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Motility tests for drugs preventing PolyQ aggregation in recombinant *Caenorhabditis elegans*.

Etudiant
Wurlod Daniel-Adrien

Tuteur
Prof. Pierre Goloubinoff
DBMV, Unil

Co-tuteur
PhD Andrija Finka
DBMV, Unil

Expert
Prof. Jean-Pierre Hornung
FBM, Unil

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Abstract

The pathological formation of proteinaceous aggregates that accumulate into the brain cells of patients are hallmarks of neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and the heterogeneous group of polyglutamine (polyQ) diseases.

In the polyQ diseases, the most upstream events of the pathogenic cascade are the misfolding and aggregation of proteins, such as huntingtin in Huntington's disease, that contain expanded stretch of glutamine residues above 35-40 repeats. This expanded polyQ stretch triggers the misfolding and aggregation of cytotoxic polyQ proteins in the neurons that cause cell death through different processes, like apoptosis, excessive inflammation, formation of free radicals, eventually leading to neuronal loss and neurodegeneration.

This study focuses on the cellular network of chaperone proteins that can prevent protein aggregation by binding misfolding intermediates and may, as in the case of HSP70, actively unfold misfolded proteins into refoldable non-toxic ones (Hinault et al., 2010; Sharma et al., 2011). The chaperones can also collaborate with the proteasome to convert stable harmful proteins into harmless amino acids. Thus, the chaperone proteins that are the most important cellular factors of prevention and curing of protein misfolding, are negatively affected by aging (Morley et al., 2002) and fail to act properly in the neurons of aged persons, which eventually may lead to neurodegenerative pathologies.

The general aim of this research was to identify least toxic drugs that can upregulate the expression of chaperone genes in cells suffering from polyQ-mediated protein aggregation and degeneration.

The specific aim of this study was to observe the effect of ten drugs on polyQ aggregation in a recombinant nematode *Caenorhabditis elegans* expressing a chimeric protein containing a sequence of 35 glutamines (Q35) fused to the green fluorescent protein in muscle cells, which causes an age- and temperature-dependent phenotype of accelerated paralysis. The drugs were selected after having proven their causing the overexpression of chaperone proteins in a previous wide screening of 2000 drugs on the moss plant *Physcomitrella patens*.

The screening that we performed in this study was on these ten drugs. It suggested that piroxicam and anisindione were good reducers of polyglutamine disease mediated paralysis. A hypothesis can be made that they may act as good enhancers of the heat shock response, which causes the overexpression of many HSP chaperones and thus reduce motility impairment of polyQ disease expressing nematodes. Piroxicam was found to have the greatest effect on reducing polyQ35 proteins aggregates mediated paralysis in a dose-dependent manner but was also found to either have a toxic effect on wild type *C.elegans*, either to change its natural motility behavior, eventually reducing its motility in both cases. Chloroform should be preferred over DMSO as a drug solvent as it appears to be less toxic to *C.elegans.*

Keywords : *Caenorhabditis elegans*, polyglutamine, piroxicam, anisindione, heat shock proteins.

Introduction

Neurodegenerative diseases are a group of pathologies of great importance. Alzheimer's disease, amytrophic lateral sclerosis, the polyglutamine diseases and Parkinson's disease are just a few examples of this huge group of diseases known as the neurodegenerative diseases. Their frequency and their severity rendered them an important medical challenge worth studying.

Neurodegenerative diseases result from progressive neuronal loss, leading to dysfunction of the nervous system. According to the American National Institute of Neurological Disorders and Stroke, there are more than 600 neurologic disorders resulting from degenerative neuronal loss, affecting about 50 million of Americans each year (Brown et al., 2005).

Because of the importance of the number of different pathologies considered as neurodegenerative disease, it's difficult to find worldwide statistics about the whole group. An indirect way to measure the importance of its general epidemiology is to focus on dementia, which is a main outcome of numerous neurodegenerative diseases. According to the DSM-IV, a diagnosis of the syndrome of dementia recquires a mnesic disorder, along with one of the following features: aphasia, apraxia, agnosia and executive functions disorder. Cognitive troubles with negativ impact on everyday life should also be part of the symptoms to establish the diagnosis of dementia (Monsch et al., 2008).

Worldwide about 35.6 million people were living with dementia in 2010. Estimations are that this number will reach 65.7 million by 2030 and 115.4 million by 2050 (Wimo et al., 2010).

In Switzerland, about 98'000 persons were affected in 2008 and 23'000 persons are developing this syndrome each year (Monsch et al., 2008). The annual cost of dementia was estimated worldwide at US$ 604 billions in 2010 (Wimo et al., 2010) and at CHF 6.3 billions for 2007 in Switzerland (Ecoplan, 2010).

As defects in the genome are essentials for the pathogenesis of these neurodegenerative diseases, their hereditary character has also to be taken into account to measure the importance of this group of disease.

Neurodegenerative diseases may cause as well many different symptoms other than dementia, sometimes in addition to it, sometimes without it.

For example amyotrophic lateral sclerosis, which causes the progressive loss of the motor neurons, will cause physical symptoms such as dysphagia, dysphonia, loss of muscular strength, amyotrophy and cramps. Parkinson's disease causes mostly movement-related symptoms, like rigidity, slowness in making movement and shaking among others. Other neurodegenerative diseases may cause other symptoms (Bertram et al., 2005).

The medical world still offers limited treatments to many of these diseases and most of the time only symptomatic treatment can be offered, without really restraining the development of the disease.

The polyglutamine diseases:

In the present study we have focused on the heterogeneous group of polyglutamine (polyQ) diseases. This group is composed of nine different pathologies described *in figure 1*. In the polyQ diseases, the most upstream events of the pathogenic cascade are the aggregation of proteins, such as huntingtin in Huntington's disease, that contain expanded stretch of glutamine residues above about 38-40 repeats. This expanded polyQ stretch triggers the misfolding and aggregation of cytotoxic polyQ proteins in the neurons that cause cell death through different processes, like apoptosis, excessive inflammation, formation of free radicals, eventually leading to a general tissue loss and neurodegeneration. The aggregates are fibrillar insoluble β-sheet-rich amyloid-like, and accumulate in inclusion bodies that cause the previously mentioned processes leading to cell death. It's worth mentioning that the different proteins affected by the mutation don't have any sequence homology except for the polyQ CAG repeats stretch itself and don't share functional abilities (Nagai et al., 2010).

As shown in *table 1*, each disease of the PolyQ group has its own location of predilection and its own quantity of glutamine repeats needed for the disease to be clinically expressed.

		CAG Repeat size			
Disease	**Mutant Protein**	**Normal**	**Disease**	**Intracellular location**	**Affected brain regions**
Huntington's Disease	Huntingtin	6-35	38-180	Cytoplasmic	Striatum, cortex
DRPLA	Atrophin-1	6-35	49-88	Nuclear and cytoplasmic	Cerebral cortex
SBMA	Androgen receptor	9-36	38-65	Nuclear and cytoplasmic	Motor neurons
SCA 1	Ataxin-1	6-44*	39-82	Nuclear and cytoplasmic	Cerebellum
SCA 2	Ataxin-2	15-31	34-64	Cytoplasmic	Cerebellar Purkinje cells
SCA 3/MJD	Ataxin-3	12-40	60-84	Nuclear and cytoplasmic	Ventral pons, substantia nigra
SCA 6	Calcium channel	4-18	21-33	Membrane associated	Cerebellar Purkinje cells
SCA 7	Ataxin-7	4-35	34-306	Nuclear and cytoplasmic	Cerebellar Purkinje cells, brain stem, spinal cord
SCA 17	Tata-binding protein (TBP)	29-42	47-55	Nuclear	Cerebellar Purkinje cells

Table 1: **Overview of the polyQ pathologies**. DRPLA: Dentatorubral-pallidoluysian atrophy; SBMA: spinobulbar muscular atrophy; SCA: spinocerebellar ataxia; MJD: Machado-Joseph disease. (Paulson et al., 2000; Nagai et al., 2010) *In SCA1, disease alleles are purely CAG repeats, whereas normal alleles > 21 repeats are interrupted by 1-3 CAT units.

Huntington's disease (HD):

Huntigton's disease is worth a detailed description, as it's probably the most famous PolyQ disease. It was thoroughly described for the first time in 1872 by George Huntington an American physician. The disease itself had been known before, but the accuracy with which he described the disease convinced the medical community to name this pathology after him. It was before called the « hereditary chorea ». The word « chorea » refers to « χορεία » in ancient Greek which means « dance » (Harper, 1991). It was used because of the typical but pathological way people affected by this pathology were stricken by uncontrolled abrupt movements.

The prevalence of HD is estimated at 3-10/100'000 among individuals of European descent. In other ethnic groups prevalence is less common (Maat-Kievit et al., 2001)

Huntington's disease is an incurable autosomal dominantly-inherited pathology of late onset. The older the patient that carries the mutated gene gets, the higher the percentage that he develops symptoms of the disease is. For example if at 30 years old you have 13.0% of chances to express the disease, the percentage rises at 71.5% at 50 years old and at 95.1 at 60 years old. Since it is an autosomal dominant condition, early diagnosis is often important so that genetic advice and prognosis can be given to relatives that may as well develop the disease. Only 10% of the patients have developed HD « de novo », the other 90% have acquired it through heredity (Bertram et al., 2005). The prognosis is rather severe, as in most studies the mean age at onset is around 36-43 years old with a mean age at death around 53-56 years old (Harper, 1991). No treatment can yet delay the outcome.

The CAG expansion that characterizes the polyQ disease is situated in the gene that encodes for the huntingtin protein located in chromosome 4. The mutated huntingtin leads to a gain of function such as excitotoxicity, overactivity or interactions with other proteins. About 38-40 repeats of glutamine (CAG) are needed to cause the onset of HD. Clinically this disease is characterized by dementia, chorea, changes in personality and behavior and progressive psychiatric disease (Maat-Kievit et al., 2001). No mortal complications are specific to HD, but the cause of the death is most of the time a combination of immobility, weight loss, tendency to broncho-aspiration and general weak condition directly imputable to HD, leaving the patient vulnerable to any intercurrent disease (Harper, 1991).

Chaperone proteins:

In this study we focused on the polyQ diseases, but most of the neurodegenerative diseases have something in common: Degeneration and neuronal loss caused by the toxic aggregation of misfolded proteins into the cells. Hence finding a way of avoiding the aggregation of proteins would probably slow down or even stop the progress of such diseases.

This leads us to the concept of the molecular chaperones. The molecular chaperones are a group of proteins that can prevent the aggregation of proteins, and some of them can also unfold misfolded proteins and thus reduce a possible damaging effect by misfolded proteins in the cell. They can be define as proteins that interact, stabilize, or help a non-native protein to acquire its native conformation, but that are not present in the final functional structure (Hartl and Hayer-Hartl et al., 2009).

Most molecular chaperones can passively prevent the toxic aggregation by binding the misfolded proteins and preventing them to form an aggregate. Others can hydrolyze ATP to convert stable toxic protein aggregates into harmless natively refoldable polypeptides.

During *de novo* protein synthesis, or protein translocation into the mitochondria, polypeptides are unfolded when they emerge from the ribosome or the translocation pores. When their hydrophobic parts are in contact with water, they tend to spontaneously collapse so that these parts will be the core of the proteins, surrounded by the hydrophilic parts (Morgan et al., 1998). This is the folding of the proteins into the « native state », which is thermodynamically more stable than the unfolded state

In a cell we can also find some folded proteins in a so called « alternative-state », for example when a protein is in an inactive albeit misfolded state, awaiting a phosphorylation to become active in the cell. That would be a physiologic harmless oligomer in opposition to the folded monomer in a « native state ». It would cost too much energy to degrade all the proteins that are misfolded or in « alter-native state » and create again natively folded, functional and harmless proteins, so the cells had to find a way to spot precisely the misfolded proteins and convert them into the desired proteins (Hinault et al., 2006). This is the function of the molecular chaperones. They are of great importance since the regulation of this tight equilibrium between functional natively folded and non-functional toxic misfolded proteins is vital to the cell. Two molecular chaperones in particular have been held responsible of this essential fold-controlling task, HSP90 and HSP70. HSP stands for « heat shock proteins », the number is the apparent molecular weight in SDS-polyacrylamid gels.

There are many different families of chaperone proteins. The principal groups are described in *table 2*.

HSP70s and co-chaperones (such as HSP40 and HSP110)	In all ATP-containing compartments of living organisms. Work with other proteins in the folding of nascent polypeptides, remodeling of native protein complexes, transduction of cellular signals, regulation of the cell cycle, proliferation, apoptosis, regulation of heat-shock response (Jolly and Morimoto, 2000)
HSP60 and HSP10	Control the proper folding of 10-15% of newly synthesized proteins in bacteria (Liberek et al., 2008)
HSP90 family and co-chaperones	Mechanism still unclear, in vitro can prevent protein aggregation in an ATP-independent manner (Wiech et al., 1992). They can form super complexes with HSP70 to modify the structure of misfolded proteins bound to its docking site (Whitesell and Linquist, 2005; Zhao et al., 2005)
Small HSPs	They have a conserved α-crystalline domain that passively binds misfolded intermediates and prevent misfolding polypeptides from aggregating, protect membrane from heat disruption (Jakob et al., 2003)
HSP100	ATPase members of the AAA+ superfamily, plays a vital role in the survival of bacteria, yeast, and plant cells exposed to chemical stresses or high temperatures (Sanchez and Lindquist, 1990).

Table 2: **Major families of molecular chaperones.** *(Content from Finka et al., 2010)*

HSP70 is a well known and highly conserved molecular chaperone protein and have multiple different functions in the protein homeostasis (see *table 2*). Through the J-domain of HSP70, the HSP40 family of co-chaperones can bind themselves, stimulating ATPase and folding activity. The other main groups structure and functions are described briefly in *table 2*.

Chaperone proteins are acting in physiological conditions already, to preserve the previously mentioned equilibrium, the proteostasis, but under certain conditions they can become greatly over expressed. Indeed many of the molecular chaperones are also stress-induced. They may thus prevent the aggregation of the abnormally elevated number of misfolded proteins in stressful conditions. A well-known stressfull condition is heat, hence their name, heat-shock proteins (HSPs) (Finka et al., 2010).

It should however be noted that most heat-shock proteins are not chaperones, and that most chaperones are not heat-induced. It's an important notion to grasp correctly, as the concepts of HSPs and chaperones are tightly connected although they belong to several distinct protein families. Indeed although less than a third of the human chaperone genes are heat-inducible, statistically a chaperone gene remains about 10 times more likely to be heat-inducible that a non chaperone gene in the human genome (Finka et al., 2010).

This stress response of the cell, inducing the activation of the heat shock proteins, many of which are chaperones, has been verified and used in many different experiences already. Saidi et al. made an interesting application of this stress response in 2007. The purpose of their study was to measure the toxicity

of organic pollutants on transgenic *Physcomitrella patens* by measuring the quantity of β-glucuronidase (GUS), produced by the activity of *GmHSP17.3B* promoter that reacted to the stress induced by the pollutants. Chlorophenols, heavy metals and sulphonated anthraquinones specifically activated soybean heat shock *GmHSP17.3B* promoter, showing a correlation with long-term toxicity. This method proved itself as a powerful method to observe the response of heat shock proteins in plants exposed to environmental or chemical stress.

In 2005, Saidi et al., had already used the same promoter *GmHSP17.3B* as a tool to address whether if it was possible to produce a controlled production of a recombinant protein encoded after the *GmHSP17.3B* promoter. They used the transitory elevation of temperature, the so called « heat shock », to trigger the stress response of the cells. Induction levels of protein production, which were tightly proportional to the temperature and duration of the heat shock treatment reached up to 3000 fold of increased cellular levels of reporter protein (Sidi et al., 2005)

Chaperone proteins can also have other functions than proteostasis regulation in the cell and some of them can be found associated to the plasma membrane. Chaperone protein J3 (DnaJ homolog 3; HSP40-like) in the case of *Arabidopsis* can influence the activity of H^+-ATPase that plays an essential role in many different vital processes of the cell. J3 activates plasma membrane H^+-ATPase activity by physical interaction and by repressing PKS5, an inhibitor kinase of H^+-ATPase activity (Yang et al., 2010).

Proteasome:

Molecular chaperones are not working alone to regulate the protein homeostasis of the cell. Indeed the mechanism by which the cell is protected from the misfolded proteins damages is completed by the proteasome activity. The proteasome is an ATP-dependent protease that cannot unfold misfolded proteins, unlike the chaperone proteins, but can degrade undesired proteins avoiding their accumulation in the cytosol. The proteasome constitutes nearly 1% of all cellular proteins. About one third of the newly made polypeptide chains are degraded directly after their synthesis. The proteasome has a cylinder-like structure, constituted of a central part, the 20S core, itself formed by multiple proteins assembled in two heptameric rings stacked tail to tail. Each end of the 20S core particle is associated with a bigger protein complex, the 19S cap, acting as a gate for the proteasome. The 19S caps can recognize the targets they have to let pass, for the misfolded proteins to be degraded are tagged by branched chains composed of a small protein called « ubiquitin ». The ubiquitin-proteasome system can recognize the misfolded proteins because the alterated structure of the badly folded proteins transiently exposes degradation signals that aren't exposed in the natural structure of the protein. The 19S caps use ATP hydrolysis to unfold the polypeptide chains of the ubiquitin-marked proteins and pass them on to the 20S core for digestion to short peptides by proteolysis (Alberts at al., 4th edition, 2002).

In short when a protein is synthesized, it can be either folded to the native state by the interaction of it's hydrophobic parts with the new environment in which it's transferred, folded or refolded with the help of a molecular chaperone, or incompletely folded or misfolded and hydrolyzed by the proteasome or create toxic aggregates with other misfolded proteins.

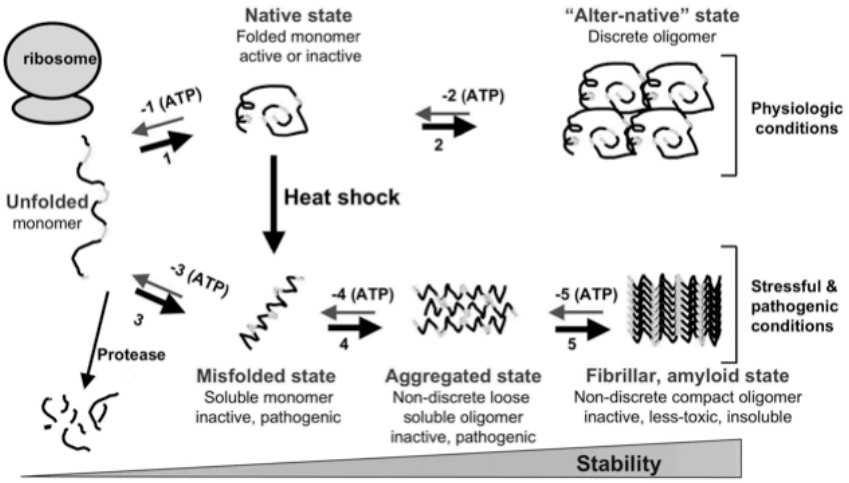


Figure 1: **Model for the role of molecular chaperones in protein unfolding (from Hinault et al., 2006).** Under physiological conditions (upper path), an unfolded newly synthetized, or translocated polypeptide may spontaneously fold (1) into a native monomer, and/or (2) further assemble into an "alter-native" discrete oligomer. ATPase unfolding chaperones can actively deoligomerize (-2) "alter-native" oligomers into monomers and further unfold (-1) near-native monomers to be translocated across membranes or degraded. Under stressful conditions (lower path), or because of mutations, a newly synthesized may spontaneously misfold (3) into a soluble, possibly toxic monomer, which may further assemble (4) into a continuum of small toxic oligomers that may further condense (5) into less toxic, compact amyloids. Specific organic molecules, may destabilize, and ATPase chaperones, actively scavenge (-5, -4) compact amyloids and fibbers into looser, albeit more toxic oligomers. ATPase chaperones may further unfold (-3) and detoxify misfolded monomers into harmless protease products or natively refolded proteins. The leftward reactions are against entropy and ATP hydrolysis may be needed for the chaperones and proteases to forcefully unfold stably misfolded or alternatively folded protein structures.

Induction of chaperones and hsp's by way of cellular membrane:

Modifications in the plasma membrane itself such as hyperfluidization caused by local anesthetic benzyl alcohol and heptanol act as a signal to the activation of the *HSP* genes, thus initiating the whole heat shock protein response (Balogh et al., 2005).

During a heat shock or when a cell is in an environmental stress, the activation of stress-induced cell response is mediated by the heat shock transcription factor HSF-1 that acts as a key coordinator to the whole process. *Figure 2* shows how HSF-1 is activated in such case and how the activation of this transcription factor triggers the heat shock proteins response.

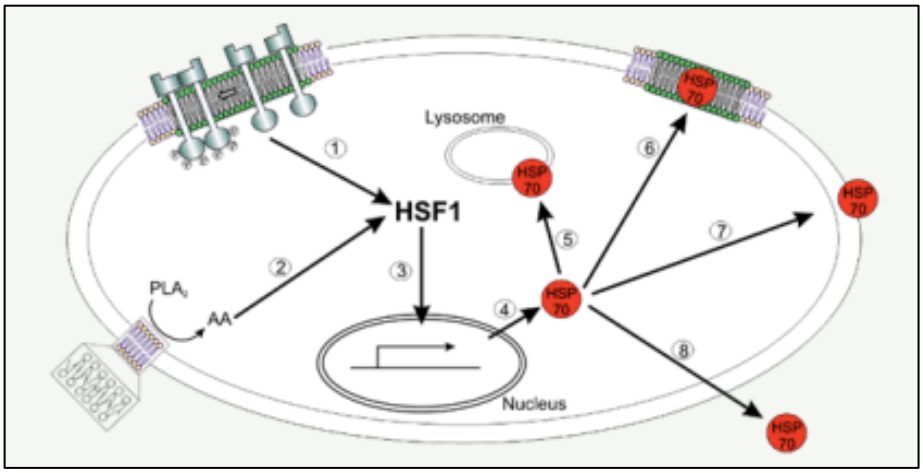


Figure 2: **Membrane Membrane-mediated stress protein response and the cellular localization of HSPs (highlighted by HSP70) in mammalian cells (from Horváth et al., 2008).** HSF1 is a key coordinator of the initiation of heat shock gene transcription, which is activated mainly by the appearance of denatured or misfolded proteins. In addition, stress sensing-signaling mechanisms operate through stress-induced membrane rearrangements. Such typical membrane-mediated changes that are evidenced to refine the expression of heat shock genes are the non-specific clustering of the growth factor receptors associated with membrane microdomains ("rafts") (1) or the activation of phospholipases (2), which sequester themselves into unsaturated-rich microdomains and cleave arachidonic acid, a known HSP inducer. Stress activation of such pathways alters the nuclear accumulation and transactivation capacity of HSF1 (3) via its covalent post-translational modifications and ultimately retailor the abundance and profile of HSPs. The function of individual HSPs (highlighted on the scheme by HSP70) depends on their intracellular, membrane bound or extracellular location. The major action of chaperone proteins in the cytosol is to maintain protein homeostasis (4). HSP70 can promote cell survival by inhibiting lysosomal membrane permeabilization via the interaction with specific lipids (5). We are currently studying the interaction of HSPs with cellular lipid droplets. Experimental evidence is accumulating in favor of the presence of HSP70 (and other HSPs) in lipid rafts as components of signaling or trafficking platforms (6). HSPs can also associate with specific lipids and proteins in the plasma membrane, inducing "membrane stabilization" and/or exhibiting an immunogenic potential (7). HSP70s of extracellular location (8) have immunomodulatory capacities and are potent agents in the activation of the innate and adaptive immune system.

It's important to precise again that most HSP's are not chaperones, and most chaperones are not HSP's. Indeed a protein overexpressed in a heat shock stress condition will be an HSP, but won't be necessarily a chaperone protein (Finka et al., 2010).

Since chaperone proteins activity can avoid the formation of misfolded proteins aggregates, finding a drug that could selectively activate their overexpression could be a gigantic step towards the cure of neurodegenerative diseases, or at least towards the control of its damaging expression.

Caenorhabditis elegans:

Saidi et al. used the *Phycomitrella patens* moss of the *Funariaceae* family as a biosensor for organic pollutants and also to see whether it could be use to produce a controlled amount of recombinant proteins through the activation of the soy bean heat shock promoter *GmHSP17.3B* (Saidi et al., 2005 and 2007).

The model bacteria *Escherichia coli* has also been widely used in different experiments on the chaperone proteins. Chang et al. observed in *E.coli* the activity of DnaK chaperone protein, which belongs to the HSP70 family and works together with DnaJ (also known as the previously mentioned HSP40). The ATPase activity of DnaK is indeed stimulated by the DnaJ co-chaperone. The purpose of the study was to identify inhibitors of the DnaK/DnaJ complex. Flavonoids were found to bind DnaK, blocking allosterically interaction of DnaK with DnaJ (Chang et al., 2011). Wisén et al. used *E.coli* as well to identify compounds that could stimulate *in vitro* functions of DnaK. Dihydropyrimidine (115-7c) was found to stimulate *in vitro* ATPase rate and protein folding, both major functions of DnaK.

Wisén et al. used also *Saccharomyces cerevisiae* yeast to test whether 115-7c could compensate for the loss of YDJ1 (*ydj1Δ*) in *S.cerevisiae* mutant. This mutation has been known to compromise severely *ydj1Δ* mutant's growth at elevated temperature (Sell et al., 1990; Caplan et al., 1993; Sahi et al., 2007). Application of 115-7c 100 µM was found to greatly enhanced *ydj1Δ* cells' growth at 34°C, an elevated temperature that normally causes the mutants' failure to thrive (Wisén et al., 2010).

Studies on neurons directly have also been made. By observing the effect of body inclusions forming mutant huntingtin on rat or mouse pups' superior cervical ganglia neurons culture, King et al. discovered that it could suppress apoptosis in sympathetic neurons, being also refractory to proteasomal and lysosomal elimination (King et al., 2008).

We choose in our study the *Caenorhabditis elegans* nematode as a model for polyQ disease.

Caenorhabditis elegans has been used already in many studies as a living organism to study the protein chaperones (such as Morley et al., 2002; Ben-Zvi et al., 2009)

Caenorhabditis elegans is a nematode from the *Rhabditidae* family. It lives in the soil in most of the temperate regions of the world and an adult measures about 1 mm. The life span is about 2-3 weeks and start producing eggs at 3.5 days. In case of hostile environment it can become a « dauer », transforming itself in a more resistant form, even resisting to desiccation. The dauer form does not age.

C.elegans can be either male or hermaphrodite. The males can produce only sperm, with which they can fertilize the oocytes of the hermaphrodites. This is called cross-fertilization. Unlike the males, the hermaphrodites can produce sperm and oocytes allowing them to self-fertilize themselves. The sperm

produced by males outcompete the sperm of hermaphrodites so cross-fertilization, that produces either males or hermaphrodites in the same proportion, is more frequent than self-fertilization that can only produce hermaphrodites (Hope, 1999). The possibility of controlling these two types of gene heredity by choosing the cross-fertilization or the self-fertilization made *C.elegans* an easy model to produce mutants, especially for producing homozygous animals carrying recessive mutations by using self-fertilization. This is the main reason why one of the main field in which *C.elegans* was used was genetics, especially in genome sequencing projects (Science N°282, 1998). *C.elegans* only requires for growth and reproduction a humid environment, an ambient temperature, atmospheric oxygen and a lawn of bacteria for food, explaining why it can be a model of choice as it's rather cheap and easy to fulfill these few conditions.

What interests us here are the advantages presented by *C.elegans* for the study of polyQ disease and chaperone proteins.

Indeed, unlike in humans where toxic polyQ-mutant proteins aggregate in the neurons, polyQ disease in *C.elegans* can be made to express itself in the body wall muscle cells causing motility impairment. This is an appreciable fact, allowing the quantification of severity of polyQ disease's expression by observing or monitoring the motility of polyQ mutant *C.elegans*. Morley et al. who studied the length-dependent threshold for aggregation and toxicity of polyQ proteins have used this particularity. Not only did they establish the link between polyQ disease expression and the length of the polyglutamine stretch but they also found that aging had a negative impact on the stress-induced response by the chaperone proteins, as it has on human Huntington's disease development (Morley et al., 2002). The way aging negatively influences the above described defense mechanisms used to avoid misfolded proteins aggregation may explain why the onset of most neurodegenerative diseases is at mid-age or older and usually not in the childhood, where chaperone proteins' expression is maximal.

Thus *C.elegans* is an animal model that can express in an easily observable way polyQ disease by observing the protein aggregates marked with GFP, and is a model where aging, an easily controlled factor, exacerbates the toxic aggregation in the cells, making *C.elegans* a model of choice for the study of the polyQ diseases.

The hydroxylamine derivative NG-094 suppresses paralysis in Caenorhabditis elegans (Haldimann et al., 2011):

This present study is the follow-up of a previous study by Haldimann et al., 2011. They used genetically modified *C.elegans* expressing in body wall muscle cells a toxic mutant protein containing a 35 CAG repeat sequence and tested NG-094 a novel hydroxylamine derivative on life span and on the progression of the impairment of motility. The recombinant protein was fused to the yellow fluorescent protein (YFP) allowing visualization by fluorescence.

As we said before, the expression of polyQ diseases is polyQ-length- and age-dependent (Morley et al., 2002). Thus the first step of Haldimann's experiment was to observe changes in *C.elegans'* motility influenced by different physiological temperatures from 15°C to 25°C. To assess motility of nematodes, 100-200 age-synchronized *C.elegans* were placed at the center of a plate where was previously deposited a 3-5mm thick ring circle made of *E.coli* OP50 strain of about 3.5 cm in diameter to attract the nematodes by chemotaxy. After 60 min, nematodes that reached the OP50 strain were counted. Nematodes were grown for three days since hatching at the mentioned temperature before the motility assays.

At temperature until 20°C, polyQ35 nematodes and polyQ0 had a similar motility, nearly the same percentage of both groups could reach the OP50 circle. Spectacular loss of motility was observed at 21°C and 22°C, where 25% and 50% of polyQ35 respectively couldn't reach the target. At 24°C just a few polyQ35 nematodes could reach the target and none could do so at 25°C (See *figure 3*).

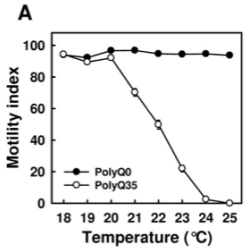


Figure 3: **Motility assays for three-days old grown nematodes at different temperatures.** At 21°C 25% of polyQ35 couldn't reach the target after an hour, 50% at 22°C. At higher temperatures, motility impairment is even greater. (From Haldimann et al.,2011)

Aggregation of misfolded proteins in body wall muscle cells was evaluated by observing mutant protein fused to YFP aggregates with fluorescence microscopy. Direct proportionality between temperature and quantity of aggregates has been observed (see *figure 4*).

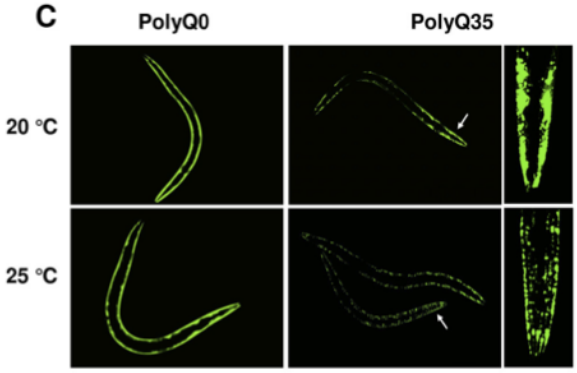


Figure 4: **Visualization of polyQ35 protein aggregates by fluorescence microscopy.** Quantity and importance of aggregates were more elevated for nematodes grown at 25°C than at 20°C, showing how temperature affects the development of polyQ disease in transgenic *C.elegans*. Homogeneous glow of GFP is replaced by easily observable pathological aggregates, at 25°C even more than at 20°C. (From Haldimann et al., 2011)

The study showed that polyQ35 were more sensitive to mildly elevated temperature during growth and that high temperatures accelerated the polyQ protein aggregation and more widely exacerbated polyQ mediated cytotoxicity. They also compared the two negatively impacting factors aging and temperature. Motility of polyQ0 nematodes was reduced by 50% either after 9 days at 20°C or after 6 days at 24°C. PolyQ35 animals' motility was reduced by 50% after 7 days at 20°C or after 3 days at 22°C (or higher temperatures). Thus elevated temperature during growth accelerates the age-dependent loss of motility, validating that *C.elegans* has a shorter life span at higher temperatures than at lower temperatures (Hirsh et al., 1976). Both negative impact of aging and high temperature growth lead to the idea that environmental factors that exacerbate the need for optimal protein quality control by increasing the demand of the misfolded protein system may increase greatly the severity of Polyglutamine diseases.

Core of the study was the test of novel hydroxylamine derivative NG-094 on polyQ35 nematodes. They compared polyQ0 animals for control with polyQ35 nematodes grown at 22°C for three days, conditions that showed motility impairment in 50% of transgenic polyQ35 nematodes. Treatment with NG-094 improved the motility of polyQ35 nematodes in a dosage dependent manner (see *figure 5*).

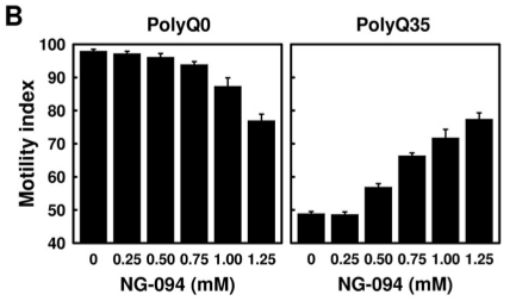


Figure 5: **NG-094 treatment increased motility index in polyQ35 nematodes in a dose-dependent manner.** (From Haldimann et al., 2011)

Figure 5 also shows that at high concentration NG-094 had a deleterious effect on polyQ0 nematodes. 1mM in the growth medium was chosen to be the concentration of NG-094 to be tested, as it had a great benefit on polyQ35 animals' motility without a too important loss in polyQ0 nematodes' motility index.

Motility assays showed many different properties of NG-094. Not only did NG-094 treatment have positive effect on loss of mobility in polyQ0 wild type nematodes suggesting an anti-aging property, but also could slow the disease progression after the onset of the disease which is most important as usually the patients discover their disease once they start to express themselves clinically.

As for the cellular mechanism responsible for the cytoprotection conferred by NG-094 treatment, the study has been able to establish the hypothesis that it involved heat shock factor HSF-1. Indeed after having knocked-down HSF-1 by RNA1 the paralysis of polyQ35 nematodes was strongly exacerbated, showing the importance HSF-1 probably has in this mechanism. The observations also permitted to suggest that the cytoprotection is not related with reduced insulin/IGF signalling. It has been already suggested that in *C.elegans*', reducing IGF-signalling which decreases transcription factor DAF-16's activity in *C.elegans* increases life span and protects against some stress-inducing factors, hence proteotoxicity (Lamitina et al., 2004; Yen et al., 2008).

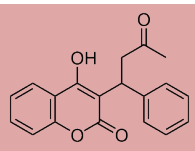
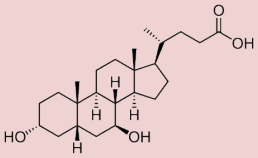
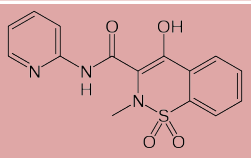
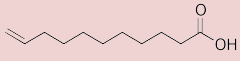
Motility tests for drugs preventing PolyQ aggregation in recombinant *Caenorhabditis elegans*:

In this general context this study aims to identify least toxic drugs that can upregulate the expression of chaperone genes in body muscle cells suffering from PolyQ-mediated protein aggregation and degeneration. First part consisted in a screening of 10 selected drugs to observe whether they could improve the motility impairment of PolyQ35 nematodes or not. Second part of the study focuses on the most promising drug following screening, piroxicam.

Material and Methods

Drugs:

The ten drugs (see *table 3*) used in the study were selected by our laboratory after having made a wide screening on 2000 drugs to find some that could overexpress or inhibit heat shock response in moss *Physcomitrella patens* (used database: The Spectrum Collection 2000 MicroSource). Drugs were provided in DMSO 10mM. Warfarin, ursodiol, piroxicam and undecyclenic acid are the drugs that have shown greatest effect in prior mentioned wide screening.

Drug	Known effects	Chemical structure
Warfarin	Anticoagulant, Rodenticide	
Ursodiol	Anticholelithogenic	
Piroxicam	Antiinflammatory, analgesic, antipyretic (NSAID's)	
Undecyclenic Acid	Antifungal	

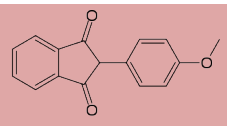
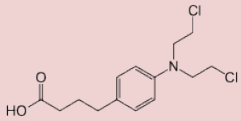
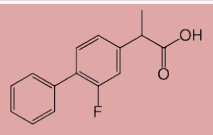
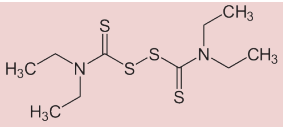
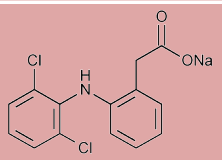
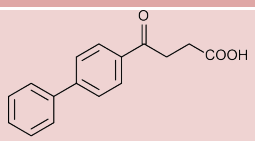
Anisindione	Anticoagulant	
Chlorambucil	Antineoplastic (alkylating agent)	
Flurbiprofen	Antiinflammatory, analgesic, antipyretic (NSAID's)	
Disulfiram	Alcohol-Dehydrogenase enzyme inhibitor	
Diclofenac Sodium	Antiinflammatory, analgesic, antipyretic (NSAID's)	
Fenbufen	Antiinflammatory, analgesic, antipyretic (NSAID's)	

Table 3: **The ten drugs tested in the motility assays.** The ten drugs were chosen among 2000 drugs in a large screening on the Spectrum Collection 2000 MicroSource database, according to their activating and inhibiting effect on chaperone proteins. The four first drugs were the ones showing best results in the screening.

Antibiogram test showed no antibacterial property. As *C.elegans* needs bacterial feeding in order to grow and survive, we were interested whether bacterial OP 50 lawn was able to grow in the medium containing drugs. In order to do so we performed prior to the motility assays an antibiogram using LB medium (Bacto agar 17g/l and Difco LB Broth 25g/l). Each drug was tested at 7 different concentrations containing always 1% of DMSO (DMSO 1% was common denominator in medium composition during the first part of the experiment): 1µM, 3 µM, 10 µM, 30 µM, 100 µM, 300 µM and 1mM. Dishes were place at 37°C for a few days and none of the drugs showed an antibacterial property. Control plate containing 100 µg/ml of Ampicillin inhibited bacterial growth.

***Caenorhabditis elegans*:**

***C.elegans* strains.** The strains used for the experiment were expressing a 35 CAG polyglutamine expansion and fused to the green fluorescent protein (GFP) in body wall muscles cells. The polyQ-GFP expressing strains are: PolyQ0 – AM134[*rmIs126(Punc-54::q0::yfp)*] and PolyQ35 – AM140[*rmIs132(Punc-54::q35::yfp)*]. The strains used in this study are the same used by Haldimann for his study with NG-094 (Haldimann et al., 2011)

Culture of *C.elegans*. Culturing and maintenance of *C.elegans* were performed according to standardized methods (Brenner, 1974)**.** Nematodes were grown on nematode growth media (NGM) dishes with *Escherichia coli* OP50 layer for feeding, placed in an incubator at 20°C. To avoid lack of food or space, transfers were made each 3-4 days by placing an approximately 4mm long square of NGM medium dish on which nematodes were growing into a new NGM Petri dish with grown OP50 layer.

Synchronization. Prior to motility assays, nematodes were age-synchronized to avoid age-related motility bias and to have the most homogenous sample possible. In order to isolate embryos from gravid adults, we collected all *C.elegans* from a Petri dish used for stock and growth and treated with solution containing equal volume of 1mM sodium hydroxide and 5% sodium hypochlorite for 8 minute. Embryos were then laid on NGM dish with OP50 (see protocol in appendix 1). In the experiments where age-synchronized nematodes were employed, NGM medium was containing drug in appropriate concentration.

Motility assays. Prior to motility assays, age-synchronized nematodes were placed on NGM medium containing tested drug in appropriate concentration for 3 days at 22°C. This temperature was chosen following Haldimann's protocol, allowing a 50% loss of motility index in polyQ35 population (Haldimann's et al., 2010). For the motility assays, age-synchronized strains treated with drugs were collected with pipette in small 1.5 ml tubes by rinsing dish where they grew with sterilized M9 solution (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 liter and sterilized by autoclaving.) With 10µl pipette, between 5-10 nematodes were then placed in the middle of 35 x 10 mm Petri dishes containing a thicker and more concentrated OP50 layer so that the traces could be easily spotted. Drop containing the nematodes was spread by moving the dish carefully in different angles. This was made to avoid the agglutination of nematodes, that would otherwise impair nematode movement during recording time and to allow M9 solution to dry up faster. Images of the traces were taken 15 minutes after drop was deposited in recording dish. During this time dishes were placed back in the incubator at 22°C to avoid ambient temperature stress. Control with polyQ35 or polyQ0 grown in same environmental conditions was made every day of assays to avoid unnecessary biological bias. Minimally 3 dishes per drug

were used and recorded for first screening and about 10 dishes were used for each treated or control strain in second part of the study.

Boxplot diagrams. Descritpive statistics has been presented through boxplot diagrams. Boxplot graphs were made using Microsoft Office 2011 Excel program (from *http://www.bloggpro.com/box-plot-for-excel-2007/)*. Middle line of the box represents the median, the top line the 3rd quartile and the bottom line represents the 1st quartile. The vertical lines show the maximum and the minimum values, regarding to their top or bottom position. This format of presentation allows an objective view on the results with no modification of numbers for statistical purposes.

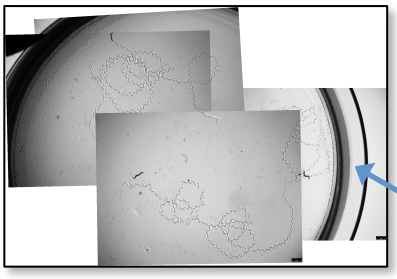
Concentrations. In the first part of the experience NGM mediums for treated *C.elegans* were all at the concentration of 64μM of drugs and dimethyl sulfoxyde 1% (DMSO 1%). For second part, NGM was at 32μM or 64μM of piroxicam and chloroform (CHCl3) 0.1% replaced DMSO 1%. Control groups were grown on regular NGM medium in same environmental conditions.

Pictures and measurements of traces length. Images were recorded with LEICA MZ16FA Microscope at minimum magnification 7.0x. At such magnification, at least 4 images had to be taken in order to have every trace recorded. Mirror of microscope was below the dish, cover was removed, and Micro 178 light of microscope was on maximum strength. Microscope program used to capture the images was Leica Application Suite. Images of same dish were then fused to form a single picture of the entire dish by using Adobe Illustrator CS5. Using neuronJ plugin of ImageJ software (*rsbweb.nih.gov/ij/*), we traced the traces and measured them by first setting a scale of pixels/mm (usually 68 pixels/mm in our study, obtained by comparing scale of microscope picture and pixels of ImageJ program) and then letting the program calculate the length of total traces in millimeters. Note that often traces of different nematodes are crossing or overlapping each other, so total traces length is easier to measure than individual trace length. *Figure 6* depicts precisely the way we treated the images and how we measured the traces.

Figure 6:

A. Fusing pictures:

1. Recorded images were assembled together in a single one showing all the nematode traces on the dish used for the motility assays. The image below shows the fused pictures composing an image of the whole dish. 4-5 pictures were needed for each dish.

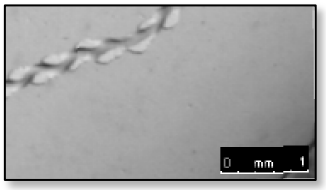


Pictures were joined to allow easier measurement with NeuronJ plugin. You can recognize the round petri dish by looking at the edges.

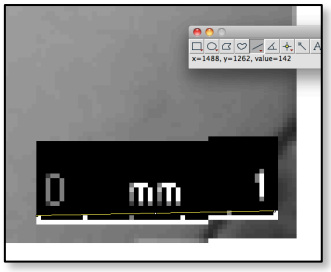
Petri dish edge

B. Obtaining the scale:

1. When taking pictures with Leica Application Suite program, configure the image so that a scale as shown appears:



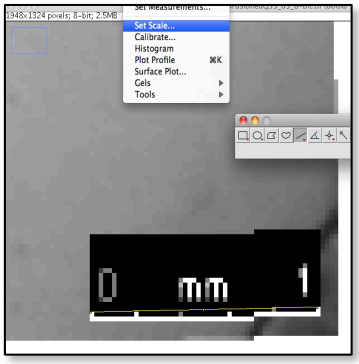
2. With ImageJ program, draw a segment that appears to be 1 mm according to the scale:



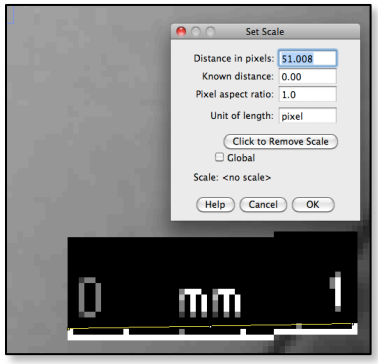
The bar in the top right corner is the first half of the application bar of ImageJ. Selected button is the one that draws the segment.

3. By selecting « Set Scale » (see *fig 3a*), find out the number of pixels needed to complete the scale and thus 1 mm (here 51 pixels for 1mm, appearing in blue in *fig 3b*).

3a.



3b.

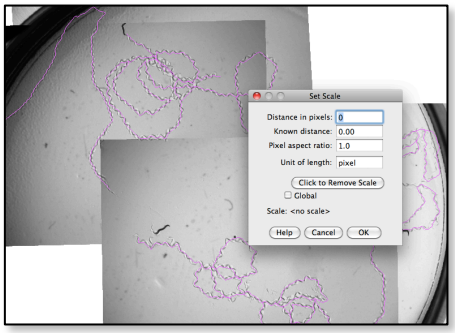


C. Traces measurements:

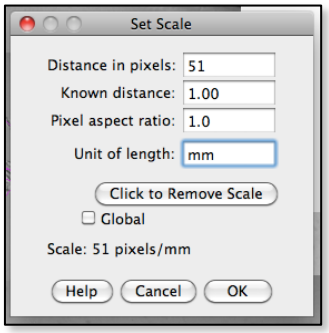
1. Open the picture with NeuronJ plugin, and draw your lines following the traces of *C.elegans* on OP50 layer. As only the total length interests us, you can draw as many different lines as you want to cover the whole nematodes' traces.

2. By choosing « set scale » in NeuronJ again (see *fig 2a*), you can set the proper scale. For 51 pixels/mm, which is appropriate for our example, fill the fields as shown in *fig 2b*.

2a.



2b.



3. Select "Measure Tracings" to obtain final length of total traces (you will get a total length of all the nematodes traces and not a length of individual traces.). In "Sum Len (mm)" of "NeuronJ:Groups" window, the presented value is used in results and in graphs.

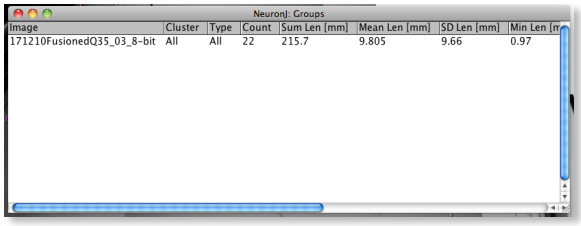
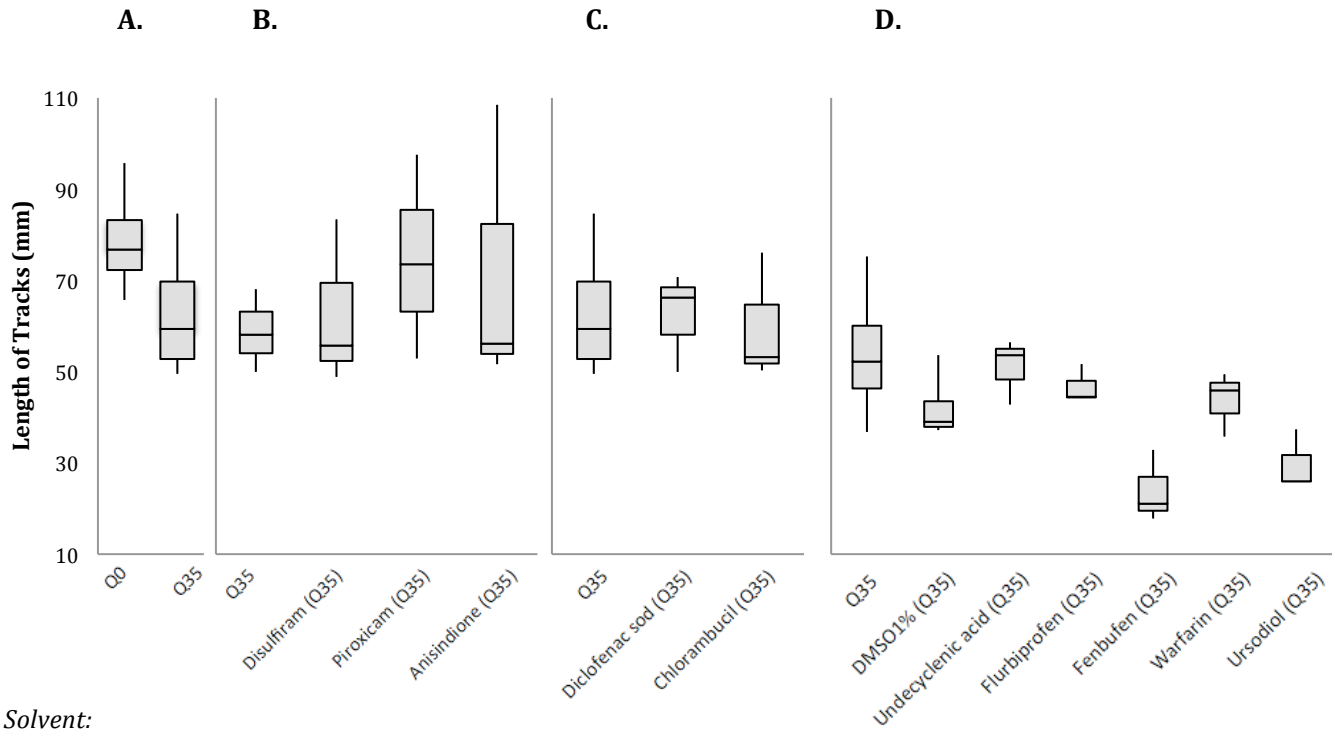


Figure 6: **Step by step explanation of tracks measurements. (A)** Pictures were fused with Adobe III Illustrator CS5 for easier measurement of tracks with NeuronJ **(B)** Obtaining scale by using ImageJ. NeuronJ plugin is not needed for this step. **(C)** Measure tracks with NeuronJ plugin after having configured the scale. Length of traces obtained is the value of the whole dish.

Images of polyQ proteins aggregates with fluorescence microscopy. Images of *C.elegans* with protein aggregates were taken with fluorescence microscopy using a LEICA DM 5500 B to verify whether we could establish a link between improvement of motility and quantity and size of toxic polyQ protein aggregates.

Results

Anisindione and Piroxicam show best improvement of motility in screening. Screening permitted to isolate two of the drugs as potentially interesting for overexpressing chaperone proteins. Anisindione and Piroxicam showed an improvement in polyQ35 nematodes' motility, respectively increasing the mean length of tracks of 22.7% and 27%. Q35 had a 24.5% reduced mean length of tracks compared to Q0, so anisindione and piroxicam could improve polyQ35 nematodes' motility nearly to the result of Q0 values or even may be slightly higher it in the case of piroxicam. *Figure 7* shows the raw results with boxplots of the length of tracks of the nematodes according to their treatment.



Solvent:																	
DMSO 1%	-	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+

Figure 7: **Results of motility assays for screening of the ten selected drugs.** Q0 are wild type healthy nematodes, Q35 are nematodes expressing polyQ disease (35 stands for the number of CAG repeats). A and C results are from the same day but this disposal with Q0 and Q35 on the left made easier the observation of drugs' effectiveness. B and D results have been recorded a different day, but each time Q35 nematodes without treatment were monitored for better comparison. Presence of solvent DMSO 1% is marked by « + », whether absence is marked by « - ». Boxplots are showing from bottom to top: minimum, 1st quartile, median, 3rd quartile, maximum.

DMSO 1% suspected of being directly toxic to *C.elegans* and/or having a negative impact on polyQ disease expression. DMSO 1% showed a reduction of 22% in polyQ35's mean length of tracks (see *figure 7*), suggesting a negative impact on polyQ disease development and expression. It also showed a loss of motility of 45.1% on polyQ0 (see *figure 9*) Since all the NGM medium used for growth contained DMSO 1% along with the drug, DMSO might have enhanced the toxic effect of some drugs, explaining partly the elevated toxicity that some drugs like fenbufen and ursodiol had in the screening. Considering DMSO potential toxicity, piroxicam and anisindione could be even more potent drugs for polyQ35 nematode motility improvement. As in the piroxicam trial using 0.1% chloroform (CHCl3) instead of 1% DMSO to dilute piroxicam didn't show a better improvement of Q35, it is possible that piroxicam may interact with DMSO molecule or prevent its toxicity. It is also possible that maximum benefit on motility was obtained with piroxicam and that presence of DMSO 1% or CHCl3 did not matter much.

Piroxicam greatly improves motility of polyQ35 nematodes in a dose-dependent like manner. 32 µM piroxicam improved mean length of tracks of 9.0% and piroxicam 64 µM of 32.3%. The motility of healthy nematodes was 33.6% better than the motility of sick nematodes (the mean length of traces results were calculated but cannot be seen in the boxplot diagrams). These results suggest that piroxicam could restore impaired motility of sick nematodes in a dose dependent response near completion.

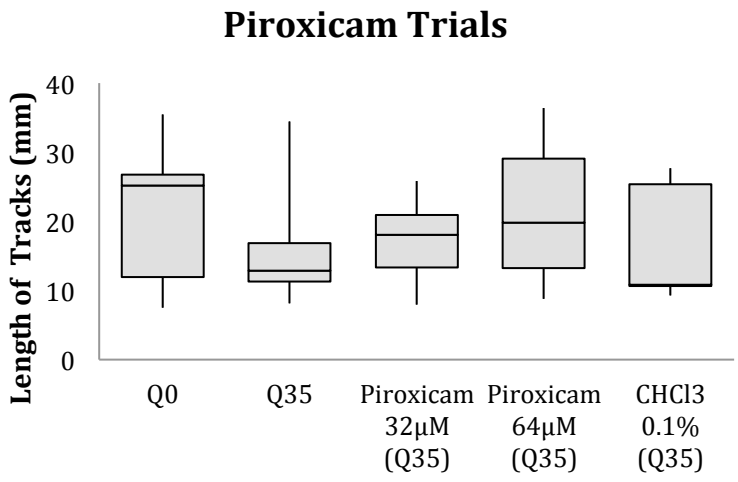
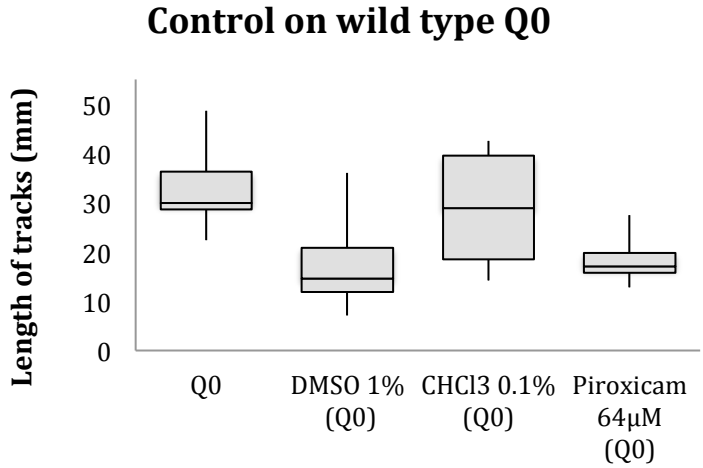


Figure 8: **Results of motility assays with piroxicam showing a dose-dependent relation between piroxicam and improvement of motility in polyQ35 nematodes.** Control was made with polyQ35 population without treatment and with polyQ0 wild type for comparison. Chloroform (CHCl3) was used as solvent as it is indicated. DMSO 1% was not used as solvent because of its potential toxicity. Boxplots are showing from bottom to top: minimum, 1st quartile, median, 3rd quartile, maximum.

Chloroform a better solvent than DMSO 1%. To verify whether 1% DMSO had a direct toxicity on *C.elegans,* we also tested 1% DMSO on Q0 wild type strain (see *figure 9*) that resulted in a loss of Q0 nematode motility of 45.1%. When 0.1% chloroform was used as a replacement of 1% DMSO for diluting piroxicam, we observed a loss of Q0 nematode motility of only 5.1%. DMSO had to be replaced because of its toxicity suspicion but also because dilution of piroxicam was impossible without precipitation in DMSO (Drugs were provided already diluted in DMSO when received in the lab for screening). This result suggests that chloroform has a lesser toxicity on *C.elegans* and should be preferred to DMSO if possible.

Piroxicam may be toxic or interfere with wild type *C.elegans'* growth development or behavior. 64 µM piroxicam was also tested on wild type Q0 nematodes for control, causing a loss of healthy nematode motility of 44.3% Therefore we conclude that piroxicam had a powerful effect on both sick and healthy strains of *C.elegans*, allowing great recovery in sick nematodes and great toxicity in healthy ones. This surprising negative effect on motility of healthy nematodes will be further discussed in discussion.



Solvent:				
CHCl3 0.1%	-	-	+	+
DMSO 1%	-	+	-	-

Figure 9: **Control motility assays on wild type Q0 for piroxicam and solvents** used to dilute drugs. Wild type Q0's length of tracks was recorded for comparison. DMSO 1% seems toxic as expected according to the results of our screening, but surprisingly piroxicam has a negative effect as well on C.elegans' motility. Boxplots are showing from bottom to top: minimum, 1st quartile, median, 3rd quartile, maximum.

Images of polyQ protein aggregates by fluorescence microscopy. PolyQ35 nematodes without treatment or treated with piroxicam seem to have the same quantity of polyQ aggregates, even though treatment with piroxicam greatly improved the motility of sick nematodes.

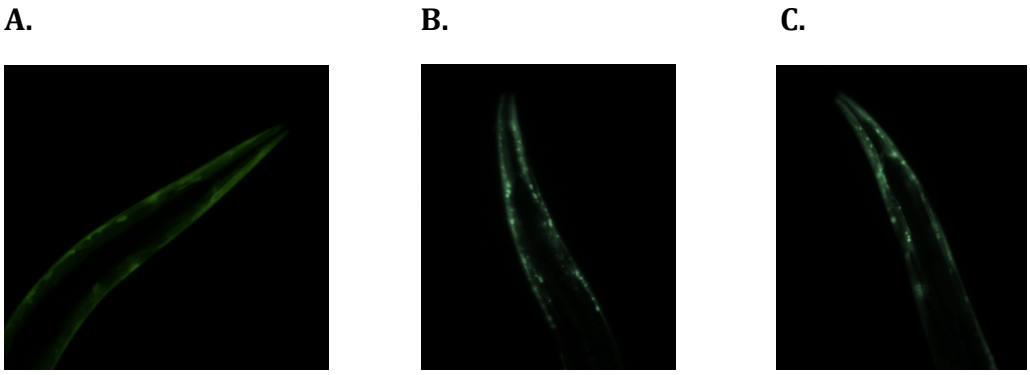


Figure 10: **Images of polyQ protein aggregates of Q0, Q35 and Q35 treated with piroxicam by fluorescence microscopy. (A)** Q0 healthy nematode with a homogeneous glow of GFP proteins. **(B)** PolyQ35 nematode**:** Homogeneous glow is modified by the presence polyQ proteins aggregates. **(C)** PolyQ35 aggregates of sick nematodes treated with piroxicam didn't show clear difference in quantity than of the untreated polyQ35 nematodes.

Discussion

At first this study was supposed to follow the method of Haldimann and colleagues (Haldimann et al., 2011). Using proclaimed method, being for a great part operator dependent, similar results were hard to obtain with conceded time. According to Haldimann, many months were needed to get stable and reproducible results. By employing the same protocol for nematode maintenance and with an operator that took part in some of the motility assays' of Haldimann's study, our results were found to be different. Motility assays with nematodes grown at 24°C did not even show a loss of motility index similar to the one for nematodes grown at 22°C according to Haldimann's results. An important part of the allowed time was spent trying to apply Haldimann's method and find similar differences between polyQ35 nematodes and wild type Q0 nematodes without success. This encouraged us to find an alternative way to measure the motility of polyQ35 transgenic nematodes. We believe the chosen method described above has the qualitative advantage of recording more precisely the treated nematodes' motility with a less time-consuming protocol. Indeed Haldimann's experiment took in account whether sick nematodes could reach a goal after a chosen length of time or not, but it didn't take in account whether nematodes lost their sensitivity to chemotaxy and/or just randomly moved without reaching the circle of OP50 while keeping physically the opportunity.

The greatest advantage is that you can record precisely the length of the traces left behind by the nematodes, being able to observe also its way of moving by looking at the quality of tracks left by the movement they make to move. This can provide good information, like whether the nematode is injured and should be removed of the analysis or if it is in good health and moves with adequate

movements. Downside is that the sample is rather small, with about 10 nematodes per dishes, compared to Haldimann's method where he could record 100 nematodes at once. But the biggest difference lies in the fact that the motility assays in this study takes directly and precisely in account the quality and quantity of tracks, and not just the capacity of reaching or not a goal in a chosen time, without knowing if those who cannot reach the goal are injured, lost their capacity of sensing the bacterial circle used as bait, or are indeed paralyzed by the polyQ disease expression.

Time constraints did not allow us to analyze further why Haldimann's results were that hard to reproduce. We already mentioned that the method was very much operator dependent, but maybe other factors could have played a role. Even though the strains used in my study were same strains they may have mutated, reducing their sensitivity to polyQ disease expression, thus reducing the difference of motility between polyQ0 wild type and polyQ35 nematodes. To verify this hypothesis, newly genetically modified nematodes strains should be used with our method and see if we could get results closer to the one of Haldimann's, without needing to struggle with the operator dependent factor.

To reflect the reality as accurately as possible, graphs are presented with boxplots. The aim of the study being a screening of ten drugs, the most important aspect to observe is a tendency of improvement or of toxicity, to see if the drug have a potential of slowing down or preventing the polyQ disease development. Boxplots can achieve this visual goal without relying on results with complicated statistical evaluations. Even with big differences between maximum and minimum values, the boxplot presentation allows us to observe well tendencies of improvement or reduction of motility.

Big differences in maximum and minimum values could be explained either by uncontrollable biological bias (such as weather or care of nematodes for example) or by the fact that maybe not all the nematodes that have received the treatment respond to it and those that do react to the treatment see their motility improved greatly, showing a great gap between the values and making taller the boxplot. This could especially be the case of piroxicam and anisindione in the first part of the experiment.

Another way to greatly improve the data acquisition of nematode motility would be to find a way of taking pictures of good quality with an instrument that could take a single picture of the whole dish, making the record of tracks easier and also avoiding the tiny bias of timing as several pictures were taken one after the other for each dish.

Next should be discussed the way piroxicam acts in the cell to improve the polyQ35 nematodes' motility. It has already been shown that in human gastric cells NSAID's could induce the expression of chaperone proteins such as an oxygen- and a glucose-related protein (Namba et al., 2010). NSAID's also increase intracellular concentration of Ca^{2+}(Tsutsumi et al., 2006), an essential step for the chaperone proteins upregulation, by blocking the Ca^{2+}-ATPase of the endoplasmic reticulum (ER) and inhibiting the intra-ER Ca^{2+} reuptake mechanism. This could explain the effect of piroxicam on polyQ35's motility improvement. However it doesn't explain why piroxicam is the only NSAID of

this study that had such an effect on polyQ35 nematodes. Not all NSAID's share the same mechanism of action so it would be wrong to generalize the effect of piroxicam to every NSAID's. Indeed it has been shown that aspirin, a NSAID cox-inhibitor can enhance HSP70 accumulation in plants (Snyman and Cronjé, 2008), that don't have the cyclooxygenases (COX) enzyme, present in humans. Piroxicam could easily have another unknown mechanism of action than COX inhibition that could explain the fact that it's the only NSAID of the study that showed such an effect on polyQ disease expressing nematodes.

Toxicity of piroxicam on wild type *C.elegans* has also been observed in the experiment (see *figure 13*). It didn't have a clear effect on size of treated *C.elegans* and it was not verified if it changed the life span or the reproductive profile of wild type *C.elegans*. The mechanism of the toxicity couldn't be explained without further experiments, but this should not make the reader think that piroxicam as a polyQ disease possible treatment isn't worth studying more because of a particular property of the studied model resulted in this negative observation. Indeed piroxicam is already a well known widely used NSAID drug in many different diseases. Our data cannot tell us if piroxicam reduced wild type nematode by toxicity or by another mechanism, for example by changing their behavior and reducing their will to move.

The concentration of 1% DMSO showed toxicity on *C.elegans* during this study. DMSO is well known to rigidify membranes and as the membranes act as a sensor activating the heat shock response, we can easily imagine that the rigidity of the membranes that DMSO causes, renders the membranes physically unable to make the stress-induced rearrangements needed to activate HSF-1 mentioned in *figure 5* (Horváth et al., 2008). Toxicity on wild type Q0 nematodes can either be explained by the fact that in the environmental conditions of the study even polyQ0 nematodes need a little heat shock response (hypothetically blocked by the action of DMSO) to keep equilibrated proteostasis, either by a direct toxicity through unknown mechanism.

Chloroform didn't show such toxicity on wild type *C.elegans* and could dilute piroxicam better, enabling us to mix it with the NGM medium and proving itself better than DMSO in this situation. This can be useful information for further study on *C.elegans*.

To complete this study many other things could have been done. If time would have permitted it, we could have measured induced RNA with microarrays and see if the expression of particular chaperone protein genes were overexpressed, we could also have made western blots to observe directly the augmentation in quantity of HSP's, HSP70 and HSP90 for example and see if they were upregulated and finally compare RNA overexpression and augmented proteins production.

As a conclusion we would recommend further studies on piroxicam as a dose-dependent elicitor of chaperone proteins, including on other models to verify its toxicity on non-polyQ expressing genotype. Anisindione would also be another possible promising drug to test. The method used for this study is recommended for quick but not very sensitive screening with *C.elegans*' model. DMSO that rigidifies membranes wouldn't be advised as a drug solvent on *C.elegans* for its previously discussed toxicity, and chloroform could be a substitute of choice if drugs dilution permits it.

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Appendix 1

Caenorhbaditis elegans' **synchronisation protocol :**

Egg preparation : removing bacterial or yeast contaminants from *C.elegans* stock plates.

Equipment and reagents

- 5M NaOH
- Household bleach (5% solution of sodium hypochloride)
- Sterile 15ml capped conical centrifuge tube
- Shaker or vortex mixer
- Table-top centrifuge
- Sterile Pasteur pipettes
- Two clean NGM plates, each seeded with an *E.coli* OP50 lawn

Method

1. Use contaminated *C.elegans* stock plates that have many gravid hermaphrodites[a]. Wash the plates with sterile H_2O. Pipette the H_2O across the plates several times to the loosen worms and eggs that are stock in the bacteria lawn.
2. Collect the liquid and worms in a sterile 15 ml conical centrifuge tube with a cap. Add H_2O to a total volume of 3.5 ml.
3. Mix 0.5 ml 5M NaOH with 1 ml bleach. Make this solution fresh before us ! Add to the centrifuge tube containing the worms.
4. Shake well or vortex the tube for a few seconds. Repeat shaking/ vortexing every 2 minutes for a total of 8 minutes.
5. Centrifuge in a tabletop centrifuge for 30 sec at 1300 *g* to pellet the released eggs.
6. Aspirate to 0.1 ml.
7. Add sterile H_2O to 5 ml. Shake well or vortex for a few seconds.
8. Repeat steps 5 and 6 two times, the second time centrifuging for 1 min.
9. Use a sterile Pasteur pipette to transfer the eggs in the remaining 0.1 ml of liquid to the edge of a clean NGM plate seeded with an *E.Coli* OP50 lawn.
10. The next day the eggs will have hatched and the larvae will have crawled into the *E.Coli* OP50 lawn. Transfer the hatched larvae to a clean NGM plate seeded with a lawn.

[a] It is embryos inside eggshells that will survive this procedure.