Biogerontology (2018) 19:109–120
https://doi.org/10.1007/s10522-017-9742-4

RESEARCH ARTICLE

Natural plant hormones cytokinins increase stress resistance and longevity of *Caenorhabditis elegans*

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Received: 20 October 2017 / Accepted: 14 December 2017 / Published online: 18 December 2017

Abstract Cytokinins are phytohormones that are involved in many processes in plants, including growth, differentiation and leaf senescence. However, they also have various activities in animals. For example, kinetin and *trans*-zeatin can reduce levels of several aging markers in human fibroblasts. Kinetin can also protect mice against oxidative and glyoxidative stress, and prolong fruit flies' lifespan. Additionally, several cytokinins are currently used in cosmetics. To extend knowledge of the breadth of cytokinins' activities, we examined effects of natural cytokinin bases on the model nematode *Caenorhabditis elegans*. We found that kinetin, *para*-topolin and *meta*-topolin prolonged the lifespan of *C. elegans*. Kinetin also protected the organism against oxidative and heat stress. Furthermore, our results suggest that presence of reactive oxygen species, but not DAF-16 (the main effector of the insulin/insulin-like growth factor signaling pathway), is required for the beneficial effects of kinetin. Ultra-high performance liquid chromatography-tandem mass spectrometric analysis showed that kinetin is unlikely to occur naturally in *C. elegans*, but the worm efficiently absorbs and metabolizes it into kinetin riboside and kinetin riboside-5′-monophosphate.

Keywords Cytokinin · Kinetin · Topolin · Zeatin · Phytohormones · Aging · *Caenorhabditis elegans*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10522-017-9742-4) contains supplementary material, which is available to authorized users.

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Introduction

Caenorhabditis elegans (*C. elegans*) is a free-living soil nematode that is used in many research fields, including biogerontology. It is a small, transparent animal with a fast life cycle, short lifespan and many other advantageous characteristics for biogerontological studies (Corsi 2006). There is significant homology between *C. elegans* and human genes,

and many human disease-related genes have counterparts in worms (Lai et al. 2000). *C. elegans* has also been used to elucidate genes and pathways that are involved in aging (Antebi 2007) and conserved among various species. The organism is routinely used to screen compounds suspected to have lifespan-prolonging activity, including many substances originating from plants. Such compounds could potentially be used medically in attempts to prevent age-associated diseases and increase elderly patients' quality of life. However, effects of cytokinins on the nematode's lifespan, which could be substantial for reasons outlined below, have not been previously evaluated.

Cytokinins are phytohormones that participate in regulation of cell division, differentiation, senescence and many other processes in plants (Kieber and Schaller 2014). Interestingly, some cytokinins have been detected in human cell extracts and urine (Barciszewski et al. 1996, 2000), and animal cells possess the metabolic pathways required to convert cytokinin bases to ribosides and ribotides (Hertz et al. 2013; Mlejnek and Kuglik 2000; Mlejnek and Doležel 2005), which also occur in plants.

Various activities of cytokinins in both mammalian cell cultures and animals have been reported (Voller et al. 2017b). The ribosides are often toxic, but cytokinin bases are mostly known to have protective effects, often ascribed to their antioxidant activity. Kinetin, a cytokinin base consisting of adenine substituted at the N^6-position with an aromatic side chain, can protect against oxidative damage in various systems, both directly by acting as an antioxidant and indirectly by inducing cells' antioxidant defense mechanisms (Olsen et al. 1999; Sharma et al. 1997; Jabłonska-Trypuc et al. 2016; Verbeke et al. 2000). For example, McDaniel et al. (2005) found that kinetin can reduce levels of reactive oxygen species (ROS), lipid peroxidation and DNA damage in cell cultures exposed to UVB. Kinetin can also ameliorate several aging markers in human cell cultures (Rattan and Clark 1994; Lee et al. 2006), prolong the lifespan of fruit flies (Sharma et al. 1995), and reduce glyoxidative stress both in vitro (Verbeke et al. 2000) and in vivo (in mice exposed to galactose) (Liu et al. 2011).

Kinetin has also been proposed to act as a hormetin (Rattan 2008), i.e. a compound capable of inducing adaptive response in lower concentration and toxic effect in higher doses.

Recently, kinetin has also been shown to directly influence other cellular processes. For example, it can reportedly correct aberrant alternative splicing of pre-mRNA in several genetic disorders (Slaugenhaupt et al. 2004; Pros et al. 2009), and initial data suggest that kinetin might be effective in patients with familial dysautonomia (Axelrod et al. 2011). Additionally, a metabolite of kinetin, kinetin riboside-5′-monophosphate, has reported neuroprotective properties by acting as a neo-substrate for PINK-1 kinase, mutations of which are linked to a hereditary form of Parkinson's disease (Hertz et al. 2013).

Trans-zeatin, a cytokinin base consisting of adenine substituted at the N^6-position with an isoprenoid side chain, can also retard aging in human fibroblasts (Rattan and Sodagam 2005), reduce the neurotoxicity of amyloid β in human cell lines, and improve the memory of scopolamine-treated mice (Choi et al. 2009; Kim et al. 2008).

Both kinetin and *trans*-zeatin are currently used in cosmetic products, as is a semi-synthetic derivative of kinetin, pyratine (6-furfuryl-amino-9-(tetrahydropyran-2-yl)purine). These products are mostly marketed as skin-protective, anti-aging or de-pigmenting agents, but lotions containing kinetin and pyratine are also effective in the treatment of photo-damaged skin (McCullough et al. 2008; Wanitphakdeedecha et al. 2015) and can reduce symptoms of rosacea (Wu et al. 2007). There is also some evidence that kinetin might be effective against psoriasis (An et al. 2017).

Furthermore, cytokinin ribosides, including kinetin riboside, have anticancer activity both in vitro and in vivo (Voller et al. 2010). Depending on concentration, length of treatment time, and cell line, this activity may be mediated by blocking cell cycle progression, or by inducing apoptosis either by depleting ATP or disrupting mitochondria (Cabello et al. 2009; Cheong et al. 2009; Voller et al. 2017a; Ishii et al. 2002).

In this study, we evaluated effects of kinetin and other cytokinin bases on *C. elegans*, particularly its longevity, and probed kinetin's metabolism and mechanism of action in the worm.

Materials and methods

C. elegans strains and maintenance

Worms were maintained at 20 °C under standard cultivation conditions (Strange 2006). The strains used in these experiments were N2, BA17 (*fem-1*) and CF1038 (*daf-16*(mu86)). All strains were provided by the *Caenorhabditis* genetic center (http://cbs.umn.edu/cgc/home).

Chemicals

Test compounds, a generous gift from OlChemIm Ltd., included cytokinin bases with either isoprenoid (*trans*-zeatin, dihydrozeatin, *cis*-zeatin, N^6-isopentenyladenine) or aromatic side chains (kinetin, N^6-benzylaminopurine, *ortho*-topolin, *meta*-topolin, *para*-topolin). Dimethylsulfoxide (DMSO) was used as a solvent. All chemicals, including juglone (5-hydroxy-1,4-naphthoquinone) used for stress assays, were purchased from Merck. Media and buffers were prepared as previously described (Solis and Petrascheck 2011).

Lifespan experiments

Lifespan experiments were performed using similar methodology to Solis and Petrascheck (2011), with several modifications. Briefly, BA17(*fem-1*) worms were bleached and age-synchronized progeny were cultivated at 25 °C until they reached adulthood. Young adults were washed from NGM plates with S-complete medium and counted, then the resulting suspension was diluted with S-complete. Worms were fed with a suspension of *Escherichia coli* OP50 in S-complete, then treated with the compounds and pipetted into 96-well plates (approximately 15 individuals per well). Plates were sealed to prevent evaporation of the medium and kept at 25 °C. Living worms were identified based on their movement, and counted using an inverted microscope three times a week. Movement was induced by a light stimulus from the microscope and by briefly shaking the plate on a plate shaker prior to counting. Worms that failed to finish normal development, worms that were injured by pipetting during preparation of the experiment and groups of worms permanently tangled together were excluded from the analysis. The seal was removed once a week for several minutes to provide the worms with sufficient oxygen. Experiments were performed under sterile conditions, so no antibiotics or fungicides were required. Experiments were performed at 25 °C because this results in all of the BA17(*fem-1*) worms developing into females, thereby preventing reproduction and avoiding the need for fluorodeoxyuridine (FuDR) treatment.

Stress assays

Age-synchronized L4 larvae were washed from the plates, counted and fed with a suspension of *E. coli* OP50 in S-complete, as in the lifespan experiments. DMSO-treated worms were used as negative controls. Worms were maintained at 20 °C. For oxidative stress assay, worms were exposed to a lethal concentration of juglone (500 µM) 3 days after pretreatment, and counted using an inverted microscope every 1–2 h. To evaluate thermotolerance, worms were incubated at 35 °C for 90 min, then counted using an inverted microscope. Animals that failed to respond to the light stimulus were scored as dead. Since N2 (WT) and mutant worm strains were used in the stress assays, the suspensions were supplemented with 25 µg/ml FUdR to prevent reproduction.

Preparation of worm extracts and cytokinin analysis by UHPLC-MS/MS

Worms were exposed to either kinetin or vehiculum alone in liquid S-complete medium supplemented with living or heat-killed bacteria (80 °C for 15 min). After 24 h, worms were washed four times with M9 buffer and snap-frozen in liquid nitrogen. *Escherichia coli* strain OP50, which was used as a food source, was cultivated overnight in LB medium containing either kinetin or DMSO. Bacteria were centrifuged and the pellets were washed with sterile water. Pellets were then re-suspended in M9 buffer and centrifuged. This process was repeated twice. The supernatant was removed, wet pellets were weighed, and samples were snap-frozen in liquid nitrogen. All samples were stored at − 70 °C prior to testing.

Cytokinin metabolites were quantified as previously described (Svačinová et al. 2012), with modifications described by Antoniadi et al. (2015). Samples (1 ml) were homogenized and extracted in 1 ml of 80% methanol (MeOH) containing a cocktail

of stable isotope-labeled internal standards (0.25 pmol of cytokinin bases and ribosides, and 0.5 pmol of cytokinin nucleotides per sample). The extracts were purified using an Oasis MCX column (60 mg/3 ml, Waters) conditioned with 1 ml each of 100% MeOH and H2O, then equilibrated sequentially with 1 ml of 50% (v/v) nitric acid, 1 ml of H2O, and 1 ml of 1 M HCOOH. After samples had been applied to the MCX column, unretained compounds were removed by washing with 1 ml of HCOOH (1 M) and 1 ml 100% MeOH. Pre-concentrated analytes were eluted in two steps, using 1 ml of 0.35 M NH_4OH aqueous solution followed by 2 ml of 0.35 M NH_4OH in 60% (v/v) MeOH solution. The eluates were then evaporated to dryness in vacuo and stored at − 20 °C. Cytokinin levels were determined using ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-MS/MS) with stable isotope-labelled internal standards as references. Three independent biological replicates were analyzed.

Concentrations were initially measured in pmol/100 worms and pmol/g of bacterial wet pellet. They were then converted to µM, assuming that each worm had the average volume of 9.54 pl reported by So et al. (2011), and the *E. coli* cells' density was 1.09 mg/ml, as they consist of 2/3 water and 1/3 other components, e.g. protein, with a typical density of 1.3 mg/ml, as reported in the BioNumbers database (Milo et al. 2009).

Statistical analysis

Descriptive statistics—mean, standard deviation, median and 90th quantile (Q90) survival—and Kaplan–Meier survival curves were calculated using the OASIS 2 online application (Han et al. 2016). The log-rank test was used to compare survival curves of control populations with those of populations treated with cytokinins. p values for each comparison in an experiment were corrected using the Bonferroni method. A two-tailed Z-test for proportions was applied (in R) when comparing effects of compounds on survival at a single time-point.

Results

The effect of cytokinin bases on the worms' lifespan

Test compounds included cytokinins with both aromatic (kinetin, N^6-benzylaminopurine, *ortho*-topolin, *meta*-topolin, *para*-topolin) and isoprenoid (N^6-isopentenyladenine, *trans*-zeatin, dihydrozeatin, *cis*-zeatin) side chains. All compounds were tested at three concentrations—200, 100 and 50 µM—except topolins, which precipitated in the medium at 200 µM and so were not tested at this concentration. None of the tested compounds were acutely toxic to *C. elegans* and treated worms displayed no behavioral or morphological abnormalities compared to control worms. Kinetin reportedly retards development in fruit flies (Sharma et al. 1995), but we observed no such effect in *C. elegans*: growth rates of worms cultivated in kinetin for three generations were indistinguishable from controls cultivated in vehicle alone (data not shown).

Four compounds—kinetin, *trans*-zeatin, *meta*-topolin and *para*-topolin—were initially identified that significantly increased the worms' lifespan (Table 1 and Supplementary Materials Table 1).

Compounds showing significant activity in the initial screening were re-tested at the concentration at which they showed the greatest efficacy. Repeated experiments confirmed the ability of kinetin, *para*-topolin and *meta*-topolin to prolong worm lifespan. Despite *trans*-zeatin yielding promising results in the initial screening, the effects were much weaker and insignificant in two repeated experiments with larger experimental populations (Table 2, Supplementary Materials Table 2). Figures 1, 2, and 3 show Kaplan–Meier survival curves for worms treated with kinetin, *meta*-topolin, and *para*-topolin, respectively.

We further characterized the protective activity of kinetin to facilitate the interpretation of any observed effects in *C. elegans* in the context of findings by other researchers, as it is the most intensively studied cytokinin known to have such activity.

Kinetin increases thermotolerance and resistance to oxidative stress

Next, as manipulations that prolong worms' lifespan also often increase their stress resistance (Zhou et al.

Table 1 Results of initial screenings of natural cytokinin bases' effects on *C. elegans* lifespan

Compound	Concentration (μM)	N	Mean—% change	Median—% change	Q90—% change	P value
kinetin	200	143	+ 21	+ 20	+ 18	5.6×10^{-7}
	100	121	+ 6	+ 5	+ 4	> 0.05
	50	75	+ 2	+ 5	+2	> 0.05
N^6-isopentenyladenine	200	107	− 2	− 6	− 1	> 0.05
	100	108	− 2	+ 2	− 1	> 0.05
	50	70	− 8	− 4	− 6	> 0.05
N^6-benzyladenine	200	111	+ 1	+ 2	− 2	> 0.05
	100	100	+ 3	+ 5	0	> 0.05
	50	91	0	− 17	+ 6	> 0.05
ortho-topolin	100	138	+ 3	+ 2	+ 15	> 0.05
	50	132	+ 6	+ 5	+ 20	> 0.05
meta-topolin	100	108	0	− 3	+ 10	> 0.05
	50	130	+ 10	+ 8	+ 27	1.2×10^{-2}
para-topolin	100	143	+ 8	+ 8	+ 21	1.2×10^{-2}
	50	111	+ 5	+ 1	+ 18	> 0.05
trans-zeatin	200	96	− 9	− 15	− 7	> 0.05
	100	115	+ 1	− 5	+ 2	> 0.05
	50	51	+ 17	+ 19	+ 15	1.2×10^{-4}
cis-zeatin	200	122	− 4	− 8	− 2	> 0.05
	100	133	− 3	− 5	− 6	> 0.05
	50	129	− 2	− 4	+ 6	> 0.05
dihydrozeatin	200	117	− 6	− 10	− 5	> 0.05
	100	126	− 1	− 5	0	> 0.05
	50	131	0	− 4	0	> 0.05

The table shows % change of mean (average) and median (time point at which 50% of the population had died) lifespan and Q90 of populations treated with the indicated compounds in comparison to control populations. Q90—the time point at which 90% of worms die—was selected as a more robust indicator of such effects than maximal lifespan (time at which the last worm dies). N indicates the size of the population. Average N for the control population was 105

Table 2 Results of confirmatory experiments with four active natural cytokinin bases selected from initial screening experiments

Compound	Concentration (μM)	Mean—% change	N	Median—% change	Q90—% change	P value
kinetin	200	+ 6	113	+ 5	+ 6	3×10^{-2}
meta-topolin	50	+ 15	113	+ 20	+ 16	1.7×10^{-2}
para-topolin	100	+ 12	128	+ 10	+ 12	4.6×10^{-2}
trans-zeatin	50	− 3	125	− 8	− 4	> 0.05
		+ 2	104	+ 4	+ 1	> 0.05

The table shows % change of mean (average) and median (time point at which 50% of the population had died) lifespan and Q90 of populations treated with the indicated compounds in comparison to control populations. N indicates the size of the population. Average N for the control population was 107

2011), we tested the possibility that the longevity-enhancing effect of kinetin is linked to increases in resistance to various stresses, in further assays involving exposing worms to 100 or 200 μM of kinetin. This pre-treatment protected the worms from oxidative stress caused by exposure to a lethal

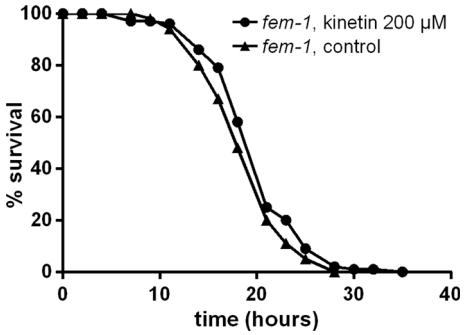

Fig. 1 Effect of 200 µM kinetin on aging of a *C. elegans* population

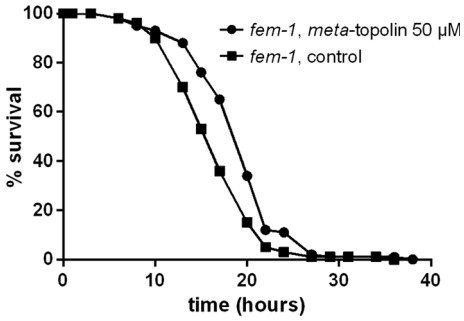

Fig. 2 Effect of 50 µM *meta*-topolin on aging of a *C. elegans* population

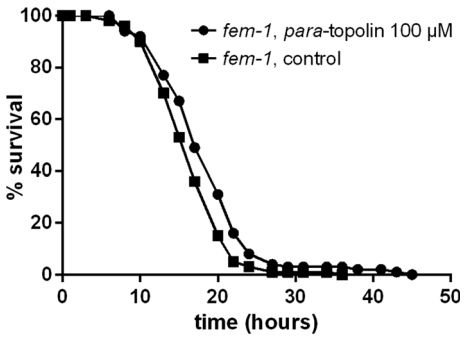

Fig. 3 Effect of 100 µM *para*-topolin on aging of a *C. elegans* population

concentration of the ROS generator juglone. In four independent experiments, 200 µM of kinetin significantly increased the survival of worms ($p < 0.05$), while the effect of 100 µM kinetin was weaker and only significant in some cases (Supplementary Materials Table 4). Representative Kaplan–Meier curves are shown in Fig. 4.

Kinetin also increased the thermotolerance of *C. elegans*. Exposure to 35 °C for 90 min resulted in an

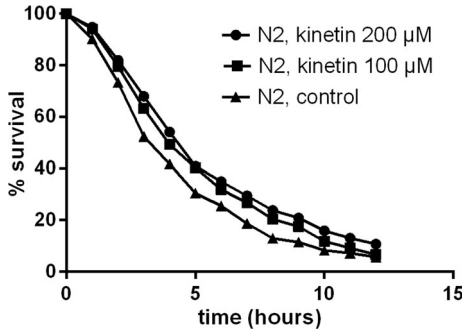

Fig. 4 Effect of kinetin on survival of worms exposed to juglone. Pre-treatment with kinetin caused a significant increase in survival ($p = 2.8 \times 10^{-4}$ and 1.8×10^{-2} for kinetin 200 µM and 100 µM respectively). Sizes of populations treated with 100 µM kinetin, 200 µM kinetin and controls were $n = 467$, 421 and 470, respectively

average increase of 14% in the survival of kinetin (200 µM)-treated worms compared to control worms ($p < 0.05$ in most cases). The increase was smaller (average increase of 5%) and insignificant in most cases for populations pre-treated with 100 µM of kinetin. These experiments were repeated four times (for overall results see Supplementary Materials Table 6).

The effect of kinetin is dependent on ROS

It was previously proposed that the beneficial effect of kinetin is a result of its antioxidant activity. However, co-treating worms with 200 µM kinetin and the antioxidant trolox (100 µM) significantly reduced kinetin's ability to protect worms against oxidative stress induced by juglone exposure. Representative results are shown in Fig. 5. This effect was observed in three independent experiments (Supplementary Materials Table 5).

Similarly, the thermotolerance of populations pre-treated with kinetin (200 µM) + trolox (100 µM) was reduced. After exposure to heat stress (35 °C for 90 min), the average survival was 15% lower than for worms treated with kinetin alone ($p < 0.05$) and even 5% lower than for untreated control worms ($p > 0.05$ in most cases). This experiment was repeated three times (Supplementary Materials Table 7).

We also decided to perform one lifespan experiment to see if the suppressive effect of trolox was reproducible also in these experimental settings. Indeed, also in the lifespan experiment the effect of

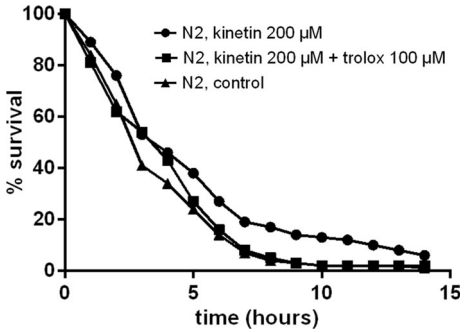

Fig. 5 Survival of worms pre-treated with kinetin and trolox after juglone exposure. Kinetin-treated worms showed significantly higher survival rates than both control worms ($p = 3 \times 10^{-3}$) and trolox co-treated worms ($p = 3.6 \times 10^{-2}$). Sizes of populations treated with 200 µM kinetin, 200 µM kinetin = trolox and controls were n = 109, 129 and 182, respectively

kinetin was suppressed by trolox (Fig. 6 and Supplementary Table 3).

Together, these results suggest that the longevity-enhancing effect of kinetin in worms is probably not based on its ability to scavenge ROS, and that ROS must be present for this effect of kinetin.

DAF-16 is not required for kinetin-induced stress resistance

DAF-16 is a transcription factor that participates in regulation of various genes involved in stress responses and lifespan extension (Tullet 2015). Thus, we evaluated the possibility that DAF-16 may mediate effects of kinetin in *C. elegans*, by assaying kinetin's ability to increase the oxidative stress resistance of *daf-16*(mu86) mutants exposed to juglone. This experiment was repeated twice. In the first experiment, using 200 µM kinetin and a small population, an increase in survival of borderline significance was observed (Supplementary Materials Table 4). In the second experiment, with a larger population, kinetin significantly prolonged the survival of *daf-16* mutants at both 100 and 200 µM (Fig. 7).

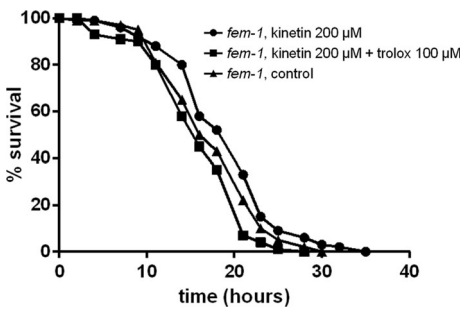

Fig. 6 Comparison of effects of 200 µM kinetin with and without 100 µM trolox on aging of *C. elegans* populations

Kinetin also increased the thermotolerance of these mutants. After exposure to 35 °C for 90 min, the survival of populations treated with 200 µM kinetin was, on average, 15% higher than in control populations ($p < 0.05$). The effect of 100 µM kinetin was smaller, a 10% increase in survival on average, and not significant in two out of three experiments (Supplementary Materials Table 6).

Our results suggest that the effect of kinetin is independent of DAF-16, the major effector of Insulin and Insulin-like growth factor (IGF) Signaling (IIS).

C. elegans worms absorb and ribosylate kinetin effectively

As already mentioned, kinetin is present in other organisms besides plants, including humans. Thus, we also compared levels of endogenous kinetin in control and kinetin-treated populations of *C. elegans*. Moreover, as cytokinin bases can be metabolized into the corresponding ribosides and riboside-5′-phosphates in both mammals and plants, to varying degrees, we also examined levels of kinetin riboside (KR) and kinetin riboside-5′-monophosphate (KMP) in these populations. The compounds were analyzed in two sets of replicate samples (each consisting of ca. 1000 worms) using UHPLC-MS/MS. Neither

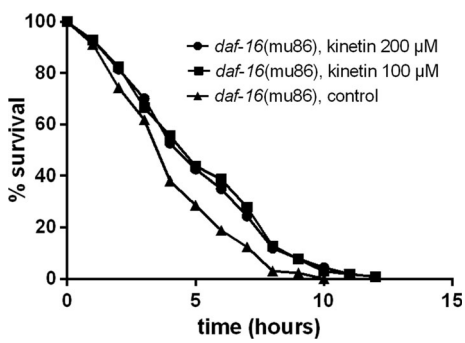

Fig. 7 Survival of *daf-16* null mutants pre-treated with kinetin after juglone exposure. Kinetin had significant effects at both 200 µM ($p = 3.9 \times 10^{-5}$) and 100 µM ($p = 6.5 \times 10^{-6}$). Sizes of populations treated with 200 µM kinetin, 100 µM kinetin and controls were n = 368, 336 and 265, respectively

Table 3 Concentrations (μM) of kinetin and its metabolites in extracts from worms and bacteria after exposure to 100 μM kinetin for 24 h

Sample type	Kinetin	Kinetin riboside	Kinetin riboside-5′-monophosphate
C. elegans, standard cultivation conditions	160.34	17.58	372.59
	970.14	71.5	508.23
C. elegans, heat-killed bacteria	33.83	9.47	30.51
	80.55	195.15	127.61
E. coli OP50	0.54	0.31	ND
	0.28	0.22	ND

kinetin nor its metabolites were detected in control samples, suggesting that the worms do not naturally produce these compounds (Table 3). However, in extracts of worms pre-treated with kinetin (100 μM, 24 h) grown under standard cultivation conditions, the parent compound was detected at levels exceeding the applied concentration, demonstrating that it is absorbed very efficiently. We also detected both KR and KMP in these worms, indicating that they metabolize kinetin. There was significant variation in both the concentrations and ratios of the three metabolites between the two sets of samples, which can be ascribed to biological variability and the loss of some worms during washing steps.

To ensure that the detected metabolites were produced by the worms and not by the *E. coli* OP50 bacteria used as food, we tested for the presence of the metabolites in these bacteria, and in worms fed with heat-killed bacteria. KR, but not KMP, was detected in the bacteria, demonstrating that KMP is not produced by bacteria. Both KR and KMP were detected in worms fed with heat-killed bacteria.

Discussion

Cytokinins have various protective activities both in vitro and in vivo. One compound from this group, kinetin, has known ability to prolong the lifespan of fruit flies (Sharma et al. 1995). Moreover, kinetin can reduce oxidative stress (Olsen et al. 1999) and glyoxidative stress both in vitro (Verbeke et al. 2000) and in vivo (Liu et al. 2011). In cell culture, kinetin can also ameliorate several markers of aging (Rattan and Clark 1994; Lee et al. 2006). In this study, we evaluate the protective effect of kinetin and other naturally occurring cytokinin bases, some of which have not previously been tested for anti-aging and cyto-protective activities, in the model organism *C. elegans*.

We found that kinetin, and two aromatic cytokinins, *meta*-topolin and *para*-topolin, can increase worms' longevity. Therefore, our data show that the lifespan-prolonging activity of kinetin is not limited to insects. It would be interesting to see whether its effect on lifespan extends to other model vertebrates such as fish and rodents. While *para*-topolin is known to have antioxidant activity in vitro (Brizzolari et al. 2016), there is limited data regarding the cyto-protective activity of topolins in cell cultures and animals. The activity we observed suggests that topolins warrant greater attention in future studies. A caveat is that the longevity-promoting effect was typically only observed at the highest tested concentrations, and these compounds' low solubility prohibited testing at higher concentrations.

Another cytokinin that has previously been extensively studied in connection with aging and age-related diseases is *trans*-zeatin. It has youth-preserving activity in cell cultures (Rattan and Sodagam 2005) and neuroprotective effects in both cell cultures and rodents (Choi et al. 2009; Kim et al. 2008). In our study, this compound showed promising results in an initial experiment, but its effect was not significant in repeated experiments using larger populations.

After initial experiments we focused on kinetin. Kinetin increased the resistance of *C. elegans* to oxidative stress caused by juglone, which generates superoxide anions by redox cycling (Inbaraj and Chignell 2004), and increased their thermotolerance.

We also performed several experiments to probe the mechanism of kinetin's protective activity in worms.

First, we tested possible involvement of the insulin/insulin-like growth factor signaling pathway (IIS), as mutations in IIS components affect the lifespan of various species (Barbieri et al. 2003), and many compounds that can prolong the lifespan and increase stress resistance in worms affect the IIS. A key effector of IIS is DAF-16/FOXO (Forkhead box protein O), a transcription factor that activates many genes involved in stress responses or *dauer* larvae formation (Tullet 2015). Reductions in IIS pathway activity result in translocation of DAF-16 into the nucleus and trans-activation of its target genes. Whereas worms with mutations in the *daf-2* gene (encoding the *C. elegans* insulin-like growth factor 1 receptor homologue DAF-2) have significantly increased lifespans and stress resistance (Kenyon et al. 1993; Lithgow et al. 1995), mutants with impaired DAF-16 have shorter lifespans than WT worms. Therefore, we tested effects of kinetin in *daf-16* null mutants, and found that it increased their stress resistance as well, showing that IIS activity is not essential for kinetin's activity.

ROS have been traditionally regarded as important pro-aging factors (the free radical theory of aging), mostly due to their ability to directly damage cells' macromolecules (Harman 1955). Recent studies suggest that their role in aging is more complex than this, but certainly no less important. For example, they can act as signaling molecules in many pathways, directly modify certain proteins involved in signal transduction, and may participate in the epigenetic modulation of gene expression (Davalli et al. 2016). ROS are also involved in many interventions that increase longevity and they activate hormetic adaptive responses—a process sometimes referred to as mitochondrial hormesis, or mitohormesis (Ristow and Schmeisser 2014).

The cyto-protective effects of cytokinins are often ascribed to their intrinsic antioxidant activities. However, we found that co-treatment with the known antioxidant trolox (an analog of vitamin E) significantly reduced or even completely suppressed the effects of kinetin. Given the ROS-scavenging activity of trolox, we hypothesize that kinetin activity requires the presence of ROS. However, the exact role of ROS in the beneficial effects of kinetin remains to be determined.

Kinetin has been previously reportedly identified in DNA extracted from human cells (Barciszewski et al. 1996) and urine from cancer patients (Barciszewski et al. 2000), suggesting that it can occur naturally under some circumstances in other organisms besides plants. Using UHPLC-MS/MS, we measured levels of endogenous kinetin in worms and compared them to its levels in a kinetin-treated population. We found neither kinetin nor its derivatives in control worms, indicating that these compounds are not normally produced by *C. elegans*, at least under our cultivation conditions.

However, exogenously applied kinetin (100 µM, 24 h) was efficiently absorbed by the worms, to levels exceeding the applied concentration. In addition to kinetin, we also detected kinetin riboside (KR) and kinetin riboside-5′-monophosphate (KMP).

The presence of kinetin metabolites at such high concentrations raises the interesting possibility that these metabolites are responsible for the beneficial effects of kinetin treatment. We propose the following hypotheses regarding these metabolites' contributions to the protective activity of kinetin based on their effects on human cells. In mammalian cells, cytokinin ribosides, including KR, are highly cytotoxic as they induce ATP depletion (Ishii et al. 2002; Cabello et al. 2009), possibly by impairing mitochondrial functions (Cheong et al. 2009). However, at low concentrations, two cytokinin ribosides, N^6-isopentenyladenosine and N^6-benzyladenosine, reportedly activate NRF-2 (Dassano et al. 2014), suggesting they may protect cells from oxidative stress. This raises the possibility that these compounds act as hormetins—compounds that induce hormetic adaptive mechanisms. We showed that the beneficial effect of kinetin is suppressed by the presence of an antioxidant. Perturbation of mitochondria in *C. elegans* by KR, as in human cells, would lead to the production of ROS and possibly activation of an adaptive response. Therefore, we speculate that kinetin might act as a hormetin precursor, a prohormetin.

Moreover, it was recently hypothesized that effects of another cytokinin riboside, N^6-isopentenyladenosine (iPR), could be mediated by activation of AMP-activated protein kinase (AMPK) by its metabolite iPR-5′-monophosphate in human cells (Pisanti et al. 2014). AMPK senses the ratio of ATP/ADP/AMP in cells, thereby providing

information about cellular energy levels (Hardie and Hawley 2001). The enzyme also plays an important role in aging, as its over-expression (Apfeld et al. 2004), or activation by compounds such as metformin (Onken and Driscoll 2010), increases longevity in worms. It is tempting to speculate that KMP might have a similar effect to iPR-5′-monophosphate. High concentrations of KMP could also activate AMPK in worms, since the enzyme is highly conserved across phylla (Hardie and Hawley 2001). AMPK activation induces respiration and catabolism (Schulz et al. 2007), thereby increasing ROS production which can be suppressed by antioxidants (Schulz et al. 2007; Mungai et al. 2011; De Haes et al. 2014). This would be consistent with our findings that ROS are required for the effects of kinetin.

Effects of exogenously applied kinetin metabolites on the lifespan of *C. elegans*, and tests of the hypotheses outlined above, will be included in planned studies. Future studies will also include further analysis of kinetin´s metabolism, for example to establish whether *C. elegans* produces higher order phosphates, which also have beneficial activity in human cells (Hertz et al. 2013).

Conclusion

The plant hormone kinetin can prolong the lifespan of fruit flies and ameliorate aging markers in human cell cultures. Here, we report that kinetin can prolong the lifespan of another model organism, *C. elegans*. We also report the longevity-enhancing effect of two other cytokinin bases, *meta*-topolin and *para*-topolin, for the first time. In addition to prolonging lifespan, kinetin increased the worms' stress resistance. Its effect was independent of the transcription factor DAF-16, and thus is not probably connected to the insulin/insulin-like growth factor pathway. However, addition of an antioxidant significantly reduced its beneficial effect, indicating a crucial role of ROS. We also analyzed endogenous levels of kinetin in *C. elegans*. Our results show that kinetin does not occur naturally in the organism, but it is effectively absorbed after exogenous exposure and, as in human cells, it is metabolized to kinetin riboside and kinetin riboside-5′-monophosphate. Kinetin's exact mechanism of action in *C. elegans* remains unclear, but we hypothesize that its metabolites play an important role, possibly via activation of adaptive responses and/or activation of AMP-activated protein kinase (AMPK).

Acknowledgements Strains used in this study were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). The authors are grateful to Hana Martínková for her help with phytohormone analyses. This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic (National Program for Sustainability I, grant nos. LO1204 and LO1304; INTER-COST LTC17 project code LTC17072). This article is based upon work from COST Action BM1408, supported by COST (European Cooperation in Science and Technology).

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