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Effects of plant hormones on the lifespan of *Caenorhabditis elegans*

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I declare this thesis is my original work which I created on my own. All sources I used during elaboration of this work are properly cited and listed in references.

In Olomouc, Alena Kadlecová

Abstract

Caenorhabditis elegans is a useful source of information about aging. As an eukaryot, it shares several crucial aging-related conservative pathways with mammals including humans. It provides a more complex picture of biological processes than cell cultures and, compared to mammal models, it is much easier and cheaper to maintain in the laboratory. This and also many other advantages make it an ideal organism to use in basic biotechnological research and also for screenings of compounds with anti-aging potential.

Cytokinins, plant hormones derived from adenine, are one group of such compounds. Some of them previously delayed senescence in cell cultures and also in flies. Moreover, they were able to suppress oxidative and glycation stress in mice. They have also been used in cosmetics for several years. However, cytokinins have never been tested on *C. elegans*.

In this thesis, 5 compounds, either natural cytokinin bases or their synthetic derivatives, along with 2 cytokinin ribosides, were tested. The results of these experiments show that cytokinins are able to prolong the lifespan of *C. elegans*, possibly via hormesis.

I would like to thank Dr. Jiří Voller and Dr. Jan Kammenga (Wageningen University) for their supervision and useful advice. I would also like to thank Joost Riksen (Wageningen University) and Kateřina Faksová for all their patience and the valuable help they gave me in the laboratory and to the whole crew from Laboratory of Nematology (Wageningen University) for their input and kindness. Last but not least, I thank Daniel Schubert for his voluntary help.

Contents

1 Aims of this thesis

The aim of this thesis was to evaluate C. elegans as model organism for screening of cytokinins for anti-aging activity.

As a part of this study I established the protocol of exposition, evaluated the acute toxicity of 7 cytokinins, introduced *C. elegans* in the Laboratory of Growth Regulators as a novel model organism for compound screenings and performed lifespan experiments with 7 compounds on *C. elegans* strain with wild type aging phenotype. Two most active compounds were than tested on oxidative stress sensitive or resistant mutant strains.

2 Introduction

Caenorhabditis elegans is a small free living soil nematode which has been successfully used as a model organism for decades. [1] It has been used particularly in the research of aging because it shares several conservative aging-related pathways and manifestations of aging with mammals including humans. It is also suitable for drug screenings due to its relatively short lifespan, cost-effectiveness and because it also enables high-throughput screening. [2]

In this thesis I used *C. elegans* for evaluation of anti-aging activity of several cytokinins. Cytokinins are plant hormones responsible (among other) for plant growth and development. They have also shown some interesting anti-aging effects in other invertebrate model organisms [3] and cell cultures [4] [5] that eventually led to clinical trials [6] [7] [8] and their application in cosmetics. The proposed mechanisms of their action in aging was their direct antioxidant activity [9] [10], ability to induce activity of organism‘s own defense mechanisms against oxidative stress [11] and hormesis [12]. Cytokinins also have other interesting biological activities – for example, they are able to protect against glycation stress [10] [13], suppress several manifestations of Alzheimer's disease [14] [15], correct splicing defects [16] [17] etc.

3 Review of the literature

3.1 *Caenorhabditis elegans*

Caenorhabditis elegans is a small but well differentiated free living soil organism. It belongs to the phylum nematoda and it feeds on bacteria.

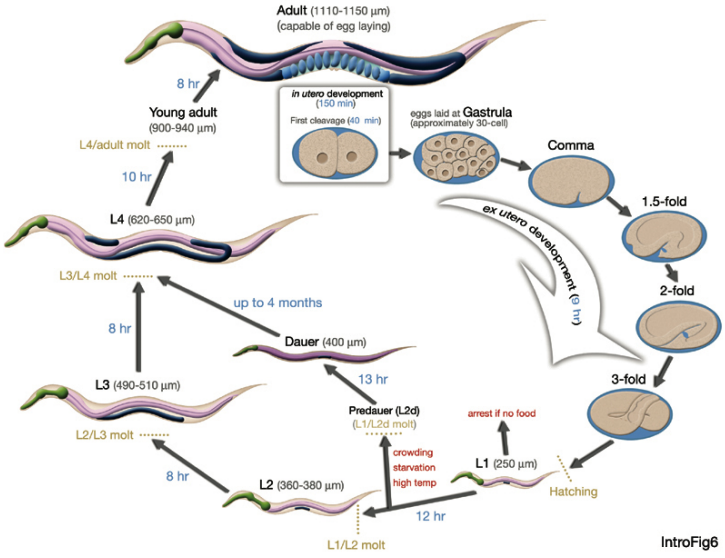
Using *C. elegans* as a model organism in developmental and later molecular biology started in 1960s. It was first proposed to be suitable for this purpose by Sydney Brenner. [1] Two Nobel prizes in physiology and medicine are linked to this worm. The first was awarded to Sydney Brenner, John Sulston and Robert Horvitz for genetics and organ development and programmed cell death, the second went to Craig Mello and Andrew Fire for describing the mechanism of RNA interference. Also a Nobel prize in chemistry for discovery and development of green fluorescent protein is associated with *C. elegans* – one of the laureates, Martin Chalfie, used *C. elegans* in his research. [1] *C. elegans* was also the first multicellular animal sequenced. [18]

Another interesting biological characteristic of this organism is its deterministic type of development. The fate of each cell in the body of this worm is more or less given. Every larval stage and also adults always have same number of cells. [19] This number is 1031 for adult males and 959 for hermaphrodites. Such non-random type of development can be found quite often in certain tissues and in certain growth stages of various species, but it is highly unusual on the whole organism level.

As mentioned above, *C. elegans* is gonochorist, meaning it has two types of sex – hermaphrodite (XX) and male (X0). It reproduces sexually – males preferentially inseminate hermaphrodites, but self-fertilization of hermaphrodites is also possible – sometimes even necessary, because males comprise only 0.1–0.2% of the population. The life cycle of *C. elegans* is very short. After embryogenesis, which takes place partly in the hermaphrodite body, partly outside, the L1 larva hatches. In the presence of food *C. elegans* goes through 4 larval stages (L1–L4) and finally develops into an adult worm. [20]

[1] www.nobelprize.org

Figure 1: Development of *C. elegans* (source http://www.wormatlas.org)



If the conditions are unfavorable, the larva might enter the dauer stage. Dauer larvae are very resistant to miscellaneous types of stresses. Genes which regulate entering into this phase are strongly related to the lifespan and aging [21] and will be discussed below. Larvae can arrest their development also in L1 stage. [22]

In the wild, adults die after 2–3 weeks. However, in the laboratory the lifespan might be longer and it depends on conditions (temperature, access to food, etc.).

There are many reasons why these worms became so popular as a model organisms. They are small both in size (they are only 1 mm long and they have 80 μm in diameter) and in genome (97 Mb), easy and cheap to maintain in the laboratory, their life cycle is very short and they are able to produce hundreds of offspring in just a few days. [1] Their transparency allows us to use fluorescent markers and dyes and easily study biological processes taking place *in vivo*.

Also, these worms are eukaryotic organisms just as mammals. In spite of many differences, there is quite a high similarity in our genetic information. They have about 60–80% of genes homologous with humans. This enables researchers to for example study functions of orthologs of disease-related genes, perform screenings for new drugs and find out more about their mechanism of action [23].

Another advantage is a simple methodology of RNAi experiments which is particulary useful for determining the functions of genes, their interactions, pathways and much more. [24]

[25] However, mutants are still needed, for example when testing medicinally potential compounds with the intention to determine their mechanism of effect. [1] Making mutants is not particularly difficult [1] and a lot of mutant strains are available from the *Caenorhabditis* Genetics Center (CGC). [2] Nowadays, one of the most important reasons of the widespread usage is the popularity itself – information, molecular tools, protocols etc. are readily available.

3.1.1 *C. elegans* and aging

Even if it might not apparent at first sight, *Caenorhabditis elegans* is a good model of human aging. We have several comparable manifestations of aging in common, for example sarcopenia [26] or the accumulation of age pigments. [27] [28]

Also, several signalling pathways influencing the lifespan and aging were identified and most of them are conserved in many species, from yeast to primates including humans. The most important one of these pathways is insulin/insulin-like growth factor signalling (IIS) pathway. [29] [30] The main role of the IIS pathway is to induce the formation of dauer larva. Activity of these genes in adult worms leads to lifespan prolongation. Also other factors influencing lifespan were identified, for example dietary restriction, mitochondrial signalling, sirtuins, some mutations in sensory neurons or several kinases like JUN kinase or target of rampamycin (TOR). All of these processes and effectors are more or less cooperating with IIS and/or each other. [31] [32]

However, these signalling pathways primarily have other functions in organism and their effect on aging might be just a byproduct. Therefore they should not be referred as "aging pathways". Mutations in components of these pathways sometimes lead to prolonged lifespan in laboratory conditions but the fitness of many long-lived mutants in natural conditions would probably be lower than the fitness of non mutated animals. [33]

The first long-lived single-gene mutants discovered were age-1, daf-2 and daf-16. All of these genes are coding the components of IIS pathway. Today dozens of mutants with prolonged lifespan are available, not only mutants in components of IIS but also mutants with slower feeding rates, certain neuronal defects or mutants with reduced germline proliferation. [32]

[2] http://www.cbs.umn.edu/cgc

Also the results from RNAi experiments provide useful insight into how the reduced level of certain gene products influences lifespan.

Prolonged lifespan is often accompanied by a decrease in fecundity. This effect is sometimes ascribed to the so called "reallocation of resources". The central idea of this theory is the trade-off between the maintenance of the adult body and reproduction because both of these processes are highly energy-consuming. However, it was demonstrated that in some long lived mutants it does not always work this way. Therefore it was proposed that mechanism might lie elsewhere, for example in signals from germline cells. [34]

Even though the amount of information grows rapidly, the molecular mechanism of aging is not understood yet. For example, when it comes to the link between aging and reactive oxidant species (ROS), which is particularly important for this thesis, the results of different studies with *C. elegans* sometimes even contradict each other. [35]

3.2 *Caenorhabditis elegans* in drug screenings

Drug screening on *C. elegans* offer many interesting options. A number of model strains representing diverse human diseases are available which allows us not only to look for potential new drugs but it might also give us insight about their metabolism and mechanisms of action in a complex eukaryotic organism. More complex data than those achieved from human cell cultures and relatively short time needed for conducting experiments plus their low price compared with experiments on mammal models make *C. elegans* an exciting option in drug research. Many researchers proposed using invertebrate model organisms as a possible bridge between cell cultures and mammals [36] and anticipate that this could improve the somewhat unsatisfying results of current drug research.

Worms can also be successfully used as hosts for infectious pathogens. These *in vivo* screenings provide wider range of information than tests of traditional microbicidal agents but they also enable us to search for compounds decreasing pathogen virulence, activating the host's immune system etc. [37].

Another possible application is research of compounds with nematocidal activity. Phytopathogenic nematodes cause significant losses of crops and have great economic impact on

agriculture [38]. Some nematodes are parasites of humans and animals. In spite of differences in lifestyle and consequential genetic adaptations, there is a number of similarities among the nematodes. When searching for antihelmitic compounds, using *C. elegans* as a model organism is safer and more comfortable than cultivating actual parasites in the laboratory. [39]

Earlier screening approaches were very laborious and therefore also low-throughput but several high-throughput methods of screening already exist. They are usually based on the optical (fluorescent) analysis of multiwell plates [40], using the liquid handling robotic stations or worm sorters (large particle analogue of flow cytometres) [3]. Also some experiments with arrays and microfluid devices [41] have been reported. Specific experiments allow using much easier methods, like measuring the chitinase activity [42] (larvae produce chitinase to be able to hatch, therefore activity of this enzyme indicates the rate of reproduction of worms) or establishing the uptake of fluorescent dyes [43] [44] [45]. High-throughput screenings have some cheaper alternatives that do not require special equipment. One of these is Wormscan analysis [46], the principle of which is scanning the plates with worms on a regular office scanner and subsequent image analysis using specialized programs (like ImageJ). Another interesting method based on a similar principle is Lifespan machine. [47]

3.2.1 Screenings of anti-aging compounds

A whole variety of potentially anti-aging compounds were tested on *C. elegans* so far – plant extracts [48] [49], polyphenols [50] [51], vitamins [52] [53] or even platinum nanoparticles [54] and medicaments like anticonvulsants [55], antidepressants, [56], antibiotics and many others. [57]

Some compounds that prolong the lifespan of worms, like resveratrol [50] [58] [59], curcumin [60] or epigallocatechin gallate [61], even already underwent [62] [63] or/and are currently going through clinical trials [4] in which their effects on several diseases is tested – for example Alzheimer's disease, diabetes or several types of cancer.

The aim of this work is to evaluate the effect of another promising group of anti-aging compounds, plant hormones cytokinins.

[3] http://www.unionbio.com/product/

[4] http://clinicaltrials.gov

3.3 Cytokinins

Cytokinins are phytohormones whose main role (among others) is to promote cell division in plants.

Apart from plants, cytokinins were found to naturally occur in other organisms [64] including humans. [65] [66]

In this work, we are investigating the effect of both isoprenoid and aromatic cytokinin bases. Some of the synthetic derivatives used in experiments also have a N^9-tetrahydropyranyl group. Also a few cytokinin ribosides were used. Unlike other tested cytokinin bases having cytoprotective effect, some of these ribosides were previously reported to be cytotoxic. [67] Although their cytotoxic effect also indicates an interesting potential for application in medicine, we tried to find out more about their possible protective impact in low concentrations.

3.3.1 Cytokinins and aging

Cytokinins are not newcomers in medical research. Their effects in organisms are quite broad and we are far from fully understanding all of them. Most relevant for this work are their anti-aging properties.

Anti-aging effects of some cytokinins, mainly kinetin, were investigated mainly in 1990s by the group of Dr S. Rattan (Department of Molecular Biology and Genetics, Aarhus University, Denmark) and led to the application of some of these compounds in cosmetics. [68]

It was discovered that kinetin delays aging of both human fibroblast cells [4] and in the fruit fly. [3] *Trans*-zeatin, another cytokin also showed to have the similar effect in human fibroblasts. [5]

Moreover, both of these compounds were found to be non-toxic and did not promote fibroblast proliferation. Increased proliferation of cells might be a sign of the risk of carcinogenic effect. Kinetin also modulates differentiation in human keratinocytes. [69]

Kinetin also went through clinical tests in which it proved its non-toxicity and showed skin-protective, anti-aging and de-pigmenting effect. [70] [71] [72] Kinetin might be also effective in the treatment of photo damaged skin [6] and some skin diseases like psoriasis or rosacea. [73]

Trans-zeatin might be able to prevent photo-aging too. It protected keratinocytes from losing water by attenuating the UV-induced loss of aquaporins (water transporters). [74]

Cytokinins might find application also in other fields than dermatology. It was previously reported that kinetin inhibited platelet formation *in vitro* [75] and it was able to protect the mice from acute pulmonary thrombosis and prevent thrombus formation in microvessels. In also showed anti-coagulation effect in rats. [76] Another interesting ability of kinetin is to correct defects in splicing in cell lines derived from patients suffering from familial dysautonomia [16] [17]. Its metabolite kinetin riboside-5′-triphosphate increases the activity of mitochondrial kinase PINK-1 whose dysfunction is linked with Parkinson's disease. [77]

Trans zeatin reduced neurotoxicity of amyloid β-protein plaques both in human cell lines and mice and also improved memory of scopolamin-treated mice. [14] [15] Amyloid β-protein plaques are the main characteristic of Alzheimer's disease.

Well known are also anti-cancer effects of several cytokinin ribosides. Kinetin riboside [78], *otrho*-topolin riboside [67], N^6-isopentenyladenosine [79] and N^6-benzyladenosine [80] showed anti-cancer effect in cancer cell lines and also mice.

While discussing various effects of cytokinins, allow me to remind that they are probably multi-target molecules and interact with several targets in cells. Due to their chemical structure it can be expected that some cytokinins might interact with purinome (proteins that use purines as their cofactors or substrates). [67]

3.3.2 Mechanisms of action in aging

The cytokinin with the best described anti-aging properties is probably kinetin. Its effect was at first ascribed to its antioxidant properties. [9] [10] This hypothesis was supported by increased activity of the enzyme catalase in kinetin fed fruit flies. [11] Kinetin can also significantly change the levels of certain other antioxidant enzymes in various rat tissues. [81] Thrombus formation which was suppressed by kinetin is tightly linked with increased ROS and kinetin lowered hydroxyl radical formation *in vitro*. [76] Kinetin was also reported to protect brain of mice from the oxidative stress induced by galactosis. [13]

The presumption that antioxidants can delay aging is based on the so called oxidative theory of aging. According to this theory, aging is a consequence of accumulation of oxidative damage

caused by ROS. This theory has been widely questioned in last few years and the amount of results which contradict it cannot be ignored. Nevertheless, some role of ROS in aging is probable. Certain new theories of aging have been proposed, for example redox stress theory in which the ROS play a role in signalling. This theory also explains certain effects like hormesis. [35]

Hormesis is an adaptive compensatory process when a low dose of stressor (certain chemicals, small repeated heat-stress or even physical exercise) leads to a beneficial effect on cells/ organisms due to the activation of protective mechanisms. Higher dose of the same stressor is toxic. [82]

The possibility of kinetin acting like a hormetic compound have been tested too. Kinetin was able to induce certain stress response pathways. [12] Therefore, kinetin is thought to work as both antioxidant and hormetic molecule at the same time. [83]

Moreover, the precursor of kinetin is one of the by-products of oxidative DNA base damage. [84] That could explain the fact that kinetin appeared in the urine of cancer patients but was not detected in the urine of healthy individuals. [65] It is interesting that it is able to incorporate itself in the DNA and it might induce reparation mechanisms. [85]

Also the effect of *trans*-zeatin in Alzheimer's disease models was partly ascribed to its ROS decreasing properties. It probably blocks the production of ROS by amyloid β-protein. [14] This proposed mechanism is logical – the brain cells are, due to high oxygen turnover and membranes rich in polyunsaturated fatty acids, very sensitive to oxidative damage. Authors of the publication [15] also discuss the possibility of *trans*-zeatin acting as an acetylcholinesterase inhibitor. It is possible that *trans*-zeatin too works as a hormetin. [83]

Other cytokinins were reported to act like antioxidants as well. [86] Still it must be taken into account that not all compounds which have antioxidant activity *in vitro* also have the desired effect in organisms [87]. But because some cytokinins were shown to have effect on lifespan of model animals and on aging in cell cultures before, it can be presumed that also other compounds from this group might have an interesting potential.

Not much is known about the mechanisms of action of ribosides. They probably vary depending on compound and, as was mentioned before, they might be also multi-target. But also kinetin riboside was reported to reduce ROS formation in leukemia cell lines. [78] Even though

it is probably not the reason of its anticancer activity, it might play a role in anti-aging properties that this work is trying to establish.

Just to demonstrate that cytokinins are multi-target molecules as stated above, I will mention here also a few of their other effects, not necessarily related to aging. Kinetin riboside and especially zeatin riboside showed the ability to interact with certain types of adenosine receptors. [88] These receptors are probably related with many pathophysiological events like neurodegenerative diseases but also cancer and many others. Some cytokinins can also interact with kinases and deaminases that regulate the function of adenine nucleosides and nucleotides which are present in cells and they are also transported by the same transporters. [67]

4 Materials

4.1 *Caenorhabditis elegans* strains

Strains used in my experiments were: N2 Bristol WT strain, thermosensitive sterile mutants BA17 (kindly provided by Laboratory of nematology, Wageningen University), and several mutant strains with altered sensitivity of oxidative stress – FX776, RB1197 and MQ887 (obtained from *Caenorhabditis* genetic center).

N2 worms were cultivated at 20°C for the experiments.

Mutants BA17 were maintained at 15°C and cultivated at 25°C – this temperature leads to their feminisation which prevents the worms from reproducing. Otherwise the phenotype of these worms is WT.

Strains MQ887, RB1197 and FX776 were maintained at 15°C and transferred to 25°C for lifespan experiments.

4.2 Buffers and media

All of the media and buffers mentioned were prepared according to [89], the book Caenorhabditis elegans: Modern biological analysis of an organism [90] or according to WormBook [5].

If not directly stated otherwise, the solutions were stored at room temperature.

4.2.0.1 LB medium

For 1000 ml:

- 10 g tryptone
- 5 g yeast extract
- 5 g NaCl
- for solid add 15 g agar
- distilled water to 1000 ml, adjust pH to 7.5 and autoclave

[5] http://wormbook.org

4.2.0.2 M9 buffer

For 1000 ml:

- 6 g Na_2HPO_4
- 3 g KH_2PO_4
- 5 g NaCl
- 0.25 g $MgSO_4 \cdot 7H_2O$
- deionized water to 1000 ml, autoclave

4.2.0.3 Potassium phosphate buffer

For 1000 ml:

- 136 g KH_2PO_4
- deionized water to 900 ml
- adjust pH to 6.0
- deionized water to 1000 ml, autoclave

4.2.0.4 Trace metals solution

For 1000 ml:

- 1.86 g Na_2EDTA
- 0.69 g $FeSO_4 \cdot 7H_2O$
- 0.20 g $MnCl_2 \cdot 4H_2O$
- 0.29 g $ZnSO_4 \cdot 7H_2O$
- 0.016 g $CuSO_4$
- deionized water to 1000 ml, autoclave and store in the dark

4.2.0.5 Nematode growth medium (NGM)

For 1000 ml:

- 3.0 g NaCl
- 2.5 g pepton
- 17 g agar
- deionized water to 1000 ml, autoclave
- after cooling down to approximately 55°C, add:
- 0.5 ml of sterile 1M $CaCl_2$
- 1 ml of cholesterol (5 mg/ml in ethanol)
- 1 ml of sterile 1M $MgSO_4$
- 25 ml of sterile potassium phosphate buffer
- use immediately after preparing, re-heating is not possible

4.2.0.6 S-basal medium

For 1000 ml:

- 5.9 g NaCl
- 50 ml of 1M potassium phosphate
- deionized water to 1000 ml, autoclave

4.2.0.7 Potassium citrate, 1M

For 1000 ml:

- 268.8 g tripotassium citrate

- 26.3 g citric acid monohydrate
- 900 mL deionized water
- adjust pH to 6.0
- deionized water to 1000 ml, autoclave

4.2.0.8 S-complete medium

For 1000 mL:

- previously prepared sterile S-basal
- 10 ml of sterile 1M potassium citrate
- 10 ml of sterile trace metals solution
- 3 ml of sterile 1M $CaCl_2$
- 3 ml of sterile 1M $MgSO_4$
- store in the dark

4.2.0.9 Stock solution of 5-fluorodeoxyuridin (FUDR)

- weigh desired amount of FUDR and dissolve in appropriate volume of S complete to achieve the stock solution of 0.6 mM
- sterilize by filtration
- store in the freezer

4.3 Cytokinins and cytokinin derivatives

Compounds used in the experiments were obtained from Laboratory of Growth Regulators (Palacky University Olomouc). 13 compounds were tested altogether (kinetin, N^9-tetrahydropyranyl

kinetin, *trans*-zeatin, N^6-benzylaminopurine, N^6-isopentenyladenine, *otrho*-, *meta*-, *para*-topolin, LGR-1796, LGR-1568, *otrho*-topolin riboside, kinetin riboside).

As a positive control I used resveratrol, a polyphenolic compound found especially in grapes and red wine, which has been repeatedly reported to have lifespan-prolonging effect on *C. elegans*.

For the experiments, compounds were dissolved in dimethylsulfoxide (DMSO) to achieve the stock solutions of the concentration 100 mM.

5 Methods

5.1 Basic methods

5.1.1 Preparing bacteria

- sterile liquid LB medium was prepared
- it was inoculated by *E. Coli* strain OP50 (provided by *Caenorhabditis* genetic center)
- suspension was cultivated overnight on shaker at 37°C
- suspension was transferred to sterile centrifugation tubes (15 ml or 50 ml) and stored in the fridge

5.1.2 Preparing the suspension of bacteria in S complete

- bacterial suspension in pre-weighed centrifugation tubes was centrifuged (3000 $\times$ G) for 10 minutes
- the excess liquid was removed carefully, weighed and re-suspended in S complete to achieve the stock solution with 60 mg of bacteria in 1 ml
- suspension was stored in the fridge (not longer than for one or two weeks) or frozen at -20°C

5.1.3 Preparing NGM plates

- NGM medium was prepared
- medium was pipetted to Petri dishes (PD) , volume was approximately 5–6 ml for 60 mm PD or 10–11 ml for 90 mm PD
- after medium solidified bacterial suspension in LB was added (30 μl for 60 mm PD, 50 μl for 90 mm PD) and spread with sterile cell spreader
- plates were sterile re-packed

- plates were cultivated overnight at 37°C or for 2 days in room temperature and stored in the fridge

5.1.4 Transfer of worms

- a piece of NGM agar which contains the worms was cut with a sterilized scalpel
- it was transferred to a new NGM plate

5.1.5 Bleaching

- plates containing pregnant adults were used
- worms were washed from the plate with 1 ml of M9
- suspension was shortly centrifuged (approximately 10 s)
- the excess M9 was removed (carefully not to disturb the worm pellet)
- 1 ml of bleaching solution was added (freshly prepared, for 10 ml: 1 ml 6–14% bleach, 0.5 ml 10M NaOH, 8.5 ml deionized water)
- suspension was shaken manually or on the thermoshaker (maximal rpm) for 5 minutes
- suspension was shortly centrifuged
- the excess liquid was removed, 1 mL of M9 was added (work quickly)
- suspension was shortly centrifuged
- for NGM plates: the pellet of eggs was pipetted to a new NGM plate
- for cultivation in S complete: washing steps were repeated 3 times with M9 and once with S complete, the worm suspension was removed from eppendorf tubes to PD/flask and corresponding amount of OP50 in S complete was added to achieve the final concentration of 6 mg of bacteria/ml

5.2 Growing worms on heat-killed bacterial food

5.2.1 Preparing the suspension of heat killed bacteria

- bacterial suspension in liquid LB was pipetted to eppendorf tubes
- heat killed bacterial suspension was prepared by shaking in thermomixer, 750 rpm
- times and temperatures chosen for the experiment were 90°C for 6, 10 or 15 minutes and 70°C for 15, 30 and 60 minutes
- suspension of heat-killed bacteria was centrifuged, re-suspended in S complete and stored as described above
- to confirm if the heat treated bacteria are really dead, one drop of bacterial suspension was cultivated overnight in 37°C on LB plate, suspension of living bacteria was used as a control

5.2.2 Growing worms on heat killed bacteria

- worms from starved plate (mostly L1 larvae) were washed with M9 and transferred to PD or 12 well plates with S complete enriched of the differently prepared heat killed bacterial suspensions in various concentrations (3, 4, 6, 8 mg/ml)
- worms were checked every day in the microscope and their behavior, growth and reproduction was compared with worms grown on living bacteria

5.3 Wormscan analysis

The protocol for using Wormscan was previously established by Rosanne Bartles and Yvonne Laven (their project was prepared as a part of the course Internship Nematology and the output report is called WormScan analysis: Now also available in Wageningen), however it was only used on worms grown on solid NGM plates.

Several changes in scanner settings and background showed to be more useful for liquid medium. These settings differ for each scanner and it is necessary to establish them every time when a different device is used.

Pictures were analysed using ImageJ.

- set the maximum memory to 850 MB or more
- open both pictures
- enter the known distance using the function Analyze → Set scale
- analyze changes in positions of worms by subtracting one picture from another (Process → Image calculator)
- de-speckle the picture (Process → Noise)
- adjust the corresponding threshold (Image → Adjust)
- count the number of particles meeting specific parameters (Analyze → Analyze particles) – these might differ on the type of analysis (for analysis of living adult worms set size to 50–500 pixels^2, circularity to 0–0.5 and check all options except Include holes and In situ Show)
- if necessary, (un)mark particles that (do not) match the worms or change the settings to better match your needs

5.4 Acute toxicity of cytokinins

5.4.1 Toxicity to bacteria: Disc diffusion test

- LB plates were fully seeded with bacteria
- 1 square cm pieces of filtration paper were prepared and rinsed in stock solution of compounds and in solvent (DMSO)
- paper pieces were carefully distributed on the plate with sterilized tweezers

- plates were incubated in room temperature for two days
- occurrence of inhibition zones was evaluated after 24 and 48 hours

5.4.2 Toxicity to worms

The following protocol is a a slightly modified protocol reported here [91] or here [44].

- pregnant adults were bleached
- the concentration of worms was estimated the next day (counting the L1 larvae in 10 drops/10 μl) and the corresponding amount of S complete + bacterial suspension was added to achieve the suspension with approximately 100 worms in 1 ml
- worms were grown for cca 2 more days until they reached adulthood
- 1 μl (2 μl respectively) of 1000 times more concentrated stock solution of the compound dissolved in dimethylsulfoxide (DMSO) was added to 1 ml of adult worm suspension to achieve the concentration required for the experiments
- concentrations used were 50, 100 and 200 μM for cytokinins and 1, 10 and 100 μM for potentially cytotoxic ribosides
- suspension with the 1 μl (2 μl respectively) of DMSO and non-treated worms were used as a negative control
- prepared suspensions were pipetted to 96 well plates, 200 μl each well, and covered with plastic sealer to prevent evaporation of medium
- living worms were calculated in inversion microscope the same day, then after 24 hours, 48 hours and after 72 hours
- the movement of the worms was triggered by short shaking on thermomixer and by strong light from microscope

5.5 Lifespan experiments

The design of lifespan experiments with BA17 strain was basically the same as in toxicity experiment only with several modifications.

- since BA17 mutant strain was used, the temperature for these experiments was 25°C
- in experiments with MQ887 strain we also used 25°C (temperature influences the lifespan of worms)
- suspension of worms was diluted to obtain the solution with approximately 60–80 worms in 1 ml – since the worms are cultivated for a longer period of time, the risk of starvation is becoming relevant and it is necessary to have less than 18 worms in each well (wells with more than 18 worms were excluded form the experiment)
- the sealer was changed once a week to make sure the worms have enough oxygen
- worms were counted in the microscope every 2–4 days

For experiments on mutant strains with altered oxidative stress sensitivity, FUDR (fluorodeoxyuridin) was added to the medium to prevent the reproduction of worms. The final concentration of FUDR was 0.12 mM.

5.6 Data analysis

- mean lifespan is the average lifespan of worms
- maximal lifespan is the average lifespan of 10% of the longest living worms
- analysis was done in OASIS (online application for survival analysis) [90]
- OASIS also provided Log-Rank P-values

A robust alternative to mean lifespan would be using median lifespan. However, in most lifespan extension studies with *C. elegans* researchers use mean lifespan because it is sensitive to shape of survival curve beyond the point when 50% of the population is dead.

Maximal lifespan usually describes the lifespan of the longest living individual. However, to obtain more robust data I decided to calculate it as an average lifespan of 10% of the longest living worms. This calculation was reported for example here [92].

6 Results and Discussion

Part of experiments described in this thesis were performed in the Laboratory of Nematology, Wageningen University. During the time I spent there, I learned the basics of work with *C. elegans*, I did most of the pilot experiments, established the protocol, evaluated the toxicity and performed the first lifespan experiment.

After my return to Palacky University, Olomouc, I introduced *C. elegans* as a new model organism for screenings here. Then I repeated the lifespan experiments and performed the lifespan experiments on oxidative stress sensitive or resistant mutants.

6.1 Heat killing

First, I needed to establish the way how to feed the experimental population of worms. Since the uptake of compounds was one of our biggest concerns, I decided to do the initial experiments with heat killed bacteria.

It has been well described that dead bacterial food influences the lifespan of worms. The reason of this is probably partly reduced food intake because worms are not able to sense dead bacteria that well and they are feeding less. Also, bacteria might produce some stress chemicals when heated which could be inhibitory to worms. [93] [94] Another reason might be the fact that bacteria proliferating in the intestine of the worm is considered to be the natural cause of death of *C. elegans*. This infection occurs due to age-related degradation of muscles in the digestive system. [95]

On the other hand, living bacteria are metabolising organisms, which might influence the uptake and the stability of compounds in medium. [96] Also, I was worried about the toxicity of high concentrations of compounds to bacteria. This could have resulted in divergent conditions in each well and influence the reliability of the data.

6.1.1 Results of heat killing

After the heat killing, no colonies occurred after 37°C overnight cultivation on LB plates. That means that the conditions of the treatment were sufficient to kill bacteria.

Bacterial suspensions were then used as a food source for worms. Growth and reproduction of worms was observed. The main points of these observations are listed below.

- All worms were growing slower than worms fed on living bacteria.
- After 3 days, worms cultivated with less that 6 mg of bacteria per ml did not grow at all.
- Worms cultivated on bacteria killed by 90°C / 10 minutes, 90°C / 15 minutes and 70°C / 60 minutes were developing significantly slower.

Longer heat treatment possibly cause the bacteria to lyse and make them inaccessible for worms to eat.

Another experiment was performed and only conditions 90°C / 6 minutes and 70°C / 15 minutes were used. Concentrations of the suspension were 6 mg/ml and 8 mg/ml. Worms were observed for 5 days.

Again, compared to worms fed by living bacteria, the development was slower. Least inhibited were worms grown at 90°C / 6 minutes in concentration 6 mg/ml. These conditions were chosen to be the most suitable for following experiments.

Higher concentrations of bacteria than 6 mg/ml might compromise the ideal conditions for worms. The fact that too much food inhibits worms is long known. [97]

6.1.2 The composition of LB medium influence the outcome of the heat killing

When new stock solution of bacterial suspension in LB was prepared, protocol for heat killing stopped working. The most probable reason to this is that the different yeast extract was used. Yeast extract contains autolysed yeast and it does not have any standardized nutrient level.

It is possible that different yeast extract contained different levels of peptides accessible to bacteria and also lower level on bivalent ions. These components are crucial for the formation of bacterial outer membrane and influence its properties. Weaker outer membrane might result in higher sensitivity to heat and the bacteria were probably lysated. [6] [98] Because of these complications I decided to switch to living bacteria after I returned to Olomouc. Living bacteria are, after all, used in most of the screening protocols available.

[6] Hiroshi Nikaido: The Limitations of LB Medium, 2009, American society of microbiology

6.1.3 Disc diffusion experiment

Change in the conditions required evaluation of toxicity of our compounds for bacteria and that is why I performed disc diffusion test.

Pieces of filtration paper were rinsed in stock solution of compounds (100 mM) which is 1000 times more than the concentrations used in experiments. Nevertheless, no inhibition zones were observed. That means that compounds do not have any toxic effect to bacteria even in very high concentrations.

6.2 Wormscan

The next issue that needed to be decided was how to analyse the survival of worms. As already mentioned in the literature review, several high-throughput methods which considerably reduce the labor-intensity of the analysis already exist. However many of these require specialized equipment which was not available in Wageningen or back in Olomouc. Still, there are some options and I decided to try a cheap and elegant method called Wormscan.

The principle of Wormscan is to take 2 pictures of the dishes with worms on a regular office scanner, one shortly after the other. Scanning is followed by comparing the changes in the position of worms on both. This gives the information about the movement of the worm which is the main parameter for evaluating if the worm is alive or dead. [46]

A working protocol was previously established in the Laboratory of nematology, Wageningen, for solid NGM plates. However, for exposure to a compound, liquid medium is more suitable. *C. elegans* have a very strong cuticule [99] and that the time needed for a food to pass its digestive system is only about 2 minutes. [100] In liquid medium worms are constantly exposed to compounds. The uptake of drugs was shown to be higher [96], the compound is distributed equally and also every potential attempt of worms to escape from the environment with xenobiotics is prevented.

I scanned adult worms in 24 well plates and on Petri dishes. The best results were obtained when:

- the setting of the scanner was changed to shadows 1000 compared to the original protocol described in WormScan analysis: Now also available in Wageningen

- dark blue paper was used as a background
- also some changes in the ImageJ setting were needed depending on each picture
- the sealer was removed from the multiwell plate

In case of 24 well plates there were problems with shadows cast by the walls of the plates. Shadows made about 1/3 of the well area impossible to analyse using ImageJ.

For Petri dishes shadows were not such an issue, only the edges of the plate could not be analysed. Worms were visible in the picture. But pictures were not clear because of the turbidity of the suspension caused by bacteria. After analysis in ImageJ it was clear that the number of worms captured on the picture and real number of worms differ significantly (about 1/4 of the worms was not detected).

Wormscan unfortunately did not provide reliable results. Better quality of scans might solve the problem but setting the scanner to higher resolution prolongs the time needed for taking one picture. Comparing 2 following pictures would probably be useless in these conditions.

Wormscan does not seem to be suitable for the analysis of worms grown in liquid medium. That also explains why, to my initial surprise, there is no mention about applying such design in literature.

Therefore I had to stick with the more laborious design and perform manual counting in microscope as reported in here [89] [56].

6.3 Solubility of the compounds

Another parameter which needed to be established was the concentration of compounds that would be used in experiments. These are *in vivo* screenings and that is why the concentrations of compounds must be quite high – the concentration in medium does not represent the level in body, animals have to ingest and absorb compounds first and they might also (partly) metabolise them.

Cytokinins were not tested on *C. elegans* before and results from previous experiments with flies and cell cultures cannot be considered reliable guidance for a completely different organism.

Based on previous experience with solubility in other media I decided to test concentrations of 50, 100 and 500 μM first. For ribosides which usually have cytotoxic effects I chose concentrations of 1, 10 and 100 μM.

Several cytokinins were forming crystals in S complete.

Table 1: Formation of crystals

Compound	c1	c2	c3
kinetin	−	−	+
N^9-tetrahydropyranyl kinetin	+	+	+
trans-zeatin	−	−	−
benzylaminopurine	−	+	+
isopentenyladenine	−	+	+
LGR-1796	−	−	+
ortho-topolin	+	+	+
meta-topolin	+	+	+
para-topolin	−	−	+
LGR-1568	−	+	+
kinetin riboside	−	−	−
otrho-topoline riboside	−	−	−

- no crystals; + crystals

For the following experiments I lowered concentration to 200 μM and I added of compounds to the medium gradually and mixed. However, in some cases crystallisation occurred anyway and that is why 7 out of 13 compounds had to be excluded from further experimentation.

The following experiments were carried out with: kinetin, N^9-tetrahydropyranyl kinetin, *trans*-zeatin, benzylaminopurine, isopentenyladenine, kinetin riboside and *otrho*-topolin riboside. I decided to use the highest concentrations possible and eventually adjust concentrations for lifespan experiments if any acute toxic effect to worms occurred.

6.4 Acute toxicity of compounds

The survival rate (% of worms alive) was established after 1, 2 and 3 days. As shown in the tables below, the survival rate was not lower than 90% in any case. Such death rate is considered to be small [101]. The average population for this experiment was 28 individuals.

Table 2: Survival rate (%) – non-treated

0 h	100
24 h	100
48 h	97
72 h	96

Table 3: Survival rate (%) – solvent (DMSO)

	0.1%	0.2%
0 h	100	100
24 h	99	100
48 h	98	97
72 h	97	93

Table 4: Survival rate (%) – kinetin

	50 μM	100 μM	200 μM
0 h	100	100	100
24 h	100	100	100
48 h	100	100	100
72 h	100	100	100

Table 5: Survival rate (%) – N^9-tetrahydropyranyl kinetin

	50 μM	100 μM	200 μM
0 h	100	100	100
24 h	100	100	100
48 h	94	94	100
72 h	94	94	100

Table 6: Survival rate (%) – *Trans*-zeatin

	50 μM	100 μM	200 μM
0 h	100	100	100
24 h	100	100	100
48 h	97	94	100
72 h	93	94	100

Table 7: Survival rate (%) – Benzylaminopurine

	50 μM	100 μM	200 μM
0 h	100	100	100
24 h	97	100	100
48 h	97	100	100
72 h	97	100	95

Table 8: Survival rate (%) – Isopentenyladenine

	50 μM	100 μM	200 μM
0 h	100	100	100
24 h	100	100	100
48 h	100	100	97
72 h	95	100	92

Table 9: Survival rate (%) – Kinetin riboside

	1 μM	10 μM	100 μM
0 h	100	100	100
24 h	100	100	100
48 h	100	100	92
72 h	97	100	92

Table 10: Survival rate (%) – *Ortho*-topoline riboside

	1 μM	10 μM	100 μM
0 h	–	100	100
24 h	–	100	100
48 h	–	100	100
72 h	–	91	100

Non-treated worms showed very similar death rate as cytokinins- and DMSO-treated worms.

From these values I also calculated viability (% of surviving worms compared to relevant DMSO control – this eliminates the effect of solutant from the results) which was not lower than 94% in any case.

Table 11: Viability (%) – 24 h

time	K	PK	*t*Z	BAP	iP	KR	oTR
c1	101	101	101	98	101	101	–
c2	101	101	101	101	101	101	101
c3	100	100	100	100	100	100	100

Table 12: Viability (%) – 48 h

time	K	PK	*t*Z	BAP	iP	KR	oTR
c1	102	96	99	99	102	102	–
c2	102	96	96	102	102	102	102
c3	103	103	103	103	100	94	103

Table 13: Viability (%) – 72 h

time	K	PK	*t*Z	BAP	iP	KR	oTR
c1	104	97	97	101	98	100	–
c2	104	97	97	104	104	104	94
c3	108	108	108	102	99	99	108

Such results most probably mean that neither the solvent nor any of compounds show any significant acute toxic effect in any of the concentration used.

Therefore I conducted lifespan experiments with all the compounds and the concentrations were not further changed.

6.5 The effect of compounds on the lifespan of *C. elegans* – the experiment with heat killed bacteria

From the data acquired from the lifespan experiment, it is possible to establish the effect of compounds on maximal lifespan and mean lifespan. The average population in this experiment was 39 individuals.

Table 14: Mean LS (days of adulthood)

Compound	50 μM	SE	100 μM	SE	200 μM	SE
kinetin	5.6	0.67	6.4	0.63	8.2	0.91
N^9-tetrahydropyranyl kinetin	6.7	0.41	6.6	0.47	5.7	0.5
trans-zeatin	5.5	0.53	5.6	0.48	5.2	0.58
benzylaminopurine	8.3	1.23	4.2	0.58	6.1	0.63
isopentenyladenine	5.9	0.51	5.7	0.53	7.4	0.68
Compound	1 μM	SE	10 μM	SE	100 μM	SE
kinetin riboside	6	0.71	5.1	0.63	5.6	0.59
otrho-topoline riboside	6.5	0.68	5.2	0.47	6	0.57
Controls	NT	SE	DMSO 0.1%	SE	DMSO 0.2%	SE
	5.3	0.49	6.5	0.71	7.2	0.49

Table 15: Maximal lifespan (days of adulthood)

Compound	50 μM	100 μM	200 μM
kinetin	12	12	21.6
N^9-tetrahydropyranyl kinetin	12	13	13.5
trans-zeatin	13	12	15.7
benzylaminopurine	20	13	12.4
isopentenyladenine	12	13	18
Compound	1 μM	10 μM	100 μM
kinetin riboside	14	17.3	12.7
otrho-topoline riboside	15.6	12	14.5
Controls	NT	DMSO 0.1%	DMSO 0.2%
	12	17.8	12.7

6.5.1 DMSO prolongs the lifespan of the worms

The first significant observation was that DMSO treated worms also showed prolonged lifespan compared to non treated control. This effect was 48% increase in mean and 25% increase in maximal lifespan for worms exposed to 0.1% DMSO and 6% increase in mean and 27% increase in maximal lifespan for 0.2% DMSO.

Until quite recently researchers discussed only a possible toxic effect of DMSO in high concentrations. In several publications DMSO is reported not to have lifespan-prolonging effect. [50] [59]

However, the ability of DMSO to prolong the lifespan of worms was previously discussed in these two publications [102] [103]. In [103], RT-PCR revealed increased expression of several protective genes which are targets of DAF-16/FOXO and were previously reported to increase lifespan. Effect was dependent on sirtuins. In the newer publication [102], authors proved the effect was independent on IIS or dietary restriction by performing experiments on several mutant lines and ascribed the effect to the ability of DMSO to affect the protein homeostasis.

Even though the effect was not statistically significant in our case it is still a phenomenon

that should not be ignored. DMSO is widely used as a solvent in drug screenings. Also potentially anti-aging drugs are often dissolved in DMSO so it is crucial to point out any of its possible effects in aging. Unfortunately, publications mentioned above contradict each other in several points (brood size unaffected/affected, oxidative stress genes upregulated/no increased resistance to ox. damage, modulating IIS/effect independent on IIS). It is known that DMSO has various effects on cell cultures, for example it is able to interact with the membranes and change their properties and permeability. [104] Both research groups used slightly different concentrations and cultivation conditions. Perhaps this is the reason why their conclusions differ. Further experiments should be done to establish if and how the DMSO really works in aging.

6.5.2 K, PK, *t*Z and iP prolong the maximal lifespan of worms

Four compounds showed additional ability to prolong the maximal lifespan of the worms in the highest concentrations when compared to the relevant DMSO control. For more clarity, results are listed in table below.

Table 16: Effects of cytokinins on the maximal lifespan (%)

Compound	increase (%)
kinetin 200	40
N^9-tetrahydropyranyl kinetin 200	6
trans-zeatin 200	19
isopentenyladenine 200	29

The effect was significant only in the highest concentration. It might be dose dependent but more experiments would have to be done to support that. It might be interesting to test also higher concentrations and see if and how far the trend would continue. However, cytokinins in higher concentrations formed crystals in the medium. This problem might be solved by considering another way of dissolving the compounds (like in HCl or NaOH).

Another way how to increase the uptake might be the use of mutants with more permeable cuticule. [105]

6.5.3 K and iP prolong mean and maximal lifespan in the highest concentrations

Not all of the compounds that prolonged maximal lifespan showed an effect when considering mean lifespan. Only 2 out of 4 previously mentioned compounds had been able to increase both of the parameters taken in account – kinetin and isopentenyladenine.

Kinetin prolonged the mean lifespan for 15%. For isopentenyladenine, it was 5%. The effect was statistically significant (P value = 0.011 for kinetin and 0.022 for iP) when compared to non-treated worms but it was not significant compared to DMSO control. The numerical difference was there and also on the Kaplan-Meier curves the effect is apparent. The problem might be a small experimental population.

On the following graphs the effect of both cytokinins is compared to the relevant DMSO control and non treated worms.

Figure 2: Kinetin 200 μM, lifespan

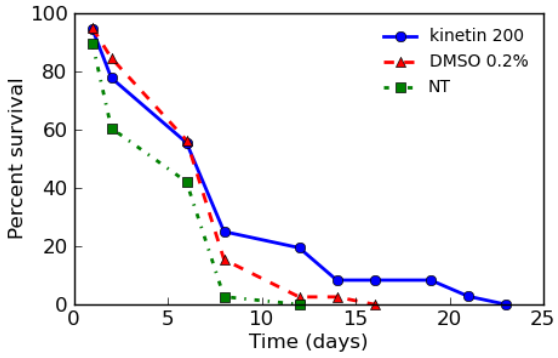
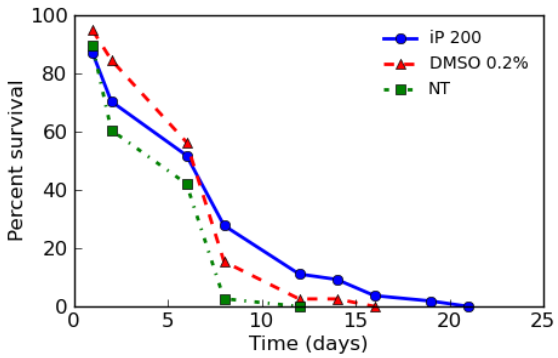


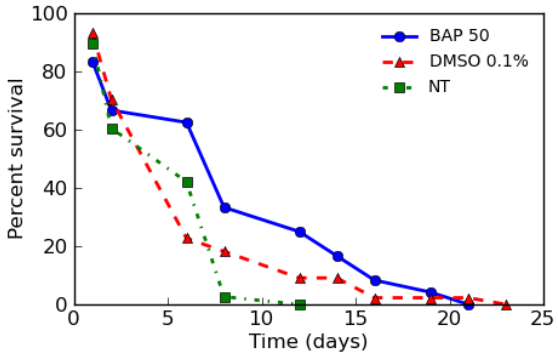
Figure 3: Isopentenyladenine 200 μM, lifespan



6.5.4 BAP prolong maximal and mean lifespan in the lowest concentration

Benzylaminopurine also showed an effect on both mean and maximum lifespan. It prolonged the maximal lifespan for 11% and mean lifespan for 13%. Also in this case the effect was statistically significant (P value = 0.009) only when compared to non-treated worms. Remarkable is that unlike other compounds, benzylaminopurine showed an effect only in the lowest concentration.

Figure 4: Benzylaminopurine 50 μM, lifespan



The highest activity in low concentration might imply hormesis. However, any discussion about the effect of BAP and why it seemingly differes form other tested cytokinins would be premature with such limited amount of data.

Neither of ribosides showed any additional lifespan-prolonging effect. It is possible that they do not have any anti-aging effect but since they also do not show any toxicity even in the highest concentration used it is possible that the absorption might be the problem here. Also, worms might be able to metabolise and inactivate the ribosides. The HPLC-MS analysis of levels and metabolism of compounds in nematodes would reveal more. Anyway, ribosides were excluded form further lifespan experiments.

Even though I was not able to statistically prove the lifespan-prolonging effect I observed some trends which suggested that *C. elegans* might be a useful model organism for screenings of biologically active cytokinins. There are also possibilities how to use worms in revealing the mechanisms of their anti-aging action. Therefore I introduced *C. elegans* in Laboratory of Growth Regulators and conducted experiments with bigger populations. Based on the results

of pilot lifespan experiment I continued only with kinetin, N^9-tetrahydropyranyl kinetin, *trans*-zeatin, benzylaminopurine and isopentenyladenine.

6.6 The effect of compounds on the lifespan of *C. elegans* – experiments with living bacteria as food

I decided to discuss results I obtained in Olomouc separately because of the change in conditions (living bacteria). Because of that I think that results obtained in Wageningen and results from Olomouc are not fully comparable and it should be considered as a different experiment rather than repetitions.

Lifespan experiments were performed twice. The average population was 60 individuals in the first and 66 individuals in the second experiment.

Table 17: Experiment 1: Mean lifespan (days of adulthood)

Compound	50 μM	SE	100 μM	SE	200 μM	SE
kinetin	20.3	0.48	20.8	0.54	21.1	0.69
N^9-tetrahydropyranyl kinetin	20.7	0.47	21.1	0.51	21.3	1.03
trans-zeatin	19.9	0.72	21.4	0.52	18.7	0.56
benzylaminopurine	19.7	0.60	18.8	0.52	20.2	1.13
isopentenyladenine	20.1	0.58	18.3	0.60	19.6	0.63
Controls	NT	SE	DMSO 0.1%	SE	DMSO 0.2%	SE
	19.5	0.63	19.8	0.49	20.3	0.93

Table 18: Experiment 1: Maximal lifespan (days of adulthood)

Compound	50 μM	100 μM	200 μM
kinetin	24	24	27.7
N^9-tetrahydropyranyl kinetin	25.6	24.3	29
trans-zeatin	24	24.6	24.9
benzylaminopurine	25.3	24.7	25
isopentenyladenine	24.4	24	27
Controls	NT	DMSO 0.1%	DMSO 0.2%
	24.8	25.4	29

Table 19: Experiment 2: Mean lifespan (days of adulthood)

Compound	50 μM	SE	100 μM	SE	200 μM	SE
kinetin	19.1	1.26	20.5	1.02	21.7	0.39
N^9-tetrahydropyranyl kinetin	22.4	0.76	19.4	0.43	20.4	0.79
trans-zeatin	21.9	0.79	18.7	0.58	16.8	0.55
benzylaminopurine	18.9	0.58	16.6	1.35	20.2	1.13
isopentenyladenine	17.3	0.6	18.7	0.51	18.9	0.95
Controls	NT	SE	DMSO 0.1%	SE	DMSO 0.2%	SE
	16.4	0.55	18.7	0.47	18.0	0.55

Table 20: Experiment 2: Maximal lifespan (days of adulthood)

Compound	50 μM	100 μM	200 μM
kinetin	25.8	25.7	27.2
N^9-tetrahydropyranyl kinetin	30	25.6	26.3
trans-zeatin	29.3	25.0	23.1
benzylaminopurine	24.5	22.1	22.9
isopentenyladenine	23.2	24.4	25
Controls	NT	DMSO 0.1%	DMSO 0.2%
	24.5	23.2	23.6

6.6.1 Effect of DMSO on the lifespan of *C. elegans*

Yet again I observed the effect of DMSO on the lifespan of the worms. In the first experiment it was smaller but nevertheless present. In the second experiment the effect of DMSO was more obvious and in some cases it even reached statistical significance. This phenomenon was already discussed, so I would just like to re-direct the reader to the subchapter 6.5.1.

Table 21: Increase in maximal and mean lifespan (%) in DMSO treated worms

Control	Mean LS – Exp. 1	Exp. 2	Max. LS – Exp. 1	Exp 2
DMSO 0.1%	1.5%	14%	2.4%	none
DMSO 0.2%	4%	10%	17%	none

6.6.2 Kinetin, N^9-tetrahydropyranyl kinetin and *trans*-zeatin prolonged the lifespan of *C. elegans* in both experiments

I observed some beneficial effect in worms treated with K, PK and *t*Z in both experiments. Most significant results are summarized in the tables below.

In the first experiment I observed just a relatively small beneficial effect of cytokinins on the lifespan. Also the effect of DMSO was just small. Compounds in concentrations that nu-

merically prolonged the lifespan compared to both controls and reached statistical significance compared to at least one control are shown.

Table 22: Experiment 1 – lifespan-prolonging effect of cytokinins compared to non-treated nematodes

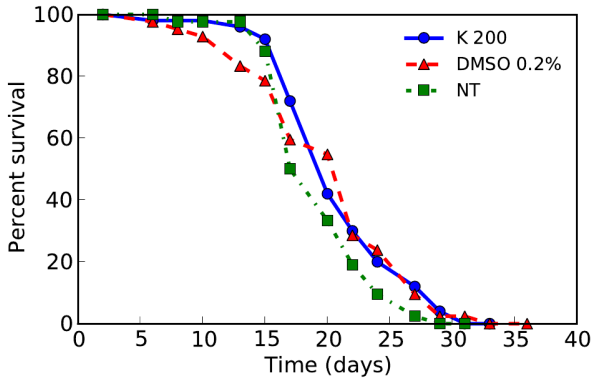
Compounds	Mean LS increase (%)	Max. LS increase (%)	P value
K 200 μM	8.5	12	0.045
PK 200 μM	9	17	0.03
*t*Z 100 μM	10	none	0.01

Table 23: Experiment 1 – lifespan-prolonging effect of cytokinins compared to DMSO control

Compounds	Mean LS increase (%)	Max. LS increase (%)	P value
K 200 μM	4	none	>0.05
PK 200 μM	5	none	>0.05
*t*Z 100 μM	8	none	0.0324

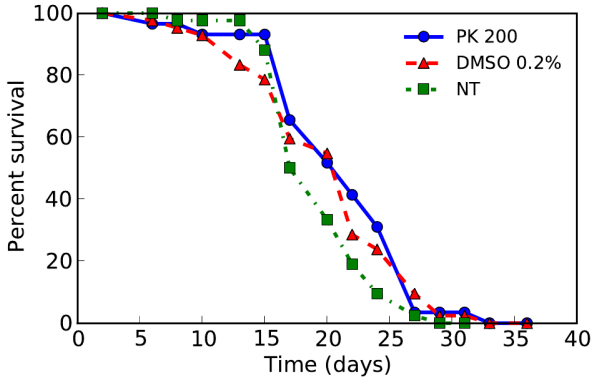
The process of the experiment and the actual effects are better demonstrated in Kaplan-Meier curves of survival.

Figure 5: Kaplan-Meier curve of survival for kinetin 200 μM



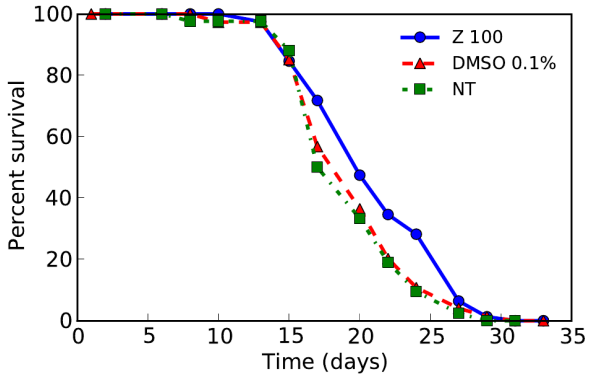
Also lower concentrations of kinetin prolonged the lifespan (effect was not statistically significant). The effect seemed to be dose-dependent.

Figure 6: Kaplan-Meier curve of survival for N^9-tetrahydropyranyl kinetin 200 μM



As in the case of kinetin, also lower concentrations of PK prolonged the lifespan in seemingly dose-dependent manner.

Figure 7: Kaplan-Meier curve of survival for *trans*-zeatin 100 μM



In case of *trans*-zeatin, the situation was a little bit different. Also 50 μM of *t*Z had a slight beneficial effect on lifespan but in higher concentration the lifespan of nematodes was lowered compared to both controls. Such effect is typical for hormesis.

In biogerontology, hormesis is understood as the increase of longevity due to mild stress which causes the activation of protective mechanisms in the cells. Low concentrations of *t*Z might have worked like this whereas the higher concentrations lead to damage and shortening of the lifespan.

Kinetin and *trans*-zeatin were previously suggested to be hormetic compounds. [83] Kinetin was able to increase levels of certain differentiation markers (lower responsiveness to these markers is one of the symptoms of aging) and it also stimulated stress response pathways. [12]

Therefore it might be possible that all cytokinins work as hormetins but *t*Z might be more active or maybe absorbed more efficiently.

In the second experiment the effect of cytokinins was much more obvious. Also the effect of DMSO control in this experiment was unfortunately much higher. That is why only the compounds that had statistically significant additional effect compared to DMSO controls are shown in this case.

Table 24: Experiment 2 – lifespan-prolonging effect of cytokinins compared to non-treated nematodes

Compounds	Mean LS increase (%)	Max. LS increase (%)	P value
K 100 μM	18	5	0.0001
K 200 μM	32	11	0.0
PK 50 μM	37	22	1.5×10^{-8}
PK 200 μM	25	7	1.6×10^{-5}
*t*Z 50 μM	33	20	3.3×10^{-5}

Table 25: Experiment 2 – lifespan-prolonging effect of cytokinins compared to DMSO control

Compounds	Mean LS increase (%)	Max. LS increase (%)	P value
K 100 μM	4	11	0.01
K 200 μM	21	15	1.3×10^{-6}
PK 50 μM	20	29	7.0×10^{-6}
PK 200 μM	14	13	0.0037
*t*Z 50 μM	17	26	2.6×10^{-5}

Again for better demonstration of the results the Kaplan-Meier curves are shown.

Figure 8: Kaplan-Meier curve of survival for kinetin 100 μM

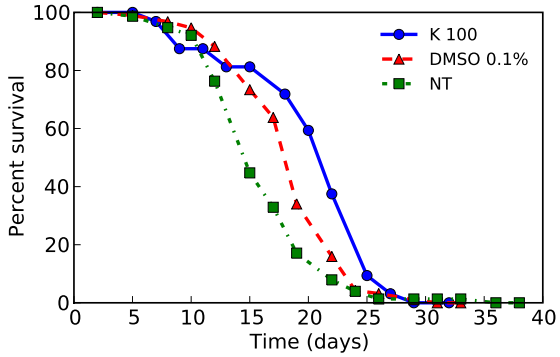
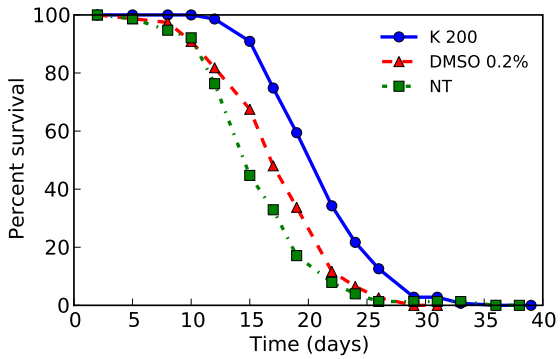


Figure 9: Kaplan-Meier curve of survival for kinetin 200 μM



Also in this experiment the effect of kinetin was dose-dependent. Also 50 μM of kinetin had slight lifespan-prolonging effect (not significant).

Figure 10: Kaplan-Meier curve of survival for N^9-tetrahydropyranyl kinetin 50 μM

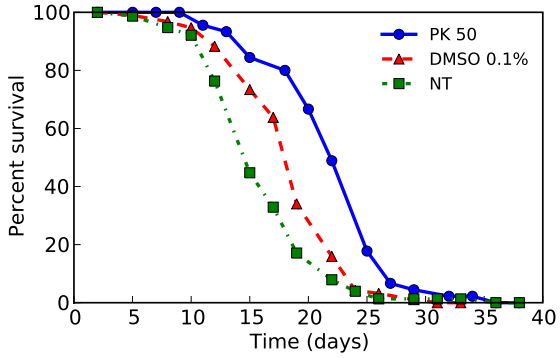
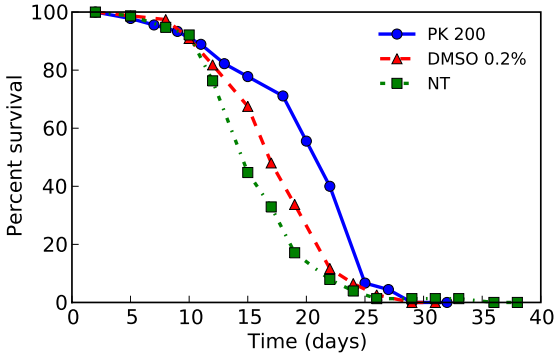


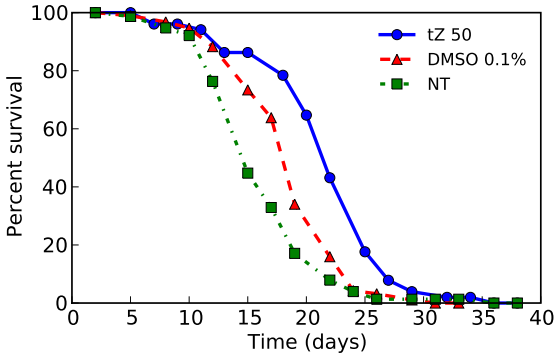
Figure 11: Kaplan-Meier curve of survival for N^9-tetrahydropyranyl kinetin 200 μM



In the case on PK the effect was different than in the first experiment – it did not seem to be dose dependent any more. Also 100 μM PK prolonged the lifespan but the effect was not significant compared to DMSO. In this case the lowest concentration of PK was the most effective.

My results are very variable and more experiments have to be done to figure out how PK really works. Also repetitions of this experiment with even bigger populations should be done.

Figure 12: Kaplan-Meier curve of survival for *trans*-zeatin 50 μM



Like in the first experiment 200 μM of *trans*-zeatin did not have any beneficial effect on lifespan and it even lowered the lifespan compared to DMSO treated worms. 100 μM of *trans*-zeatin prolonged the lifespan but its effect was not significant and it was smaller than effect of 50 μM of *t*Z in this case. Again it seems that *t*Z works probably as a hormetin.

6.6.3 Resveratrol had just a very small effect on the lifespan of *C. elegans*

In parallel with the second experiment I performed one lifespan experiment with 100 μM resveratrol (such concentration was first reported here [50] and it was used also in most of later studies investigating the effect of resveratrol on *C. elegans*) so results of cytokinins could be compared with a compound with known lifespan-prolonging effect.

In this experiment, resveratrol was able to prolong the lifespan of nematodes compared to non-treated worms.

Table 26: Mean and maximal lifespan

Compound	Mean LS	SE	Max. LS
Resv 100 μM	19.0	0.45	24.4

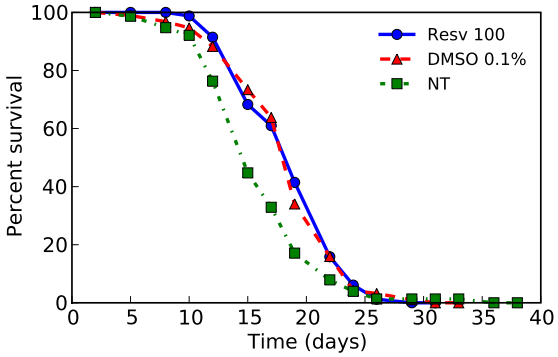
However the effect compared to DMSO treated nematodes was just very small. The statistical significance was not reached.

Table 27: Lifespan prolonging effect of resveratrol 100 μM

x NT	Mean LS increase (%)	Max. LS increase (%)	P value
Resv 100 μM	16	none	0.0014
x DMSO	Mean LS increase (%)	Max. LS increase (%)	P value
Resv 100 μM	1.5	5	>0.05

The small additional beneficial effect on lifespan can be seen also in the Kaplan-Meier curve.

Figure 13: Kaplan-Meier curve of survival for resveratrol 100 μM



Even though resveratrol was repeatedly reported to prolong the lifespan of nematodes [50] [58] [59] in my experiments its effect was just very poor. It did not provide results good enough to serve as a positive control. In some cases researchers do report somewhat non-unified effect of resveratrol on lifespan [106] and it is true that I tested it only once – further experiments maybe also with concentration scale might give better results. But I think for the future experiments it should be a priority to perform a screening just with compounds with reported lifespan-prolonging effect and pick more effective positive control.

Benzylaminopurine and isopentenyladenine did not show any or showed just small effect on the lifespan in both experiments. It was not statistically significant in any concentration. It is possible that absorption was the problem here since I used living bacteria in these experiments. [96] Also results of the lifespan experiment conducted in Wageningen could be overrated due to the small population.

6.7 Effects of cytokinins on the lifespan of strains with altered oxidative stress sensititity

Since it was previously suggested that the mechanisms of action of cytokinins might be based on their antioxidant properties or/and their ability to induce defense mechanisms against ROS (discussed in more details in subchapter 3.3.2) I also decided to test what effect cytokinins have in strains sensitive and resistant to oxidative damage.

Due to the limited time I decided to continue only with K and PK which seemed to be the most effective in previous experiments. The concentration used was 200 μM.

6.7.1 Cytokinins were toxic to the strains with increased sensitivity to oxidative stress

Strains used in these experiments were FX776 and RB1197. The first strain has a deletion in superoxiddismutase-1 gene, the second in calatase-1 gene. If cytokinins can directly protect the worms against oxidative stress, under concentrations which were beneficial for BA17 worms I expected even more pronounced effect on lifespan of these mutants.

If the activity of cytokinins is dependent on the particular enzyme deleted in a mutant their positive effect on lifespan can be missing. However, *C. elegans* dispose of several variants of these enzymes [35] so I could still expect to see a prolonged lifespan due to the increased activity of other type of superoxiddismutase or catalase.

Surprisingly, what I observed was a mortality rate close to 100% after 24 hours. Worms in the WT control were alive. Concentrations beneficial for WT worms are highly toxic to strains sensitive to oxidative stress.

It does not seem probable that cytokinins can directly protect against oxidative stress. This effect can be however explained by hormetic effect of cytokinins. If they work as a mild stressors, the level of stress which was just enough to trigger defense mechanisms in BA17 might have been way too high for these sensitive strains.

It would be good to see how would cytokinins do in lower concentrations. Unfortunately, due to the limited time I did not have a chance to conduct such experiments in time for this thesis.

6.7.2 Cytokinins prolonged the lifespan of MQ887 mutants resistant to oxidative stress

The strain MQ887 bears the mutation in isp-1 gene which encodes a Rieske iron sulfur protein which is a part of subunit III in respiratory chain [7]. This mutation leads to decreased oxygen consumption which results in slower development and movement but on the other hand it makes these worms more resistant to oxidative stress.

[7] http://wormbase.org

As with the sensitive mutants, I anticipated that if the main mechanism of function of cytokinins was their antioxidant activity any effect on this strain would be much smaller.

But cytokinins prolonged the lifespan of this strain. The average population in this experiment was 60 individuals.

Table 28: Mean lifespan (days of adulthood) – MQ887

Compound	200 μM	SE
kinetin	12.6	0.54
N^9-tetrahydropyranyl kinetin	12.1	0.53
Control		SE
NT	11.2	0.48
0.2%	10.6	0.54

Table 29: Maximal lifespan (days of adulthood) – MQ887

Compound	200 μM
kinetin	16.9
N^9-tetrahydropyranyl kinetin	17.1
Control	
NT	14.6
0.2% DMSO	16.0

Table 30: Strain MQ887 – lifespan-prolonging effect compared to non-treated nematodes

Compounds	Mean LS increase (%)	Max. LS increase (%)	P value
K 200 μM	12.5	16	0.0242
PK 200 μM	8	17	<0.05

Table 31: Strain MQ887 – lifespan-prolonging effect compared to DMSO control

Compounds	Mean LS increase (%)	Max. LS increase (%)	P value
K 200 μM	19	5.5	0.014
PK 200 μM	14	7	0.0448

In these mutants DMSO did not prolong the lifespan – it even decreased it a bit comparing to non-treated worms. In one of the papers focused on mechanism of action of DMSO in *C. elegans* aging [103] researchers indeed suggested its effect might be connected to oxidative stress but in the second paper this effect was not observed. [102] The divergent effect in BA17 and MQ887 strain is definitely interesting but any discussion about the possible mechanism of action of DMSO would be preliminary at this point. Unfortunately due to the limited time this experiment was held only once and the population was quite small. In the future, repetitions of these experiments should be held and if I could to see the same phenomenon again it would definitely be a good lead for further investigation of the lifespan-prolonging effect of DMSO.

Due to this, both of the compounds reached the statistical significancy compared to DMSO control. But when compared to non-treated worms P value for PK was 0.075. That is slightly more than what is considered to be acceptable, even though there is a numerical difference.

Kaplan-Meier curves of survival are shown below.

Figure 14: Kaplan-Meier curve of survival for kinetin 200 μM – MQ887 strain

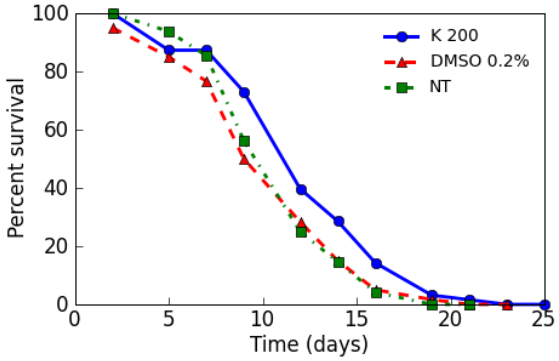
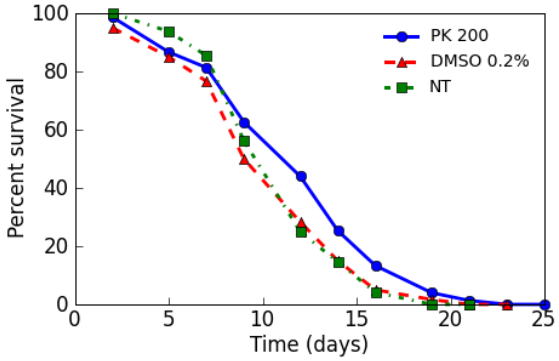


Figure 15: Kaplan-Meier curve of survival for PK 200 μM – MQ887 strain



From these results in can be concluded that it is quite unlikely that the effect of cytokinins in aging is based mainly on their antioxidant properties. Hormesis might again be the possible explanation.

6.7.3 Summary of the results and future prospects

Several selected cytokinis showed consistent anti-aging activity even though the statistical significancy was not reached in all cases. However, my results are quite variable among experiments. Some differences could be ascribed to biological variability among experimental populations but most probably it is not only that.

Even though populations in these experiments were bigger than in my first experiments in Wageningen it is still just dozens of individuals and I am concerned about the significant influence of extremes. Also, if the effect is just small it is impossible to reach statistical significancy on a small population. It would be best to perform screenings with hundreds of individuals. However I am afraid that such screenings would require a change of methodology. Even though Dr. Petrascheck who designed the methodology I used in this thesis designated this method as high-throughput and performed a screening of thousands of compounds using it [57], I am afraid that the throughput I describe in this thesis is near the limit of what single person is able to perform in the lab.

There are several possibilities of how to improve the throughput. The laboratory I worked in does not have equipment for high-throughput methods which would allow us to perform automated assays in liquid medium (wormsorter, automated microscopy, liquid handling robotic

station etc.). It would be possible to switch to solid medium assays but as I already described above, one of my concerns was the uptake of compounds. [96] To make sure if the solid medium would be suitable for this purposes, I sent several samples to analytical lab whose employees kindly agreed to perform analysis of uptake of cytokinins in worms grown in solid and liquid medium using UPLC-MS. These tests should also tell us more about the metabolism of PK and ribosides. Unfortunately results were not finished soon enough for this thesis.

Solid-based assays which could be then considered for the lifespan analysis would be Wormscan or Lifespan machine which would not demand any initial investment (or just a relatively small one). Also there could be a possibility to use a phenotyping system available in Centrum Haná for future experiments. Together with researches from Photon System Instruments, the developer of the system, we performed several pilot tests which confirmed that this system should indeed be suitable for this purpose.

Results, especially of the statistical analysis, are also probably distorted by the lifespan-prolonging effect on DMSO. It is clear that I have to consider using different solvent in the future. I already performed several pilot experiments with dissolving in NaOH and HCl. It is less comfortable than using DMSO due to the necessity to control pH carefully, neutralisation and addition of several more steps to ensure sterility. Also, the choice of solvent has to be careful – there is a risk it might degrade compounds. On the other hand these solvents do not show any toxic effect on the worms (the product of neutralisation is after all water and NaCl which are regular components of the medium) and it seems that I might be able to reach higher experimental concentrations without precipitation. This might also enable me to perform experiments with a wider range of concentrations, create relevant dose-dependence curves and see if they really would be typical for hormesis.

7 Conclusion

The aim of this study was to evaluate *C. elegans* as model organism for screening of cytokinins for their anti-aging activity. In lifespan experiments, anti-aging activity was evaluated as the ability of compounds to prolong mean and/or maximal lifespan of worms and it is established from Kaplan-Meier survival curves.

During the optimisation of screening conditions I experimented with various ways of feeding the worms. To prevent metabolization of the compounds by bacteria, heat-killed bacteria were used in first experiments. [96] However, the heat-killing procedure has to be careful – bacteria need to be dead but not lysated or else they are not accessible for worms to eat. The outcome of heat-killing is significantly influenced by the composition of LB medium in which bacteria grow – accessible nutrients are crucial for the strength of their outer membrane. Several components of LB medium do not have any standardized nutrient levels and when a different brand of chemicals is used, the protocol of heat-killing needs to be re-established. That is why this way of feeding was abandoned after the first lifespan experiment and I used living bacteria instead.

There are two ways how to maintain the experimental population – in liquid and solid medium. For compound screening liquid medium is much more suitable – it should ensure better uptake [96], tested compounds are distributed equally and any attempt of worms to escape from environment with xenobiotics is prevented. Since the uptake of cytokinins was one of my biggest concerns, I decided to use liquid medium and subsequential data analysis had to be adjusted to that.

I experimented with two different ways of worm counting. Firstly, I tested the method Wormscan [46]. This method is elegant, simple and it is working well for solid medium assays, however, it showed to be unsuitable for liquid medium. Therefore I analysed the lifespan by counting the living worms in a microscope [89]. This method is standard but allows processing of only limited number of samples. Slight effects in a small population may not reach statistical significance.

Several cytokinins formed crystals in the medium and had to be excluded from further experiments. Toxicity and lifespan experiments were conducted with 7 compounds – kinetin,

N^9-tetrahydropyranyl kinetin, *trans*-zeatin, benzylaminopurine, isopentenyladenine, kinetin riboside and *otrho*-topolin riboside.

Studied cytokinins were toxic neither to *C. elegans* with WT aging phenotype nor to the bacteria worms are fed with.

DMSO is a widely used solvent in life-extension studies. However, in my experiments, it prolonged the lifespan of the BA17 strain. This phenomenon was previously investigated by two research groups with different conclusions regarding to mechanism of action. [102] [103] Any interference of solvent in lifespan experiments is a problem. Effects of DMSO could be overcome by changing the solvent in future experiments, which should not be a problem. In follow-up experiments, I plan to use another solvent with no effect on lifespan.

I have observed reproducible effect of 3 cytokinins – kinetin, N^9-tetrahydropyranyl kinetin and *trans*-zeatin – on the lifespan of *C. elegans*. Benzylaminopurine and isopentenyladenine showed quite promising results in the pilot lifespan experiment but the effect was not reproducible in later experiments with bigger populations. Two ribosides which I tested – kinetin riboside and *ortho*-topolin riboside – did not have any lifespan-prolonging effect.

The mechanism of action of cytokinins in aging was previously ascribed mainly to their antioxidant properties. They should be able to directly reduce oxidative damage [9] and also induce an organism's own defense mechanisms [11]. Two of the compounds – kinetin and *trans*-zeatin – were also proposed to be hormetins [83]. Therefore I decided to test the two most active compounds – kinetin and N^9-tetrahydropyranyl kinetin – on mutant strains with altered oxidative stress sensititity. If cytokinins really work as antioxidants, I expected to see even more pronounced lifespan-prolonging effect in strains sensitive to oxidative stress and reduced effect in a strain resistant to oxidative damage.

Surprisingly both of the tested compounds were highly toxic to the strains sensitive to oxidative damage. Moreover, the lifespan of the strain resistant to oxidative damage was prolonged. This suggests that anti-aging effects of cytokinins might not be mainly a result of their antioxidant activity. My results could be however explained by the other proposed mechanism – hormesis. In biogerontology, hormesis is understood as the increase of longevity due to a mild stress which causes the activation of protective mechanisms in cells. Some of my data support this hypothesis. I observed repetitive beneficial effect of *trans*-zeatin in low concen-

tration whereas in higher concentration it slightly reduced the lifespan. Such effect is typical for hormesis. Also, if cytokinins work as mild stressors, it could explain their toxic effects to the sensitive strains. The level of stress caused by concentrations of cytokinins which were beneficial to WT worms might simply have been too high for these sensitive mutants. However, these results are preliminary and further experiments are required to find out more about how cytokinins work in aging.

Overall, my results suggest that some cytokinins have life-extending effects in *C. elegans*. Follow-up experiments in larger populations are necessary to confirm these observations. The experimental design should also reflect some pitfalls identified in this thesis, above all the effect of DMSO on lifespan.

It is clear that there is still plenty to optimise but there is a reasonable solution for most of the problems.

C. elegans could become a very useful tool for searching for another, possibly more effective, cytokinins (and maybe also other plant hormones) with anti-aging effect and defining their mechanism of action using mutants. Also searching for compounds active against a variety of stresses and diseases using model strains and many other applications would be possible.

List of abbreviations

*t*Z *Trans*-zeatin

BAP Benzylaminopurine

DMSO Dimethylsulfoxide

FUDR 5-fluorodeoxyuridin

IIS insulin/insulin-like growth factor signalling

iP Isopentenyladenine

K Kinetin

KR Kinetin riboside

LB Lysogeny broth

LS Lifespan

NGM Nematode growth medium

NT non-treated

OASIS Online application for survival analysis

oTR *Ortho*-topoline riboside

PD Petri dish

PK N^9-tetrahydropyranyl kinetin

ROS Reactive oxygen species

RT-PCR Real-time polymerase chain reaction

SE Standard error

TOR target of rampamycin

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