring until death of all individuals within a population. Death was determined when the worms did not move after 2 min shaking on a microplate-shaker or exposure to

strong light. Many parameters influencing the nematodes' survival and well-being such as the population density and feeding frequency were adapted based on previous findings[39].

Nile red assay. Triacylglycerol stores can be stained with the solvatochromatic dye Nile red mixed into the worms' bacterial OP50 diet. In aqueous medium Nile red is almost non-fluorescent. By mixing it with OP50 bacteria and upon feeding, it accumulates in the nematodes' subcellular compartments, the so-called lysosome related organelles. In this environment rich in polar lipids Nile red exposes a strong red to yellow-shifted fluorescence[40–42]. The intensity of this fluorescence has been shown to correlate with the worms' fat content, why this method has been used for chemical and genetic screens for small molecules and genes that affect fat metabolism[11,43–46]. Compared with biochemical and chromatographic methods the vital Nile red assay is more suitable for rapid large-scale screenings in a miniaturized format and can be performed in S-complete medium filled in 96-well plates. *C. elegans* mutant strain SS104, bearing a temperature-sensitive mutation in valyl aminoacyl tRNA synthetase *glp-4(bn2ts)*, was employed for screening at the restrictive temperature of 25 ± 0.5 °C. *Glp-4(bn2ts)* mutant worms develop normal at 16 °C but are sterile at the experimental temperature of 25 °C[47,48].

Optimization of treatment time. For the determination of an ideal treatment time L4 worms were incubated at 25 °C for up to 7 days, and Nile red fluorescence was measured each day. An increase of fluorescence was observed from day 2 to 6 followed by a decline (Fig. S1). This finding is in accordance with results of Shen et al.[49], who determined an increased fat accumulation of N2 worms from day 2 to 6 measured with biochemical methods. However, for chemical screens not only the time of fat accumulation itself but also the accumulation and the persistence of chemicals in the worms have to be considered. It has been shown that drug concentrations in worms gradually decrease after the first day of treatment[50]. Based on these findings and our observations we selected a treatment of 5 days starting from L4 staged worms. This time point guaranteed a high Nile red fluorescence indicating a sufficient fat accumulation as well as persistence of chemicals in worms. Furthermore, an additional feeding within these 5 days was not necessary.

Optimization of DMSO concentration. Low solubility of NPs is a well-known issue[51], but complete solubility as a prerequisite of bioavailability is a crucial requirement for testing bioactivity in a phenotypic-based assays[52,53]. Dimethyl sulfoxide (DMSO) guarantees chemical stability and dissolves samples with a wide range of chemical properties[54,55]. However, DMSO itself has shown to effect *C. elegans* phenotypes[56,57]. It was reported that a DMSO concentration higher than 0.60% shortens the lifespan of *C. elegans* in liquid culture and should be avoided in survival studies[39]. Other sources indicate that concentrations between 0.80 – 1.00% DMSO lead to a significant increase in median lifespan (by 15%) when using DMSO from egg-stage throughout life, although these effects did not occur when worms were treated after egg-laying period[57]. To find the optimal concentration of DMSO for both the survival assay and Nile red assay, we tested different concentrations of DMSO and could observe a lifespan prolonging tendency (without significance) ranging from 0.00 to 1.00% of DMSO treatment (Fig. 2A). The mean DT_{50} value of untreated worms in S-complete medium was 15.33 days compared to 15.83, 15.17, 17.83 and 17.50 days for worms treated with DMSO concentrations of 0.20%, 0.33%, 0.60% and 1.00%, respectively (Table S2). Those results are consistent with the study of Wang et al.[57], who has shown that nematode growth media (NGM) plates containing DMSO concentrations from 0.01% to 2.00% led to a dose-dependent lifespan extension, reaching its maximum at 0.5%. The effect of DMSO on Nile red staining of SS104 nematodes was minor when tested up to concentrations of 1.00% with a mean fluorescence of 105.30 ± 13.28% compared to untreated worms. The two highest tested concentrations, 1.50 and 2.00% DMSO, caused an insignificant reduction of fluorescence (Fig. 2B). Thus, it can be concluded that 1.00% DMSO can be used for chemical screens without significantly influencing the nematodes' survival or Nile red fluorescence.

Positive controls. *Survival assay.* A number of different drug substances have been reported to prolong the nematodes' lifespan (e.g. the antidepressant mianserine has previously been shown to extend the nematodes' lifespan by 31% when given throughout adult life at a concentration of 50 µM[2]). Interestingly, this effect was only present at 20 °C and abolished when the survival assay was performed at 25 °C[58]. The anti-hypertensive drug reserpine has been reported to increase *C. elegans* lifespan by 31% and 64% when worms were treated with 30 µM reserpine from embryo stage onwards and from young adult stage on, respectively. This effect was also temperature dependent and only present at 25 °C. No significant lifespan extension was observed at 20 °C[59]. Further, the xanthine caffeine has been reported for its lifespan extending potential when nematodes were exposed to various concentrations of caffeine ranging from 10 to 200 µg/mL at 20 °C[60] (corresponding to 51.5–1,000 µM). For validation of the presented assay conditions, mianserine 50 µM, reserpine 30 µM and caffeine 50 µM were tested (Fig. 3). In our assay settings, no significant DT_{50} extension was observed for the antidepressant mianserine in comparison to the control group; for the natural stimulant caffeine a moderate DT_{50} extension was recorded. In line with the previously mentioned data, reserpine at 30 µM resulted in a significant DT_{50} extension of 31.43% ($p < 0.05$, Table S3) and was henceforth used as positive control in all lifespan experiments.

Nile red assay. For assay validation we tested three pharmacological agents reported to influence mammal and *Caenorhabditis elegans* fat stores (Fig. 4) i.e. olanzapine, fluoxetine and 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR). Worms treated with olanzapine exhibited an increased Nile red fluorescence of 135.3% and 129.3% at 10 and 25 µM, respectively, compared to vehicle treated worms. On the contrary, fluoxetine showed a dose dependent fluorescence-reducing effect. At 100 µM fluoxetine significantly reduced the mean fluorescence to 56.7% compared to vehicle treated worms. AICAR similarly reduced Nile red fluorescence but at higher concentrations. Treatments of 250 and 100 µM AICAR significantly reduced the mean Nile red fluorescence

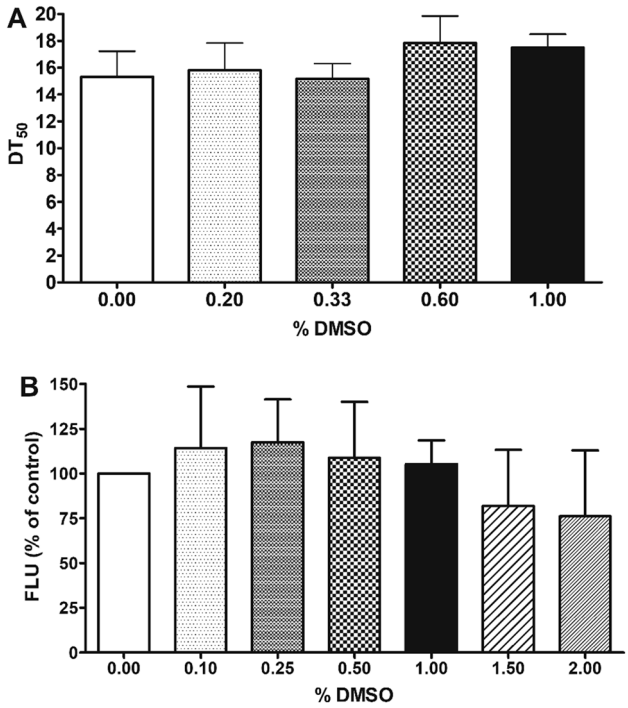

Figure 2. Effect of different DMSO concentrations (0.00—2.00% in S-complete medium) on (**A**) the survival and (**B**) vital Nile red staining of *C. elegans*; (**A**) Bars represent the mean DT_{50}s of N2 worms in days ± SD. Survival assay tested in parallel triplicates. (**B**) Bars represent the mean vital Nile red fluorescence intensities of SS104 worms of five independent experiments expressed as % of control worms (0% DMSO) ± SD.

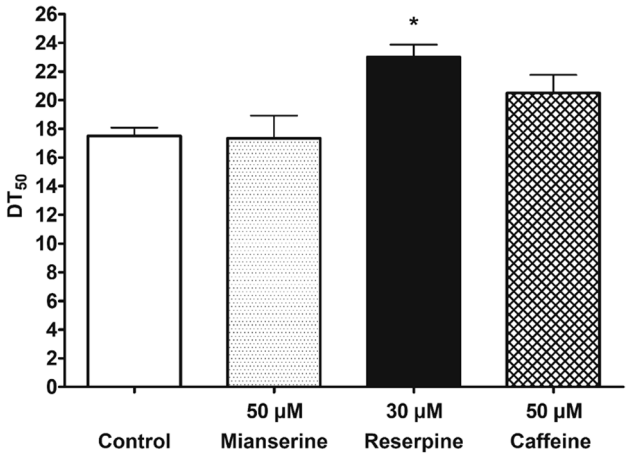

Figure 3. Screening of positive controls. Bar charts of mean DT_{50} values of *C. elegans* treated with mianserine (50 µM), reserpine (30 µM) and caffeine (50 µM) in comparison to vehicle control. Reserpine at 30 µM significantly extended the DT_{50} of wild type *C. elegans* by 31.4%. Bars represent the DT_{50} value in comparison to the control group ± SD of three parallel experiments. Significance was assessed by one way ANOVA with Dunnett post-test (*$p < 0.05$).

to 46.7% and 66.4%, respectively, compared to vehicle treated worms. The response to the three compounds suggests that the *C. elegans* Nile red assay has a certain degree of translatability to mammals as all three tested pharmacological agents are known to modulate mammalian fat similar, i.e. olanzapine is known to exacerbate hyperlipidemia and type-2 diabetes and induce fat accumulation in humans[61–63]; the serotonin reuptake inhibitor fluoxetine is known to lead to weight reduction in obese patients[64] and the investigational drug and AMPK activator AICAR blocked high-fat diet induced body weight gain in mice[65].

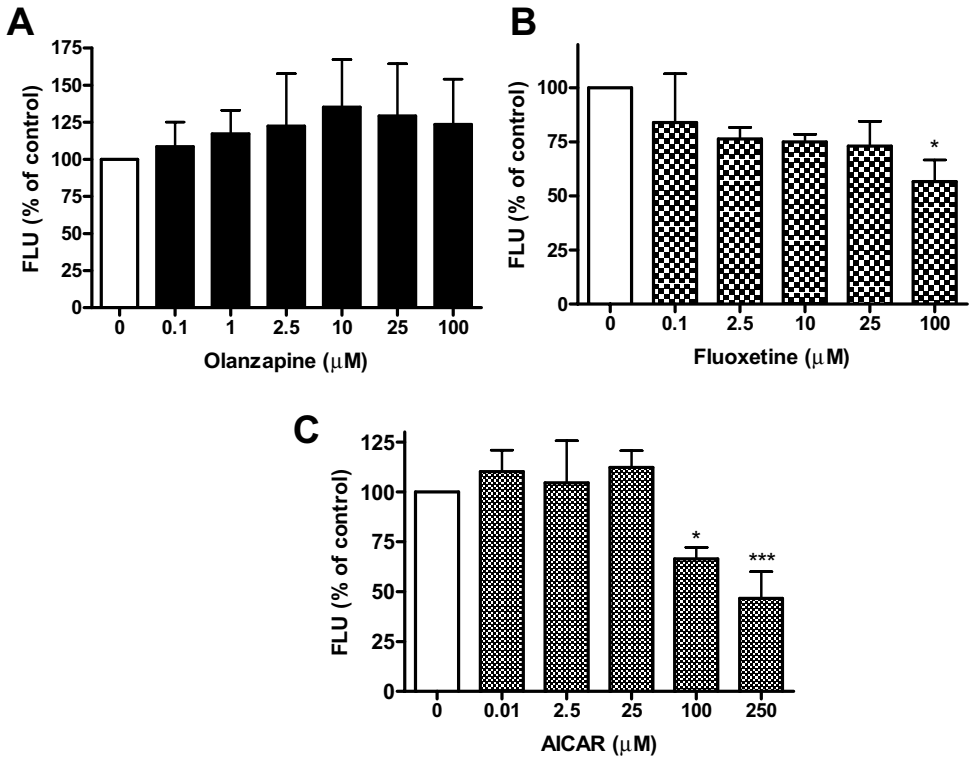

Figure 4. Effects of drugs on Nile red fluorescence of SS104 *C. elegans*. Bar charts represent the mean Nile red fluorescence of three independent experiments (± SD). Worms were treated with different concentrations of (**A**) olanzapine, (**B**) fluoxetine, and (**C**) AICAR normalized to vehicle treated control worms. Statistical significance assessed by one way ANOVA and Bonferroni post-test (*$p < 0.05$, ***$p < 0.001$).

Extract preparation adapted to phenotypic screening. Although nature has been one of the most prolific resources for drug discovery[66], the isolation of their bioactive compounds and their physicochemical characterization are often tedious. Screening of extracts is a useful approach for the selection of natural starting material worth a phytochemical work-up. However, the complexity[51,67] of an organism's metabolome and its matrix can account for profound issues when screened in biological systems. Assay perturbation, aggregating metabolites and dynamic residual complexities are prone to give false-positive or false-negative results[33]. The concept of front-loading an extract with drug- or lead-like properties was previously described by Camp et al.[67]. This process facilitates drug discovery from natural sources by enrichment of compounds that meet the physicochemical properties of drug-like molecules. Additionally, the depletion of highly apolar constituents (e.g. waxes, lipids) and oligo- and polymeric polyphenols (e.g. tannins), not only significantly reduces problems arising during the extracts' analysis and chromatographic work-up (e.g. clogging of columns), but also diminishes the risk of assay interferences[23]. Complex mixtures comprising compounds with very different molecular weights and polarity may have challenging implications when it comes to solubility, especially when handling a liquid assay[68]. Further, it has to be considered that minor bioactive constituents within multi-component mixtures may easily been overseen, and accordingly ask for assays with high sensitivity[33,52,68]. To integrate extracts to our liquid-based screening platform, we aimed to establish a standardized small-scale extraction procedure with an enriched yield of drug-like secondary metabolites and depleted primary metabolites and assay-interfering compounds. A detailed extraction procedure is provided in the Supplementary Information (Protocol for the preparation of optimized small-scale extracts). Small-scale dichloromethane (CH_2Cl_2) and methanol (MeOH) extracts were generated as previously described[67,69,70], with some alterations. Briefly, dried natural materials were ground and defatted with *n*-hexane twice. The remaining materials were successively extracted with CH_2Cl_2 and MeOH. Both extracts were combined. A depletion of potentially included tannins was performed with solid-phase extraction (SPE). These small-scale extracts optimized for their application in the miniaturized *C. elegans* assays were transferred to pre-weighted and labeled Eppendorf tubes, dried in a desiccator and the total extract weight was determined using an analytical balance. Aliquots of the transferred extract were dissolved in DMSO to a concentration of 10 mg/mL and stored at − 20 °C until use.

NPs applicability on established screening platform. The phenotypic screening was exemplarily performed with 24 optimized small-scale extracts of herbal and fungal materials (Table 1), which have either been traditionally used against symptoms related to the metabolic syndrome or described to be active in any kind of animal model in this disease area. All extracts were tested at two concentrations (25 and 100 µg/mL) for their effect on survival and Nile red staining in our liquid-based *C. elegans* screening platform (Table S1). Six

Species	Family	Organ	Source/sample location	Voucher specimen/charge number
Andrographis paniculata (Burm.f.) Nees	Acanthaceae	herb	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20150313-A1 Ch.Nr.: 780,672
Azadirachta indica A.Juss	Meliaceae	fruits	Padma AG, Wetzikon, Switzerland	JR-20150615-A1 Ch.Nr.: 2,021,108,301
Calendula officinalis L.	Asteraceae	flowers	Padma AG, Wetzikon, Switzerland	JR-20150615-A9 Ch.Nr.: 21,348,300
Cetraria islandica (L.) Ach	Parmeliaceae	lichen	Padma AG, Wetzikon, Switzerland	JR-20150615-A7 Ch.Nr.: 20,885,300
Cynanchum paniculatum (Bunge) Kitag. ex H.Hara	Apocynaceae	roots	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20150313-A2 Ch.Nr.: 840,274
Cynanchum stauntonii (Decne.) Schltr. ex H.Lév	Apocynaceae	rhizomes	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20101012-A1 Ch.Nr.: 310,051
Drynaria fortunei (Kunze ex Mett.) J.Sm	Polypodiaceae	rhizomes	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20150313-A3 Ch.Nr.: 030,161
Eriobotrya japoinica (Thunb.) Lindl	Rosaceae	leaves	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20110421-A1 Ch.Nr.: 030,724
Euphrasia officinalis L.	Orobanchaceae	herb	Kottas Pharma GmbH, Vienna, Austria	JR-20090625-A1 Ch.Nr.: KLA90309
Fomitopsis pinicola (Sw.) P. Karst. (strain 10)	Fomitopsidaceae	fruit body	Viggartal, Ellbögen, Austria (grown on dead spruce trunk); Ursula Peintner	FompinE0010
Ganoderma lucidum (Curtis) P. Karst	Ganodermataceae	fruit body	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20150313-B1 Ch.Nr.: 680,898
Gardenia jasminoides J.Ellis	Rubiaceae	fruits	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20150313-A1 Ch.Nr.: 11 0,284
Gloeophyllum odoratum (Wulfen) Imazeki	Gloeophyllaceae	fruit body	Oberperfuss, Austria (grown on spruce) Ursula Peintner	JR-20140310-A1 GloodoE0054
Imperata cylindrica (Nees) C.E.Hubb	Poaceae	rhizomes	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20150313-A4 Ch.Nr.: 81 0,178
Inonotus obliquus (Ach. ex Pers.) Pilát	Hymenochaetales	sclerotia	Finnland, 1998 Ursula Peintner	UP-20121212-A1
Peucedanum ostruthium (L.) W.D.J.Koch	Apiaceae	roots /rhizomes	Kottas Pharma GmbH, Vienna, Austria	JR-20180119-A2 Ch.Nr.: P17301770
Pimenta dioica (L.) Merr	Myrtaceae	fruits	Padma AG, Wetzikon, Switzerland	JR-20150615-A4 Ch.Nr.: 21,362,100
Piptoporus betulinus (Bull.) P. Karst. (strain 39)	Fomitopsidaceae	fruit body	Vahrn bei Brixen, Italy, grown on birch, Ursula Peintner	PipbetE0039
Potentilla aurea L.	Rosaceae	herb	Padma AG, Wetzikon, Switzerland	JR-20150615-A3 Ch.Nr.: 21,161,301
Scutellaria barbata D.Don	Lamiaceae	herb	Plantasia GmbH, Oberndorf bei Salzburg, Austria	JR-20150313-A5 Ch.Nr.: 76 0,577
Sida cordifolia L.	Malvaceae	herb	Padma AG, Wetzikon, Switzerland	JR-20150615-A2 Ch.Nr.: 20,981,300
Syzygium aromaticum (L.) Merr. & L.M.Perry	Myrtaceae	flowers	Padma AG, Wetzikon, Switzerland	JR-20150615-A8 Ch.Nr.: 21,321,101
Terminalia chebulia Retz	Combretaceae	fruits	Padma AG, Wetzikon, Switzerland	JR-20150615-A5 Ch.Nr.: 21,324,301
Valeriana officinalis L.	Caprifoliaceae	roots	Padma AG, Wetzikon, Switzerland	JR-20150615-A6 Ch.Nr.: 21,388,100

Table 1. Plant and mushroom materials for extraction.

extracts exerted a significant reduction of Nile red fluorescence (i.e. *Inonotus obliquus*, *Terminalia chebula*, *Valeriana officinalis*, *Imperata cyclindrica*, *Gardenia jasminoides* and *Scutellaria barbata*), and nine extracts showed a pronounced increase in the nematodes' lifespan; the 50% survival rate of wild-type worms was significantly extended by six small-scale extracts tested at 100 µg/mL (i.e. *Gloeophyllum odoratum*, *Pimenta dioica*, *I. obliquus*, *Syzygium aromaticum*, *Cynanchum stauntonii* and *G. jasminoides*) and one extract even at 25 µg/mL (i.e. *Cynanchum paniculatum*). Two extracts, i.e. those of *Euphrasia officinalis* herbs and *Eriobotrya japoinica* leaves were capable of significantly increasing the 50% survival rate at both tested concentrations. On the contrary, the root extract of *Peucedanum ostruthium* drastically decreased the nematodes' DT_{50} at both test concentrations. Correspondingly, this extract was not evaluable at 100 µg/mL in the Nile red assay, since most worms were already dead. From all the tested samples only two extracts significantly reduced the worms' Nile red fluorescence at both tested concentrations, 25 and 100 µg/mL (i.e. *I. obliquus* and *I. cyclindrica*). The reduction of Nile red fluorescence by the extract of *V. officinalis* was however accompanied by a decreased worm survival. *T. chebula*, *I. cyclindrica* and *S. barbata* decreased Nile red fluorescence and increased (although not significantly) survival of worms. Intriguingly, the extracts of *I. obliquus* and *G. jasminoides* showed both, a significantly increased survival rate and reduced Nile red fluorescence (Fig. 5).

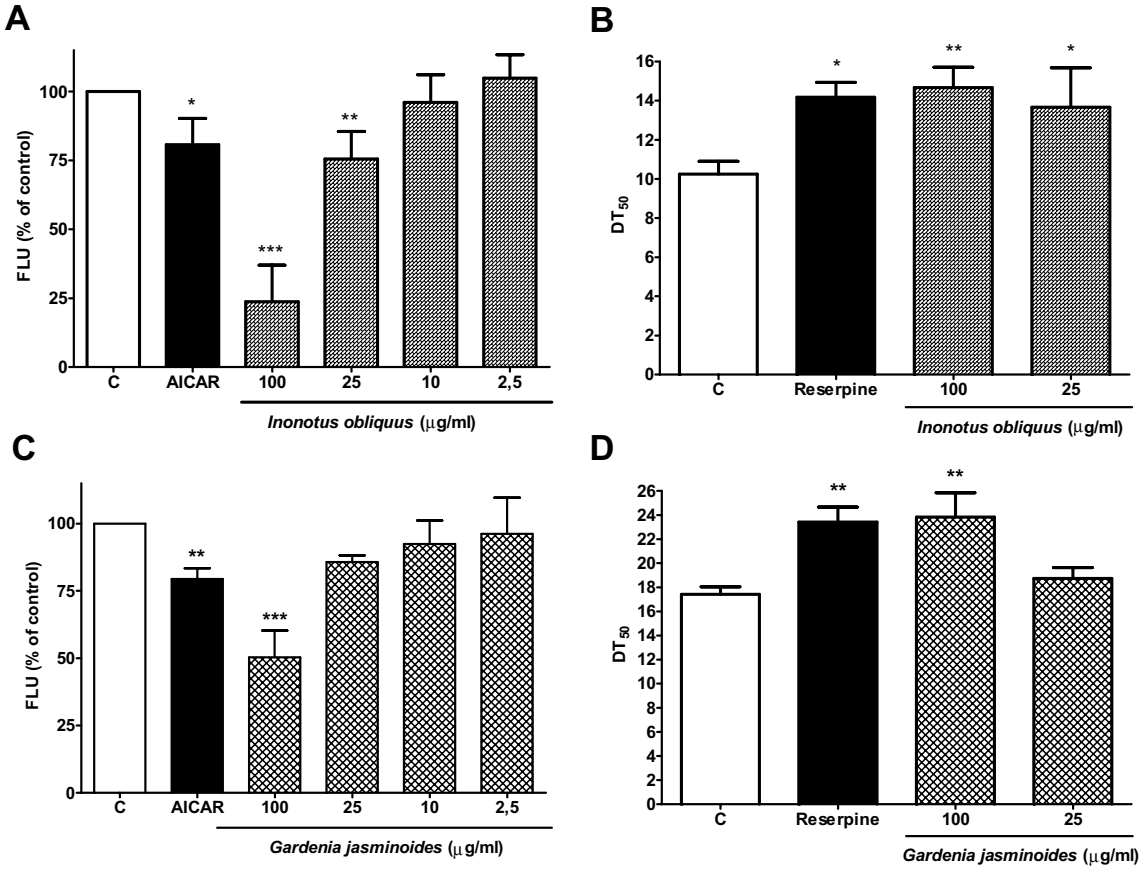

Figure 5. Effect of *I. obliquus* and *G. jasminoides* extracts on Nile red fluorescence (A, C) and survival (B, D) of *C. elegans*. Nile red assay (**A**, **C**): Vital Nile red fluorescence of *C. elegans* treated with control, AICAR (100 µM) and extracts of *I. obliquus* (**A**) and *G. jasminoides* (**C**). Bars represent the mean fluorescence intensities of at least three independent experiments expressed as % of control worms ± SD. Significance was assessed by One-Way ANOVA and Bonferroni post-test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). Survival assay (**B**, **D**): Worms were treated with control, reserpine (30 µM) and extracts of *I. obliquus* (**B**) and *G. jasminoides* (**D**). Bars represent the mean DT_{50} ± SD of three parallel experiments. Significance was assessed by One-Way ANOVA and Dunnett's post-test (*$p < 0.05$; **$p < 0.01$).

		Mean DT_{50} ± SD	*N*	DT_{50} extension (%)	*p*-value
Control		10.33 ± 0.66	220	–	
Reserpine	30 µM	14.17 ± 0.76	138	38.21	$p < 0.05$
I. obliquus	100 µg/mL	14.67 ± 1.04	119	43.09	$p < 0.01$
I. obliquus	25 µg/mL	13.67 ± 2.02	112	33.33	$p < 0.05$

Table 2. Survival analysis upon treatment with *I. obliquus*. *N* is the total number of worms assayed for survival. One way ANOVA with Dunnett's post-test was used for statistical evaluation. *P*-value < 0.05 was considered as statistically significant.

Inonotus obliquus is a parasitic mushroom belonging to the Hymenochetaceae family. Its sclerotia, commonly known as chaga conks, grow on hardwood trees. Chaga decoctions are traditionally used in Eastern Europe and Russia for different therapeutic indications such as cancer, inflammation and metabolic disease[71,72]. In our screening, the extract (HPLC chromatogram Fig. S2) significantly inhibited the Nile red fluorescence at 100 and 25 µg/mL and extended the mean survival rate of worms with a significant DT_{50} increase of 43.1% and 33.3%, at 100 and 25 µg/mL, respectively (Table 2). Our findings are in line and complement recent preclinical studies on the beneficial effects of *I. obliquus* extracts in different mammal models of obesity and diabetes[73–76]. Longevity effects *of I. obliquus* in *C. elegans* were already reported by Scerbak et al.[77,78], and in *Drosophila melanogaster* by Zhang et al.[79].

Gardenia jasminoides is an evergreen shrub belonging to the Rubiaceae family. Its fruits are traditionally used in China because of their diuretic, cholagogue, anti-inflammatory and hypoglycemic properties[80,81]. The extract

		Mean DT_{50} ± SD	*N*	DT_{50} extension (%)	*p*-value
Control		17.42 ± 0.63	213	–	
Reserpine	30 µM	23.42 ± 1.23	208	34.45	$p < 0.01$
G. jasminoides	100 µg/mL	23.83 ± 2.02	97	36.84	$p < 0.01$
G. jasminoides	25 µg/mL	18.75 ± 0.90	88	7.66	ns

Table 3. Survival analysis upon treatment with *G. jasminoides*. N is the total number of worms assayed for survival. One way ANOVA with Dunnett's post-test was used for statistical evaluation. P-value < 0.05 was considered as statistically significant.

(HPLC chromatogram Fig. S3) significantly increased the 50% survival rate of N2 worms by 36.84% (Table 3) and inhibited Nile red fluorescence at 100 µg/mL. *G. jasminoides* and its isolated constituents e.g. geniposide, genipin, crocetin and crocin are already known to have anti-obesity, hypolipidemic and hypoglycemic effects in different mammal models[82–84]. Although some beneficial effects of genipin/geniposide on ageing such as cognition in aged fruit-flies and age-related insulin resistance in rats are reported[85,86], it is the first report of a longevity promoting effect described for the extract of *G. jasminoides* fruits.

Conclusion

The preclinical evaluation of efficacy of multicomponent mixtures is a crucial step in drug discovery from nature. For the selection of the herein probed NPs, we decided to consider the ethnopharmacological relevance, since such samples are supposed to be more likely active[87,88] and—proven by a long-standing application—they are considered to be safer and in turn less toxic[89] compared to samples from randomly collected species. Starting from crude extracts it might be a tedious and costly process often obstructed by many pitfalls due to the presence of highly abundant bulk substances (e.g. lipids, waxes, chlorophyll, tannins) that are commonly known to interfere with biological assays[24]. With this study, we emphasize the need to perform a thoroughly performed phyto-/mycochemical extraction procedure that allows the separation of assay interfering compounds and facilitates the enrichment of putatively bioactive metabolites in a multicomponent mixture. The herein presented 96-well plate liquid format enables a robust, time- and resource-efficient, thus cost-effective screening of extracts. For the presented survival assay (counting up to one month) roughly 240 samples (including controls) can be managed by one operator (evaluated by three wells in parallel). For the Nile red assay, in one month about 400 samples (including controls) are manageable per operator (evaluated by six wells in parallel). These numbers refer to a setup without any technically challenging devices (except for a fluorescence microscope). With more sophisticated and costly instrumentation the herein presented *C. elegans* assays are adaptable to automatization for screenings of larger collections[20]. These collections could also include samples from less researched resources, e.g. marine organisms, terrestrial microbes or randomly selected plants and fungi[90].

Several parameters have been optimized in this study to overcome challenges usually posed by assaying multicomponent mixtures in a phenotypic screening[34]. *C. elegans* is an established living model for obesity and aging research[49]. Adapted for probing of extracts in miniaturized form (on 96-well plates) this phenotypic screening workflow serves as valuable preclinical tool using even small quantities of multicomponent mixtures (less than 1 mg) to assess their potential to interfere in lipid metabolism and age-related diseases. A further big advantage is the concurrent toxicity prescreening in the *C. elegans* lifespan assay[37]. Intriguingly, in our experiments only one out of 24 extracts showed a significant nematotoxicity, namely the root extract of *Peucedanum ostruthium*. The nematodes' DT_{50} value was decreased by roughly 45% and 60% at 25 and 100 µg/mL, respectively, when compared to the untreated control.

Although the observed efficacy in *C. elegans* has to be critically validated to stand the test also in higher animal models, this workflow enables the rapid identification of auspicious extracts for further chemical, pharmacological and toxicological investigations. The tractability of *C. elegans* to genetic manipulations and the availability of genetic and biochemical tools further facilitates a straight-forward identification of involved pathways and genes[91,92].

The screening applied with optimized extracts (tested at 25 and 100 µg/mL) from 24 herbal/fungal drugs underlines the suitability of the generated workflow. The data revealed a considerable amount of extracts significantly increasing the worms' lifespan (9 out of 24 at least at one of the tested concentrations) and decreasing the worms' Nile red fluorescence (6 out of 24 at least at one of the tested concentrations).

Two extracts even exerted both, a reduction in *C. elegans* Nile red fluorescence and an increase in the nematodes' survival. The most pronounced dual activities were observed for the extracts of Chaga and *G. jasminoides* fruits, thus offering a superb starting point for future investigations in the search for natural products improving healthy aging and the discovery of natural lead structures able to conquer metabolic diseases and age-related pathologies.

Methods

Natural materials. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy, University of Vienna, Austria.

Generation of extracts. Optimized small-scale extracts were prepared as previously described in Kratz et al.[69] adapted from Camp et al.[67]. In short, ~ 300 mg of dried, pulverized material were defatted twice with 5 mL *n*-hexane (AnalaR NORMAPUR ACS, ≥ 95%). The remaining material was extracted successively with 7 mL CH_2Cl_2 (GPR RECTAPUR, ≥ 99%) and 13 mL MeOH (AnalaR NORMAPUR ACS, ≥ 99.8%) at RT under sonication for 15 min. The extracts were filtered, combined and dried under vacuum. For tannin depletion, the dried CH_2Cl_2-MeOH extract was restored in 4 mL MeOH, loaded onto a 3 mL solid-phase extraction cartridge (phenomenex, AH0-7001) filled with polyamide gel (CC-6; 900 mg) and washed two times with MeOH. The tannin-free sample was dried again under vacuum to deliver the final extract. Dried small-scale extracts were dissolved in DMSO (Rotipuran ≥ 99.8%, p.a.) to a final concentration of 10 mg/mL. Samples were stored at -20 °C until used.

***Caenorhabditis elegans* strains, maintenance and synchronization.** *Caenorhabditis elegans* wildtype var. Bristol N2, the mutant SS104 *glp-4(bn2ts)*, and *E. coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota). OP50 were grown in LB medium for eight hours at 37 °C, harvested through centrifugation, washed with double distilled water (ddH_2O) and were air-dried. Then bacteria were suspended in S-complete medium at a concentration of 100 mg/mL. Flasks were stored at 4 °C until use. Hermaphrodite animals were maintained on nematode growth medium (NGM) agar plates seeded with 200 µL of OP50 solution at 16 °C according to standard protocols[93]. For maintenance worms were transferred to new plates every week and cultures were monitored on a regular basis. For preparation of a synchronized worm population N2 worms were chunked three days, SS104 worms were chunked four days before synchronization. Synchronization was performed by bleaching technique[39,94,95]. Briefly, worms were washed of the plates with ddH_2O and treated with bleaching solution for approximately 5–10 min. The lysis of the worms was controlled under a dissecting microscope. Then isolated eggs were pelleted and washed twice with M9 buffer and S-complete medium. Eggs were kept in S-complete medium for 42 h with gentle agitation and sufficient aeration until the synchronized population of nematodes hatched.

Experimental procedures. *Survival assay.* Survival experiments of wildtype *C. elegans* strain N2 in 96-well microtiter plates were performed as previously described[39] with some alterations: since nematodes grow faster at higher temperatures[49], we performed our survival experiments at 25 °C, we omitted the use of antibiotics and antimycotics and we tested our samples with a final concentration of 1.00% DMSO as a solubilizing agent of extracts. Briefly, 5–18 age-synchronized L1 nematodes were transferred by pipetting to 96-well microtiter plates with 6 mg/mL air dried OP50 in 120 µl S-complete medium, where they grow for 24 h at 25 °C until all worms reach the L3 stage. 5-Fluorodeoxyuridine (FUdR; 0.12 mM final; Sigma-Aldrich, F0503) is added in order to sterilize the worms and to keep the population synchronized[38,95]. The following day (day 0), test samples dissolved in DMSO were added to the sterilized adult worm culture. Reserpine 30 µM (Sigma-Aldrich, 83580) was used as positive control[59]. Nematodes were oxygenized every three days and 5 µL OP50 (c = 100 mg/mL) were added on day 5 of adulthood to prevent starvation. All assays were performed in parallel triplicates. To prevent evaporation, the outer wells were filled with S-complete medium and plates were sealed with parafilm. Plates were stored at 25 °C under light exclusion.

Data analysis survival assay. Raw data of the survival assay was recorded with MS Excel 2013 to keep track of living/dead population per well. Survival curves for each plate were determined based on the percentage of living nematodes per well plotted versus time. For each condition at least 3 wells per trial were used. The time point, when 50% of worms were dead/alive (DT_{50}) was determined upon survival curves. Mean DT_{50}s are given based on the survival curves obtained from three parallel experiments. The deviation of lifespan in comparison to the vehicle control is given as increase/decrease in percentage [DT_{50} extension/reduction (%)]. For better visualization of the DT_{50} extension/reduction all results were depicted as bar charts (GraphPad Prism 4.03). The data values were reported as the mean ± SD. In order to determine whether the differences between control and treated groups were statistically significant an ANOVA (analysis of variance) with Dunnett's post-test was performed. Significant activity is based on $p < 0.05$.

Nile red assay. Synchronized SS104 L1 worms were put on fresh agar plates and kept overnight at 16 °C, then transferred to 25 °C. Experiments were started at L4 stage. 3 to 10 worms were put into the wells of a 96-well plate in S-medium containing 10 mg/mL washed and air dried OP50 bacteria and 100 nM Nile red. Vehicle control and test samples were added to reach a final concentration of 1% DMSO. To prevent evaporation the outer wells were filled with S-complete medium and plates were sealed with parafilm. Worms were kept under light exclusion at 25 °C for 5 days. Worms were paralyzed for image acquisition with NaN_3.

Image acquisition and processing. A Zeiss Z1 Axio Observer inverted fluorescence microscope equipped with a Rhodamine filter and an Axio Cam MRm system was used for imaging. Every living worm was recorded using the same settings and saved as tiff-image in RGB format. The open source software ImageJ[96] was used for image processing and quantification of fluorescent units. At first, the tiff-images were converted into 8-bit grey scale images and subsequently brightness and contrast adjusted to a mean of 1.2 and a standard deviation of 6.6. For segmentation of the image, the plug-in "Trainable Weka Segmentation"[97] was used to classify every pixel of the image into "worm" or "background". Prior performing the classification in ImageJ, the machine learning algorithm *J48 pruned tree classifier*[98] was trained based on the results of an entropy filter as basis for the classification decision. For an improvement of the results and avoidance of false-positive areas like bacteria and artefacts belonging to the class "worm", all areas smaller than 3,000 pixels were deleted, followed by manual control.

Subsequently the improved results of the classification process were multiplied with the normalized versions of the original images. Binarization of the images was performed with the default threshold-function in ImageJ, resulting in an image that can be quantified. Finally, the total area of white pixels per image were assigned to the fluorescent units of a single worm.

Data analysis of the Nile red assay. Mean fluorescent units of worms that have been treated with one condition, repeated in 5–6 replicates, were calculated. The mean fluorescence intensity of vehicle treated control animals was set to 100% and values were expressed as % of control. The presented values show the mean of at least three independent biological experiments. GraphPad Prism 4.03 software was used for all statistical analyses. In order to determine whether the differences between control and treated groups were statistically significant an ANOVA (analysis of variance) with Bonferroni post-test was performed.

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Author contributions

J.Z. performed the survival experiments; B.K. and T.L. performed the Nile red experiments; B.K., A.T. and T.L. developed the image acquisition and processing method; J.Z., B.K., D.P. and J.R. designed the experiments. J.Z., B.K., T.L. and J.R. interpreted results. J.Z., B.K. and J.R. wrote the manuscript text and prepared all figures and tables. All authors reviewed the manuscript.

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Prolongation of metallothionein induction combats Aß and α-synuclein toxicity in aged transgenic *Caenorhabditis elegans*

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Neurodegenerative disorders (ND) like Alzheimer's (AD), Parkinson's (PD), Huntington's or Prion diseases share similar pathological features. They are all age dependent and are often associated with disruptions in analogous metabolic processes such as protein aggregation and oxidative stress, both of which involve metal ions like copper, manganese and iron. Bush and Tanzi proposed 2008 in the 'metal hypothesis of Alzheimer's disease' that a breakdown in metal homeostasis is the main cause of NDs, and drugs restoring metal homeostasis are promising novel therapeutic strategies. We report here that metallothionein (MT), an endogenous metal detoxifying protein, is increased in young amyloid ß (Aß) expressing *Caenorhabditis elegans*, whereas it is not in wild type strains. Further MT induction collapsed in 8 days old transgenic worms, indicating the age dependency of disease outbreak, and sharing intriguing parallels to diminished MT levels in human brains of AD. A medium throughput screening assay method was established to search for compounds increasing the MT level. Compounds known to induce MT release like progesterone, $ZnSO_4$, quercetin, dexamethasone and apomorphine were active in models of AD and PD. Thioflavin T, clioquinol and emodin are promising leads in AD and PD research, whose mode of action has not been fully established yet. In this study, we could show that the reduction of Aß and α-synuclein toxicity in transgenic *C. elegans* models correlated with the prolongation of MT induction time and that knockdown of MT with RNA interference resulted in a loss of bioactivity.

Alzheimer's-, Parkinson's-, and Huntington's or Prion diseases are age-dependent disorders which are characterized by an accumulation of misfolded proteins and neuronal cell death. All current approaches for the treatment of AD provide only temporary symptomatic relief and do not inhibit the underlying disease mechanisms because they have mainly been developed based upon a notion that has been dominating the AD field for the past two decades—'the amyloid cascade hypothesis'[1]. Several investigational drugs that target Aβ have failed to show any correlation between a reduction in amyloid burden and improvement of cognitive functions in large-scale clinical trials[2]. Recently it has been shown that the metalloprotein Aß becomes amyloidogenic upon treatment with stoichiometric amounts of Zn^{2+} and Cu^{2+}[3]. In the metal hypothesis[5], Bush and Tanzi claimed in 2008 that the interaction of metals with the major protein components of NDs like Aß, α-synuclein, huntingtin or prion proteins is the underlying cause of the corresponding diseases[4]. This is not merely due to increased (i.e., toxic) levels of metal exposure, but rather due to a breakdown in the homeostatic mechanisms that compartmentalize and regulate these metals[5]. Chemical agents that restore metal homeostasis could be effective drugs against ND[6]. It has been reported that calcium, copper, iron and manganese increase as a function of age in *C. elegans*, while potassium and phosphorus levels decrease. Further increases in dietary iron accelerated age-related accumulation of insoluble proteins. Metal chelation by CaEDTA attenuated proteotoxicity in an Aß expressing *C.*

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elegans model and promoted lifespan and health span[7]. The approved use of several medical chelators is limited to genuine situations of metal overexposure (e.g. Wilson's disease or lead toxicity) or rheumatoid arthritis because the removal of essential metal ions leads to serious adverse effects (e.g. iron-deficiency anaemia), and their use is any case complicated because chelators cannot cross the BBB due to their hydrophilic nature[8]. Bush and Tanzi developed small molecules with more sophisticated properties (e.g. metal-protein attenuation compounds (MPACs)) that serve as metal exchangers and ionophores[5]. The first-generation of MPACs was based on clioquinol (CQ; 5-chloro-7-iodo-8-hydroxyquinoline). CQ was initially shown to dissolve synthetic Aβ-Cu^{2+}/Zn^{2+} aggregates and amyloid deposits from post-mortem AD brain[9]. It is the prototype of the novel drug PBT2, which has been effective in Phase 2 clinical trials for AD and HD (10; 11). Both compounds translocate Cu^{2+} and Zn^{2+} into the cell thereby initiating neuroprotective signalling cascades like PI3K and upregulation of metalloproteases. Moreover neurite extension is promoted and dendritic spine density is increased. The mechanism of action prevents the effects of breakdown of metal homeostasis but also rectifies the misbalance[6]. A prominent response pathway involved in the chelation of metal ions involves MTs[12]. MTs are a heterogeneous superfamily of endogenous multipurpose proteins that participate in the transport, homeostasis, and detoxification of heavy metals[13]. While there is considerable variation in MTs within the animal kingdom, all MTs share similarities of being cysteine-rich, and showing a regulatory response to essential and non-essential metal exposure. The primary mode of action for MTs is formation of metal-thiolate bonds and subsequent removal of the metal from the cytoplasm[14]. In AD, rising metal concentration leads to reactions with Aß to form oligomers and aggregates. Metallothionein-III (MT3) released into the cleft by neighbouring astrocytes has the potential to ameliorate this adverse interaction, but this release is decreased in AD[5]. The metal-exchange process between Zn7MT-III and Aβ1-40 Cu^{2+} has recently been elucidated[15]. Many experimental data demonstrated that MTs have a close relation with neuroprotection and neurological diseases in mammals[16–19]. MT-III was shown to be markedly diminished in brains in AD, amyotrophic lateral sclerosis (ALS), PD, prion disease, brain trauma, brain ischemia, and psychiatric diseases[16]. The down-regulation of MT-III in patients as well as in a transgenic mouse model of AD has been proposed to alter copper homeostasis in the brain and then lead to extracellular amyloid pathology[20,21]. Double transgenic mouse models overexpressing MT-III and human SOD1 (modelling amyotrophic lateral sclerosis) exhibited normal levels of copper ions in spinal cords and showed prolonged survival with significant suppression of motor neuron death[22]. Effects of MT-III expression on ALS mice were also explored by using a retrograde viral delivery system[23]. Even when injection of the adenovirus encoding MT-III gene started at the mean age of disease onset in ALS mice (~20 weeks), MT-III expression was found to prevent further loss of motor neurons and prolonged lifespan. The importance of MT in maintaining metal homeostasis was further demonstrated in studies involving exposure to heavy metals in MT-1/2 knock out mice, which led to metal toxicity. In contrast, MT-1/2 overexpressing mice were relatively well protected from heavy metal toxicity[24]. In a study of Xu et al., the role of Zn_7MT3 to protect against AD was investigated by treating APP/PS1 mice with sustained drug release of Zn_7MT3 directly to the central nervous system. The results demonstrated that Zn_7MT3 can significantly ameliorate cognitive deficits, improve the morphology and function of hippocampus, regulate metal homeostasis, abolish Aß plaque load, and reduce oxidative stress and neuronal cell apoptosis in APP/PS1 transgenic mice. Therefore investigators assumed that Zn_7MT3 has potential for applications in AD therapy[25]. Many experiments showed that some agents (e.g. apomorphin, propofol) exert neuron protective effects mainly via up-regulation of MTs[16,26]. Treatment of SHSY-5Y cells with dexamethasone reduced Cu-dependent α-synuclein aggregates significantly by MT induction[27]. Miyazaki and colleagues showed that the expression of MT-III and its mRNA was up-regulated in the healthy aged rat brain. Lipopolysaccharide (LPS) treatment induced expression of MTIII and its mRNA only in young but not in aged rat brain regions. These results suggested that the reduced inducibility of brain MT-III against oxidative stress with aging is related to vulnerability and neurodegeneration of aged brain tissue[28]. There is strong evidence that activation of failed MT induction in age-dependent ND like AD or PD is a promising novel therapeutic target[29,30]. To find promising drug candidates against NDs based on the metal hypothesis many variables must be considered to conduct a single definitive assay: these include the complex interplay between metals, the magnitude of metal: protein interactions and the non-linear change in metals during aging and during the course of disease[6]. These factors are best explored by using a whole animal screening model.

The availability of transgenic mouse models is a major step forward in research for NDs, although the associated costs and ethical concerns are a clear drawback[31]. To bridge the gap between in vitro high throughput screening methods and the validation of compounds in mammalian models we used transgenic *C. elegans* models to answer the following question: Can we identify compounds against NDs, which exert their effect via prolongation of MT induction? Therefore, we implemented a robust *C. elegans* medium-throughput assay to monitor MT content during ageing and to investigate the influence of compounds on it. The fully sequenced genome of the nematode *C. elegans* contains two MTs: CeMT-1 is constitutively active in the pharyngeal bulb and CeMT-2 is mainly induced in intestinal cells[12,32]. *C. elegans* is widely used in studies of metal homeostasis, aging and NDs[7,33]. Results from age-related analyses of the metallome indicated that aging of *C. elegans* is associated with the accumulation of iron, copper and manganese. Supplementation with metals can affect worm physiology in different ways. Depending on the concentration, they are able to lengthen or shorten the lifespan and to decrease or increase the pathology of Aß transgenic worms. Iron supplementation enhanced toxicity in both Aß and PolyQ-associated models of protein aggregation[7]. Using transgenic *C. elegans* models of AD and PD we screened several compounds, which have been previously reported to be endowed with ND protective properties. In our experiments in the Aß expressing strain CL2120, where the content of GFP tagged MT can be visualized, the level of MT was increased until day 6 and is followed by a breakdown at day 8. In the healthy control strain CL2122, MT was only slightly induced (Fig. 1). This led to the question of whether the accumulation of metals and the decrease of MT content with ageing might be responsible for the outbreak of AD or PD. To test this, we established a *C. elegans* based medium-throughput screening assay to search for compounds able to prolong

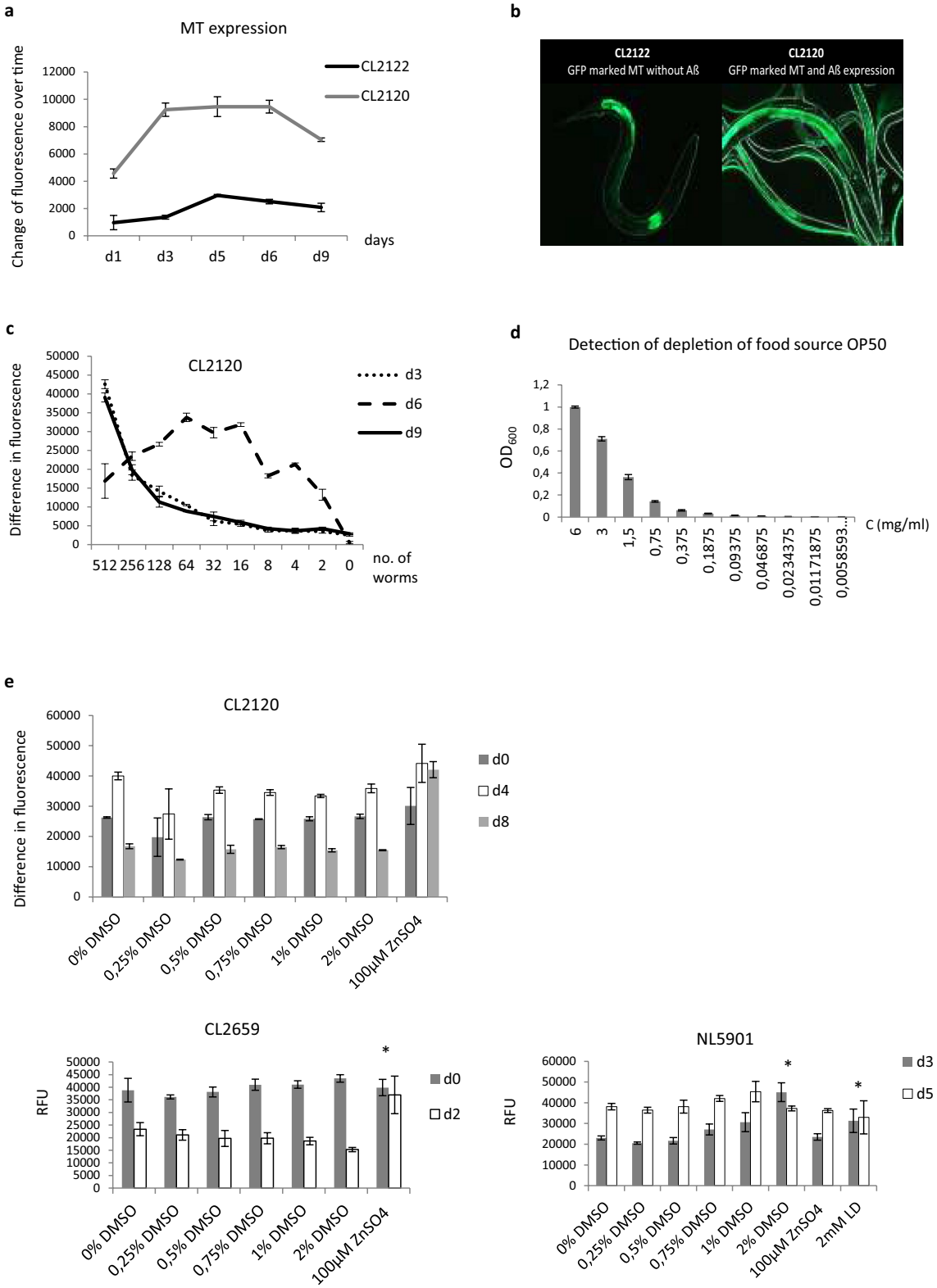

Figure 1. Preliminary tests for a MT medium throughput screening assay based on transgenic *C. elegans*. (**a**) Fluorescence of MT::gfp in strains CL2122 and CL2120 was measured by a fluorescence multiwell plate reader at em/ex 450/535. (**b**) Fluorescence in strains CL2120 and CL2122 was detected at day 6 by fluorescent microscope (magnification ×20). (**c**) Serial dilution (1:1) of CL2120 L4 larvae in a 96 well plate starting with 512 worms/well in triplicates. Fluorescence of reporter was measured after 3, 6 and 9 days. (**d**) Serial dilution (1:1) of *Escherichia coli* strain OP50 in a 96 well plate starting with a concentration of 6 mg/ml. Absorbance was detected by a multiwellplate reader at 600 nm. $OD_{600} = 0{,}8$ at a concentration of 5 mg/ml. (**e**) Influence of different DMSO concentrations on fluorescence of GFP reporter in strains CL2120, CL2659 and NL5901. *$p \leq 0.05$; **$p \leq 0.005$.

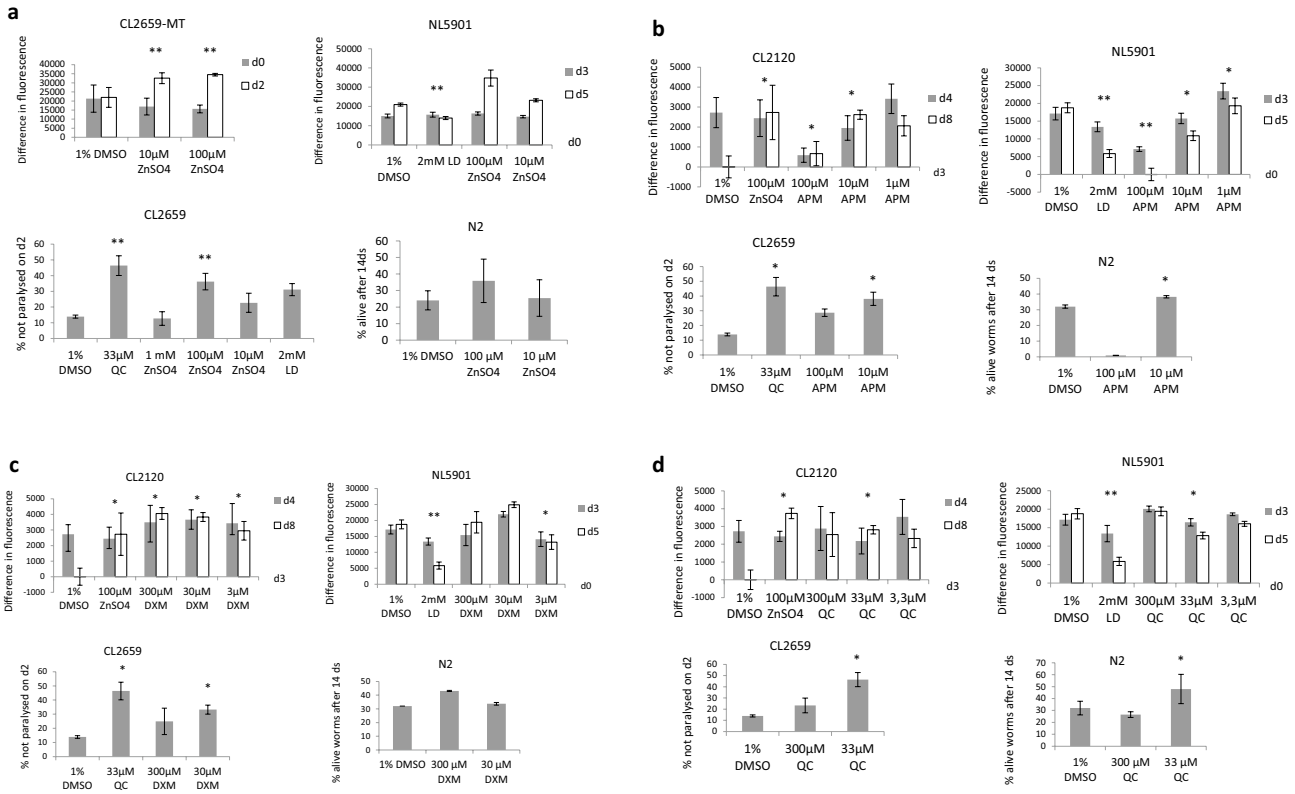

Figure 2. Influence of different concentrations of MT inducing test compounds on strains CL2120, CL2659, NL5901 and N2 was measured by fluorescence multiwell plate reader at em/ex 450/535. Each concentration was tested in triplicates. Change in fluorescence of test compounds was compared to vehicle control 1% DMSO in all assays. (**a**) $ZnSO_4$ (**b**) apomorphin (APM) (**c**) dexamethasone (DXM) (**d**) quercetin. Error bars show s.d. *p ≤ 0.05; **p ≤ 0.005; *QC* quercetin, *LD* levodopa, *d* day.

MT release in ageing worms. $ZnSO_4$, quercetin, apomorphin and dexamethasone induced MT, decreased proteotoxicity of Aß and α-synuclein in strains CL2659 and NL5901 and prolonged lifespan in the wild type strain N2 (Fig. 2). Compounds for novel neurotherapeutics against NDs, such as clioquinol (CQL) thioflavin T (Th T) and emodin decreased proteotoxicity by MT induction and all but clioquinol prolonged lifespan in the wild type strain N2 (Fig. 3). Sesamin was not able to induce MT (Table 1). Knockdown of MT even resulted in a loss of function of the bioactivity of emodin in the AD assay with strain CL2659 (Fig. 3, Table 2).

Results and discussion

Breakdown of metallothionein induction in aged Aß expressing worms. In 2002 Mijazaki et al.[28] showed that the expression of MT and its mRNA was up-regulated in the healthy aged rat brain, whereas treatment with lipopolysaccharide (LPS)-induced expression of MT only in young but not in aged rat brain regions. Based on this study, we monitored MT expression in transgenic worms with (CL2120) and without (CL2122) Aß expression. We could show that MT in healthy organisms slightly increases with age whereas in Aß expressing worms an intense induction in the young adults was followed by a breakdown during ageing (Fig. 1a–c) accompanied by an accumulation of iron, copper and manganese[7]. Therefore, we hypothesized that prolonging the time span of MT release might be a promising therapeutic target in NDs.

Establishment of a novel medium throughput screening assay method to search for compounds that prolong time of MT induction in aged transgenic *C. elegans* model of AD. To screen compounds for their ability to prolong MT induction we established a robust medium throughput screening assay based on *C. elegans* strain CL2120. CL2120 MT is GFP tagged and can be detected by fluorescence in a multiwell plate reader at em/ex 450–535 nm. By a serial dilution step in a multiwell plate, we determined the optimal worm density for a strong signal to be between 30 and 50 worms per well (Fig. 1c). The first measurement (day 0) was performed when larvae in the 4th stage were transferred from the petri dish to the 96 well plate containing media with compounds. The second time point indicated the highest MT level and was between d4 and d7. The last time point indicated the breakdown of MT induction between d7 and d9 (Fig. 1a, c). To guarantee optimal feeding of the worms throughout the whole experiment, we measured the optical density at 600 nm (OD_{600}) for the determination of the bacterial clearance. In this way, the change in optical density of bacteria over time was quantified and the necessary amount of *E. coli* OP50 was added based on the absorbance value[34]. The optimal concentration of the *E. coli* food source was determined to be 5 mg/ml with an OD600 of 0.9 (Fig. 1d). A higher concentration might be toxic, whereas less food induces caloric restriction which is known to

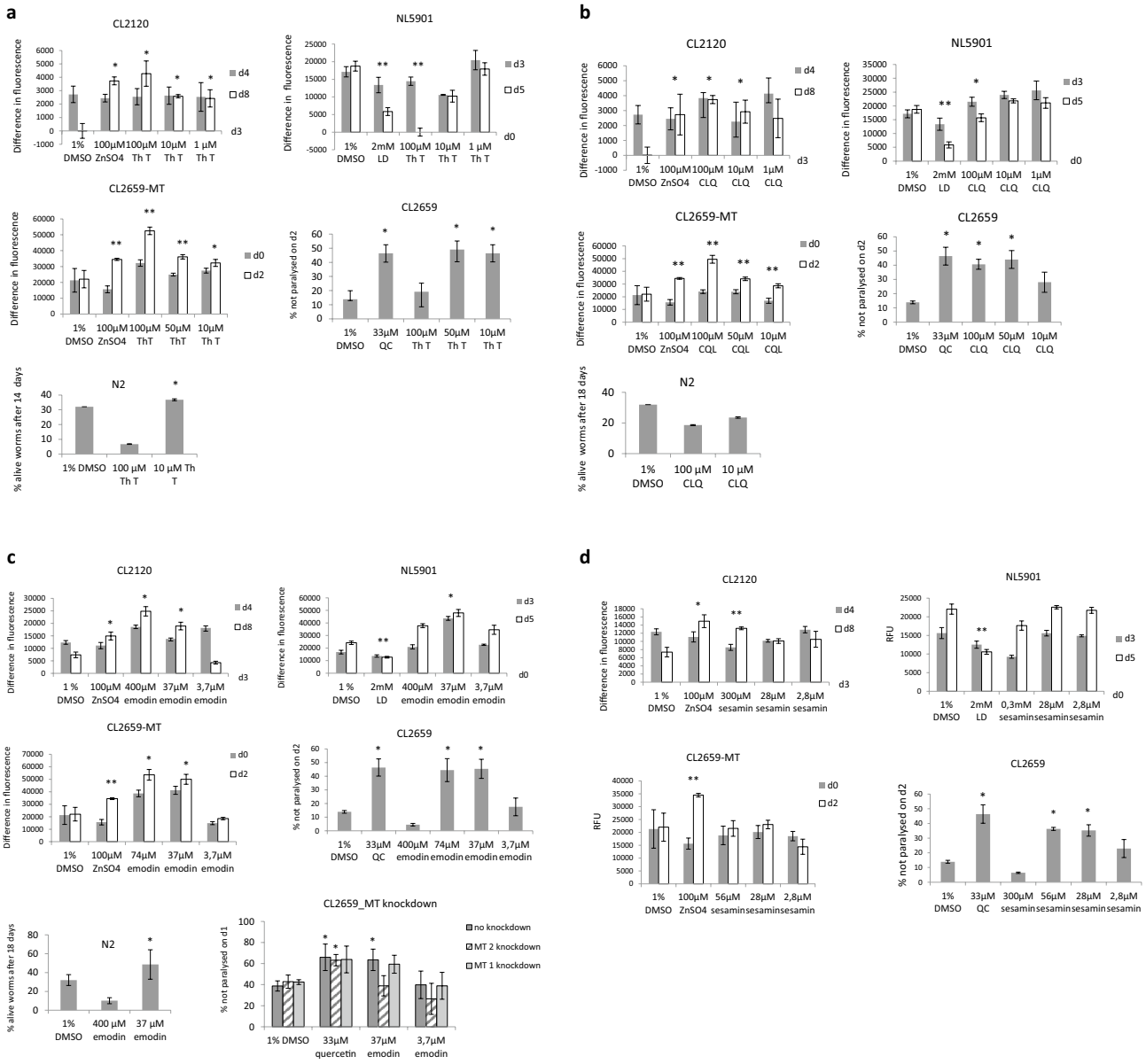

Figure 3. Influence of different concentrations of neuroprotective test compounds on strains CL2120, CL2659, NL5901 and N2 was measured by fluorescence multiwell plate reader at em/ex 450/535. Each concentration was tested in triplicates. Change in fluorescence of test compounds was compared to vehicle control 1% DMSO in all assays. (**a**) thioflavin T (Th T) (**b**) clioquinol (CQL) (**c**) emodin (**d**) sesamin. Error bars show s.d. *$p \leq 0.05$; **$p \leq 0.005$; *QC* quercetin, *LD* levodopa, *d* day.

interfere with several pathways[34]. To enable a broad range of chemically diverse test compounds to be dissolved for assaying, we have chosen DMSO for stock solutions to be diluted for the final test concentration. Accordingly, we screened several test concentrations of DMSO starting with 0, 1–2% in the MT assay and in assays of Aß and α-synuclein toxicity (Fig. 1e). Only a little effect was observed. When assaying α-synuclein toxicity with strain NL5901 the concentration of 2% DMSO turned out to be toxic. Therefore, all test compounds were dissolved in 1% DMSO.

Compounds known to induce MT protected worms against Aß- and α-synuclein toxicity and prolonged lifespan. For the assay evaluation procedure, we hypothesized that the efficacy of compounds in prolonging the time of MT induction in strains CL2120 or CL2659 will correlate with a reduction in Aß and α-synuclein toxicity burden.

MTs are mainly induced by heavy metals like Zn, Cu, Cd, Hg and others. Constantinidis and Burnet hypothesized that supplementation with zinc could prevent or delay the onset of dementia[35,36]. Many human trials were undertaken e.g. Constantinidis reported about improved memory, understanding, communication and social interaction of AD patients aged between 56 and 86 years when Zn was administered[11]. In our experiment with the transgenic strain CL2659 the supplementation of growth medium with $ZnSO_4$ was able to significantly increase

Compound	Strain				
	CL2120	CL2659	NL5901	CL2659-MT	N2
1% DMSO	1	1	1	1	1
100 µM $ZnSO_4$	0.013	0.015	0.778	0.002	0.738
10 µM $ZnSO_4$	n.t	0.124	n.t	0.011	0.017
2 mM levodopa	0.773	0.012	0.001	0.531	n.t
100 µM clioquinol	0.038	0.018	0.037	0.001	0.085
50 µM clioquinol	n.t	0.018	n.t	0.001	n.t
10 µM clioquinol	0.018	0.010	0.013	0.002	0.011
1 µM clioquinol	0.245	n.t	0.049		n.t
100 µM thioflavin T	0.011	0.018	0.000	0.000	0.003
50 µM thioflavin T	n.t	0.018	n.t	0.001	n.t
10 µM thioflavin T	0.035	0.010	0.201	0.036	0.009
1 µM thioflavin T	0.045	n.t	0.063	n.t	n.t
100 µM apomorphin	0.044	0.005	0.006	n.t	0.085
10 µM apomorphine	0.013	0.009	0.025	n.t	0.009
1 µM apomorphine	0.163	n.t	0.018	n.t	n.t
300 µM dexamethason	0.030	0.177	0.328	n.t	0.662
30 µM dexamethason	0.035	0.005	0.584	n.t	0.317
3 µM dexamethason	0.056	n.t	0.183	n.t	n.t
300 µM quercetin	0.067	0.128	0.149	n.t	0.051
33 µM quercetin	0.018	0.011	0.007	0.011	0.076
3.3 µM quercetin	0.136	n.t	0.010	n.t	n.t
0.3 µM sesamin	0.005	0.002	0.052	n.t	n.t
56 µM sesamin	n.t	n.t	n.t	0.342	n.t
28 µM sesamin	0.055	0.008	0.109	0.155	n.t
2.8 µM sesamin	0.965	0.123	0.339	0.138	n.t
400 µM emodin	0.008	0.0.003	0.0.014	n.t	0.087
74 µM emodin	n.t	0.023	n.t	0.011	n.t
37 µM emodin	0.018	0.015	0.018	0.040	0.020
3.7 µM emodin	0.002	0.432	0.187	0.014	n.t

Tabel 1. p-values of the Parkinson-, of the metallothionein-, of the paralysis- and the lifespan assay using student's t-test. *n.t* Not tested.

p-values/RNA interference with CL2659			
	No knock down	MT 2 knock down	MT 1 knock down
1% DMSO	1	1	1
33 µM quercetin	0.05	0.01	0.10
37 µM emodin	0.03	0.59	0.07
3.7 µM emodin	0.90	0.19	0.68

Table 2. p-values of the paralysis assay with RNA interference using student's t-test.

the MT level and even prolong timespan where MT is increased in Aß transgenic worms (Fig. 2a: CL2659-MT). Simultaneously we could show that lower concentrations (< 100 µM) decreased whereas higher concentrations (> 100 µM) increased the pathology of Aß (Fig. 2a: CL2659) and α-synuclein (Fig. 2a: NL5901) transgenic worms. 10 µM further prolonged the lifespan of wild type worms N2 (Fig. 2a: N2) Exposure to 100 µM and 200 µM $ZnSO_4$ caused cell death in cultured cortical neurons too[37]. Pre-treatment of cortical neurons with 20 µM apomorphin (APM), a dopamine receptor agonist, rescued them from Zn^{2+} toxicity in a dose- and time-dependent manner[37]. Apomorphine is used in clinics for the therapy of PD. It has pleiotropic biological functions because it is antioxidative and upregulates NGF synthesis in cultured mouse astrocytes[38]. Another study reported that apomorphine stimulates degradation of intracellular Aß in a mouse model of AD[39]. The compound also exerts protective effects on neurons mainly via up-regulation of MT[16]. 10 µM apomorphin increased MT in our assay with strain CL2120 too and protected worms from proteotoxicity in both Aß (Fig. 2b: CL2659) and α-synuclein (Fig. 2b: NL5901) assay. Furthermore, the same concentration even prolonged the lifespan in the wild type strain (Fig. 2b: N2).

Glucocorticoids like progesterone are able to induce MT expression[40]. Pre-treatment with the synthetic glucocorticoid analogue dexamethasone (DXM) suppressed the formation of α-synuclein cytoplasmic aggregates in neuroblastoma cells after incubation with copper[27]. In our experiments, 30 µM and 3 µM DXM prolonged MT induction (Fig. 2c: CL2120), reduced proteotoxic burden in strains CL2659 (Fig. 2c: CL2659) and NL5901 (Fig. 2c: NL5901) and 300 µM and 30 µM prolonged the lifespan in the wild type strain N2 (Fig. 2c: N2).

The flavonoid quercetin (QC) was able to induce MT in hepatoma cells and protected them against oxidative stress at a concentration of 10 µM[41]. In *C. elegans* quercetin prolonged the mean lifespan by 15% by increasing stress resistance[42]. Further studies showed that quercetin ameliorated Alzheimer's disease pathology and protected cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Extracellular β-amyloidosis, tauopathy, astrogliosis and microgliosis in the hippocampus and the amygdala have been decreased after treatment with quercetin[43]. QC at 33 µM prolonged timespan in which MT content was elevated (Fig. 2d: CL2120) and reduced proteotoxicity in our assays with strains CL2659 (Fig. 2d: CL2659) and NL5901 (Fig. 2d: NL5901). Further the same concentration significantly prolonged lifespan in the wild type strain N2 (Fig. 2d: N2). However, bioactivity of QC in the paralysis assay with CL2659 has not been lost after knockdown of MT with RNA interference (Fig. 3c: CL2659: MT knockdown). One explanation for this could be that QC mediated lifespan extension in *C. elegans* is modulated by age-1, daf-2, sek-1 and unc-43[44]. There has been shown that daf-2 and age-1 are not only responsible for longevity of *C. elegans* but further provide heavy metal resistance[45]. Therefore, we assume that MT induction is not the solely mode of action of QC in AD and PD transgenic nematodes.

Novel neuroactive lead compounds decreased proteotoxicity of Aß and α-synuclein by prolonging of MT induction in *C. elegans*. After assay evaluation, we investigated if the mode of action of thioflavin T (Th T), clioquinol (CQL), sesamin and emodin against Aß toxicity is based on prolonging the time of MT release. Alavez and colleagues showed that the amyloid binding compound Th T was able to maintain protein homeostasis during aging and extended lifespan and suppressed human Aß associated toxicity in *C. elegans* models depending on the protein homeostasis network regulator heat shock factor 1 (HSF-1), the stress resistance and longevity transcription factor SKN-1, molecular chaperones, autophagy and proteosomal functions[46]. Further treatment with Th T prevented Aß fibrillation in double transgenic AD mice[47]. In our study, Th T prolonged MT induction in both strains CL2120 (Fig. 3a: CL2120) and CL2659 (Fig. 3a: CL2659-MT) at concentrations between 1 µM or 10 µM and 100 µM. We could show for the first time that in the Parkinson assay 100 µM Th T was able to reduce α-synuclein (Fig. 3a: NL5901). In the paralysis assay performed with strain CL2659 (Fig. 3a: CL2659), 50 and 10 µM Th T prolonged the time until the paralysis phenotype indicating a reduction in Aß toxicity. Interestingly 10 µM Th T prolonged whereas 100 µM shortened the lifespan in the wild type strain N2 (Fig. 3a: N2).

CQL is the prototype of the novel drug PBT2, which is effective in phase 2 clinical trials for AD and HD[48]. CQL has been shown to be neuroprotective, by decreasing brain aggregate load and restored reduced insulin levels in R6/2 HD mice[49]. A double-blind phase 2 clinical trial demonstrated the efficacy of clioquinol treatment in producing effects on plasma Aβ and zinc ion (Zn^{2+}) levels. The drug was well tolerated and inhibited cognitive decline in patients who, untreated, otherwise experienced deterioration[50]. PBT2 delayed the onset of paralysis in a *C. el*egans model of PolyQ overexpression[51]. In our study 10–100 µM CQL induced MT release in strains CL2120 (Fig. 3b: CL2120) and CL2659 (Fig. 3b: CL2659). At 100 µM, CQL reduced α-synuclein fluorescence in strain NL5901 (Fig. 3b: NL5901). At 100 µM and 50 µM CQL significantly prolonged the time until paralysis in strain CL2659 (Fig. 3b: CL2659). Neither 100 µM nor 10 µM CQL were able to prolong the lifespan in the wild type strain N2. Both concentrations even shortened it (Fig. 3b: N2). We studied the gene expression of metallothionein-1 (mt-1) and -2 (mt-2) after the CQL treatment in strain CL2120. The mt-1 and mt-2 expression remained unchanged in treated worms showing that the increased MT-2 protein level observed in CL2120 strain (Fig. 3b) must be a result of post-transcriptional regulation (Fig. 4a, b).

Emodin is a bioactive antraquinone present in some prescriptions of traditional Chinese medicine for cerebral protection activity. Cognitive deficits of hyperhomocysteinemia rats were improved by emodin. The animals had better behavioral performances, so that in the hippocampi the neuron loss decreased and synapse-related proteins increased. Further Aß overproduction and tau hyperphosphorylation were eliminated by emodin[52]. Emodin inhibited zinc-induced neurotoxicity in neuroblastoma SH-SY5Y cells[53]. Further Aloe-emodin has been shown to be neuroprotective to NMDA-treated retinal ganglion cells by Cu–Zn superoxide dismutase[54]. Anthraquinone-2-sulfonic acid prevents death of primary neurons by mechanisms like caspase inhibition and AKT activation. This compound may be a lead to develop a novel neurotherapeutic antraquinone-based drug[55]. In our study, emodin induced and prolonged MT release at 400 µM, 74 µM and 37 µM in CL2120 (Fig. 3c: CL2120) and CL2659 (Fig. 3c: CL2659-MT). α-synuclein expression was decreased with 37 µM emodin (Fig. 3c: NL5901) and when treated with 74 µM and 37 µM emodin the time until Aß expression-induced paralysis was prolonged in strain CL2659 (Fig. 3c: CL2659). At the highest dose (400 µM), emodin induced MT (Fig. 3c: CL2120), but was toxic in both CL2659 paralysis assay (Fig. 3c: CL2659) and NL5901 based Parkinson assay (Fig. 3c: NL5901). Bioactivity of 37 µM emodin in the paralysis assay has been lost when MT 2 has been knocked down with RNA interference and has been reduced when MT 1 has been knocked down (Fig. 3c: CL2659 with MT knockdown). Further 37 µM emodin prolonged lifespan in the wild type strain N2 (Fig. 3c: N2). We also studied the mt-1 and mt-2 expression in emodin treated CL2120 strain (Fig. 4a, b). We found a modest increase in mt-1 expression (Fig. 4a). The mt-1 expression showed an increasing trend in three and five day treated worms, while a reverse trend was observed for mt-2 (Fig. 4c). Our observations suggest that mt-1 and mt-2 may act differently to regulate the metallothionein levels in worms.

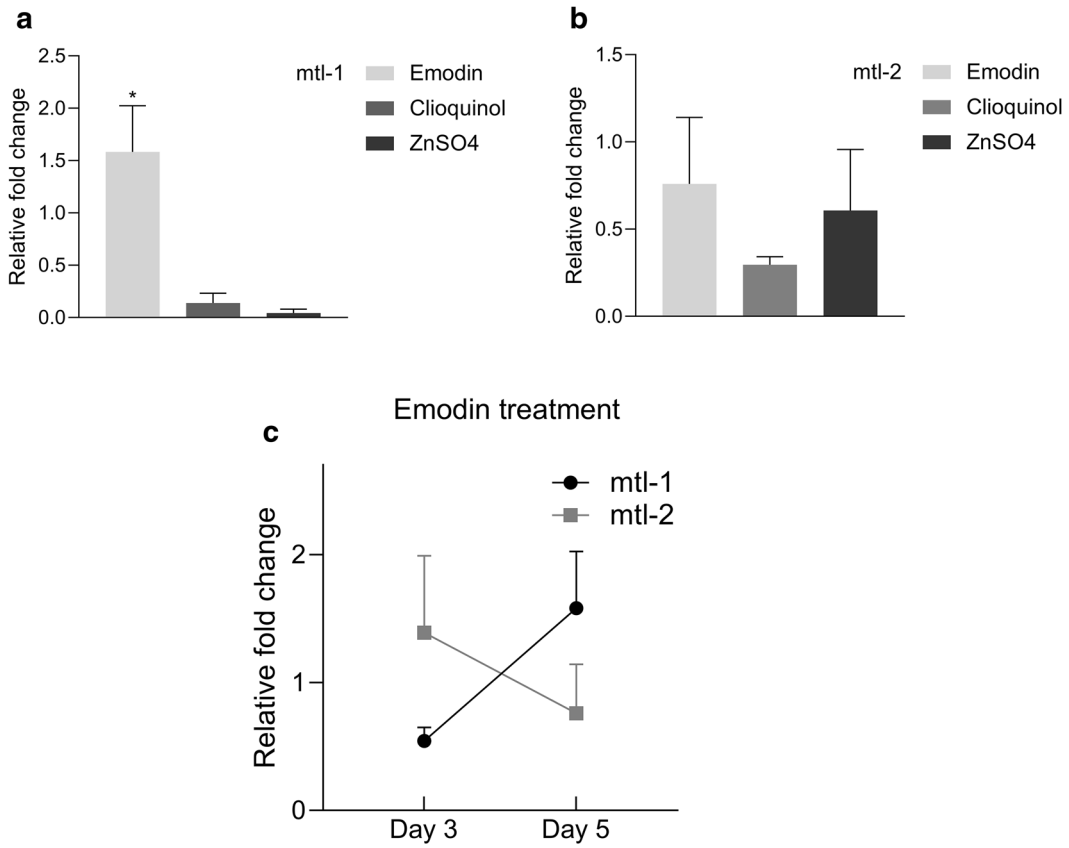

Figure 4. Relative expression of mt-1 (**a**) and mt-2 (**b**) following treatment with test compounds. 150 worms of CL2120 strain were treated with 40 µM emodin, 100 µg/ml clioquinol and 100 µM $ZnSO_4$. Relative fold change was measured using comparative ΔΔCT method. Gene expression data was normalized to rps-18. (**c**) 150 worms of CL2120 strain were treated with 40 µM emodin for a period of 3 and 5 days and quantitative RT PCR was performed to measure relative mt-1 and mt-2 expression. One-way ANOVA followed by Tukey's multiple comparisons test was used to measure statistical significance (*$p \leq 0.01$). All experiments were performed in triplicates. Data represents means ± SEM.

The test compound sesamin has been shown previously to act protective against Aß toxicity and to extend the lifespan in *C. elegans*[55]. We wanted to clarify if these effects were due to an increase of MT. Only a high dosage (300 µM) was able to induce MT expression (Fig. 3d: CL2120), but resulted in a significant toxicity in the PD assay (Fig. 3d: NL5901) and in the paralysis assay (Fig. 3d: CL2659). As reported before[55] 56 µM and 28 µM sesamin were protective against Aß toxicity (Fig. 1d: CL2659) in our study, but α-synuclein fluorescence was unaltered (Fig. 3d: NL5901). Therefore, we assume that sesamin is protective through another mechanism than MT activation.

The paralysis assays performed with strain CL2659 showed that knockdown of either MT-1 or -2, alone, did not worsen the paralysis phenotype (Fig. 3c). Furthermore, quercetin (33 µM) treatment rescued the paralysis phenotype after MT-1 and MT-2 knockdown in CL2659 strain. Emodin (37 µM) mediated rescue was partly affected after MT-1 knockdown and was completely abolished after MT-2 knockdown in CL2659 strain. These observations indicate that MT-1 and MT-2 may show functional redundancy, however further studies are required to evaluate this hypothesis. Also, pharmacological compounds may act differently to ameliorate the paralysis symptoms, and precise mechanism of actions will be addressed in future work.

Materials

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).

Strains. All strains in this work were provided from CGC (Caenorhabditis Genetic Stock Centre), University of Minnesota, St. Paul, U.S.A. OP50: Uracil auxotroph Escherichia coli strain. 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. This strain further expresses human Aß peptide. CL2122: dvIs15 [(pPD30.38) unc-54(vector) + (pCL26) mtl-2::gfp]. Control strain for CL2120. NL5901: pkIs2386 [unc54::α -synuclein::yfp unc-119(+). Expression of human a-synuclein fused to yellow fluorescent protein (YFP) in the body wall muscle of C. elegans, where it accumulates into "Lewy bodies" with increasing age. CL2659: dvIs770 [myo-3::Aß 1–42 wt::3′ UTR(long) + mtl-2::GFP]). N2: wild type strain. All substances were provided by Merck KGaA, Darmstadt, Germany.

Escherichia colis HT115(DE3) has been purchased from GE Dharmacon (www.horizondiscovery.com).

Methods

Maintenance of *C. elegans*. *Caenorhabditis elegans* were maintained according to the protocol of the CGC (Caenorhabditis Genetics Center), University of Minnesota, Minneapolis, MN 55455 USA. Cultivation and preparation of media and agar plates were done according the protocol of Stiernagle[56]. All worms are kept at 16 °C. *C. elegans* were growing on plates containing nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 (CGC) as described[57]. Large numbers of developmentally synchronized worms grew in solid culture and were harvested at L3 stage (CL2659) or L4 stage (CL2120, CL2122, NL5901). They were suspended at defined density in 96 well plates containing SOF medium (S-medium with OP50 and 5-fluorodeoxyuridine (FUdR)) and test compounds[58,59]. In vivo fluorescence was measured with a fluorescence microplate reader—phenotypical changes like paralysis with a stereo light microscope. In order to maintain an age-synchronized population we used the egg prep method from the CGC. To prevent the population from producing progeny, 5-fluorodeoxyuridine was used. In our procedure, a synchronized population is exposed to 60 µM FUdR just as it reaches sexual maturity[60].

Measuring the depletion of *E. coli* food source. OP50 were diluted 1:1 in liquid broth (LB) in a 96 well plate starting with a concentration of 6 mg/ml in triplicates and absorbance was measured at 600 nm (OD_{600})[34] (Fig. 1d).

Determination of optimal worm density and MT expression pattern. To follow protein expression in *C. elegans*, we used the transgenic strain CL2120, where MT is tagged to GFP and Aß is expressed. As control strain CL2122 was used. Real-time fluorescence intensity was measured with a fluorescence multiwell plate reader. For high throughput assays, the protocol of Leung et al.[59] was used, where defined densities of developmentally synchronized fluorescent worms were added to 96-well plates (Fig. 1c). To determine the optimal worm concentration we serially diluted CL2120 L4 1:1 in a 96 well plate starting with a concentration of 256 worms per well and measured the fluorescence at 450/535 nm daily.

MT assay with strain CL2120. 50 µl SOF medium were added to each well of a 96 well plate. 10 µl of compounds dissolved in 1% DMSO were added in triplicates in different concentrations. L4 larvae were harvested from the NGM agar plates and suspended in SOF medium. 40 µl of worm suspension were added to each well of the 96 well plates at the concentration of 30–50 worms/well. MT expression was followed by measuring GFP at d0, d4, d8 by the fluorescent multiwell plate reader at 450/535 nm. OD_{600} was measured on d0 and d3 and d6 to observe depletion of food source. OP50 were added after 3 days at the time after the food source was usually depleted.

Assaying α-synuclein toxicity with strain NL5901. The handling for the assays of alpha-synuclein toxicity followed the same protocol as used in the MT assay.

α-Synuclein expression was followed by measuring GFP at d0, d3 and d5 by the fluorescent multiwell plate reader at 450/535 nm. OD_{600} was measured on d0 and d3 to observe depletion of food source. OP50 were added after 3 days because this was the time after which food source was usually depleted.

Assaying Aß toxicity CL2659: paralysis assay. In this strain Aβ expression can be induced by temperature upshift in muscle cells[59]. A correlation between the increase of neurotransmission and progression of paralysis has been reported previously[62]. This strain has wild-type movement at the permissive temperature of 16 °C but becomes paralysed upon temperature upshift to 25 °C within approximately 48 h in liquid culture. Treatments that inhibit Aβ toxicity in this model (e.g. exposure to *Ginkgo biloba* extracts[65] alter the rate of paralysis in these worms. In accordance to the work of Dostal et al.[63] with some alterations, we used the screening protocol for measuring the rate of paralysis. The inducible Aβ expression does not lead to amyloid deposits and the paralysis phenotype appears independent of amyloid deposition[64]. Therefore, the acute toxicity of induced Aβ expression resulting from the accumulation of soluble oligomeric Aβ can be measured. Simultaneously, real time MT expression was followed by GFP fluorescence. For a high throughput screening method 50 µl SO medium were added to each well of a 96 well plate. 10 µl of compounds dissolved in 1% DMSO were added in triplicates in different concentrations. L3 larvae were harvested from NGM plates and suspended in SO medium. 40 µl of worm suspension were added to each well of the 96 well plates in the concentration of 10–20 worms/well. Aβ transgene expression in muscle cells was induced by temperature upshift from 16 to 25 °C and lasts until the end of the paralysis assay. Usually on d0 (before temperature upshift) and d2 (48 h after temperature upshift) the number of paralysed worms was scored under the dissecting microscope. The percentage of non-paralysed worms on d0 and d2 is shown in a bar graph using MS-Excel 2010.

MT induction in CL2659-MT. Fluorescence of GFP-tagged MT expressing worms in the paralysis assay was measured on d0 and d2 by the fluorescent multiwell plate reader at 450/535 nm and MS-Excel 2010 was used for all calculations and plotting of data. The changes in fluorescence between wells with worms treated with the test compound and vehicle control on two different days were analysed using two tailed Student's t-test (n = 10–20/well).

Lifespan assay with N2. The lifespan assays were performed with some alterations according the protocol of Solis and Petraschek[65]. L4 worms were washed from the agar plates, pelleted and transferred to a 96 well plate containing S-medium with OP50, FUdR and compounds in triplicates. Vehicle control contained 1% DMSO. Immediately number of worms were counted per well. Usually we used 10–20 worms/well. Counting after transferring worms to the 96-well plate marked time point 0. Counting of living worms was repeated after 18 days. After 6 days of transferring bacterial food source OP50 were added. After 18 days percentage of compound treated living worms were compared to worms from the vehicle control.

MT 2 and MT 1 knockdown with RNA interference. Induction of RNA interference by feeding was performed according the protocol of Conte et al.[67]. Clones carried in E. coli HT115(DE3) were purchased from GE Dharmacon (www.horizondiscovery.com) . Clone Id for MT 1 is: K11G9.6 ORF and clone Id for MT 2 is T08G5.10 ORF. Synchronized L1 larvae of CL2659 were transferred to agar plates inoculated with the *E. coli* strain carrying the double stranded RNA for RNA interference. L3 larvae were harvested and paralysis assay was performed as usual.

Gene expression analysis. Total RNA was extracted using TRIzol reagent[68] (ThermoFisher scientific, Austria). Quantitative RT PCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories G.m.b.H., Austria) as per manufacturer's instructions. Complementary DNA (cDNA) was synthesized with the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs GmbH, Germany) using primers described by Chiang et al.[31]. Gene expression was standardized against rps-18 and comparative ΔΔCT method was used to measure the relative gene expression changes. All the experiments were performed in triplicates.

Statistical evaluation of paralysis. Raw data of the paralysis assay were analysed in Excel to keep track of paralysed populations in each well. For each well the coordinates in the plate, strain, drug and the total number of animals paralysed on day 0 (d0) and day2 (d2) were recorded. For generation of bar graphs, the median fraction of non-paralysed worms was given as percentage at d0 and d2. Compound treated and vehicle treated worms were compared using two tailed Student's-test (n = 10–20/well).

Statistical evaluation of gene expression. For gene expression analysis, Graphpad Prism 8 was used to perform One-way ANOVA followed by Tukey's multiple comparisons test.

Fluorescence analysis. The fluorescence intensity of each well was measured with a microplate reader with the appropriate emission and excitation wavelength (Filter for our assay: GFP 450/20ex 535/20ex). The difference of fluorescence increase or decrease between compound treated wells and vehicle control on two different days was analysed using two tailed Student's t-test (n = 30–50/well).

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

D.P. designed and carried out the experiment, interpreted the results and wrote the manuscript. A.K., T.H. and A.S. designed the qPCR and A.K. further carried out the experiment and interpreted the results of qPCR. J.R., A.S.,T.W., L.K.,M.G., M.M., and A.P. supported the work.

Competing interests

The authors declare no competing interests.

Additional information

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Effects of Azepine-Indole Alkaloids from *Psychotria nemorosa* on Neurodegenerative Disorders Evaluated in Transgenic *Caenorhabditis elegans*

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Nemorosine A (**1**) and fargesine (**2**), the main azepine indole alkaloids isolated from the *Psychotria nemorosa* alkaloid fraction (PNAF), were explored for their pharmacological profile on neurodegenerative disorders (NDs) applying a combined *in silico – in vitro – in vivo* approach. By using **1** and **2** as queries for similarity-based searches of the ChEMBL database, structurally related compounds were identified to modulate the 5-HT_{2A} receptor; *in vitro* experiments confirmed an agonistic effect for **1** and **2** (24 and 36% at 10 μM, respectively), which might be linked to cognition-enhancing properties. On *C. elegans* transgenic strain CL4659, which expresses A*ß* in muscle cells leading to a phenotypic paralysis, PNAF (at 10 μg/mL), **1**, and **2** (at 10 and 100 μM) reduced A*ß* proteotoxicity by increasing the percentage of unparalyzed worms up to 49%. Both alkaloids were further able to significantly extend the time of metallothionein induction, which is associated with reduced neurodegeneration of aged brain tissue. An NL5901 strain, in which α-synuclein is YFP-tagged, was used as Parkinson's disease model. PNAF (10 μg/mL), and both alkaloids (10 μM) significantly reduced the α-synuclein expression. These results add to the multi-target profiles of **1** and **2** and corroborate their potential in the treatment of ND.

Indian tribes from Amazonia have been using leaves from *Psychotria viridis* (in combination to *Banisteriopsis caapi*) for the preparation of Ayahuasca, a decoction applied in rituals for the treatment of various health problems, including mental diseases.[1] Since the early 1990s, research groups have investigated the use of Ayahuasca for the treatment of anxiety, depression, alcohol addiction, and neurodegenerative diseases (NDs).[2,3] The pharmacological properties of Ayahuasca are attributed to the indole alkaloids identified in the beverage: tryptamine derivatives from *P. viridis* and *β*-carboline derivatives from *B. caapi*.[1] Stimulated by the research on Ayahuasca, further *Psychotria* species became the subject of pharmacological and pharmacobotanical studies. Several indole alkaloids have been isolated and evaluated regarding their bioactivity on proteins related to Alzheimer's disease (AD) and Parkinson's disease (PD).[4–8]

AD and PD are multifactorial NDs; for which the etiology is poorly understood.[9,10] AD is characterized by cognitive impairment. Commonly applied medicines include cholinesterase inhibitors, e.g. donepezil, galantamine, and rivastigmine, the *N*-methyl-D-aspartate-receptor-antagonist memantine, and *Ginkgo biloba* extract EGb 761.[11,12] However, none of these therapeutics is able to alleviate the causative disorder, but only delay the progression of symptoms related to AD. For PD, the situation is similar. PD is characterized by motor deficits caused by the degeneration of dopaminergic neurons, therefore, several dopaminergic therapies are used as symptomatic treatment, e.g. levodopa (dopamine precursor), selegiline (monoamine oxidase B inhibitor), entacapone (catechol *O*-methyltransferase inhibitor), and pramipexole (dopamine agonist).[13,14]

In recent years, the drug discovery paradigm has shifted from single-target pharmacology to the recognition and utilization of multi-target pharmacology, whereby clinical activity is expressed as the sum of several pharmacodynamic effects on multiple targets.[15] With increasing knowledge of drug-target interactions, more effective drugs, which modulate multiple targets, might be discovered for diseases such as PD and AD.[16,17]

Recently, it was demonstrated that the alkaloid fraction obtained from the leaves of *P. nemorosa* Gardner (PNAF) is able to inhibit the activities of butyrylcholinesterase (BuChE) and monoamine oxidase A (MAO-A).[5,8] Chemical analysis led to the isolation of several azepine-indole alkaloids, including nemorosine A (**1**), and fargesine (**2**) as active compounds, among others.[5]

This study has two objectives:

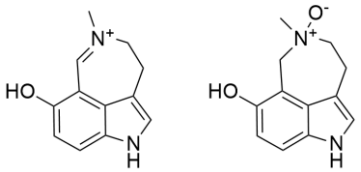
(i) To broaden knowledge of the potential multi-target effect of representative alkaloids from *P. nemorosa*. Thus, an *in silico* study was performed to predict bioactivity profiles of **1** and **2**. The results show that both compounds under investigation interact on the 5-HT_{2A} receptor.

(ii) To gain insight into the alkaloids' efficacy against NDs. For this reason, PNAF and its two main alkaloids, **1** and **2**, were tested in *C. elegans*-based AD and PD models.

C. elegans is a nematode with a rapid life cycle, transparent body (ideal for fluorescent markers), whose genome is fully sequenced with 60-80% homology with human genes. These features make *C. elegans* a suitable model to study human diseases.[18,19] Its applicability to study age-related diseases, such as neurodegeneration, has been highlighted in the literature.[16] Three transgenic *C. elegans* strains were used as models:

(1) CL2659 expressing A*ß* in muscle cells shows a paralysis phenotype after 48 hours at 25°C and was applied to study treatments that encounter soluble A*ß* oligomerization leading to a delayed onset of the paralyzed phenotype; (2) Strain NL5901 endowed with YFP-tagged α-synuclein was used as a PD model;. its expression can be monitored by a fluorescent reader; (3) Strain CL2120 with GFP-tagged metallothionein (MT) and constitutively expressed A*ß* was applied to investigate a functional mode of action. MT is an intracellular, low molecular, cysteine-rich protein participating in the transport, homeostasis, and detoxification of heavy metals. Its expression and regulation is related to progressive ND.[20]

Here we demonstrate that **1** and **2** are not only inhibitors of MAO-A and BuChE, but also agonists of the 5-HT_{2A} receptor. The isolates themselves and a fraction enriched with them (i.e., PNAF), showed a distinct positive effect on the three *in vivo* ND models, significantly reducing Aβ and α-synuclein proteotoxicity.



Results and discussion

To identify the putative targets of **1** and **2**, the alkaloids of interest were compared to a large set of compounds with measured bioactivities for a total of 4600 proteins. This bioactivity data set is a subset of the ChEMBL database[21,22] that was compiled and curated by some of us previously.[23] The screening engine ROCS[24] was employed for pairwise comparisons of aligned, 3D molecular shapes, taking also the alignment of

chemical features such as hydrogen bond donors, hydrogen bond acceptors and hydrophobic moieties into account. ROCS has previously been shown to perform well even in cases where the compounds of interest are only remotely related to compounds in the reference set.[25]

By screening **1** and **2** against the reference set, a rank-ordered list of the 4600 proteins covered by bioactivity data set was obtained. The top-20 targets are reported in Supplementary Tables S1 and S2. Intriguingly, for each compound, 14 out of the top-20 predicted targets are known to be of significance to the treatment of ND. These targets include indolamin-2,3-dioxygenase (IDO), poly[ADP-ribose]polymerase-1 (PARP1) and 5-hydroxytryptamine receptors (5-HTR). Moreover, the two known targets of **1** and **2**, MAO-A and BuChE, were assigned lower ranks, but still among the top 150. More specifically, MAO-A was ranked 90 and 45 for **1** and **2**, and BuChE 105 and 137, respectively. The lower ranks of these targets can be explained by the fact that all the active compounds for these targets recorded in the reference set (and even in the unprocessed, complete ChEMBL database) are structurally clearly dissimilar from **1** and **2**.

The prediction of the 5-HTR interaction was considered particularly promising to us, given the good quality of 3D alignment of the alkaloids to known modulators of the 5-HT_2 receptor, such as 6-methoxy-*N*,*N*-dimethyl-1,3,4,5-tetrahydrobenzo[*cd*]indol-4-amine

(ChEMBL49309) (Figure 1). ChEMBL49309 has been reported as an antagonist on rat 5-HT_1 and 5-HT_2 receptors.[26] Accordingly, the isolated alkaloids were subjected to experimental evaluation on the (human) 5-HT_{2A} receptor in cellular functional assays.

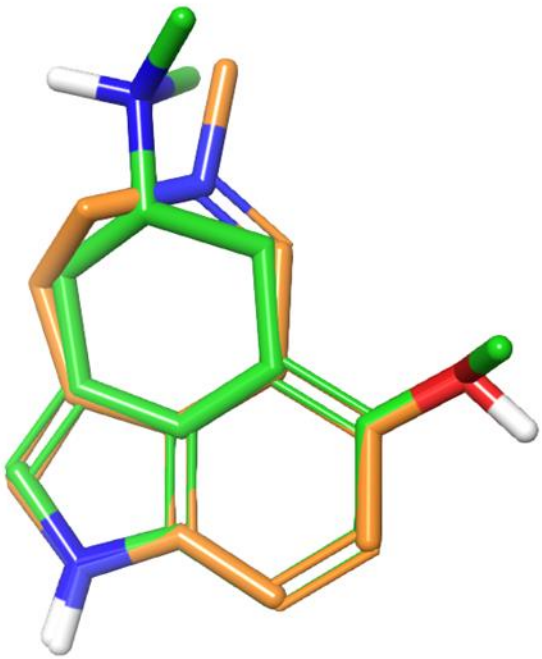


Figure 1. Nemorosine A (**1**) aligned with 6-methoxy-*N*,*N*-dimethyl-1,3,4,5-tetrahydrobenzo[*cd*]indol-4-amine (ChEMBL49309), a known antagonist of the 5-HT_1 and 5-HT_2 receptors.

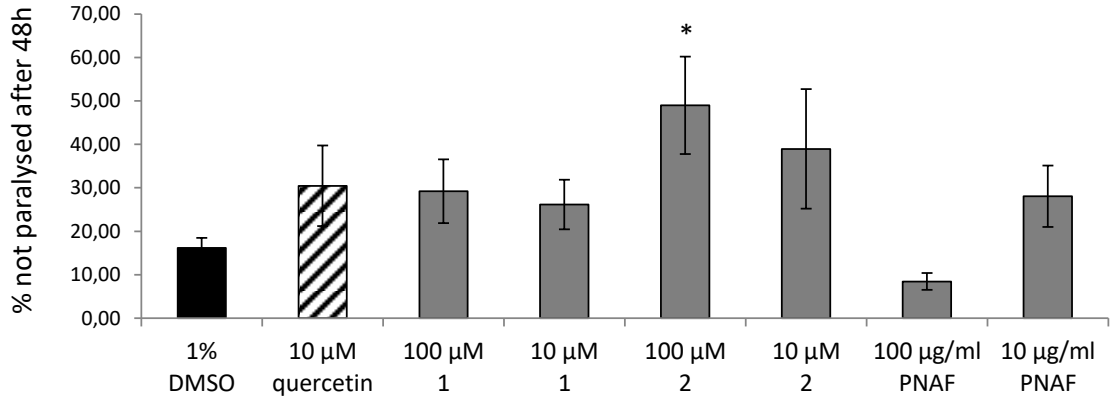
Agonistic and antagonistic effects were evaluated for nemorosine A (**1**) and fargesine (**2**) using functional assays on 5-HT_{2A} receptors at concentrations ranging from 0.01 to 10 μM. No significant antagonistic effect was observed for the isolated alkaloids. On the other hand, **1** and **2** (at 10 μM) exhibited agonistic effects on the 5-HT_{2A} receptor in the order of 24.0 ± 2.2% and 35.0 ± 2.4% of serotonin response, respectively. 5-HT_{2A} is widely distributed in the brain, and agonists are related to cognitive-enhancing and hallucinogenic activities. However, it was demonstrated that the neocortical 5-HT_{2A}

receptor binding is decreased in patients with AD, which is correlated to the cognitive impairment.[20] This result reinforces the hypothesis that the 5-HT_{2A} receptor might be an important target in AD treatment.

With the exception of 5-HT_3, serotonin receptors are G protein-coupled receptors (GPCR) with an important role in neurotransmission; this serotoninergic signaling system is largely conserved between nematodes and humans.[27,28] In *C. elegans* it plays an important role in pharyngeal pumping, locomotion, and learning, and regulates longevity and behavioral aging.[27,29,30] Several 5-HT-sensitive GPCR are known from *C. elegans*: SER-1 is likely coupled to a Gαq protein and a Ca^{2+} mediated signaling pathway and resembles 5-HTR_2.[31,32] SER-4 attenuates and SER-7 stimulates adenylate cyclase activity upon serotonin binding, resembling 5-HTR_1 and 5-HTR_7, respectively.[33,34]

The predicted and experimentally confirmed target profiles prompted us to evaluate the isolated alkaloids and PNAF for their effects in ND models of *C. elegans*. As a first step, PNAF as well as **1** and **2** were evaluated on strain CL2659. Unlike the human amyloid precursor protein (APP) gene, the *C. elegans* homologue gene apl-1 cannot produce the neurotoxic peptide A*β*. The strain CL2659 has been engineered by Link et al.[35] to inducibly express A*β* upon temperature upshift in muscle cells, which causes a paralyzed phenotype within 48 hours. Treatments that counter A*β* toxicity in this model, e.g. by exposure to *Ginkgo biloba* extracts,[36] modify the rate of paralysis in these worms. In our experiments, PNAF at 10 μg/mL and the two alkaloids **1** and **2** at 10 μM and 100 μM, significantly reduced Aß proteotoxicity in *C. elegans* similar to our positive control quercetin (10 μM), and increased the percentage of non-paralyzed worms (Figure 2 and Supplementary Table S3).

A


Alzheimer Assay
% not paralysed after 48h
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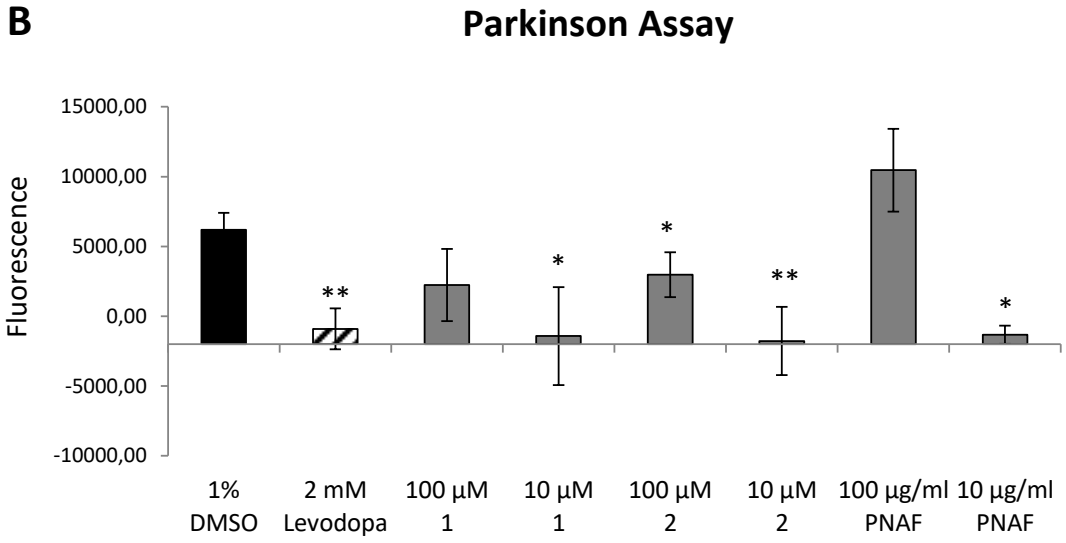
100 µM 1
10 µM 1
100 µM 2
10 µM 2
100 µg/ml PNAF
10 µg/ml PNAF

B


Parkinson Assay
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100 µM 1
10 µM 1
100 µM 2
10 µM 2
100 µg/ml PNAF
10 µg/ml PNAF

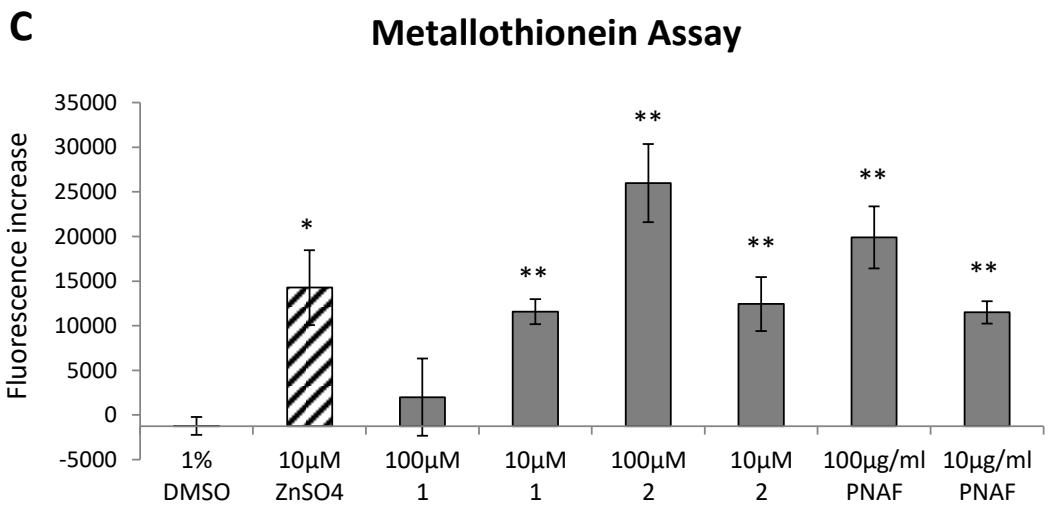

Figure 2: A) Alzheimer assay with strain CL2659. Bars represent % of non-paralyzed worms in vehicle control group (1% DMSO), positive control group (quercetin, 10 μM) and treatment groups 48h after Aβ induction, as mean ± SD of three parallel experiments. Significance was assessed by student´s t-test (*, $p<0.05$).

B) Parkinson assay with strain NL5901. Fluorescence of YFP-tagged α-synuclein was measured by multi-well plate reader and the increase/decrease of fluorescence between day 3 and day 6 is shown. Bars represent the mean fluorescence change of three parallel experiments expressed as % of control worms ± SD. Significance was assessed by student´s t-test (**, $p<0.01$; *, $p<0.05$).

C) Metallothionein assay with strain CL2120. Bars represent the mean GFP derived fluorescence changes between day 3 and day 6 derived from worms treated either with vehicle control (1 % DMSO), positive control ($ZnSO_4$, 10 μM) expressed as % of control worms ± SD. Significance was assessed by student´s t-test (**, $p<0.01$; *, $p<0.05$).

The stimulation of serotonin receptors has previously been reported to play an important role in astrocytes by the upregulation of MTs, leading to a neuroprotective effect.[37,38] Sequeira et al.[39] observed a correlation between a decreased expression of both 5-HT_{2A} receptors and MTs in mood disorder patients. MTs are divided into four isoforms.[40] These proteins play an important role in the transport, storage, and homeostasis of some essential metal ions, such as Zn^{2+} and Cu^{2+}, as well as the detoxification of heavy metals.[20,40,41] It has been observed that MT-1 and MT-2, protective factors against neuronal damage and ROS, are overexpressed in patients with AD.[40] On the other hand, MT-3 expression is controversial.[20,40–42] MT-3 also exerts important protective effects. However, this isoform seems to play a more complex role, since it is also involved, for example, in Aβ endocytosis in the astrocytes by modulating actin polymerization.[40,41] Recently, Pretsch et al.[20] demonstrated that the prolongation of MT induction time is correlated to a reduction of A*β* toxicity in *C. elegans*. They observed that, in Aβ-expressing worms, MT is overexpressed in young adults, but its levels are largely reduced during ageing. On the other hand, healthy worms, not expressing Aβ, show a discrete increase in MT levels with age. Therefore, the role of MT in the positive results derived from the paralysis assay was investigated, using the *C. elegans* transgenic strain CL2120, where MT is GFP0tagged and A*ß* is expressed constitutively. The treatment with PNAF (10 μg/mL), **1** and **2** (10 μM) significantly reduced A*β* proteotoxicity and prolonged MT induction time in *C. elegans* (Figure 2 and Supplementary Table S3), similar to the results obtained previously for quercetin, apomorphine and other known MT-inducers.[20]

MTs also play a neuroprotective role in PD.[37,38] It was demonstrated that MTs are overexpressed in astrocytes and reduced in neurons of PD patients.[43,44] In addition to the antioxidant role, MTs modulate the bioavailability of metals, such as Cu^{2+} which accumulates in the brain during ageing and binds to α-synuclein and aggregating as well as originating Lewy bodies.[44] These are markers of PD, localized mainly in dopaminergic neurons, hampering dopamine transmission.[45] Therefore, increasing the expression of MTs is hypothesized as a possible mechanism of action of disease modifying therapies.[44] Recently, Pretsch et al.[20] corroborated this hypothesis by demonstrating that the prolongation of MT induction reduces α-synuclein toxicity in *C. elegans*. For this reason, the effect of *P. nemorosa* alkaloids was evaluated on *C. elegans* transgenic strain NL5901 endowed with YFP-tagged *α*-synuclein. Similar to the positive control, i.e. levodopa at 2 mM, PNAF at 10 μg/ml, and the two isolated alkaloids **1** and **2**, at 10 μM, significantly reduced the α-synuclein toxicity after 6 days of treatment (Figure 2 and Supplementary Table S3).

To summarize, in this study a combined *in silico*, *in vitro* and *in vivo* approach was performed to assess the impact of *P. nemorosa* and its alkaloids nemorosine A (**1**), and fargesine (**2**) on ND. The data obtained from the different nematode models and the target-based assays corroborate the multifunctional profile of these azepine-indole alkaloids both in AD and PD. They modulate symptomatic[5] and disease modifying targets. The results of this study underline the potential therapeutic effects of the investigated *Psychotria* species and reinforce the importance of *Psychotria* genus as a promising source of chemical entities with new scaffolds for the search of bioactive compounds on targets related to ND.

Methods

Molecular target prediction. The ChEMBL database version 24 was downloaded and the chemical structures prepared following the protocol previously reported.[23] Compounds **1** and **2** were prepared accordingly. Next, 3D conformer ensembles were generated with OMEGA version 3.0.1.2 (all settings kept default),[46] and screening was performed with ROCS version 3.3.0 using the TanimotoCombo similarity measure (all settings kept default).

Human 5-HT_{2A} agonistic and antagonistic effects. The agonistic and antagonistic effects of **1** and **2** were evaluated by functional assays on 5-HT_{2A} receptors using HEK-293 cells. The assays were provided by Eurofins Cerep, using the protocols described previously.[47] Compounds were evaluated at 0.01, 0.1, 1.0, and 10 μM, using serotonin (for agonistic) or ketanserin (for antagonistic) as positive controls.

Chemicals. The alkaloid fraction PNAF was obtained from the leaves of *P. nemorosa* as previously described (Voucher number FURB41750 – Dr. Roberto Miguel Klein Herbarium; SisGen/Brazil code AA26CBC).[5] For nemorosine A (**1**), and fargesine (**2**), isolation and elucidation procedures were described before.[5] All other substances were obtained from Merck KGaA, Darmstadt, Germany.

Materials for *C. elegans* based assays. Zeiss Z1 Axio Observer inverted fluorescence microscope (Carl Zeiss, Austria), phase contrast microscope (Nikon TMS, Germany), incubator (Lucky Reptile, Austria), Bigger Bill Digital Orbital Shaker—

Barnstead Thermolyne M73735 (Clarkson laboratory & supply inc., USA), freezer 4°C (Liebherr ProfiLine, Germany), Multiwell Plate Reader - Tecan Infinite 200 pro, (Tecan AG, Switzerland) Freezer 16°C (Öko-Santo Superelectronic, AEG, Austria), All strains were obtained from CGC (Caenorhabditis Genetic Stock Centre), University of Minnesota, St. Paul, U.S.A. OP50: Uracil auxotroph *Escherichia coli* strain. 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. This strain further expresses human Aß. NL5901: pkIs2386 [unc54::α -synuclein::YFP + unc-119(+)]. Expression of human α-synuclein fused to YFP in the body wall muscle of *C. elegans*, where it accumulates into "Lewy bodies" with increasing age. CL2659: dvIs770 [myo-3::Aß 1–42 wt::3′ UTR(long) + mtl-2::GFP].

Sample preparation for *C. elegans* based assays. The alkaloid fraction PNAF and its isolated compounds were dissolved in DMSO and diluted with S-Medium to concentrations of 100 μg/ml and 10 μg/ml, or 100 μM and 10 μM with a final concentration of 1% DMSO. Positive controls were 2 mM levodopa for the PD assay, 10 μM quercetin for the AD assay, and 10 μM $ZnSO_4$ for the MT assay, each containing a final concentration of 1% DMSO, analogous to the test samples.

Cultivation of *C. elegans.* Cultivation of *C. elegans* has been done according to the protocol of the CGC. Media and agar plates have been prepared according to Pretsch et al.[20] All strains were grown on plates containing nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 (CGC) at 16°C as described. Age-synchronization has been done by the egg prep method from the CGC. Developmentally synchronized worms were harvested at L3 stage (CL2659) or L4 stage (CL2120,

NL5901) and suspended at defined density in 96-well plates containing SO medium (S-medium with 5mg/mlOP50, and for strains CL2120 and NL5901 60 μM 5-fluorodeoxyuridine (FUdR) to prevent the population from producing progeny) and test compounds.

MT and Parkinson assays with *C. elegans.* MT assays with strain CL2120 and PD assays with strain NL5901 have been performed according to the work of Pretsch et al.[20] Here we describe the procedure in short. Ninety μl worm suspension containing L4 larvae in SO medium at a concentration of about 50 worms/well was added to each well of a 96 well plate. Ten μl compound dilutions were added in triplicates in given concentrations. MT expression was followed by measuring GFP at day 0, 3, 6 and α-synuclein expression was followed by measuring YFP at day 0, 3 and 6 by the fluorescent multiwell plate reader at 450/535 nm. The difference in fluorescence increase or decrease between compound treated wells and vehicle control on two different days was analysed using a two-tailed Student's t-test (n = 30–50/well).

Alzheimer assay with *C. elegans.* For the AD assay we used the strain CL2659 where Aβ expression was induced by temperature upshift from 16°C to 25°C, which resulted in a paralysis phenotype within approximately 48 h in liquid culture. Treatments that inhibit Aβ toxicity in this model alter the rate of paralysis in these worms. In accordance to the work of Pretsch et al.[20], we used the screening protocol for measuring the rate of paralysis. Here the test procedure is described in short. Fifty μl SO medium was added to each well of a 96-well plate. Ten μl compound dilutions were added in triplicates. L3 larvae were harvested from NGM plates and suspended in SO medium. Forty μl worm suspension were added to each well of the 96-well plates at the

concentration of 10–20 worms/well. Aβ transgene expression in muscle cells was induced by temperature upshift. Usually on day 0 (before temperature upshift) and day 2 (48 h after temperature upshift) the number of paralyzed worms was scored under the dissecting microscope. For generation of bar graphs, the median fraction of non-paralyzed worms was given as percentage at day 0 and 2. Compound treated and vehicle treated worms were compared using two tailed student's-test (n = 10–20/well).

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

L.C.K.J. and D.P. designed and carried out the experiment, interpreted the results and wrote the manuscript. B.K., Y.C., and S.C. carried out part of the experiments (*in vivo*, *in silico*, and chemical assays, respectively). A.L.G., Y.V.H., P.C., J.K., A.T.H., and J.M.R. supported the study, as well as reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Supplementary Information

Tables S1 and S2 give the top 20 ranked targets for nemorosine A (**1**) and fargesine (**2**), based on ROCS screening of a curated ChEMBL subset.

Table S3 gives the results of the Parkinson and the Alzheimer assays with mean values, standard deviations and level of significance using Student´s t-test (n=3).

Effects of Azepine-Indole Alkaloids from *Psychotria nemorosa* on Neurodegenerative Disorders Evaluated in Transgenic *Caenorhabditis elegans*

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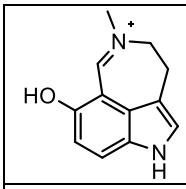
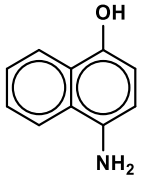
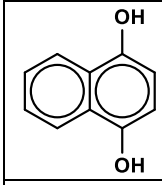
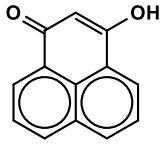
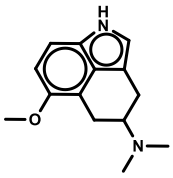
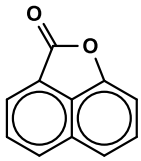
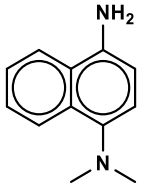
Table S1. Top 20 ranked targets for nemorosine A (**1**).

	**Chembl ID**	**TC score (1)**	**Target**	**Role**	**Significance against neurodegenerative disease**
	CHEMBL576321	1,60	IDO CHEMBL4685	tryptophan-metabolism	[1–3]
	CHEMBL206816	1,59	IDO1 CHEMBL1075294	tryptophan-metabolism	
	CHEMBL1446983	1,54	female germline-specific tumor suppressor gld-1 CHEMBL1293302	entry into meiosis (*C. elegans*)	-
	CHEMBL49309	1,52	5-HTR 2 CHEMBL2093870	serotonergic system	[4–7]
			5-HTR 1 CHEMBL2095159	serotonergic system	
	CHEMBL2323363	1,52	thymidylate synthase CHEMBL1952	DNA biosynthesis	-
	CHEMBL1998253	1,51	DYRK1A CHEMBL2292	cell cycle control cell differentiation	[8]
			NEK2 CHEMBL3835	cell cycle control	-
			LIMK1 CHEMBL3836	cytoskeleton architecture	-
			CLK4 CHEMBL1998253	spliceosome regulation	-
			CHECK1 CHEMBL4630	DNA damage response cell cycle control	-

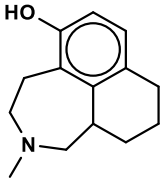
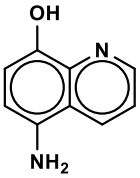
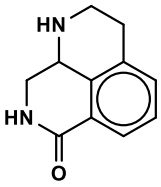
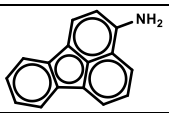
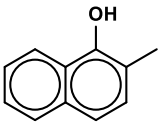
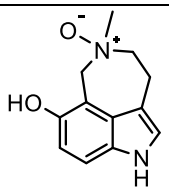
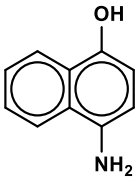
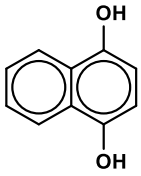
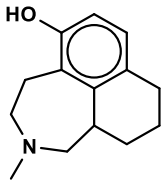
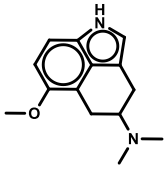
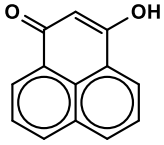
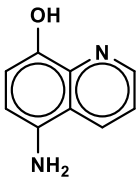
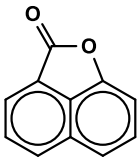
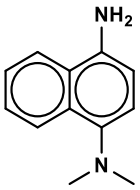
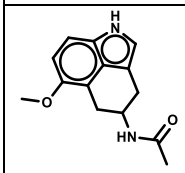
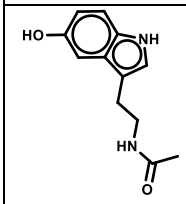
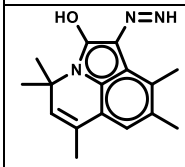
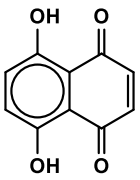
	CHEMBL1203602	1,51	adrenergic receptor α2 CHEMBL2093864	norepinephrine system	[9]
			5-HT1aR CHEMBL273	serotonergic system	[4–7]
	CHEMBL449298	1,51	TDO CHEMBL1075307	tryptophan-metabolism	[10,11]
			HSP90 CHEMBL2095165	chaperone	[12]
			proteasome macropain subunit MB1 CHEMBL4662	protein degradation	[13]
	CHEMBL608994	1,50	PARP1 CHEMBL3105	DNA repair	[14–16]
	CHEMBL103583	1,48	CRF-BP CHEMBL3885546	stress response	[17,18]
	CHEMBL122451	1.48	COX CHEMBL2096674	inflammation	[19,20]
		1,48	5-LO CHEMBL312	inflammation	[21,22]

Table S2. Top 20 ranked targets for fargesine (**2**).

	**Chembl ID**	**TC score (2)**	**Target**	**Role**	**Significance against neurodegenerative disease**
	CHEMBL576321	1,47	IDO CHEMBL4685	tryptophan-metabolism	[1–3]
	CHEMBL206816	1,47	IDO1 CHEMBL1075294	tryptophan-metabolism	
	CHEMBL1203602	1,44	adrenergic receptor α2 CHEMBL2093864	norepinephrine system	[9]
			5-HT1aR CHEMBL273	serotonergic system	[4–7]
	CHEMBL49309	1,44	5-HTR 2 CHEMBL2093870	serotonergic system	[4–7]
			5-HTR 1 CHEMBL2095159	serotonergic system	
	CHEMBL1446983	1,41	female germline-specific tumor suppressor gld-1 CHEMBL1293302	entry into meiosis (*C. elegans*)	-
	CHEMBL449298	1,40	TDO CHEMBL1075307	tryptophan-metabolism	[10,11]
			HSP90 CHEMBL2095165	chaperone	[12]
			proteasome macropain subunit MB1 CHEMBL4662	protein degradation	[13]

	CHEMBL2323363	1,38	thymidylate synthase CHEMBL1952	DNA biosynthesis	-
	CHEMBL1998253	1.38	DYRK1A CHEMBL2292	cell cycle control cell differentiation	[8]
			NEK2 CHEMBL3835	cell cycle control	-
			LIMK1 CHEMBL3836	cytoskeleton architecture	-
			CLK4 CHEMBL1998253	spliceosome regulation	-
			CHECK1 CHEMBL4630	DNA damage response cell cycle control	-
	CHEMBL61418	1.38	melatonin receptor CHEMBL2094268	entraining sleep/wake	[7,23]
	CHEMBL33103	1,37	melatonin receptor CHEMBL2095154	entraining sleep/wake	[7,23]
	CHEMBL1328384	1,37	streptokinase A	thrombolytic medication	-
	CHEMBL274056	1,37	5-LO CHEMBL2980	inflammation	[21,22]
			CDC25B CHEMBL4804	cell cycle control	-
			DUSP6 CHEMBL5511	negative feedback of MAPK pathway	[24]
			DUSP1 CHEMBL5623	negative feedback of MAPK pathway	

Abbreviations.

5-HTR, 5-hydroxytryptamine receptor; 5-LO, arachidonate 5-lipoxygenase; CHECK1, serine/threonine-protein kinase Chk1, CDC25B, dual specificity phosphatase CDC25B; CLK4, dual specificity protein kinase CLK4; COX, cyclooxygenase; CRF-BP, corticotropin-releasing factor receptor 2/corticotropin-releasing factor-binding protein; DUSP1, Dual specificity protein phosphatase 1; DUSP6 Dual specificity protein phosphatase 6; DYRK1A, dual-specificity tyrosine-phosphorylation regulated kinase 1A; HSP90, heat shock protein 90; IDO, indolamin-2,3-dioxygenase; LIMK1, LIM domain kinase 1; NEK2, serine/threonine-protein kinase NEK2; PARP1, poly[ADP-ribose]polymerase-1; TC, Tanimoto combo; TDO, tryptophan 2,3-dioxygenase

Table S3. Results of the Parkinson and the Alzheimer Assays with Mean Values, Standard Deviations and Level of Significance Using Student´s t-test (n=3).

	Concentration	**Alzheimer Assay**	**Parkinson Assay**	**MT Assay**
		% not paralyzed on day 2	**GFP Fluorescence**	**GFP Fluorescence**
Vehicle ctrl	1% DMSO	16,14 ± 2,33	6177,67 ± 1217,33	-1223,67 ± 991,08
Levodopa (pos ctrl PD)	2 mM	N.T.	-901,33 ± 1468,08**	N.T.
Quercetin (pos ctrl AD)	10 μM	30,48 ± 9,29	N.T.	N.T.
$ZnSO_4$ (pos ctrl MT)	10μM	N.T.	N.T.	14272,33 ± 4202,34*
PNAF	10 μg/mL	28,03 ± 7,07	-1329,67 ± 2614,34*	11497,33 ± 1255,69**
	100μg/mL	8,45 ± 1,96	10459,33 ± 3289,46	19902,33 ± 3474,99
Nemorosine A (**1**)	10 μM	38,96 ± 13,77	-1774,33 ± 658,18*	12432 ± 3016,67**
	100 μM	49,00 ± 11,20*	2970,67 ± 2960,7	25982 ± 4363,3**
Fargesine (**2**)	10 μM	26,17 ± 5,70	-1420,67 ±	11572,33 ±

			2441,97*	144,49**
	100 μM	29,20 ± 7,34	2237 ± 1601,9*.	1998 ± 4321,92

N.T.: not tested. * $p<0.05$; ** $p<0.01$

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Abnormal metal homeostasis as a common drug target to combat neurodegenerative diseases

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As life expectancy increases, the prevalence of age-related diseases will also increase. There has been an estimation that 50 million individuals are living with dementia in 2019. This number will increase to 75 million in 2030 and 131.5 million by 2050 (McGill-Carter, 2020). Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson´s disease (PD) and Huntington disease (HD) disproportionately affect older adult populations, inflicting a considerable physical, emotional, and economic burden.

Current treatments target only the symptoms, and not the causes, because for those disorders no target has yet been identified. In AD, several investigational drugs that target amyloid-β (Aβ) have failed to show any correlation between a reduction in amyloid burden and an improvement of cognitive functions in large-scale clinical trials. Elevated cortical Aβ concentrations were not solely responsible for the deposition of Aβ. Otherwise it would be difficult to explain why Aβ deposits are focal (related to synapses and the cerebrovascular lamina media) and not uniform in their distribution, especially because the amyloid precursor protein and amyloid-β are ubiquitously expressed. Moreover, to attribute Aβ accumulation to the presence of Aβ alone is problematic because the peptide is a normal component of healthy cerebrospinal fluid. Finally, whereas Aβ deposition is an age-dependent phenomenon, Aβ production does not appear to increase with age. So it is clear that for the search of new drugs against neurodegenerative disorders, other considerations have to be made.

We are used to think that neurodegenerative disorders are different clinical entities that target different brain regions and have separate pathology and symptoms. But when having a look towards the genetic or the molecular and cellular mechanisms, we should realize that several players and patterns appear consistently. All neurodegenerative disorders show an early vascular dysfunction, an accumulation of misfolded proteins, a selective sensitivity of specific neurons, and an activation of immune responses, to name but a few (Editorial, 2018). Accumulation of misfolded proteins in the brain is one of the most prominent pathological sign: extracellular oligomers of the peptide Aβ form the notorious plaques found in AD. The protein α-synuclein accumulates within dopaminergic neurons in PD and the misfolded huntingtin protein oligomerizes in HD.

Another commonality is that each of these proteins interacts with several kinds of metal ions *in vitro*, for example with copper or iron (Xiao et al., 2013). Under physiological requirements Cu and Fe control important mechanisms of the central nervous system like neurotransmitter synthesis, myelin production, oxygen transportation and synaptic signalling to name but a few. To achieve this, enzymes, receptors and neurotransmitters have to be regulated structurally and catalytically. Cu and Fe provide redox properties for most of these cellular functions, but on the other hand they are also a source for the formation of reactive oxygen species too. Consequently, reactive oxygen species promotes neurodegeneration through the oxidation, misfolding, and aggregation of particular proteins. Another fact is that ageing promotes accumulation of metals in brains of humans and mice (Acevedo et al., 2019).

Bush and Tanzi (2008) stated in the "Metal Hypothesis of Neurodegenerative Disease", that an abnormal metal homeostasis during ageing promotes deleterious metallic reactions in the brain and this may provide a more tractable therapeutic target to inhibit disease progression. Many preclinical and clinical studies showed that metal modifying complexes and chelators have therapeutic potential by reducing neurodegeneration and improving clinical symptoms (Acevedo et al., 2019). Copper chelation has been examined as a means to fight free-radical damage previously, with clioquinol shown to reduce aggregation of Aβ and α-synuclein in transgenic mice (Adlard and Bush, 2018). However there must be further mode of actions than merely chelation of clioquinol because the use of the relatively specific copper chelator D-penicillamine did not show any protection in a mouse model of PD, and in the presence of diethyldithiocarbamate, another effective copper chelator, enhanced neurotoxicity was observed in the same experimental model (Zhang et al., 2013). Clioquinol is the prototype of the novel drug PBT2. Both compounds translocate Cu^{2+} and Zn^{2+} into the cell, thereby initiating neuroprotective signaling cascades and consequently preventing the effects of breakdown of metal homeostasis (Adlard and Bush, 2018).

Metallothioneins (MTs) provide another possibility for the chelation of metal ions. MTs consist of a diverse superfamily of endogenous multipurpose proteins and their function is the transport, homeostasis, and detoxification of heavy metals. There has been shown that MT-III was markedly reduced in brains in AD, PD, amyotrophic lateral sclerosis, prion disease, brain trauma, brain ischemia, and psychiatric diseases (Zhang et al., 2013). Contrary, other work demonstrated that MTs were more highly expressed in Parkinsonian astrocytes (Michael et al., 2011). Miyazaki and colleagues unravelled the mystery by showing that the expression of MT-III and its mRNA was up-regulated in the healthy aged rat brain. After treatment with lipopolysaccharides, expression of MT-III and its mRNA has been increased only in young but not in aged rat brain regions. Therefore the scientists assumed that the reduced induction of brain MT-III against oxidative stress with aging is related to vulnerability and neurodegeneration of aged brain tissue (Miyazaki et al., 2000).

Therefore we hypothesized that drugs which are able to induce MT in aged model organisms of AD, PD or other neurodegenerative diseases could provide a novel promising therapy. But how can we easily evaluate the impact of compounds on MT induction in a high throughput screening assay?

In the search for new drugs, many variables must be considered if we want to conduct a single definitive screening assay which is based on the metal hypothesis: these include the complex interplay between metals, the magnitude of metal:protein interactions and the non-linear change in metals during ageing and during the course of disease (Barnham and Bush, 2014). These factors are best explored by using a whole animal screening model. The murine model Mus musculus is limited by its costs in large scale therapeutic screenings. By contrast, the model organism *C. elegans* is now gaining momentum as a host for screening tools. These nematodes combine genetic amenability, low cost and culture conditions

that are compatible with large-scale screens. Human diseases can be artificially engineered by expressing the human disease gene in *C. elegans*. By the use of such disease models, compounds which are able to suppress the disease phenotype can be identified after treatments. Hits can be further investigated for target identification by easily knocking down single genes using RNAi. In this way, new molecular mechanisms and targets could be found. With *C. elegans* we can bridge the gap between hit identification in cell based assays and the validation in mammalian models. *C. elegans* is widely used in studies of metal homeostasis, aging and neurodegenerative diseases. Results from age-related analyses of the metallome indicated that aging of *C. elegans* is further associated with the accumulation of iron, copper and manganese (Klang et al., 2014). There already exist many transgenic *C. elegans* models of neurodegenerative diseases which can be easily ordered from the Caenorhabditis Genetic Stock Center at the University of Minnesota. In our company, we use different transgenic *C. elegans* models for the initial screening and the mechanistic evaluation of potential new drugs for aging and neurodegenerative diseases. With the strain CL2659, we are looking for compounds which are able to reduce $A\beta_{42}$ toxicity because the expression of $A\beta_{42}$ in the muscle cells leads to a phenotypic paralysis after 48 hours. It has been shown that compounds that encounter AD prolong the time until paralysis (Pretsch et al., 2020). For the PD assay, we use the *C. elegans* strain NL5901, where α-synuclein is GFP-tagged and a reduction in α-synuclein burden can be monitored by a fluorescent reader. After screening several compounds in those models of neurodegenerative diseases, we found out that most hits in the AD screen were active in the PD assay too. To further examine these compound´s influence on metalhomeostasis, we used the transgenic strain CL2120 where $A\beta_{42}$ is expressed and MT is GFP tagged so that MT induction through disease progression can be monitored.

Like in the above mentioned study of Miyazaki, where murine models have been used, we were able to show that in the healthy *C. elegans* control strain CL2122, MT slightly increases with age, whereas in the $A\beta_{42}$ expressing strain CL2120, we observed an induction in the young adults until the 6th day of their lifespan whereas after 9 days a breakdown followed (Pretsch et al., 2020). *C. elegans* has a lifespan of 14 to 20 days. Compounds that were active in both, in the AD and PD assays, like emodin and clioquinol, were further able to prolong the time of MT induction in strain CL2120. When knocking down MT-2 by RNAi, active compounds lost their ability to reduce proteotoxicity in the AD and PD assays. Targeting the failed MT induction in aged nematodes with an AD or PD phenotype reduced the proteotoxicity by presumably restoring the metal homeostasis (Pretsch et al., 2020).

In my opinion, it is worth to have this target in mind when searching for new therapeutics against neurodegenerative diseases, and here I am not only referring to AD and PD. MT promotion should be further taken into account for HD, amyotrophic lateral sclerosis, prion disease, brain trauma, brain ischemia, and psychiatric diseases, as it has been shown that MT is diminished in those disorders too (Michael et al., 2011). Therefore our newly established MT assay could provide a first stage selection of compounds which should be further investigated in the particular disease model.

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