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Review

The plant hormone kinetin in disease therapy and healthy aging

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ABSTRACT

It has been more than 60 years since the discovery of kinetin, the first known member of a group of plant hormones called cytokinins. In this review we summarize the health-promoting activity of kinetin in animal systems, ranging from cells cultured *in vitro* through invertebrates to mammals. Kinetin has been shown to modulate aging, to delay age-related physiological decline and to protect against some neurodegenerative diseases. We also review studies on its mechanism of action, as well as point out gaps in our current knowledge.

1. Discovery of kinetin and its activity in plants

In 1955, Miller et al. isolated a new bioactive compound, N^6-furfuryladenine (Fig. 1A), from an autoclaved DNA sample from herring sperm. Due to its ability to stimulate cytokinesis in various plant tissues, the compound was given the name kinetin (K) (Miller et al., 1956, 1955a, b; Amasino, 2005). It was the first member to be identified from the group of phytohormones which were later named cytokinins (Skoog et al., 1965). Chemically, cytokinins are adenines substituted with either an isoprenoid or an aromatic side chain at the N^6-position. Since their discovery, it has been established that cytokinins regulate plant growth, development, leaf senescence, apical dominance, seed germination, water and nutrient mobilization, flowering and many other processes (Kieber and Schaller, 2014).

2. Occurrence of kinetin in nature

For a long time, kinetin was believed to be an artificial product of DNA rearrangement (Hall and De Ropp, 1955), although some researchers suspected that it might be spontaneously formed *in vivo* from DNA (Skoog, 1994). The first evidence of its natural occurrence was reported in 1996, when K was identified in extracts from root nodules of *Casuarina equisetifolia* infected by the bacterium *Frankia* (Raman and Elumalai, 1996). In the same year, Barciszewski et al. found K in plant cell extracts (0.1 ng/g of dried material), in DNA isolated from human fibroblasts cultured *in vitro* and in commercially available calf thymus DNA (concentrations not reported) (Barciszewski et al., 1996). Later, K was also found in human urine (0.12 nM in urine of cancer patients, 10–100 times less in healthy subjects) (Barciszewski et al., 2000), in plant material (0.34 nM in coconut water) (Ge et al., 2005) and in brain (up to 17.2 nM) and liver (up to 63.7 nM) tissue of transgenic mice models of familial dysautonomia (Shetty et al., 2011).

It should be pointed out that in a study conducted by our group, K was not detected in native extracts from *Caenorhabditis elegans* and *Escherichia coli* (Kadlecová et al., 2018). Orr et al. were unable to detect K in native brain tissue of mice and rats (Orr et al., 2017). We have also been unable to identify K in various plant materials (unpublished data), suggesting that the question of kinetin's natural origin is probably more complicated than was previously thought.

The proposed mechanism of formation of kinetin from DNA is based on a reaction of the amino group of adenine with the aldehyde group of furfural (Barciszewski et al., 1997a, b). The resulting base is then excised from the DNA backbone (Wyszko et al., 2003). The authors presumed that furfural arises from oxidized deoxyribose by a mechanism suggested by Pratviel and collaborators (Pratviel et al., 1991). Pratviel et al. did indeed detect furfural in DNA exposed to manganoporphyrin/$KHSO_5$ artificial nuclease, but only when the samples were heated to 90 °C for 15 min. However, as furfural is ubiquitously present in nature and also occurs in various foods (Hoydonckx et al., 2000), it may be present in cells from other sources.

It has also been previously proposed that K can be formed in plants only under certain specific conditions, for example in wounded tissues

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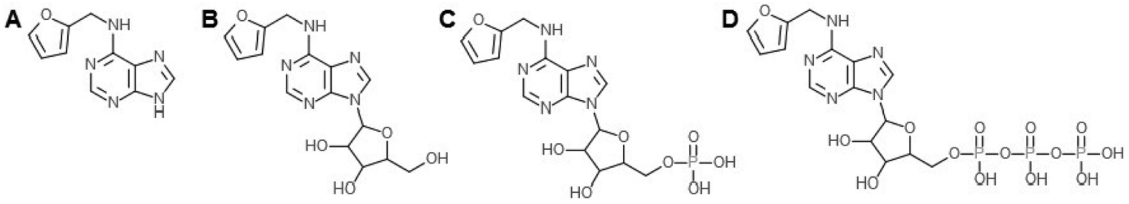

Fig. 1. Structures of kinetin and kinetin ribotides. A) kinetin. B) kinetin riboside. C) kinetin riboside-5′-monophosphate. D) kinetin riboside-5′-triphosphate.

(Skoog, 1994) which undergo a spike in reactive oxygen species (ROS) production. In humans, kinetin was detected at much higher concentrations in the urine of cancer patients, suggesting that its formation may be linked with the increased oxidative damage that accompanies the disease or its treatment (Barciszewski et al., 2000). If the formation of K does, indeed, follow an increase in ROS production, this could partly explain its absence from native plant and animal/microorganism samples in some studies. It might be fruitful to further investigate whether kinetin is present in extracts prepared from plant and animal materials subjected to different types of stress, and compare them to native samples. Metabolization of kinetin excised from DNA or its ribosides and ribotides by enzymatic deamination could also explain its absence from some materials. Cytokinins are substrates of cytokinin oxidase/dehydrogenase in plants and adenosine deaminase in human cells.

3. Protective effects of kinetin in animal models

When considering the effect of K in plants, researchers soon started to wonder what activity it would have in other organisms. Initial experiments in tissue cultures derived from healthy human skin and in breast carcinoma connective tissue fibroblasts cultivated *in vitro* showed that K at low concentrations (approximately 0.05–0.3 μM) stimulated outgrowth but it had an inhibitory effect at higher concentrations (approximately 10–50 μM) (Orr and McSwain, 1957). The latter finding led to a further study of K's potential anti-cancer effect, but K had no effect on outgrowth of tissues derived from breast carcinomas (Orr and McSwain, 1960). The same, however, was not true for kinetin riboside (KR, Fig. 1B) (Orr and McSwain, 1960; Hampton et al., 1956). Subsequent studies have also shown that some natural cytokinin ribosides are highly cytotoxic to cancer cell lines while their corresponding bases have no or limited toxicity (Grace et al., 1967; Fleysher et al., 1969; Doležal et al., 2007; Voller et al., 2010, 2019). Nevertheless, cytokinin bases including kinetin might still find a place in cancer therapy, as they induce differentiation in some leukemic cell lines (Ishii et al., 2002, 2003; Honma and Ishii, 2002). However, research on kinetin and other cytokinin bases has focused primarily on their (cyto)protective activity, which will be discussed in more detail in the following sections.

3.1. Anti-aging and protective effects of kinetin on skin

The anti-senescence activity of K in plants inspired Rattan and Clark to investigate its effect on aging in animal cells. Continuous cultivation of human mammary skin fibroblasts with K (40–200 μM) positively influenced several markers of aging, including cell size and morphology, levels of autofluorescence and cytoskeleton organization. K treatment helped late passage fibroblasts to successfully complete cytokinesis, as indicated by a 10-fold reduction in the number of cells containing multiple nuclei. No effect of K on growth rates and replicative lifespan was reported in skin fibroblasts from two different donors as well as in MRC-5 embryonic lung fibroblasts (Rattan and Clark, 1994). K was also reported to increase the total amount of DNA and [^{3}H] uridine incorporation in human fibroblasts (Kowalska, 1992).

In addition to skin fibroblasts, keratinocytes have also been used to investigate the anti-aging effect of K. Proper functioning of keratinocytes, especially normal proliferation and differentiation is indispensable for maintaining the epidermis and it is adversely affected by aging. Several studies reported that K treatment led to increased expression of various differentiation markers in keratinocytes exposed to high levels of Ca^{2+} (Berge et al., 2006, 2008). The authors reported that efficacy was greatest at concentrations of 40 and 80 μM (Berge et al., 2006, 2008). Higher doses reportedly led to slower growth of early passage cells, although the change was not accompanied by reduced viability or impaired DNA synthesis (Berge et al., 2006). In another study, the effect of K on keratinocytes exposed to UVB irradiation was investigated. The authors reported a 36% reduction in thymine dimer formation in the DNA of cells treated with 100 μM K (McDaniel et al., 2005).

Interestingly, K was also shown to modulate abnormal behavior in primary keratinocyte cultures derived from psoriatic patients. It induced their differentiation, as demonstrated by reduced proliferation rates, heterogeneous cell morphology and larger numbers of cells that developed cornified envelopes (Bolund et al., 1991). Clinical evidence that K might improve the symptoms of psoriasis is, however, still lacking.

The beneficial effects of K have also been reported from 3D reconstructed skin equivalents (Vicanova et al., 2006). Here, topical application of 0.1% K resulted in induction of both proliferation (Ki-67) and differentiation (filaggrin) markers. The authors only reported an overall effect, without specifying in which keratinocyte layer proliferation/differentiation occurred. In addition, K showed some activity in deeper layers of the skin model. It stimulated the formation of the basement membrane as demonstrated by increased levels of laminin 5. The study also showed an increase in the content of elastin, fibrillin-1 and collagen type I in the elastic network in the upper dermis and that they were organized perpendicular to the dermal-epidermal junction.

3.1.1. In vivo studies and clinical trials

The reports of beneficial effects in skin cells led to an interest in the application of K in cosmetic treatments and dermatology. During aging, skin undergoes structural and functional deterioration. This results in increased risk of mechanical damage, exacerbated by a decrease in the ability to induce wound healing responses, a higher risk of infection due to decreased immune function and increased incidence of other skin disorders. The deterioration is enhanced by environmental factors, such as chronic UV exposure.

Effects of K *in vivo* have been studied in 10-year-old dogs descended from Mexican hairless dogs whose skin shows age-related changes similar to those occurring in humans. Dogs were treated topically, with lotions containing from 10 to 10,000 μM and 2% K. Authors reported apparent improvements in skin texture, reduced wrinkling and a decrease in pigmentation. After 100 days of treatment, improvements were apparent even in the skin treated with the lowest K concentrations. Histological evaluation of the skin revealed a decrease in the thickness of the corneal layer and a smaller number of melanin granules. In dermis, the authors reported an increased number of collagen and elastic fibers and observed that their alignment was more orderly. Changes in pigmentation were reversible after discontinuation of the treatment (Kimura and Doi, 2004). In a later study, the effect of a lotion containing 0.5% of K, as well as a commercially available cream (0.1% of K), was evaluated on pig skin. The lotions were applied daily for 4

days and treatment was followed by UV irradiation (at intensities of 5 mW/cm^2 of UVB and 40 mW/cm^2 of UVA). K did not reduce erythema generated in response to UV, nor did it reduce the number of "sun-burned" cells in the skin. These results suggest that short-term application of K-containing cosmetic cannot substitute for the use of sunscreen products (Tournas et al., 2006).

The safety and efficacy of K containing products has been assessed in several clinical studies on human volunteers (McCullough and Weinstein, 2002; Wanitphakdeedecha et al., 2015; Chiu et al., 2007; McDaniel et al., 2005; An et al., 2017; Thornfeldt and Rizer, 2016). K, although not necessarily the most active substance in comparative studies, was generally reported to be well tolerated and to improve parameters of photodamaged skin, including facial skin erythema, moisture, hyperpigmentation and texture. Interestingly, a similar beneficial effect and lack of adverse reaction could be observed not only in healthy subjects, but also in patients suffering from rosacea. The patients also reported significant improvement in several symptoms accompanying the disease, such as burning and stinging sensations and dryness of the skin (Wu et al., 2007). Some studies have also focused on increasing the efficacy of K, either by combining it with other bioactive compounds (Chiu et al., 2007; Maia Campos et al., 2012) or by increasing its absorption by incorporating the molecule into solid lipid nanoparticles (Goindi et al., 2015) or liposomes (Maia Campos et al., 2012).

3.2. Neuroprotective activity of kinetin

A decline in neuronal function and development of neurodegenerative diseases can undoubtedly be regarded as one of the most debilitating signs of aging. The prevalence of neurodegenerative diseases is rising, mostly due to an increase in life expectancy, but the current treatment options are rather limited. K showed promising results in studies focusing on the treatment of hereditary Parkinson's disease (PD), Huntington's disease (HD), familial dysautonomia (FD) and neurofibromatosis 1 (NF1), as well as in models utilizing stressors for induction of certain symptoms of (neuro)degeneration.

3.2.1. PINK-1 activation and Parkinson's disease (PD)

K showed a promising effect in *in vitro* models of the hereditary form of early onset PD, caused by a loss-of-function mutation (G309D) in PTEN-induced kinase 1 (PINK1) (Hertz et al., 2013). This kinase plays a crucial role in mitochondrial quality control by targeting depolarized mitochondria for degradation by a Parkin-dependent mechanism. The authors showed that a metabolite of K, kinetin riboside-5´-triphosphate (KRTP), is able to function as a neo-substrate of PINK-1, boosting its activity (details below). The authors pre-treated human neuroblastoma cell line SH-SY5Y with 50 μM K and then exposed the cells to carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a toxin which causes depolarization of the mitochondrial membrane. In K-treated cells, they observed a PINK1-dependent increase in phosphorylation of Bcl-xL. In HeLa cells transfected with either wild-type (WT) or mutant PINK1, treatment with K resulted in faster PINK1-dependent recruitment of Parkin, and in an increase in its phosphorylation levels. In SH-SY5Y and Parkin-transfected HeLa cells pre-treated with K, the authors reported reduced cleavage of caspase 3/7 following exposure to a proteasome inhibitor. This is in agreement with yet another described biological activity of PINK1 – its anti-apoptotic effect in cells undergoing proteasomal stress (Klinkenberg et al., 2010). A smaller number of apoptotic SH-SY5Y cells was also detected. K also decreased the mobility and velocity of axonal mitochondria in rat hippocampal neurons – a decrease in these parameters is considered to be a first step in PINK1-regulated removal of damaged mitochondria. Furthermore, a K concentration of 50 μM had no demonstrable toxicity on cultured dopaminergic neurons even after a relatively long period of treatment (10 days).

A subsequent study attempted to validate these results *in vivo* (Orr et al., 2017). Here, the authors re-introduced WT or mutant PINK1 into the brain of PINK1 knock-out (KO) rats with the intention of studying the effect of K (approximately 300 mg/kg body weight/day, administered in food). The model behaved rather surprisingly, as the authors did not observe any loss of dopaminergic neurons in the original PINK1 KO animals, but they reported neuronal loss after reintroduction of both WT and mutant PINK1, suggesting unexpected toxicity on the part of either PINK1 overexpression or the vector used. The authors also reported a small, statistically insignificant trend towards an increase in the number of dopaminergic neurons in K-treated animals and they highlighted the need for development of a new *in vivo* model of this type of familial PD. They also studied the effect of K in transgenic rats and mice overexpressing α-synuclein. K (administered in food, 400–600 or 300 mg/kg/day on average for mice or rats respectively) did not show any protective activity in either model. In rats, the authors reported similar rates of degeneration of dopaminergic neurons as in non-treated control animals. K treated mice showed neither reduced levels of phosphorylated α-synuclein in hippocampus and cortex nor correction of behavioral abnormalities. The lack of activity might be influenced by dosage, route of administration or induction of clearance mechanisms. The authors pointed out that the daily estimated dose rate for K decreased slightly over time as the animals gained weight, and they also showed a significant 2 to 5-fold decrease in K levels in the brain tissue of the older animals (from the original 800–1000 pg/mg of tissue at the start of treatment) (Orr et al., 2017). It would be informative to further investigate the effect of K in PD using different experimental settings and other models of both hereditary and sporadic PD.

3.2.2. Huntington´s disease (HD)

A recent study also reported K as a potential modulator of HD (Bowie et al., 2018). Here, researchers showed that K is capable of increasing the phosphorylation of the N17 subunit of mutant huntingtin in striatal cells derived from transgenic knock-in HD model mice. Such modification was shown to reduce the detrimental changes accompanying the disease in both *in vitro* and animal models of HD (Gu et al., 2009; Atwal et al., 2011). As discussed below, the mechanism of action involves an increase in the activity of casein kinase 2. In further experiments, 0.1–1 μM K increased the viability of mouse cortical neurons transfected with a fragment of mutant huntingtin in a dose dependent manner. Next, the authors studied the effect of K in transgenic HD model mice expressing mutant human huntingtin containing 128 CAG repeats. The characteristic phenotype of this model is impairment of motor functions and anxiety-like behavior. Animals receiving 0.83 or 4.17 mg/kg/day of K intraperitoneally (i.p.) fared better in tests measuring their motor performance on a rotarod. The effect was dose dependent. To assess the level of anxiety, the authors chose to administer K in the diet (653 mg/kg of chow; concentrations of K in animal tissues were not reported) to avoid stress caused by regular painful injections. K prevented anxious behavior in 2 out of 3 tests carried out. In mice fed with K, the authors did not observe any improvement in motor function, probably due to the lower dose and decreased absorption of K using this route of administration. Orally treated animals showed a decrease in levels of insoluble mutant huntingtin in the cortex, but not in the striatum. A similar trend was observed in mice receiving i.p. injections, with a small, statistically insignificant decrease in huntingtin levels in the striatum as well (Bowie et al., 2018).

3.2.3. Familial dysautonomia

K also showed a protective effect in yet another hereditary neurodegenerative disorder – familial dysautonomia (FD). This rare but debilitating disease is caused in more than 99% of cases by a point mutation (T → C/ IVS20 + 6T→C) in the splice site of intron 20 of the gene encoding elongator complex protein 1 (ELP1). This leads to decreased splicing efficiency and skipping of exon 20 in a pre-mRNA transcript, resulting in synthesis of truncated ELP1. The protein regulates transcription of multiple genes, including some encoding

proteins involved in cytoskeleton function and cell movement which are necessary for normal cell growth and development. The mutation does not have full penetrance and while some tissues are able to compensate and produce a sufficient amount of functional protein, nervous tissue is severely affected. FD is therefore characterized by impaired development and survival of neurons in both sensory and autonomic nervous system, resulting in progressive neurodegeneration (Shohat and Hubshman, 2014).

In 2004, Slaugenhaupt et al. found that K could correct the aberrant splicing of ELP1 in a lymphoblast cell line derived from FD patients. The effect was dose dependent, with the highest non-cytotoxic concentration tested (400 μM) being the most effective. The results were consistent across various cell lines (Slaugenhaupt et al., 2004). A similar effect was observed in a subsequent study, where the authors demonstrated that treatment of FD lymphoblast cells with 100 μM K restored the amount of WT ELP1 at both mRNA and protein level to that similar to non-treated controls. Interestingly, K also increased the levels of WT ELP1 mRNA in cell lines derived from carriers of the disease and from healthy controls (Hims et al., 2007). Although a later study reported an increase in WT ELP1 only at the mRNA and not at the protein level, it is possible that the relatively short 3-day treatment may have accounted for the difference (Sinha et al., 2018).

A positive effect of K on ELP1 expression was also observed in cells of neuronal lineage, including olfactory ecto-mesenchymal stem cells collected from FD patients (Boone et al., 2010), neural crest precursors (Lee et al., 2009) and sensory neurons derived from induced pluripotent stem cells created from fibroblasts of FD patients (Zeltner et al., 2016). Treatment also increased the differentiation of neural precursors (Lee et al., 2009) and protected sensory neurons against degeneration (Zeltner et al., 2016). The effective concentrations ranged from 25 to 200 μM. On the other hand, K did not correct the migration deficits of the cells (Lee et al., 2009; Boone et al., 2010).

Correction of aberrant splicing by K was also observed *in vivo*. In mice expressing humanized FD and WT ELP1 and fed with 400 mg/kg/day of K for 30 days, Shetty et al. reported an increase in exon 20 inclusion in various tissues, including brain, liver, kidney, blood, spleen, lung, heart and eye. K was detected in the serum, liver and brain tissue (Shetty et al., 2011). This suggests that K is absorbed and, importantly, can even cross the blood-brain barrier.

The promising results of these studies, as well as favorable results in pre-clinical pharmacokinetics and safety studies in rodents (Axelrod et al., 2011), eventually led to clinical trials. The pharmacokinetics, tolerability and efficacy of oral K treatment were first evaluated in 29 asymptomatic heterozygous carriers of the mutation. The treatment lasted for 8 days and K was supplied once per day at doses ranging from 3.35 to 23.5 mg/kg/day. The majority of the subjects receiving 23.5 mg/kg/day of K reached the desired plasma levels of 2150 ng/mL (10 μM). K was quickly absorbed and eliminated according to first order elimination kinetics. The treatment led to a dose dependent increase in normal ELP1 mRNA in peripheral leukocytes. The most common adverse effect was mild to moderate nausea. More serious adverse effects included severe nausea, vomiting, diarrhea, rash or headache and resulted in 4 subjects withdrawing from the study after administration of the first dose (Gold-von Simson et al., 2009).

In a follow-up study, 8 FD patients received 23.5 mg/kg/day of K for 28 days in total. In the 7 patients who completed the study – one of them withdrew due to reasons unrelated to the treatment – the mean improvement in exon 20 inclusion was 17% in white blood cells. All patients except one reached the target plasma levels. Side effects were mild, and included two cases of diarrhea, three cases of mild nausea and three cases of headache. Elevated liver enzymes in three patients and one case of decrease in platelet and white blood cell count were also reported. These changes were reversed after treatment was discontinued. According to ClinicalTrials.gov, K is currently in phase II of clinical trials (Identifier: NCT02274051).

Kinetin´s favorable toxicity profiles observed in preclinical and clinical studies so far are encouraging, especially given that it contains a furan moiety. The furan moiety is a potential toxicophore as it can be metabolized by cytochrome P450 enzymes to produce reactive electrophilic species (Bakhiya and Appel, 2010). Many furan-containing compounds are therefore hepatotoxic (Peterson, 2012).

The ability of K to modulate aberrant splicing, although highly specific as discussed below, is not limited solely to familial dysautonomia. Another disease frequently linked with mutations leading to aberrant splicing (in 30–40 % of cases) is neurofibromatosis type I (NF1). This disorder is characterized by the presence of "café au lait" spots and neurofibromas. Less common but more serious manifestations include, for example, the development of malignant tumors (Friedman, 2018). In their study, Hims et al. created an NF1 model by transfecting human embryonic kidney (HEK) cells with minigenes containing part of the WT or mutated NF1 sequence. One of the mutant constructs modelled the splicing defect and was responsive to K – after treatment, the authors observed increased incorporation of exon 36 (Hims et al., 2007). In a later study, Pros et al. examined the effect of K in both short term and immortalized lymphocyte cell lines derived from 22 patients carrying 19 different NF1-splicing defects. A decrease in aberrantly spliced product following treatment with 100 μM K was observed for 4 different mutations. The effect was also reproducible in patient-derived fibroblasts, suggesting that it is not limited to a specific cell type (Pros et al., 2010). It must be noted, however, that unlike in FD, where the vast majority of patients harbor the same mutation, more than 1000 different mutations that cause NF1 exist and K treatment would therefore be of potential benefit to only a small proportion of patients.

3.2.4. Protective activity of kinetin in models of CNS stress

A common characteristic of the majority of neurodegenerative diseases is an increase in oxidative stress (Liguori et al., 2018). K has also been reported to protect neurons and nervous tissue against various type of stressors.

Radhakrishna et al. investigated the effect of K on radiation-induced behavioral changes (Radhakrishna et al., 2017). Mice, orally treated with K for 5 days, showed increased survival following exposure to a lethal dose of radiation (10 Gy), with 100 mg/kg of K being the most effective dose. This dose was then used in subsequent experiments, in which they evaluated behavioral deficits in mice exposed to lower, sublethal doses of radiation (6 Gy). The treated animals displayed reduced anxiety levels and improved learning ability. The authors speculated that this could be due to the protective effect of K in dopaminergic neurons.

The effect of K was also investigated in glial cells, specifically in a primary culture of rat astrocytes. Treatment with K (50–200 μM) protected the cells against D-galactose (D-gal) induced toxicity and prevented their morphological deterioration (Liu et al., 2011). Exposure to high D-gal doses leads to glycoxidative damage and degenerative changes resembling those occurring with aging (Sadigh-Eteghad et al., 2017).

The (neuro)protective effect of K was also studied in mice receiving a treatment in which D-gal (subcutaneous injections, 100 mg/kg body weight) was combined with $AlCl_3$ (0.1088 mg/mL in drinking water) (Wei et al., 2017). Some researchers use aluminum to induce neurodegeneration with some features resembling those of Alzheimer´s disease, although the relevance of such a model is questionable (Lidsky, 2014). In this study, 5, 10 and 20 mg/kg of K were orally administered to the mice. After 90 days of treatment, researchers evaluated the spatial learning and memory of mice and showed improvement in K treated groups. Further experiments showed that K attenuated several histopathological changes in the CA3 region of the hippocampus, which plays a crucial role in spatial memory, restored acetylcholine content and decreased the activity of acetylcholinesterase. The authors also showed reduced levels of Aβ 1–42 containing plaques in the cortex and hippocampus and a decrease in levels of expression of amyloid precursor protein, β-secretase, γ-secretase and amyloid beta 1–42. The

effect was dose dependent in all experiments.

In a recent study, Wei et al. used the immortalized mouse hippocampal cell line HT22 to assess the ability of K to prevent glutamate-induced oxidative damage. The authors observed that 8 -h pre-incubation of the cells with 5 mg/mL (approximately 23 μM) of K protected them against toxicity induced by 5 mM of glutamate, as demonstrated by increased cell viability, a decrease in the number of apoptotic and necrotic cells and reduced cytolysis. They also reported inhibition of apoptosis signal-regulating kinase 1 and other downstream members of its signaling pathway. Moreover, K-treated cells maintained normal mitochondrial function – the treatment prevented mitochondrial membrane depolarization and restored the ATP content (Wei et al., 2018).

3.3. Protective effect of kinetin in cardiovascular and other systems

Cardiovascular diseases are a leading cause of death worldwide. Here too, aging is a major risk factor. Age-related deterioration of endothelial cells contributes to a decrease in the plasticity of blood vessels and is linked to the development of vascular diseases (Donato et al., 2015). Interestingly, continuous cultivation with 50 μM K was reported to have a protective effect in dermal microvascular endothelial cells HDMEC. K protected the cells against apoptosis and increased the number of dividing cells in the culture. The late passage cells maintained morphology resembling those in the earlier passages. There were also fewer senescent cells. As a result, 7 more population doublings were achieved (Lee et al., 2006).

In another study, K inhibited platelet aggregation in suspensions activated by several different inducers in a dose-dependent manner (Sheu et al., 2003). A subsequent investigation demonstrated that a similar effect can also be observed *in vivo*. Intravenous application of 4 or 6 mg/kg of K increased the number of platelets in plasma and prevented death in mice with acute pulmonary thrombosis induced by ADP. The same dosage of K also increased bleeding time in rats with severed mesenteric arteries. Moreover, 13–16 mg/kg of K prolonged the time needed for thrombus formation in microvessels of mice exposed to fluorescein and light irradiation (Hsiao et al., 2003).

Some evidence that K might positively influence the symptoms of certain metabolic diseases, such as diabetes, also exists. Treatment with K resulted in an increase in glucose uptake in cultured myocytes (0.3–7.5 μM) and in *ex vivo* epitrochlearis rat muscle (0.5 and 2.5 μM). The authors of this patent also prepared cytokinin-enriched extracts from sprouted barley. The extracts had a beneficial effect on glucose absorption *in vitro*, and, remarkably, decreased blood glucose levels in rats in which diabetes was induced by streptozocin. The same extracts were then orally administered to patients with type 2 diabetes for 90 days, resulting in decreased serum glucose levels, increased glucose tolerance, reduced levels of glycosylated hemoglobin and an improved LDL/HDL ratio. Although the authors note that they confirmed the presence of K in the extract by HPLC and LC/MS analysis, they did not report the exact concentrations of various cytokinins and they measured only the beneficial effect of the combination of all phytochemicals present in the extracts (Mijikovic et al., 2007). The extent to which kinetin contributed to the observed effects is therefore unclear.

Other studies were conducted with D-gal treated rodent models of accelerated aging, focusing on organs other than the brain. In rats exposed to D-gal, administration of K in subcutaneous injections for 45 days delayed atrophy of the spleen, with the highest dose used, 20 mg/kg/day, being the most effective. The histological profile and appearance of the spleen cells were more similar to those of healthy control animals. K also restored serum levels of interleukin-2 and interleukin-6, which play pivotal roles in immune response modulation. Increased levels of several immunoglobulins (IgA, IgM, IgG) were detected. In spleen lymphocyte suspension isolated from the animals, the authors detected fewer apoptotic and more proliferating cells. Mitochondria maintained their membrane potential, and the ratio of the pro-apoptotic protein Bcl-2 to the anti-apoptotic protein Bax was similar to that in healthy control rats (Li et al., 2014a,b,c). K also protected the reproductive organs of D-gal treated female mice (Sun et al., 2013). Animals receiving 25 or 50 mg/kg/day of K by intragastric injections for 40 days showed a decrease in atrophy of the ovaries and uterus. Mice also displayed higher levels of estrogen, a shorter estrous cycle and an increased number of mature oocytes.

3.4. Lifespan studies in invertebrate models

Reduced rates of aging following K treatment are not limited to cell cultures and rodent models of accelerated aging; they have also been reported in invertebrate models. Fruit flies of the species *Zaprionus paravittiger* and *Zaprionus indianus* fed with 25 ppm K (approximately 120 μM) showed prolonged lifespan, slower development (Sharma et al., 1995, 1997) and decreased fertility (Sharma et al., 1997). Higher concentrations either resulted in smaller beneficial effects or were toxic to the animals (Sharma et al., 1995). 200 μM K significantly increased longevity in *Caenorhabditis elegans* (Kadlecová et al., 2018). The K-treated worms also showed greater resistance to oxidative and heat stress.

It is possible that the effect of kinetin on aging in different models may be due to the induction of the same, highly conserved mechanism. However, we currently lack definitive experimental evidence of the exact mechanism, apart from direct radical quenching which, as discussed below, might be responsible. As K is probably a multi-target molecule, it is possible that its effects in various models, ranging from dividing human cells to post-mitotic worms, could result from different mechanisms.

4. Mechanism of action

As summarized in the previous sections, various protective effects of K in diverse models have been reported. However, our knowledge about the mechanism of action remains relatively limited. It might be tempting to create hypothetical links between the activity of K in animals and that in plants, especially as it seems that some of its effects – such as the promotion of differentiation and protection against oxidative stress – are shared between both kingdoms. But such reasoning is supported by little experimental evidence. Apart from the other vast molecular and physiological differences, the receptors and signaling systems that mediate the activity of cytokinins in plants are not present in animals (Voller et al., 2017).

4.1. Protection against oxidative stress

In many studies, the effect of K has been ascribed to its ability to reduce oxidative stress. Reactive oxygen species (ROS) play an important role in the pathogenesis of multiple diseases and possibly also in aging (Liguori et al., 2018). They have the ability to damage macromolecules, which, over time, might lead to a deterioration at the cellular, and subsequently tissue and organ, levels (the free radical theory of aging; see Harman, 1955; Perez et al., 2009)). Although we now know that ROS also act as essential signaling molecules (Davalli et al., 2016), the fact remains that any decline in cellular protection mechanisms, resulting in excessive amounts of ROS, is detrimental.

Initially, it was proposed that K acts as an antioxidant. It could directly scavenge ROS in several possible ways. It might form complexes with copper that have superoxide dismutase-like activity (Parvez and Birdsall, 1990). The radicals could also abstract hydrogen from the α-carbon of the amine bond (Barciszewski et al., 1999) or possibly from the electron-rich furan ring. Furan derivatives are known scavengers of ROS (Okada and Okajima, 1998; Lemke et al., 2014; Okada et al., 1996). The extent of kinetin's ROS scavenging capability has been measured in multiple studies using several different biochemical assays. Brizzolari et al. reported that K had some radical scavenging activity in

an ORAC (oxygen radical absorbance capacity) assay and a deoxyribose degradation assay but did not observe any effect in a TEAC (Trolox equivalence antioxidant capacity) assay (Brizzolari et al., 2016). In another study, the antioxidant capacity of K was measured using a Photochem system. Here, K scavenged ROS only at a concentration of 1 μM, the highest one tested, and performed less well compared to several other known antioxidants such as idebenone, DL-α-tocopherol, L-ascorbic acid or ubiquinone. No intrinsic antioxidant activity of K was observed in a FRAP (ferric reducing antioxidant power) assay at concentrations of up to 0.5 μM (Othman et al., 2016). In a recent study, the ROS scavenging ability of K was measured in 5 different assays – DPHH (1,1-diphenyl-2-picrylhydrazyl), FRAP, Fe^{2+} chelation, superoxide radical inhibition and hydroxyl radical inhibition assays. K performed poorly in all of them (Wei et al., 2018). Taken together, these results suggest that although K probably has some ROS scavenging effect, it is unlikely to be a particularly strong antioxidant.

Apart from scavenging ROS directly, K could also protect cells by inducing anti-oxidative enzymes, such as superoxide dismutase (SOD), catalase (CTL) or glutathione peroxidase (GP). Protection by low doses of K (100 nM) against glutathione depletion caused by the stressor patulin, as well as reduced levels of intracellular ROS following 4-nitroquinoline 1-oxide treatment, were observed in the human promyelocytic cell line HL-60 (Othman et al., 2016). K also suppressed ROS production in collagen-activated platelets (Hsiao et al., 2003). In the mouse hippocampal cell line HT22, K decreased the ROS levels as well. This may be ascribed to the reported translocation of the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) to the nucleus and subsequent expression of its targets, including heme oxygenase-1 (HO-1) (Wei et al., 2018). Nrf2 is a major regulator of xenobiotic metabolism, oxidative and electrophilic stress responses, *etc.,* and its target genes include a number of protective enzymes, such as HO-1 (Ma, 2013).

Interestingly, the content of HO-1, as well as the activity of SOD, CTL and GP, was also increased in the brains of K and D-gal treated mice (Wei et al., 2017). These results are in agreement with those of an earlier study, in which the authors also reported induction of SOD and GP in brain tissue of D-gal and K treated animals (Liu et al., 2011). Increased activity of antioxidant enzymes was also observed in the reproductive organs (Sun et al., 2013) and spleen of rodents exposed to K and D-gal (Li et al., 2014a,b,c).

As outlined above, increased oxidative stress is associated with damage to macromolecules. K has been reported to protect DNA against Fenton reaction-mediated oxidative damage in a biochemical assay. Kinetin (100 μM) significantly reduced the formation of 8-hydroxy-2-deoxyguanosine, which results from hydroxyl radical attack on the C8 of guanine and is frequently used as a marker of oxidative DNA damage. The authors ascribed this reduction to either the intrinsic antioxidant activity of K, or its possible interaction with iron, which is used as a catalyst in the reaction (Olsen et al., 1999). Pre-treatment with 100 nM K reduced the number of DNA breaks after exposure to the genotoxic agent 4-nitroquinoline 1-oxide in freshly isolated human leukocytes and 3 different cell lines: HL-60 (a human promyelocytic cell line), HaCat (a human keratinocyte cell line) and NRK (an epithelial rat kidney cell line) (Othman et al., 2016). Reduction in levels of 8-hydroxy-2-deoxyguanosine was also observed *in vivo*, in the brains of K treated mice exposed to D-gal (Wei et al., 2017).

K has also been shown to prevent protein oxidation and glyoxidation *in vitro*. Verbeke et al. incubated bovine serum albumin with glucose – a less reactive sugar causing slower glycation/glyoxidation – or a more aggressive combination of ribose, arabinose and glyoxal. K (50 and 200 μM) prevented the formation of advanced glycation age products (AGEs) and carbonylated proteins. K also prevented aggregation of proteins, which results from the cross-linking and fragmentation induced by the damage (Verbeke et al., 2000). Remarkably, formation of AGEs in D-gal treated mouse brain tissue was also prevented by K treatment (Wei et al., 2017).

Lipids undergo oxidative damage as well. Markers of lipoperoxidation include lipid hydroperoxides (LP) and malondialdehyde (MDA). *In vitro*, treatment with 100 μM K completely prevented the formation of LP in low density lipoproteins isolated from human blood and induced by $CuSO_4$. It also slightly reduced MDA formation (to 76% of the control) in microsomal preparations from rat liver induced by the pro-oxidant system NADPH/ADP/Fe^{3}+ (McDaniel et al., 2005). Some decrease in levels of membrane lipoperoxidation products was observed in K treated fibroblasts (Jabłonska-Trypuc et al., 2016). In this case, the effect was observed *in vivo* as well – decreased levels of lipoperoxidation markers were detected in the brain (Wei et al., 2017; Liu et al., 2011), spleen (Li et al., 2014a,b,c) and reproductive organs of mice exposed to D-gal (Sun et al., 2013).

In a study focused on late-passage endothelial cells after long-term cultivation with K, Lee et al. described some K-mediated changes that took place at the protein level. Utilizing 2-D electrophoresis, they showed several dozen protein spots altered in K-treated cells and then identified some of these proteins by MALDI-TOF MS. Differentially expressed proteins included those involved in antioxidant defenses, cytoskeleton function, intracellular trafficking, cell-cycle progression, translation and protein turnover, coagulation and collagen maintenance (Lee et al., 2006).

4.2. Hormetic activity

It has also been proposed that kinetin can act as a hormetin (Rattan, 2002, 2008). Hormesis can be defined as a beneficial biological effect which appears after stimulation of cellular protective mechanisms by mild stress (Mattson, 2008). As outlined above, K has been reported to stimulate antioxidant enzymes in multiple models, and it induced heat-shock proteins (Hsp27 and Hsp70) and HO-1 in human keratinocytes (Berge et al., 2008). Activation of the Nrf2 pathway in mouse hippocampal cells (Wei et al., 2018) could be a result of kinetin´s hormetic effect. How exactly kinetin stimulates the hormetic pathways is not clear at this point. The only insight into the mechanism of action comes from a study on *C. elegans*. We observed that co-treatment of these worms with 200 μM K and 100 μM of the antioxidant Trolox led to suppression of kinetin's ability to prolong the lifespan and induce stress resistance (Kadlecová et al., 2018). This suggests that the presence of ROS is necessary for kinetin to have this effect.

An interesting question is whether it is K itself or some of its metabolites that are responsible for the hormetic activity. In worms, we observed efficient metabolization of K into kinetin riboside (KR) and kinetin riboside-5´-monophosphate (KRMP) (Kadlecová et al., 2018). Kinetin ribotides were also detected in human cell lines after treatment with K (Hertz et al., 2013; Bowie et al., 2018; our unpublished data). KR is a known cytotoxic compound which induces ATP depletion in cells (Cabello et al., 2009), possibly by impairing mitochondrial function (Cheong et al., 2009). Formation of a small amount of riboside in cells after K treatment could therefore cause mild oxidative stress and trigger an adaptive response. The fact that other cytokinin ribosides in low doses have been previously reported to act as Nrf2 inducers (Dassano et al., 2014) provides some support for this theory. We also observed induction of HO-1 after KR treatment of skin fibroblasts and keratinocytes (unpublished). It is necessary to note that low doses of other metabolites – such as those formed by metabolization of the furan ring present in the molecule – could possibly act as hormetins. Electrophilic α,β-unsaturated dialdehydes known to arise from furan ring opening (Peterson, 2012) may also activate Nrf2.

Explaining any hormetic mechanism of action has its possible pitfalls. The hormetic concentration – *i.e.*stress inducing but not yet toxic – may differ not only between individual cell and tissue types but also between the same cells/tissues under various physiological conditions. If kinetin is indeed a hormetin precursor (a pro-hormetin), differences in metabolism among various cells/tissues could further complicate the picture. Cells/tissues may also differ in their ability to accumulate the

(pro-hormetin. Differences in the accumulation of kinetin in mouse brain and liver tissue have already been reported (Shetty et al., 2011). However, analysis of a broader spectrum of tissue types, including detailed analysis of kinetin metabolites, is still lacking.

4.3. Kinetin as a mediator of stress response

As K is widely considered to be a natural molecule, formed in DNA during oxidative damage, it was previously proposed that its formation could itself be a protective response to a stress stimulus (Barciszewski et al., 1997b; Maiuri et al., 2018). The presence of K in the DNA could possibly activate DNA repair enzymes. K has also been proposed to act as an antioxidant, responding to the oxidative stress that triggered its formation in a negative feedback loop. However, as discussed above, K was reported to perform rather poorly in multiple *in vitro* assays measuring its ROS scavenging ability. Moreover, K was typically detected at nM concentrations in native tissues (Barciszewski et al., 2000; Shetty et al., 2011). Tens or even hundreds of μM of K are usually required for a protective effect in *in vitro* assays and doses of several mg/kg/day need to be administered in *in vivo* experiments. Even when considering the possible increase in formation of K under stress and the disturbance of the intracellular environment that accompanies pathological states, it should be carefully considered whether naturally formed K (and the KRTP that may form subsequently) could reach sufficient levels to induce a meaningful protective response.

4.4. Interaction with protein kinases

Hertz et al. proposed a very exciting mechanism of action for K in their study focused on hereditary PD caused by mutations in PINK1. They suggested that the active compound is kinetin riboside-5´-triphosphate (KRTP, Fig. 1D), which acts as a PINK1 neo-substrate. In *in vitro* assays, they showed that instead of ATP, KRTP can act as a substrate for PINK1, boosting its activity (Hertz et al., 2013). A later study based on molecular modelling showed that this might be due to the N^6-furfuryl group causing steric hindrance to the main chain of the hinge region connecting the N- and C-lobes. This might influence their spatial arrangement and subsequently the kinase activity (Okatsu et al., 2018). Because charged KRTP cannot cross cell membranes, Hertz et al. used K in cell-based assays and demonstrated that it can be transformed first into kinetin riboside-5´-monophosphate (KRMP, Fig. 1C) by adenine phospho-ribosyl transferase, and then to higher phosphates, evidenced by the fact they detected KRTP in HeLa cells. Moreover, K boosted the activity of wild-type PINK1. It is an interesting question whether this activation of wild-type PINK1 could protect cells against oxidative stress due to more efficient maintenance of a healthy mitochondrial population, or whether it could prove detrimental in the end. Nevertheless, the work of Hertz et al. suggests an interesting new strategy – using neo-substrates to increase the activity of kinases – which could potentially be utilized in treating many diseases (Hertz et al., 2013; Kleiner and Kapoor, 2013). A similar idea was adopted in a study by Maiuri et al. focused on Huntington´s disease. They proposed that KRTP serves as a more efficient neo-substrate for casein kinase 2, an increase in the activity of which leads to phosphorylation of the N17 subunit of mutant huntingtin, and this has a protective effect (Maiuri et al., 2018).

Because KRTP is consumed during the kinase reaction, to have any meaningful protective activity in the cells its initial concentration must be sufficiently high in order to compete with ATP for the binding site of the kinase. Although Hertz at al. demonstrated that KRTP can effectively compete with ATP as a PINK1 substrate *in vitro* if it is present at a concentration 4 times lower than that of ATP, in HeLa cells they detected it at a 200-times lower concentration (68 ± 13 μM, compared to 1950 ± 421 μM of ATP) (Hertz et al., 2013). In our own experiments, using capillary electrophoresis we detected even lower concentrations of KRTP (< 10 μM) in 5 different cell lines treated with 100 μM K for 24 h. Low intracellular concentrations of KRTP could explain why we did not observe any protection against various mitochondrial toxins, including CCCP, conferred by kinetin (unpublished data).

The KRTP content can be increased by using prodrugs aimed at delivering KRMP to cells, thus overcoming the limited efficiency of the base transformation. A recent study described the development of ProTides (one of the prodrug types developed for delivery of nucleoside 5′-monophoshates into cells) designed to deliver KRMP into cells, and their ability to activate PINK1 in HEK cells (Osgerby et al., 2017). The authors reported that KR at a concentration of 50 μM can also activate PINK1. This is not unexpected, since it was previously reported that cells are able to efficiently transform KR into KRMP (Mlejnek and Doležel, 2005), but high cytotoxicity and low bio-availability make KR unsuitable as a drug candidate.

In their patent, Mijikovic et al. showed that another kinase, AMP-activated protein kinase (AMPK), was regulated by kinetin (Mijikovic et al., 2007). This enzyme detects intracellular AMP/ADP/ATP ratios and subsequently regulates a number of metabolic processes. It also plays an important role in the modulation of aging and dysfunction of the enzyme is implicated in multiple diseases (Jeon, 2016). Using immunoblotting, the authors reported that treatment of cultured myocytes with 0.1–10 μM K led to an increase in the phosphorylation of Thr172 (*i.e.*activation) of AMPK. Activation of two of its downstream targets, protein kinase B and glucose transporter 4, was also observed. Interestingly, KR was identified as an AMPK activator as well. It is tempting to speculate that metabolization of K and KR to KRMP is required for APMK activation. It was previously suggested that another cytokinin, N^6-isopentenyladenosine-5´-monophosphate, is able to bind to the gamma subunit of AMPK, functioning as its activator instead of AMP (Pisanti et al., 2014). It is therefore possible that KRMP might have a similar effect. It should be noted, however, that activation of AMPK may also be due to a decrease in ATP levels caused by cytokinin ribosides.

4.5. Splicing modulation

The exact mechanism by which K modulates splicing in familial dysautonomia and neurofibromatosis 1 is not yet fully understood. It has been shown that K does not generally increase inclusion of alternatively spliced exons (Slaugenhaupt et al., 2004; Hims et al., 2007). Moreover, microarray evaluation of the activity of K in olfactory ectomesenchymal stem cells showed that K altered the expression of only a rather small number of genes. Among these were *SNRPA*, encoding a core component of the snRNP U1, and *LUC7L*, which encodes a putative subunit of snRNP U1. The authors suggested that K reinforces 5' splice site (ss) recognition by improving recruitment of splicing factors (Boone et al., 2012). In their study aimed at finding a sequence within exon 20 required for sensitivity to kinetin, Hims et al. created a library of exon 20 deletion constructs. They pinpointed the critical sequence as being the last 3 nucleotides, a CAA motif, on the 5' ss itself. The authors also examined whether splicing of other genes could be modulated by K. They selected over 40 candidates based on a literature search for transcripts with a single alternatively spliced exon and on unpublished microarray data, where they identified genes differentially expressed after K treatment. They found two genes – *BMP2* and *ABI2* – that showed small but consistent increases in internal exon inclusion following treatment with K. In both cases the genes possess the CAA motif on 5' ss. The K-responsive NF1 construct also shared the same motif, although misplaced by one base (Hims et al., 2007). A later study identified one more sensitive transcript, *ZNF280D*, which has the same 5' ss motif (Boone et al., 2012).

It is somewhat surprising that the microarrays showed only a limited number of differentially expressed genes (Boone et al., 2012), given that due to its structure, K could be expected to interact with various members of the cell´s purinome and subsequently modulate the activity of multiple pathways. We expect that Next-Generation Sequencing experiments will better capture transcriptome changes induced by

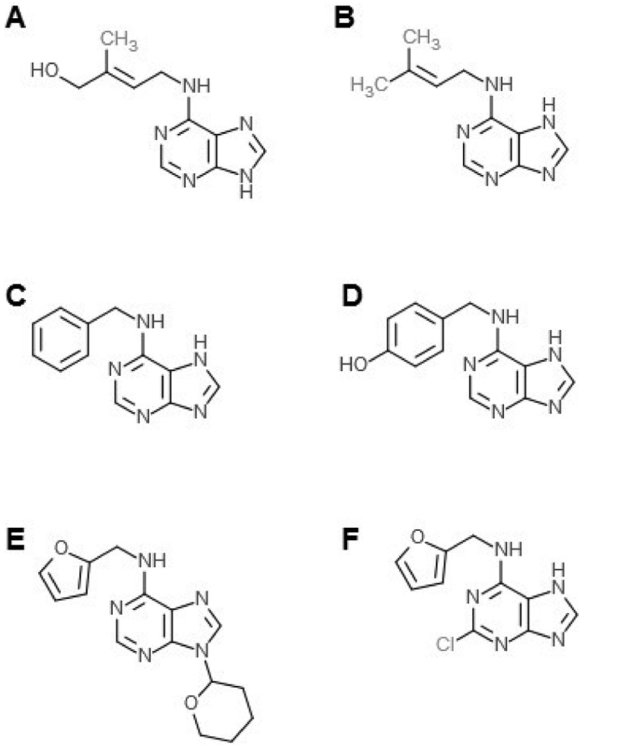

Fig. 2. Structures of natural and synthetic cytokinins. A) *trans*-zeatin. B) N^6-isopentenyladenine. C) N^6-benzylaminopurine. D) *para*-topolin. E) 6-furfurylamino-9-(tetrahydropyran-2-yl)-9H-purine (Pyratine) F) 2-chloro-N-(furan-2-ylmethyl)-7H-purin-6-amine (RECTAS).

kinetin, including splicing modulation. Such studies could also provide insights into whether kinetin's protective effect and ability to modulate aging could be related to alternative splicing of pre-mRNA.

5. Protective effect of other cytokinins

Reports of kinetin having protective activity in animal systems prompted interest in the effects of other cytokinins and their synthetic derivatives. Some of their activities have recently been reviewed elsewhere (Voller et al., 2017).

Natural compounds such as *trans*-zeatin (tZ, Fig. 2A), N^6-isopentenyladenine (iP, Fig. 2B), N^6-benzyladenine (BA, Fig. 2C) and *para*-topolin (Fig. 2D) were reported to have antioxidant activity *in vitro* (Choi et al., 2009; Jabłonska-Trypuc et al., 2016; Brizzolari et al., 2016).

*t*Z is another cytokinin used as an active ingredient in cosmetics, although as far as we know, results of clinical tests of its safety and efficacy in humans have not yet been published. Nevertheless, *t*Z was able to modulate the aging of fibroblasts in a manner similar to K (Rattan and Sodagam, 2005), and it protected skin cells against UV irradiation, increased the expression of aquaporin 3 and improved the healing/migration capability of cells in a scratch assay (Ji et al., 2010; Yang et al., 2009). Some studies have also shown that *t*Z has neuroprotective activity – it protected the rat pheochromocytoma cell line PC12 against toxicity induced by β-amyloid treatment and improved cognitive functions in mice with scopolamine-induced amnesia (Choi et al., 2009; Kim et al., 2008).

Both *ortho*- and *para*-topolin prolonged the lifespan of *C. elegans* (Kadlecová et al., 2018). Moreover, a recent study showed that topical application of the latter compound improved the appearance of photodamaged skin in humans (Garcia et al., 2018).

Cytokinins may also find applications in the treatment of skin hypopigmentation disorders such as vitiligo. In B16 melanoma cells, BA was shown to induce melanogenesis by activation of protein kinase A and subsequent up-regulation of microphthalmia-associated transcription factor and tyrosinase expression (Kim et al., 2009). We observed a similar effect for *para*-topolin (unpublished data).

Some derivatives of K have been developed and their protective effects investigated in animal models as well. A notable example is Pyratine® (6-furfurylamino-9-(tetrahydropyran-2-yl)-9H-purine; a 9-tetrahydropyranyl derivative of kinetin, Fig. 2E), another commercially used cosmeceutical, which reduced the symptoms of rosacea and improved the appearance of photodamaged skin in clinical trials (McCullough et al., 2008; Tremaine et al., 2010; Ortiz et al., 2009). Recently, our institute developed more efficient cytokinin derivatives able to protect cells against UV irradiation *in vitro* and increase the stress resistance of worms (Hönig et al., 2018). Our laboratory is involved in several projects focused on the development of cytokinin-inspired molecules with protective activity, with possible applications in treating skin and metabolic disorders. Our aim is to prepare more active derivatives, as well as to improve their pharmacokinetics Our recent results show that various cytokinin derivatives either protect against, or sensitize *C. elegans* to, oxidative and/or heat stress. The protective activities of cytokinin derivatives with substitutions at position C8 of the purine ring seem to be especially promising (Fig. 3). Several of these active compounds also protected fibroblasts derived from patients with Friedreich ataxia against oxidative stress (unpublished, Fig. 3). Friedreich ataxia was selected as a model of mitochondrial disease, because of its complex phenotype including oxidative stress, ATP depletion and mitochondrial iron overload. However, we envision the use of cytokinins in treating not only Friedreich ataxia but also other mitochondrial diseases.

The ability of K to correct aberrant splicing in FD models and patients has led to increased interest in the development of more active analogs. The first reported compound showing approximately 25-fold higher efficacy than K was 2-chloro-N-(furan-2-ylmethyl)-7H-purin-6-amine, also known as RECTAS (Fig. 2F) (Yoshida et al., 2015). In a recent study, Salani et al. tested 520 cytokinin derivatives and reported that 214 of these new compounds were more active than K. However, structures were shown only for two of the active compounds – 2-chloro-8-((3,3-difluorocyclobutyl)methoxy)N-(thiazol-2-ylmethyl)-9H-purin-6-amine and 2-chloro-8-(2-methoxyethoxy)-N-(pyrimidin-4-ylmethyl)-9H-purin-6-amine (Salani et al., 2019).

Protective activity has also been reported for some cytokinin ribosides. These compounds are typically cytotoxic at higher concentrations, which is why most research has focused on their potential application as cancer chemotherapeutic drugs. Nevertheless, N^6-isopentenyladenosine, N^6-benzyladenosine (Dassano et al., 2014) and other cytokinin ribosides, with the notable exception of 6-(2-hydroxy-3-methoxybenzyl)adenosine (unpublished), are activators of the Nrf2 pathway. *Ortho*-topolin riboside (Huang et al., 2011), *trans*-zeatin riboside, and possibly also kinetin riboside (Lee et al., 2012) have been proposed to act as agonists of adenosine A2A receptors, which are a potential target of multiple neurodegenerative and other diseases (Pisanti et al., 2014).

6. Conclusions

N^6-furfuryladenine, otherwise known as kinetin (K), is a well-known member of the cytokinins, a class of plant hormones. Although widely considered to be a natural molecule, present not only in plants but in animals as well, inconsistencies in studies focused on its detection in various materials have made us wonder under which circumstances it really occurs in nature. Apart from a variety of activities in plants, K has also been shown to have a health-promoting effect in animal systems. It was able to modulate aging *in vitro* and in invertebrates and it prevented deterioration in multiple tissues in rodent models of accelerated aging. What is lacking, however, are data describing its effect on natural aging in mammals, as well as studies focused on an increase in

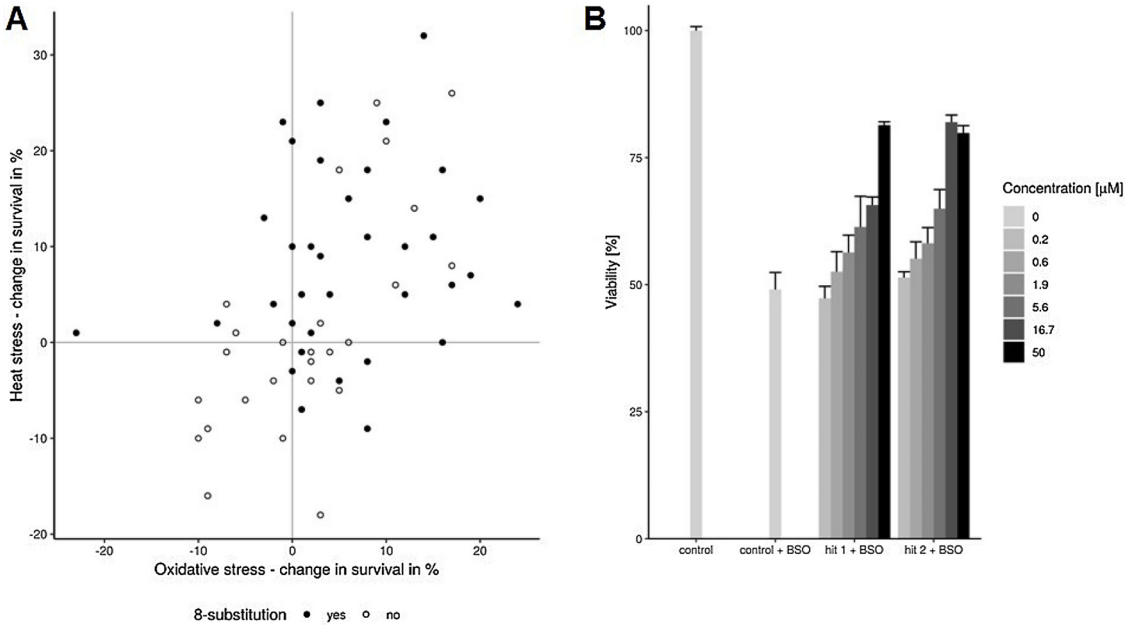

Fig. 3. Protective effect of cytokinin derivatives. A) 3-day pre-treatment of *C. elegans* with cytokinin derivatives protects against/sensitizes to oxidative (500 µM juglone, 5 h) and heat (35 °C, 6 h) stress. The set tested is enriched for compounds with protective activity, the majority of which have a substitution in position 8 of the purine ring. B) A 6 -h pre-treatment with 2 screening hits partially protects fibroblasts derived from a patient with Friedreich ataxia against stress caused by 48 -h exposure to 15 µM of the inhibitor of glutathione synthesis L-buthionine sulfoximine in a resazurin assay.

healthspan – a disease free period preceding an age-related physiological decline – rather than lifespan itself. We would argue that inclusion of K in programs such as the Interventions Testing Program could be of benefit[1] . K has also been reported to possess some protective activity in neurodegenerative, cardiovascular and metabolic diseases *in vitro* and *in vivo* and it corrects splicing of the ELP-1 gene, mutations in which cause familial dysautonomia.

K has been reported to induce antioxidant defenses and directly quench ROS. It modulates alternative splicing of certain pre-mRNAs, most likely by increasing recognition of the 5' splice site. In cells, K can be transformed into KR, KRMP, KRDP and KRTP. The latter was reported to act as a neo-substrate of the kinases PINK1 and casein kinase 2, boosting their activity. The base, the riboside, or other products of K´s metabolism, may also induce adaptive hormetic responses. It is unclear at this point whether, and if so how, these various activities are connected and whether the effects observed in various organisms and tissues, although sometimes similar in nature, result from a shared mechanism of action.

These promising results have also led to an interest in creating more active derivatives of K. Examples include the development of ProTide prodrugs aimed at delivering KRMP to cells, the preparation of more active modulators of splicing or our own projects aimed at developing novel cytokinin-inspired molecules for treating skin and metabolic diseases. K may be a multitarget molecule, and it is therefore possible that focusing on the improvement of a given activity would lead to the loss of other beneficial effects. A particularly interesting question is whether the reactivity of the furan ring is important for K´s protective activity, since finding a bioisosteric replacement would be challenging. Nevertheless, such attempts are logical, as the activity of K is rather low. The fact that typically concentrations of tens or even hundreds of µM of K are required in *in vitro* assays makes it challenging to provide effective doses *in vivo*.

On the other hand, the toxicity of K seems to be remarkably low as well, as indicated by the limited cytotoxicity it displays even after long-term cultivation experiments. Moreover, K has been used as an active ingredient in cosmetics for years and it has generally been reported as being safe and well tolerated after topical application. It is also in phase 2 of clinical trials for treatment of familial dysautonomia. Results from pre-clinical studies and from initial clinical evaluation seem to be encouraging so far. K has even been detected in mouse brain tissue, which shows it can cross the blood brain barrier. In patients, short-term oral administration of K did not cause any serious adverse effects. It remains to be seen whether this promising trend will be maintained in long-term studies, especially given the presence of the furan moiety, which has been associated with hepatotoxicity. However, taking into account the protective effect of K shown in various assays, further stages of clinical trials and the subsequent use of K for the treatment of FD or other approved indications may also provide information about its health-promoting "side effects" in humans.

It is clear that we still need more data to even begin to understand the precise mechanism of kinetin´s action. Detailed evaluation of its effects in various tissues and organisms could be beneficial. Still, hopefully it will not prove out of place to allow ourselves a little optimism, and hope that in the near future, K could bring much-needed relief to patients suffering from certain debilitating and currently incurable disorders.

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