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Doctoral Thesis

Synthesis and study of mechanism of action of new cytokinin derivatives

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Mgr. Lucie Plíhalová, Ph.D.

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Abstrakt	V rámci disertační práce byla připravena řada C2-chlor a N9-(tetrahydrofuran-2-yl) a N9-(tetrahydropyran-2-yl) substituovaných cytokininových derivátů, s modifikovaným C6 substituentem. Tyto látky byly připraveny již dříve popsanou dvoukrokovou syntézou, která byla optimalizována pro potřeby konkrétních cytokininových derivátů. K C2 a N9 substituovaným derivátům byly připraveny také odpovídající volné báze. Byla navržena syntéza N9 substituovaným derivátů s N-acetylglucosaminem a kyselinou glukuronovou, tedy jednotkami polysacharidu kyseliny hyaluronové a dále s fluorescenční značkou Cyanine 5. U některých látek byly pomocí senescenčního testu na ustřižených listech pšenice odhaleny silné antisenescenční účinky. Připravené látky také vykazovaly fotoprotektivní a antioxidační účinky na lidské kožní buňky a modelový organismus *Ceanorhabditis elegans*.
Klíčová slova	Cytokininy, kinetin, senescence, oxidativní stres, UVA/UVB fotoprotekce
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Abstract	In this thesis, cytokinin derivatives with modified C6 substituent, C2-chloro and/or N9-(tetrahydrofuran-2-yl) and N9-(tetrahydropyran-2-yl) substitutions were prepared and known procedures were optimized to obtain compounds in decent yields and purity. New routes for cytokinin conjugates with N-acetylglucosamine and D-glucuronic acid were implemented as well as the methods for the preparation of Cyanine5 labeled cytokinins. Prepared compounds were tested in wheat leaf senescence bioassay where some possessed strong antisenescent properties. Furthermore, photoprotective and antioxidant activities of prepared derivatives were shown on human skin cells and model organism, *Caenorhabditis elegans*.
Keywords	Cytokinins, kinetin, senescence, oxidative stress, UVA/UVB photoprotectivity
Number of pages	65
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Language	English

Declaration of Authorship

Hereby I declare that the Ph.D. thesis and the work presented in it is my original work except where otherwise indicated.

In Olomouc, 24.05.2019

Martin Hönig

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Content

Abbreviations

2,3-DHF	2,3-dihydrofuran
3,4-DHP	3,4-dihydropyran
AHK	arabidopsis histidine kinase
AHP	histidine-containing phosphotransfer protein
AllBr	allyl bromide
APX	ascorbate peroxidase
ARR	Arabidopsis response regulator
BAP	6-(benzylamino)purine
BSA	Bis(trimethylsilyl)acetamide
CAT	catalase
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
CRF	cytokinin response factor
Cy5	Cyanine5 fluorescent dye
cZ	6-(Z)-(4-hydroxy-3-methylbut-2-enylamino)purine (*cis*-zeatin)
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCE	dichloroethane
DCM	dichloromethane
DHZ	6-(4-hydroxy-3-methylbutylamino)purine (dihydrozeatin)
DIAD	diisopropyl azodicarboxylate
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
ENT	equilibrative nucleoside transporter
Et_3N	triethylamin
EtOAc	ethyl acetate
EtOH	ethanol
HPLC	high-performance liquid chromatography
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
HaCaT	spontaneously immortalized human keratinocyte cell line
iP	6-(2-isopentenylamino)purine
Kin	6-(furfurylamino)purine (kinetin)

LPO	lipid peroxidase
MemT	6-(3-methoxybenzylamino)purine (*meta*-methoxytopolin)
MeOH	methanol
MeoT	6-(2-methoxybenzylamino)purine (*ortho*-methoxytopolin)
MDA	malondialdehyde
mT	6-(3-hydroxybenzylamino)purine (*meta*-topolin)
NHS	N-hydroxysuccinimide
NBD	4-chloro-7-nitrobenzofurazan
NHDF	normal human dermal fibroblasts
ORAC	oxygen radical absorbance capacity
$Pd(PPh_3)_4$,	tetrakis(triphenylphosphine)palladium(0)
Ph_3P	triphenylphosphine
PUP	purin permease
oT	6-(2-hydroxybenzylamino)purine (*ortho*-topolin)
pT	6-(4-hydroxybenzylamino)purine (*para*-topolin)
Py	pyridine
ROS	reactive oxygen species
SAGs	senescence-associated genes
SAR	structure activity relationship
SOD	superoxide dismutase
TCS	two-component signal system
TDZ	1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (thidiazuron)
TFA	trifluoroacetic acid
THF	tetrahydrofuran-2-yl
THP	tetrahydropyran-2-yl
THFP	9-(tetrahydrofuran-2-yl)purine
THPP	9-(tetrahydropyran-2-yl)purine
TLC	thin-layer chromatography
TMS	tetramethylsilane
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
tZ	6-(*E*)-(4-hydroxy-3-methylbut-2-enylamino)purine (*trans*-zeatin)
WLSA	wheat leaf senescence assay

Introduction

Cytokinins (CKs) are a class of plant hormones that affect various aspects of plant growth and development, such as, cell division, leaf senescence, response to biotic and abiotic factors and many others (Kieber and Schaller, 2014). Since their discovery in the 1950s and synthesis of Kin by Miller *et al*. (1955), a wide range of CKs have been synthesized (Matsubara, 1980; Plíhalová et al., 2016; Skoog et al., 1967). Prepared compounds were usually tested for CK activity in various bioassays such as callus proliferation assays, pigment formation assays, leaf senescence delaying assays and many others, in various plant species (Gyulai and Heszky, 1994). Biological data from classical CK assays and later on from CK response (Romanov et al., 2002) and receptor binding bacterial (*E. coli*) assays (Spíchal et al., 2004) enabled researchers to start establishing complex structure activity relationships (SAR). From these, highly active CK derivatives were synthesized and utilized in agriculture (Koprna et al., 2016) or in tissue culture (Aremu et al., 2012a, 2012b; Madzikane-Mlungwana et al., 2017; Moyo et al., 2018).

In the 1990s CK 6-furfurylaminopurin (kinetin, Kin) was found to affect human fibroblasts (Rattan and Clark, 1994). Later on CKs, were shown to possess antioxidant properties in various animal cells and influence the activity of several antioxidant enzymes (Jabłońska-Trypuć et al., 2016; Olsen et al., 1999). A life-prolonging effect of CK was found in an *in-vivo* experiment with *Zaprionus fruitflies* (Sharma et al., 1997).

Kin derivative 6-furfurylamino-9-(2-tetrahydropyran-2-yl)purine was prepared by the introduction of a tetrahydropyranyl group to the N9 atom of Kin moiety (Szüčová et al., 2016). This compound possessed high activity in wheat leaf senescence assay (WLSA) and also delayed the senescence of human fibroblasts (Szüčová et al., 2016). Moreover, topical application to the human face improved the appearance of photodamaged skin (McCullough et al., 2008).

This work deals with the preparation and biological properties of Kin derivatives and/or analogues with a modified furfuryl ring including compounds accompanied by either THF (tetrahydrofuran-2-yl) or THP (tetrahydropyran-2-yl) protective groups. Prepared compounds were primarily studied for their antisenescent activity in plants and protective properties in human skin cells.

Besides CK conjugates with two subcomponents of hyaluronic acid, D-glucuronic acid and N-acetyl-D-glucosamine were also prepared to test their biological activity as the conjugation of CKs with different sugars in the N9 position of the purine moiety can dramatically change their activity including increased efficiency in antisenescent tests (Doležal et al., 2018, 2007; Holub et al., 1998).

Aims and scope

A large number of CK derivatives with various structural modification of adenine moiety have been prepared to date. Even small structural change can lead to complete loss of CK-like activity. Hence well-described structure-activity relationship is necessary for the preparation of highly active compounds. New CK derivatives were prepared and their biological activity was studied to reveal highly active molecules with possible practical application.

The overall aims of this doctoral thesis are:

- Preparation of Kin derivatives and/or analogues with modification of the furfuryl ring.
- Preparation of N9-purine substituted kinetin-like compounds with THF and THP protection groups.
- Preparation of CK sugar conjugates.
- Formulation of structure-activity relationship and identification of key structural features.
- Evaluation of the biological activity of newly prepared compounds in both plants and animals.

Literature review

Plant hormones, also referred to as phytohormones, are a group of naturally occurring compounds with unique biosynthesis, metabolism and perception and which influence physiological processes at very low concentrations (Davies, 2010). Plant hormones are divided into several groups and they differ in chemical structure, occurrence, ways of transport and of course, in their effect on plant growth and development. Since the discovery of auxin (indole-3-acetic acid, IAA) in 1920s (Enders and Strader, 2015), several hormonal groups have been described, namely: CKs, gibberellins, ethylene, abscisic acid, brassinosteroids, jasmonates and strigolactones (Davies, 2010).

Besides these basic phytohormone groups, there are compounds with regulatory activity which however do not fit the definition of plant hormones as postulated at the beginning of this chapter. These are salicylic acid, peptides, polyamines and karrikins (Chiwocha et al., 2009; Davies, 2010). In this thesis, I focused on the group, cytokinins, and these are described below in more detail.

1. Cytokinins

CKs were discovered as compounds that are able to promote cell division in the presence of auxin (Davies, 2010). The first identified CK was Kin: an artificial product of autoclaved DNA (Miller et al., 1956). However, the first discovered naturally occurring CK, zeatin, was identified much later in immature maize endosperm (Letham, 1973). Since then, CKs have been found to be associated with almost all aspects of plant growth and development, from embryonic development to leaf senescence together with the response to biotic and abiotic stress (Kieber and Schaller, 2014).

CKs can be divided according to their structure into adenine-type CKs substituted at the N6-position with either an isoprenoid or aromatic side chain (Fig. 1). Another group is represented by phenylurea-type CKs. Adenine-type CKs consist of both endogenous and synthetic compounds while phenylurea-type CKs are all artificial (Davies, 2010).

The most common CK in higher plants is zeatin, an isoprenoid side chain cytokinin that occurs in two geometric isomers. *Trans*-zeatin (*t*Z, 6-(E)-(4-hydroxy-3-methylbut-2-enylamino)purine), together with another isoprenoid CK 6-(2-isopentenylamino)purine (iP),

play a major physiological role in *Arabidopsis thaliana* and many other species (Kieber and Schaller, 2018). C*is*-zeatin (*c*Z, 6-(E)-(4-hydroxy-3-methylbut-2-enylamino)purine), has lower CK activity and affinity to its receptor in *Arabidopsis thaliana* (Romanov et al., 2006; Spíchal et al., 2004). Although in some plants including rice, it is the most abundant form (Gajdošová et al., 2011), and well-recognized by *Zea mays* cytokinin receptors, its physiological role has not been fully elucidated (Osugi and Sakakibara, 2015).

6-(2-Hydroxybenzylamino)purine (ortho-topolin, oT) and 6-(3-hydroxybenzylamino)purine (meta-topolin, mT), and their corresponding 9-substituted derivatives, were identified as naturally occurring aromatic CKs in various plant species (Jones et al., 1996; Strnad et al., 1997). Later on, 6-(2-methoxybenzylamino)purine (ortho-methoxytopolin, MeoT), 6-(3-methoxybenzylamino)purine (meta-methoxytopolin, MemT) and their 9-β-D-ribofuranosyl derivatives were found to occur in plants as well (Tarkowská et al., 2003). Both topolins and methoxytopolins possessed high activity in CK biotests (Holub et al., 1998; Tarkowská et al., 2003, Doležal e tal. 2006) and are preferred for use in plant tissue culture (Aremu et al., 2012a).

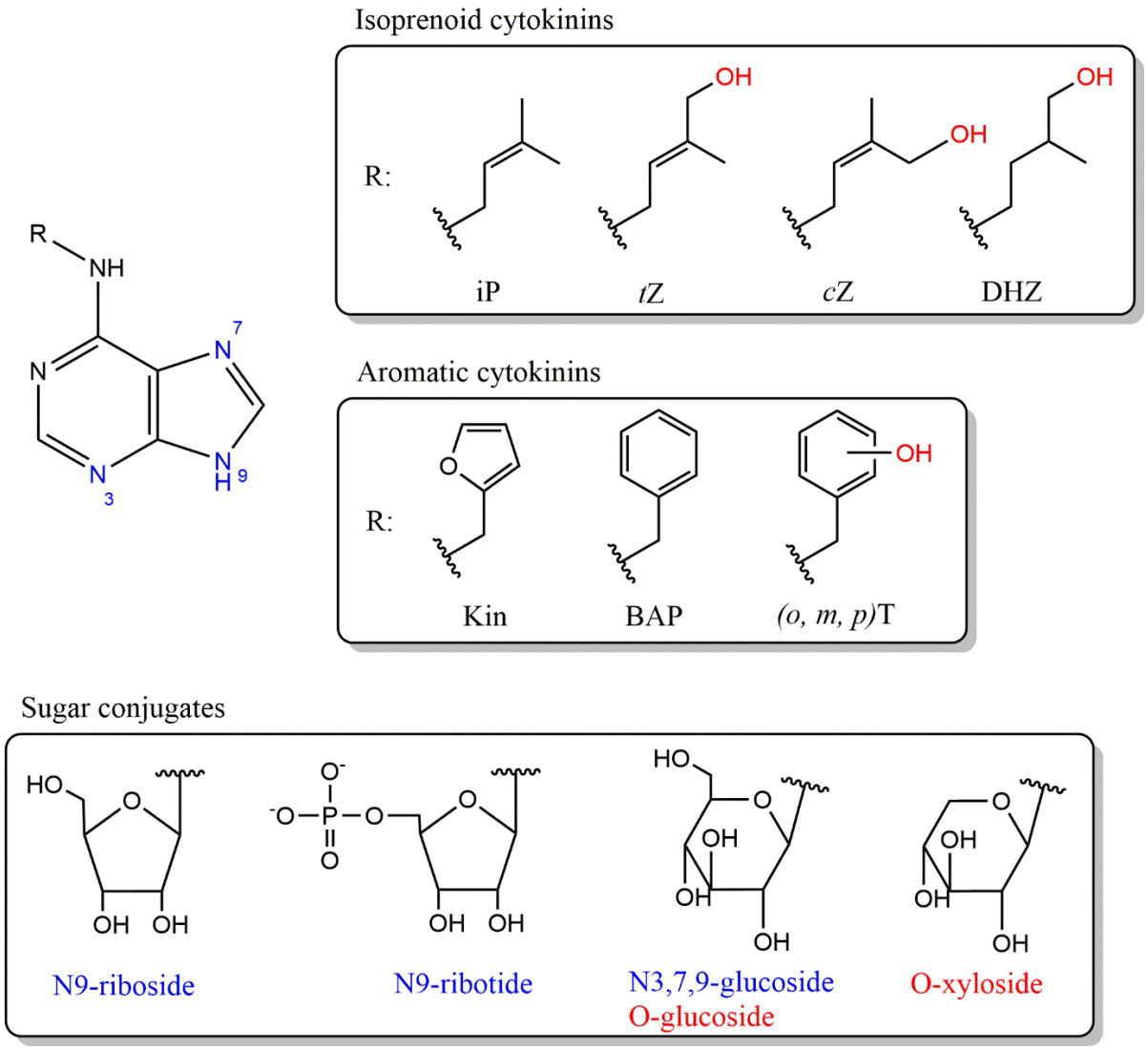


Figure 1 *Structure of CKs and their sugar conjugates. Abbreviations: iP, 6-(2-isopentenylamino)purine; tZ, 6-(E)-(4-hydroxy-3-methylbut-2-enylamino)purine (trans-zeatin); cZ, 6-(Z)-(4-hydroxy-3-methylbut-2-enylamino)purine (cis-zeatin); DHZ, 6-(4-hydroxy-3-methylbutylamino)purine (dihydrozeatin); Kin, 6-(furfurylamino)purine (kinetin); BAP, 6-(benzylamino)purine; oT, 6-(2-hydroxybenzylamino)purine (ortho-topolin); mT, 6-(3-hydroxybenzylamino)purine (meta-topolin); pT, 6-(4-hydroxybenzylamino)purine (para-topolin)*

1.1. Biosynthesis and metabolism of CKs

CKs are present in plants in nanomolar concentrations and those may vary in different plant organs and can be influenced by growth conditions (Osugi and Sakakibara, 2015). The active forms of CKs are usually free bases (Lomin et al., 2015). These can be inactivated *via* conjugation with sugars or amino acids (Bajguz and Piotrowska, 2009; Davies, 2010). There are N9, N7 and N3 adenine rings conjugates with glucose that are irreversibly synthetized by N-glucosyltransferase. CKs with the hydroxyl group in their side chain, such as *t*Z, *c*Z, DHZ

and topolins can also form reversible storage form with glucose or xylose in the reaction catalyzed by O-glucosyltransferase or O-xylosyltransferase (Sakakibara, 2006). Both N- and O- cytokinin glucosides were found to be non-active in receptor binding assay (Spíchal et al., 2004). However, unlike N-glucosides, O-glucosides can be transferred into active form by enzyme β-glucosidase (Brzobohatý et al., 1993).

Biosynthesis of isoprenoid CKs starts with the formation of iP-ribotides *via* the reaction of dimethylallyl diphosphate (DMAPP) and adenine nucleotides (ATP or ADP), catalyzed by adenosine-phosphate isopentenyltransferase (IPT) (Frébort et al., 2011; Kasahara et al., 2004; Sakakibara, 2006; Takei et al., 2001) The isoprenoid side chain of the resulting iP-ribotides could be subsequently hydroxylated by Cytochrome P450 monooxygenase CYP735A to corresponding *t*Z-ribotides (Takei et al., 2004). In the last step, active forms of CKs are formed from the cytokinin-riboside monophosphates in the reaction catalyzed by the CK riboside 5'-monophosphate phosphoribohydrolase (LONELY GUY, LOG) (Kurakawa et al., 2007; Kuroha et al., 2009).

CKs can also be inactivated by the enzyme cytokinin oxidase/dehydrogenase (CKX) which irreversibly degrades CKs by cleavage of the side chain in the N6 position. CKX is able to cleave isoprenoid CKs with unsaturated side chains such as iP and *t*Z as well as their corresponding nucleosides (Kieber and Schaller, 2014). On the other hand, DHZ type CKs, aromatic CKs like Kin or 6-benzylaminopurine (BAP) or urea based CKs such as thidiazuron (TDZ) are resistant to the cytokinin oxidase cleavage (Kieber and Schaller, 2014). Modification of the side chain such as O-glucosylation can also inhibit degradation by CKX (Galuszka et al., 2007; Nisler et al., 2018; Zalabák et al., 2014). Both CK conjugation and degradation play an important role in the control of CK homeostasis and concentration level (Kieber and Schaller, 2014).

1.2. CK transport

In plants, CKs are synthesized in various cell types in both root and shoot and then transported locally as well as through long-distance transport from root to shoot, and vice versa (Davière and Achard, 2017; Durán-Medina et al., 2017; Kieber and Schaller, 2018). Both efflux and influx transporters capable of CK transport have been identified (Kieber and Schaller, 2018). Efflux transporter ABCG14 was shown to be involved in root-to-shoot translocation *via* xylem

(Ko et al., 2014; Zhang et al., 2014) while purine permeases (PUP) and equilibrative nucleoside transporters (ENT) are capable of CK uptake (Bürkle et al., 2003; Hirose et al., 2005).

The biosynthetic precursor, tZ-riboside is the most abundant CK form transported acropetally *via* the xylem. However, the biologically active form, *t*Z is transported as well. Moreover in an experiment with grafted mutant plants it was reported that root-derived *t*ZR and *t*Z play different roles in Arabidopsis shoot (Davière and Achard, 2017; Osugi et al., 2017). In another experiment, acropetal transport of aromatic CK 3-methoxy(-6-benzylamino)purine (3MeOBAP) was significantly enhanced by tetrahydropyranyl substitution at the N9-position of the adenine moiety. Moreover, this modification slowed inhibition of 3MeOBAP by conjugation with glucose. (Podlešáková et al., 2012).

In contrast, *iP*-derived CKs, including free base and conjugates are most abundant in phloem, playing the major role in basipetal transport (Hirose et al., 2008; Kudo et al., 2010).

CKs thus function as long distance messengers, although local changes in CK levels also play a significant role in mediating the response (Kieber and Schaller, 2014).

1.3. CK perception and signal transduction

Plants use a two-component signal system (TCS) which is analogous to bacterial TCS that senses and responds to environmental signals to sense and transduce CK signals. This signaling system consists in a lipid-membrane localized receptor which senses the CK signal and response regulator that propagates the signal. Signal transduction follows multistep phosphotransfer between His residue in the sensor kinase and Asp residue in the receiver domain of the response regulator (Kieber and Schaller, 2018).

The TCS in CK signaling consists of three groups of proteins in Arabidopsis: histidine kinases (AHKs), histidine-containing phosphotransfer proteins (AHPs), type-A and type-B response regulators (ARRs, Fig. 2) (Kieber and Schaller, 2014; Osugi and Sakakibara, 2015).

Three cytokinin receptors have been discovered in *Arabidopsis thaliana* so far. They are designated CRE1/AHK4, AHK3 and AHK4 (Kakimoto, 2003). These receptors share similar structure (Kieber and Schaller, 2014) but they can differ in their ligand specificity (Spíchal et al., 2004). Since the discovery of the first receptor CRE1/AHK4 in 2001 (Inoue et al., 2001), several homologues have been identified in other species including *Zeya mays* (Yonekura-Sakakibara, 2004), *Oryza sativa* (Ding et al., 2017), *Lotus Japonicus* (Held et al., 2014), *Medicago truncatula* (Laffont et al., 2015), *Brassica napus* (Kuderová et al., 2015), *Nicotiana*

attenuate (Schäfer et al., 2015), *Malus domestica* (Daudu et al., 2017) and *Solanum tuberosum* (Lomin et al., 2018b). Individual AHK homologues can differ in ligand affinity (Lomin et al., 2012) and can be in some aspects, species-specific (Lomin et al., 2018b).

CKs are bound into the extracytosolic CHASE domain of the AHK receptor that triggers activation of the cytosolic histidine kinase domain and autophosphorylation on the conserved His residue, the phosphoryl group is then internally transferred to the conserved Asp residue in the attached receiver domain (Davies, 2010; Inoue et al., 2001; Kieber and Schaller, 2018). CK receptors are primarily localized in the ER membrane, however they were also found in the plasma membrane (PM) (Lomin et al., 2018a; Wulfetange et al., 2011; Zürcher and Müller, 2016). Although ER is considered the predominant site of CK signal perception, signal initiation at the PM may be relevant as well (Romanov et al., 2018)

The phosphate is then transferred to the AHP protein which moves to the nucleus where it can transfer phosphate to the receiver domain of type-A or type-B ARRs. Type-B ARRs then regulate the expression of many target genes, including type-A ARRs (Schaller et al., 2014).

Type-B ARRs contain a receiver domain, a DNA-binding domain (GARP) and a glutamine rich domain for transcriptional activation. Their phosphorylation is required to bind DNA and to activate the transcription of targeted genes (Hwang and Sheen, 2001; Sakai, 2001; Sakai et al., 2000).

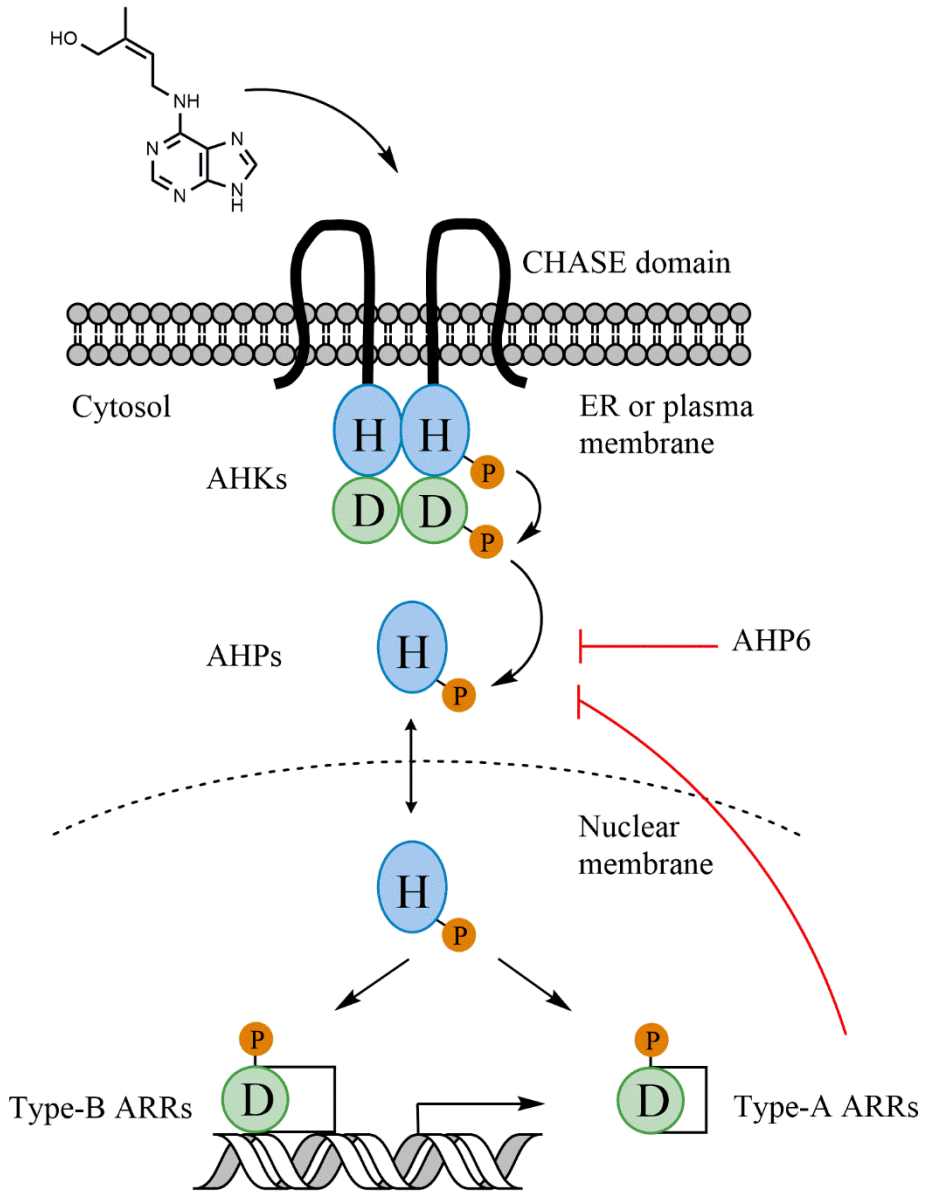


Figure 2 *CK signal is received by CHASE domain on the extracytosolic side of ER or plasma membrane. CK binding is followed by autophosphorylation of His (H) residue and internal transfer of phosphate to Asp (D). AHPs then transfer phosphate to the ARRs in the nucleus. Type-B ARRs are transcription factors that regulate expression of CK response genes including Type-A ARRs. Hovewer, type-A ARRs lack a DNA-binding domain and operate in a negative feedback loop being phosphorylated by AHPs. Pseudo-histidine phosphotransfer protein AHP6 which lack a histidine residue also negatively regulate the CK response* (Kieber and Schaller, 2018, 2014).

1.4. Senescence, antioxidant defense and photosynthesis

CKs play a crucial role in the regulation of leaf senescence (Gan and Amasino, 1997). Leaf senescence is the final stage of leaf development during which leaf cells undergo highly coordinated changes in cell structure, metabolism and gene expression.

The concentration and activity of endogenous CKs decrease during senescence (Van Staden, 1988) while expression of senescence associated genes (SAGs) increases (Gan and Amasino, 1997). Senescence and SAG expression can be inhibited by the application of exogenous CKs (Van Staden, 1988) as well as by the activation of CK production in genetically engineered plants (Gan and Amasino, 1997). Leaf senescence is characterized by increased levels of reactive oxygen species (ROS) and decrease in antioxidants. CKs are reported to induce the activity of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) together with secondary antioxidant metabolites, while lipid peroxidation and H_2O_2 formation are decreased (Hönig et al., 2018, **II**; Zavaleta-Mancera et al., 2007).

CK activity is connected to photosynthesis and maintenance of the photosynthetic apparatus (Hönig et al., 2018, **II**; Talla et al., 2016). CKs affect chloroplast differentiation (Harvey et al., 1974) and division (Okazaki et al., 2009), grana production (Wilhelmova and Kutik, 1995) and chlorophyll biosynthesis (Cortleven and Schmülling, 2015). CKs are able to protect chloroplasts against changes related to senescence progression as well as prevent decrease in chlorophyll level (Wojciechowska et al., 2018).

From a molecular biology point of view, the antisenescence properties of CKs appear to be regulated mainly by the AHK3 receptor (Kim et al., 2006). Downstream of the AHK3 receptor cascade, type-B response regulator, ARR2 was reported to regulate leaf longevity, together with cytokinin response factor 6 (CRF6; Kim et al., 2006; Zwack et al., 2013), which is also involved in retardation of senescence. On the other hand, protection against lipid peroxidation in *Arabidopsis* detached leaves was maintained mainly by the CRE/AHK4 receptor. (Janečková et al., 2018).

1.4.1. Antisenescent activity of CK derivatives in wheat leaf senescence assay (WLSA)

Various CK derivatives (including BAP, Kin, tZ, cZ, iP etc.) with modified C2, C8, N6 and N9 atoms of the adenine moiety were prepared. The potential antisenescent activity of these derivatives was tested in a wheat leaf senescence assay performed in dark (Holub et al., 1998). Based on numerous SAR studies published over the past few decades, key structural motifs potentially responsible for antisenescent properties have been proposed. The substitution at the N6 atom seems to be a crucial structural feature. Concurrently, the N6-H hydrogen must stay unsubstituted and "free to operate". Substituents at the N6 atom must contain an oxygen atom in the form of oxo-, hydroxyl or methoxy groups and/or halogen atoms, such as fluorine or chlorine. N9 substituted derivatives are active in WLSA only in the presence of proper N6 substituent (Hönig et al., 2018, **II)**.

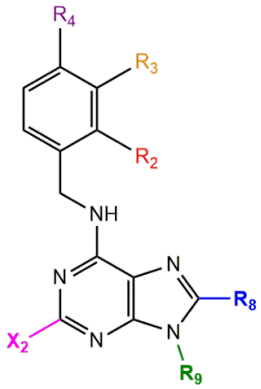
Isoprenoid endogenous CK tZ as well as aromatic CK mT possess higher activity in WLSA than BAP or Kin (Hönig 2018 et al., **II)**, however endogenous CK iP as well as its derivatives showed only low or no activity in a wheat leaf senescence bioassay (Mik et al., 2011b).

Both C2 and C8 substitutions in the majority of cases lowered or completely reduced the antisenescent activity of modified compounds (Hönig et al., 2018b, **I**; Zahajská et al., 2017).

The activity of BAP derivatives in WLSA is summarized in Table 1. Ortho (R_2) and meta (R_3) hydroxy BAP derivatives, also referred to as ortho (oT) and meta (mT) topolins, are potent antisenescent agents (Holub et al., 1998). Moreover, equivalent methoxy derivatives also possess antisenescent properties (Tarkowská et al., 2003). Fluorine, chlorine and methyl substitution in the ortho position increased BAP activity in WLSA. However only florine was beneficial for antisenescent activity in the meta position (Doležal et al., 2006). In general,subtitutions in the *meta* position of the benzyl ring possesed highest antisenescent activities (Doležal et al., 2006, 2007; Szüčová et al. 2009; Vylíčilová et al., 2016). The activity of topolins was significantly decreased by N9 glucosylation (R_9, Holub et al., 1998). On the other hand, BAP β-D-ribofuranoside (Doležal et al., 2007), β-D-arabinofuranoside, β-D-2'-deoxyribofuranoside and their derivatives were shown to be very effective in chlorophyl retention (Doležal et al., 2018). THP and THF derivatives were active in WLSA only in the presence of proper N6 substituent. However, these derivatives showed none of the negative aspects of CK treatment such as root inhibition or problems with aclimatization during micropropagation (Aremu et al., 2017; Podlešáková et al., 2012; Szüčová et al., 2009). The presence of chlorine at the C2 atom did not reduce the antisenescent properties (Vylíčilová et al., 2016) compared to coresponding ribosides (Doležal et al., 2007), however bromine, iodine

or chlorine at the C8 atom (Zahajská et al., 2017) had negative effect on the antisenescent properties of modified compounds. In contrast, the oxo group at C8 atom increased the activity of BAP in WLSA (Zahajská et al., 2017).

Table 1 *Relative activity of BAP derivatives in WLSA. Arrows illustrate higher* (↑), *lower* (↓) or comparable (↕) *biological activity as BAP. Three upward arrows (↑↑↑) show the highest activity while three downward arrows indicate (↓↓↓) complete loss of antisenescent activity. Abbreviations: Ribose, β-D-ribofuranose; Arabinose, β-D-arabinofuranose; Deoxyribose, β-D-2'-deoxyribofuranose; THP, tetrahydropyran-2-yl*

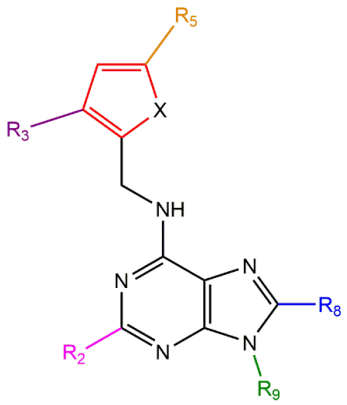


R_2	R_3	R_4	R_8	R_9	C_2	Activity	Literature
OH	H	H	H	H	H	↓↓	(Holub et al., 1998)
H	OH	H	H	H	H	↑↑	(Holub et al., 1998)
MeO	H	H	H	H	H	↑↑	(Tarkowská et al., 2003)
H	MeO	H	H	H	H	↑↑	(Tarkowská et al., 2003)
H	H	MeO	H	H	H	↓	(Doležal et al., 2006)
F	H	H	H	H	H	↑↑	(Doležal et al., 2006)
H	F	H	H	H	H	↑↑↑	(Doležal et al., 2006)
H	H	F	H	H	H	↕	(Doležal et al., 2006)
Cl	H	H	H	H	H	↑	(Doležal et al., 2006)
H	Cl	H	H	H	H	↓	(Doležal et al., 2006)
H	H	Cl	H	H	H	↓	(Doležal et al., 2006)
CH_3	H	H	H	H	H	↑↑	(Doležal et al., 2006)
H	CH_3	H	H	H	H	↑	(Doležal et al., 2006)
H	H	CH_3	H	H	H	↓↓	(Doležal et al., 2006)
OH	H	H	H	Ribose	H	↕	(Holub et al., 1998)
H	OH	H	H	Ribose	H	↑↑	(Holub et al., 1998)
MeO	H	H	H	Ribose	H	↑↑↑	(Tarkowská et al., 2003)
H	MeO	H	H	Ribose	H	↑↑↑	(Tarkowská et al., 2003)
H	H	MeO	H	Ribose	H	↓	(Doležal et al., 2007)
F	H	H	H	Ribose	H	↑	(Doležal et al., 2007)
H	F	H	H	Ribose	H	↑↑↑	(Doležal et al., 2007)
H	H	F	H	Ribose	H	↑↑	(Doležal et al., 2007)
Cl	H	H	H	Ribose	H	↑	(Doležal et al., 2007)

R_2	R_3	R_4	R_8	R_9	C_2	Activity	Literature
H	Cl	H	H	Ribose	H	↓	(Doležal et al., 2007)
H	H	Cl	H	Ribose	H	↕	(Doležal et al., 2007)
H	I	H	H	Ribose	H	↓↓	(Doležal et al., 2007)
CH_3	H	H	H	Ribose	H	↑↑	(Doležal et al., 2007)
H	CH_3	H	H	Ribose	H	↑↑	(Doležal et al., 2007)
H	H	CH_3	H	Ribose	H	↓↓	(Doležal et al., 2007)
F	H	H	H	Ribose	Cl	↑↑	(Vylíčilová et al., 2016)
H	F	H	H	Ribose	Cl	↑↑↑	(Vylíčilová et al., 2016)
H	H	F	H	Ribose	Cl	↑↑↑	(Vylíčilová et al., 2016)
OH	H	H	H	Glucose	H	↓↓↓	(Holub et al., 1998)
H	OH	H	H	Glucose	H	↓↓↓	(Holub et al., 1998)
OH	H	H	H	THP	H	↓↓	(Szüčová et al., 2009)
H	OH	H	H	THP	H	↑↑	(Szüčová et al., 2009)
H	H	OH	H	THP	H	↓↓↓	(Szüčová et al., 2009)
MeO	H	H	H	THP	H	↕	(Szüčová et al., 2009)
H	MeO	H	H	THP	H	↓	(Szüčová et al., 2009)
H	H	H	Cl	H	H	↓↓	(Zahajská et al., 2017)
H	H	H	O	H	H	↑	(Zahajská et al., 2017)
H	H	H	Br	THP	H	↓↓↓	(Zahajská et al., 2017)
H	H	H	I	THP	H	↓↓↓	(Zahajská et al., 2017)
H	MeO	H	H	Arabinose	H	↑	(Doležal et al., 2018)
H	F	H	H	Arabinose	H	↑↑↑	(Doležal et al., 2018)
H	H	H	H	Deoxyribose	H	↑↑	(Doležal et al., 20018)

The activity of Kin derivatives in WLSA is summarized in Table 2.The substitution of hydrogen atoms of the furan ring significantly reduced the antisenescent properties observeable in Kin (Hönig 2018 et al., **I**). The same effect followed substitution of the oxygen atom of the furan ring by sulphur or carbon. However, saturation of the furan ring had no effect in reducingthe activity of the prepared Kin derivative (Hönig 2018 et al., **I**). Halogens, amino or oxo group substitution at C8 atom of adenine moiety (R_8) eliminated or partially reduced Kin antisenescent activity. Hovewer, C8 methoxy and 2-hydroxyethyloxy derivatives maintained their antisenscent activity (Zahajská et al., 2017). N9-subtituted Kin derivatives with THF and THP protective groups were prepared (Szüčová et al., 2016, 2011) and both compounds possessed higher antisenescent properties in comparison to Kin. N9-Ethoxyethyl and N9-chloroethyl Kin derivatives were also highly active in WLSA (Mik et al., 2011a). β-D-arabinofuranose substituted at N9 atom increased the antisenescent properties of Kin, as well (Doležal et al., 2018). The addition of chlorine at the C2 atom of adenine (R_2) lowered and even completely abolished the antisenescent activity (Hönig et al, 2018, **I**) in contrast to BAP derivatives (Tab. 2, Vylíčilová et al., 2016).

Table 2 *Relative activity of Kin derivatives in WLSA. Arrows illustrate higher (↑), lower (↓) or same (↕) biological activity as BAP. Three upward arrows (↑↑↑) show the highest activity while three downward arrows indicate (↓↓↓) complete loss of antisenescent activity. Abbreviation: Arabinose, β-D-arabinofuranose; THP, tetrahydropyran-2-yl; THF, tetrahydrofuran-2-yl; Unsatur., unsaturated heterocycle; Satur., saturated heterocycle*



Heterocycle	X	R_9	R_8	R_2	R_5	R_3	Activity	Literature
Unsatur.	S	H	H	H	H	H	↓	(Hönig et al. 2018, **I**)
Satur.	C	H	H	H	H	H	↓	(Hönig et al. 2018, **I**)
Satur.	O	H	H	H	H	H	↑	(Hönig et al. 2018, **I**)
Unsatur.	O	THP	H	H	H	H	↑↑	(Szüčová et al. 9999)
Unsatur.	O	THF	H	H	H	H	↑	(Mik et al., 2011)
Unsatur.	S	THF	H	H	H	H	↑	(Hönig et al. 2018, **I**)
Satur.	O	THF	H	H	H	H	↑	(Hönig et al. 2018, **I**)
Unsatur.	O	THF	H	Cl	H	H	↓	(Hönig et al. 2018, **I**)
Unsatur.	S	THF	H	Cl	H	H	↓↓↓	(Hönig et al. 2018, **I**)
Satur.	O	THF	H	Cl	H	H	↑	(Hönig et al. 2018, **I**)
Unsatur.	O	THF	H	H	CH_3	H	↑	(Hönig et al. 2018, **I**)
Unsatur.	O	THF	H	H	H	CH_2-OH	↓	(Hönig et al. 2018, **I**)
Unsatur.	O	Chloroethyl	H	H	H	H	↑	(Mik et al., 2011)
Unsatur.	O	Arabinose	H	H	H	H	↑↑↑	(Doležal et al., 2018)
Unsatur.	O	H	Cl	H	H	H	↓↓↓	(Zahajská et al., 2017)
Unsatur.	O	H	Br	H	H	H	↓↓↓	(Zahajská et al., 2017)
Unsatur.	O	H	I	H	H	H	↓↓↓	(Zahajská et al., 2017)
Unsatur.	O	H	MeO	H	H	H	↕	(Zahajská et al., 2017)
Unsatur.	O	H	O	H	H	H	↓	(Zahajská et al., 2017)
Unsatur.	O	H	MeS	H	H	H	↓↓	(Zahajská et al., 2017)
Unsatur.	O	THP	Cl	H	H	H	↓↓	(Zahajská et al., 2017)
Unsatur.	O	THP	MeS	H	H	H	↓↓	(Zahajská et al., 2017)

2. 6-Furfurylaminopurine (Kinetin, Kin) and its activity in animal and human cells and tissues

Kin is a plant hormone classified as ArCK, which is well-known for a number of biological activities. Although, Kin is a plant hormone, it was found to possess various effects in animal and animal cells (Barciszewski et al., 2007). Kin was discovered in 1955 as an artificial product of autoclaved DNA (Miller et al., 1956) and up to now identified as a naturally occurring compound in fresh DNA preparations from human cell cultures, normal human plasma, human urine (Barciszewski 2000) and coconut milk (*Cocos nucifera L.;* Ge et al., 2005).

The antioxidant properties of Kin have been reported in both *in vitro* and *in vivo* experiments (Jabłońska-Trypuć et al., 2016; Olsen et al., 1999). Human skin tissue possesses a very effective protective antioxidative system that includes enzymes glutathione peroxidase (GPX), glutathione reductase (GR), CAT and SOD (Jabłońska-Trypuć et al., 2016). This is important given that there is a direct connection between oxidative stress and simultaneous overexpression of SOD and CAT as determined in transgenic *Drosophila melanogaster* (Sohal and Weindruch, 1996). Kin showed stimulatory effect on the activity of CAT, an enzyme with high antioxidative activity, during the development and adult life of *Zaprionus fruitflies* (Sharma et al., 1997, 1995). Stimulation of CAT activity was also observed in Kin treated normal human skin fibroblasts (Jabłońska-Trypuć et al., 2016). This aside, it has been shown that Kin stimulated other antioxidative enzymes such as GPX and GR and maintained constant reduced glutathione (GSH) content in fibroblasts, caused decrease in membrane phospholipid peroxidation and exhibited protective properties against malondialdehyde (MDA) production (Jabłońska-Trypuć et al., 2016). In a model of aging rats, the application of Kin significantly decreased the level of oxidative stress markers such as MDA and lipid peroxide (LPO), while it increased the activity of antioxidative enzymes SOD, GPX and CAT (Li et al., 2016).

Kin is reported to be a compound, able to influence the response of immune system in animals, affecting the content of immunoglobulin, interleukin-2 (IL-2) and interleukin-6 (IL-6((Li et al., 2016, 2014). Kin retarded aging and prolonged the lifespan of Kin-fed *Zaprionus fruitflies* used as a model organism, but also slowed down development and delayed the maturation of insects in the larval and pupal stages (Sharma et al., 1997, 1995).

It was also found to delay age related characteristics of human fibroblasts without any increase in the cell culture lifespan in terms of increased proliferative capacity *in vitro.* It also reduced the accumulation of the auto-fluorescent compound lipofuscin in aging human fibroblasts (Rattan and Clark, 1994).

The significant effect of topically applied Kin both on epidermis and dermis formation and development was observed using reconstructed skin equivalent (SE) Mimeskin® (Vicanova et al., 2006). Kin treatment also increased the amount of laminin 5 at the dermal-epidermal junction and stimulated formation of fibrillin-1 and elastin deposition that positively affected the formation of basement membrane and the elastic network in the upper dermis (Vicanova et al., 2006).

Clinical studies on volunteers have been described where topical treatment with Kin significantly ameliorated the clinical signs of photodamaged skin such as fine wrinkles, rough skin texture and mottled hyperpigmentation (McCullough and Weinstein, 2002). Twice-daily application of 0.1% Kin lotion successfully reduced signs and symptoms of mild to moderate inflammatory facial rosacea (Wu et al., 2007).

Kin N9-derivative 6-furfurylamino-9-(2-tetrahydropyran-2-yl)purine (Pyratine) prepared and patented by the Institute of Experimental Botany AS CR and launched by the American company Senetek PLC is curently utilized in cosmetics under the trade mark Pyratine-6® and marketed by Pyratine LLC company as a compound with the ability to delay skin senescence and aging (Szüčová et al., 2016, 2011). In a 12 week human clinical study, Pyratine reduced symptoms and signs of photodamaged skin, such as fine wrinkles, roughness and mottled hyperpigmentation significantly more efficiently than Kin. Pyratine also reduced transepidermal water loss and thus increased the level of moisturein the skin. Moreover, significant reduction in erythema at 2 weeks and further reduction at weeks 4, 8 and 12 of Pyratine treatment, was found. (McCullough et al., 2008).

In contrast, Kin-N9-riboside, which includes the tetrahydrofuranyl ring, displayed high cytotoxicity (Berge et al., 2006; Doležal et al., 2007; Griffaut et al., 2004).

6,9-disubstituted and 2,6,9-trisubstituted Kin derivatives were found to possess photoprotective properties in human skin cells against both UVA and UVB irradiation (Hönig et al., 2018, **I**). In the same study, two Kin derivatives 6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine and 2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine showed antioxidant effects against 5-hydroxy-1,4-naphthalenedione (juglone) induced oxidative stress in an *in-vivo* experiment with *Caenorhabditis elegans.* (Hönig et al., 2018, **I**).

3. Cytokinin synthesis

Various synthetic approaches were used to modify classical CK molecules by substitutions in various positions of the adenine moiety (Plíhalová et al., 2016).

3.1. Monosubstituted aromatic cytokinins

Kin was first synthesized from 6-methylmercaptopurin and freshly distilled furfurylamine. The reaction mixture was heated at 115-120 ºC for 9h. After recrystallization from ethanol, colorless platelets were obtained (82% yield) (Miller et al., 1956). Sattsangi et al. prepared Kin from adenine in 1980. 2-Furaldehyde was portion wise added in 2h intervals to an aqueous solution of adenine in the presence of cyanohydridoborate and HCl for 28 hours at 55 ºC. Kin was purified using column chromatography and recrystallization from ethanol (85% yield). Two years later, in 1982, Girgis and Pedersen used hypoxanthine as starting material. Hypoxanthine was gradually added to the mixture of furfurylamine, phosphorus pentoxide and N, N´-dimethylcyclohexamine and heated to 170 ºC for 24h. The final product was obtained after crystallization from ethanol in 21% yield. Miller's original method was modified by Villar and Motta (2000): 6-methylmercaptopurine in the first step was oxidized by trichloroisocyanuric acid to form 6-methylsulfonylpurine and then refluxed with furfurylamine in n-butanol for 45 min to give a crude reaction mixture. The desired product was then obtained after recrystallization from ethanol (82% yield) (Fig 3). In 1956, Daly and Christensen proposed synthesis of Kin from 6-chlorpurine in n-butanol with a large excess of furfurylamine. This modified approach has been used up to now. The excess of furfurylamine was replaced by the addition of large portion of trimethylamine to secure alkaline conditions (Wang et al., 2018).

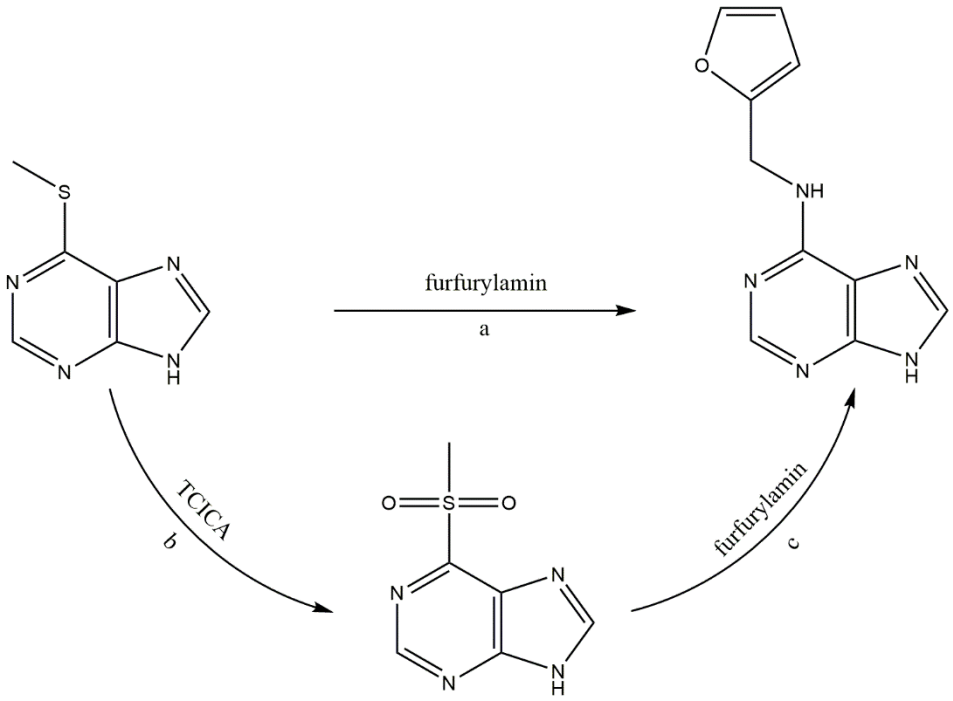


Figure 3 *Preparation of Kin from 6-methylmercaptopurine as described in* Miller et al., (1956) *and* Figueroa Villar and Motta, (2000). *(a) 120°C, 9h; (b) methanol-water solution, 10 °C, 1.5 h; (c)n-butanol, 120°C, 45min. TCICA, trichloroisocyanuric acid*

BAP was synthesized for the first time shortly after Kin (Daly and Christensen, 1956). BAP was prepared from 6-chlorpurine (Daly and Christensen, 1956), 6-methylmercaptopurin (Okumura et al., 1957), hypoxanthine (Girgis and Pedersen, 1982) as well as 6-methylsulfonylpurine (Figueroa Villar and Motta, 2000) according to the protocol described for Kin preparation using benzylamine instead of furfurylamine. BAP was also obtained from adenine in the reaction with an excess of sodium benzyl oxide (55% yield). Adamska et al (2012) first prepared the Shiff base of the BAP precursor in the reaction of adenine with benzaldehyde in anhydrous methanol with an excess of magnesium methanolate. The obtained imine was reduced to BAP by sodium tetrahydroborate (Fig. 4).

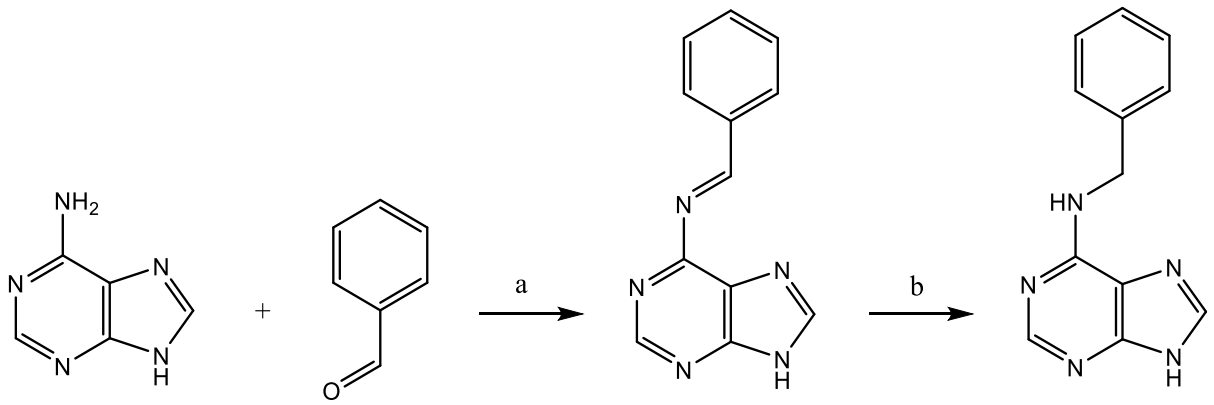


Figure 4 *Preparation of BAP via formation of Shiff base and subsequent reduction. (a) $Mg(MeO)_2$, MeOH, 55◦C, 3 h; (b) $NaBH_4$, 0.5 h, MeOH, rt* (Adamska et al., 2012).

7-Benzyl-6-benzylamino-2-chloropurine was used as a starting material for BAP preparation in the procedure published by Kochergin et al. (2000). The reaction was done in ethanol in the presence KOH and a palladium catalyzer. Daly's method of BAP preparation from the 1950s was enhanced using microwave assisted syntheses. BAP was obtained at 95% yield as 6-chlorpurine reacted with 10 equivalents of benzylamine for only 5 minutes at 100 °C (Huang et al., 2007).

Back in 1959, Okumura et al. prepared a library of fourteen BAP derivatives with variously substituted phenyl rings utilizing condensation of 6-(methylmercapto)purine with corresponding amines. The library was extended by Doležal at al. (2006) using a more efficient method with 6-chlorpurine with trimethylamine in *n*-butanol. In this publication almost forty different amines were attached to the adenine moiety utilizing this effective method (Fig. 5).

3.2. 6,9-disubstituted and 2,6,9-trisubstituted cytokinin derivatives

The procedure described for the synthesis of Kin and BAP (Fig. 5) *via* nucleophilic substitution on 6-chlorpurine was also used for the preparation of disubstituted CKs. In Doležal et al (2007), forty-eight 6-benzyladenosine derivatives were prepared following this procedure using commercially available 6-chloropurine riboside as starting material. Similarly, Vylíčilová et al (2016) prepared a series of 2-chloro-N^6-(halogenobenzylamino)purine ribosides using a slightly modified method. In this study, 2,6-dichloropurine riboside was used as a starting material, *n*-propanol as a solvent and Et_3N was used to strengthen alkaline conditions.

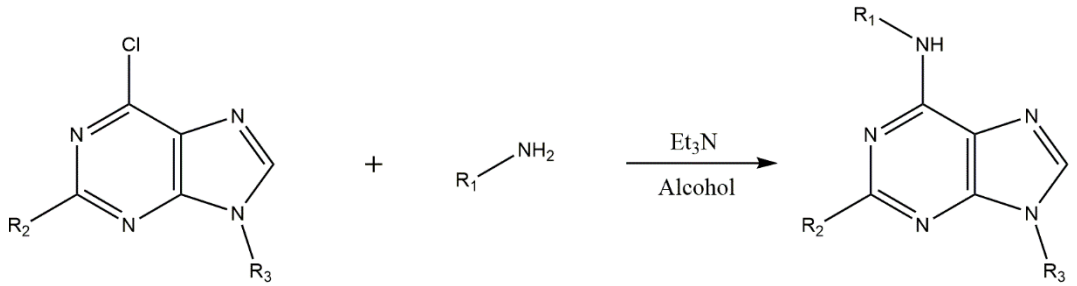


Figure 5 *Recently, the most utilized method for CK preparation via nucleophilic substitution of 6-chlorpurine by amine. R_2: H, chlorine; R_3:THP, THF, halogenalkyls, sugars*

A method for the preparation of various 9-(tetrahydropyran-2-yl)purines (THPP) was published by Figueroa Villar and Motta in 2000. In this procedure, 6-methylsulfonylpurine reacted with the appropriate amine as depicted in Fig 4. The obtained product was then protected by a THP group in the reaction with *p*-toluenesulfonic acid in dry 4-methyl-2-pentanone. A different approach was introduced by Szüčová et al in 2009. Thirty-three 6-benzylamino-9-tetrahydropyran-2-ylpurine and 9-tetrahydrofuran-2-ylpurine (THFP) derivatives, with variously positioned hydroxy and methoxy functional groups on the phenyl ring, were prepared *via* nucleophilic substitution (Szüčová et al., 2009). However, the 6-chloro-9-(tetrahydrofuran-2-yl)purine and 6-chloro-9-(tetrahydropyran-2-yl)purine precursors were prepared from 6-chlorpurine in the reaction with trifluoroacetic acid (TFA) and 2,3-dihydrofuran (2,3-DHF) or 3,4-dihydropyran (3,4-DHP) in ethyl acetate (Fig 6, Szüčová et al., 2009). In the next step, chlorine in C6 position was substituted by the appropriate amine under alkaline conditions by nucleophilic substitution (Szüčová et al 2009). This approach was much more efficient than the procedure described in Figueroa Villar and Motta (2000).

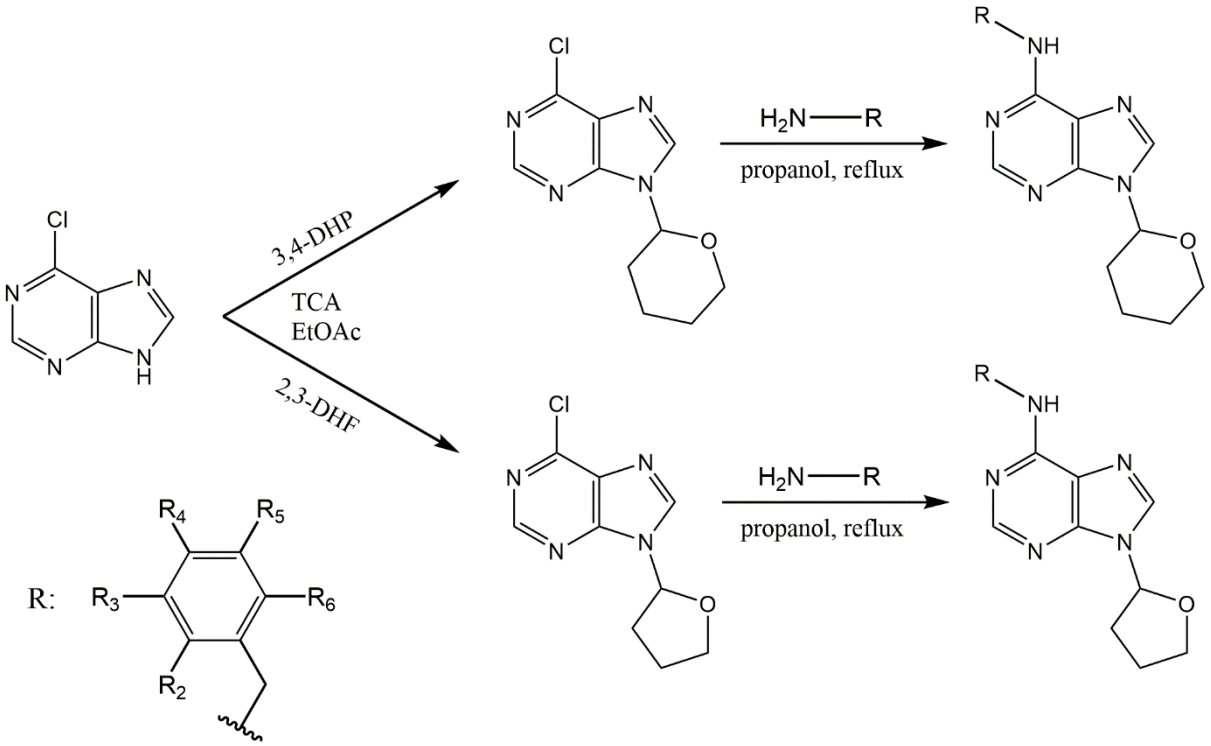


Figure 6 *Preparation of 6-benzylamino-9-(tetrahydropyran-2-yl)purine (THPP) and 9-(tetrahydrofuran-2-yl)purine (THFP) derivatives as described by Szüčová et al (2009). 3,4-DHP, 3,4-dihydropyran; 2,3-DHF, 2,3-dihydrofuran; TCA, trifluoroacetic acid; EtOAc, ethylacetate; R_2-R_6 are various positioned –OH and/or –OMe groups*

The synthetic pathway for the preparation of N9-halogenalkyl derivatives of Kin as well as iP was described by Mik et al., (2011a, 2011b). N6 substituted CK reacted with the appropriate halogenalkylbromide in the presence of potassium carbonate in DMSO or DMF.

The synthetic route proposed by Szüčová et al., (2009, Fig 6) was exploited for the preparation of Kin-THF derivatives with modified furan ring. 2,6-dichlorpurine was also used in this study to obtain the appropriate 2-chloro substituted derivatives (Hönig et al., 2018, **I**).

Method described earlier in the text for BAP preparation *via* Shiff base formation, was also used for the preparation of 2'-Deoxy-N^6-furfuryladenosine from commercially available 2'-deoxyadenosine (Adamska et al., 2012).

6-chloro-9-β-D-glucopyranosylpurine and 6-chloro-9-β-D-glucofuranosylpurine were prepared from chloromercaptopurine and acetbromoglucose or 2,3,5,6-tetra-*O*-acetylglucofuranosyl chloride, respectively. After careful deacetylation in methanolic ammonia, in the subsequent reaction with appropriate amines were prepared 9-glucosides/ribosides of zeatin and BAP (Cowley et al., 1978). In the same study, direct alkylation of 6-chlorpurine with acetbromoglucose in the presence of potassium carbonate in

propylene carbonate was proposed. 9-Glucoside was obtained together with corresponding 7-glucoside, using this method (Cowley et al., 1978).

Preparation of N9-glucosides and N9-galactosides was also described with peracetylated β-D-glucose or β-D-galactose used as a starting material. Peracetylated sugars were transformed with trimethylsilyl tri-fluoromethanesulfonate (TMSOTf) into reactive cations, which were combined with silylated 6-chloropurine to yield peracetylated-glucosyl- and galactosyl-6-chloropurine derivatives. Methanolic ammonia at 0°C was used for the subsequent deacetylation (Ando et al., 2007). The method described with some modification can also be used for [$^{15}N_4$]purine labeled CK glycosides (Tranová et al., 2019)

Schwarz et al. (2014) optimized synthesis utilizing methyl peracetylated or perbenzylated glycoside precursors in the presence of Lewis acid (TMSOTf) to couple 6-chloropurine with D-glucosyl, D-galactosyl and D-mannosyl residues. These authors also quantified the yields and product ratio of the obtained α/β-stereoisomers and N7/N9-regioisomers for microwave assisted synthesis of these purine nucleosides.

[$^{15}N_4$]purine labeled CK glycosides of zeatins and topolins containing a 9-β-D, 7-β-D-glucopyranosyl, or 9-β-D-ribofuranosyl group were prepared as potential internal standards for phytohormone analysis (Tranová 2018). The synthetic route *via* acetbromoglucose (Cowley 1987) was optimized to prepare both N7/N9-regioisomers of 6-chlorpurineglycoside precursors in one pot. ^{15}N labeled CKs containing 9-β-D-ribofuranosyl group were further prepared by direct enzymatic transglycosylation from labeled CK precursors (Tranová 2018).

Fluorescently labeled CKs were also prepared as a tool for study of CK receptor properties (Kubiasová et al., 2018, **III**). Different fluorescent labels were attached to the C2 or N9 atom of purine moiety *via* a C2 or C6 carbon linker. Two carbon linkers were attached to the N9 carbon of 6-chlorpurine *via* the Mitsunobu reaction as Boc protected 2-aminoethanol. The fluorescent label was then attached after N6 substitution and subsequently the Boc deprotection. C2-linker was coupled with 2-chloro-iP in the reaction with ethane-1,2-diamine or hexane-1,6-diamine. Fluorescent labels were connected afterwards in the reaction with free amino group on the linker. (Kubiasová et al., 2018, **III)**

Material and methods

General procedure

The chromatographic purity and mass spectra of the prepared compounds were analyzed using the HPLC-PDA-MS method. Samples (10 µl of 3.10^{-5} M in 1% methanol) were injected onto a reverse-phased column (Symmetry C18, 5 µm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 25 °C. Solvent (A) consisted of 15 mM ammonium formate adjusted to pH 4.0. The solvent (B) consisted of methanol. The flow-rate was set to 200 µL/min. A binary gradient was used: 0 min, 10 % of B; 24 min; 90 % of B; 34 min; 90 % of B; 45 min; 10 % of B using the *Waters* Alliance *2695* Separations *Module* (Waters, Manchester, UK). The effluent was then introduced to the Waters 2996 PDA detector (Waters, Manchester, UK) (scanning range 210–700 nm with 1.2 nm resolution) and a tandem mass analyser *Q-Tof micro Mass* Spectrometer (Waters, Manchester, UK) with an electrospray. The cone voltage was set to 20 V. Analyses were performed in positive mode (ESI+) or negative mode (ESI-) therefore molecular ions were recorded as $[M + H]^+$, $[M-H]^-$ or ESI adduct ions. ^{1}H NMR spectra were measured on a Jeol 500 SS spectrometer operating at a temperature of 300 K and a frequency of 500.13 MHz The samples were prepared by dissolving the compounds in DMSO-d_6. Tetramethylsilane (TMS) was used as an internal standard. Thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck). $CHCl_3$/MeOH (9:1, v/v) or EtOAc/MeOH/NH_3 (34:4:2, v/v) were used as the mobile phase. Purification *via* column chromatography was carried out using silica gel Davisil R LC60A 40-63 micron.

Synthetic procedure I

6-chloro-9-(tetrahydrofuran-2-yl)purine (**A**) or 2,6-dichloro-9-(tetrahydrofuran-2-yl)purine (**B**) were prepared according to modified protocols from Szüčová et al. (2009) and Plíhalová (2016), respectively. Only 1.5 equivalents of 2,3-dihydrofurane were used in both reactions (instead of 2.5 eq.) which allowed subsequent crystallization in EtOH at -20 °C. Thanks to this modification, a pale yellow solid compound **A** was obtained..

In the next step, 6-chloropurine, **A** or **B** was refluxed with the appropriate amine (1.2 eq.) in the n-propanol with an excessive amount of triethylamine (3 eq) for 4-6 hours as summarized in the reaction scheme (Fig. 7). The crude reaction mixture was then concentrated *in vacuo* and the product was extracted into EtOAc using liquid-liquid extraction (water : EtOAc). The ethyl

acetate phase was then dried over $MgSO_4$ and concentrated under vacuum. The crystallization technique as well as column chromatography were used for final product purification and are described in detail in Hönig et al (2018, **I**)

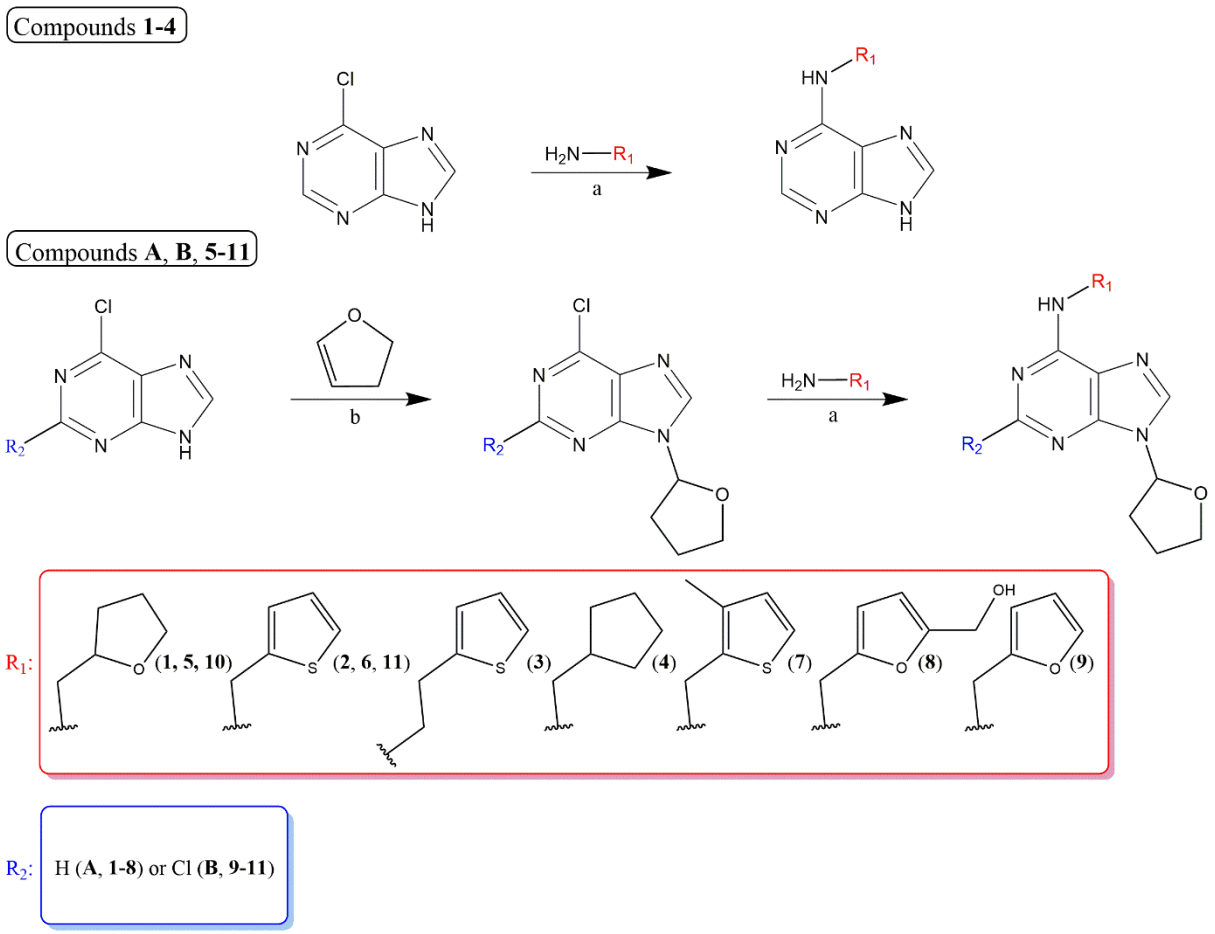


Figure 7 *Reaction scheme for the synthesis of prepared 6-substituted, 6,9-disubstituted and 2,6,9-trisubstituted purine derivatives with 9-THF protecting group published in Hönig et al., (2018,* ***I****). a) Et3N, propanol, 100 °C, 4 h (**1**, **2**, **5**, **9**, **10**), 5 h (**3**, **4**, **6**, **8**) and 6 h (**7**); b) EtOAc, CF3COOH, NH3, RT, 3.5 h.*

Synthetic procedure II

6-chloro-9-(tetrahydropyran-2-yl)purine (**C**) or 2,6-dichloro-9-(tetrahydropyran-2-yl)purine (**B**) were prepared according to modified protocols as published in Szüčová et al. (2009) and Bíbová et al. (2018) respectively. A smaller equivalent of 3,4-dihydropyran (1.5 instead of 2.5) was used which allowed us to obtain **C** as a solid compound after crystallization in EtOH at -20 °C overnight.

In the following step, 6-chloropurine, 2,6-dichloropurine, **C** or **D** was refluxed with appropriate amine (1.2 eq.) and excessive amount of triethylamine (3 eq.) for 5 h in n-propanol. The crude reaction mixture was then concentrated *in vacuo* and the product was extracted into EtOAc using liquid-liquid extraction (water : EtOAc). The ethyl acetate phase was then dried over $MgSO_4$ and concentrated under vacuum. Crystallization in different solvents or solvent mixtures was preferably used as a last purification step. The reaction scheme (Fig. 8) and reaction conditions are described in detail in the section Unpublished results.
Preparation of 6-(thiophen-2-ylmethylamino)-9-(tetrahydropyran-2-yl)purine (**14**) has been described in the thesis of Ryšavá (2019). However, Ryšavá used a longer reaction time and utilized diethyl ether instead of ethanol for final crystallization to obtain a compound of similar purity and yield.

Synthetic procedure III

Acetylation of N-Acetyl-D-glucosamine was performed as described in Kong et al. (2016) for the acetylation of N-Acetyl-D-mannosamine using acetic anhydride in pyridine. Peracetylated N-Acetyl-D-glucosamine was attached to the purine moiety as described in Ando et al. (2007) for reaction of 6-chloropurine with 1,2,3,4,6-penta-O-acetyl-β-D-glucose. However, crystallization was preferably used for product purification instead of silica gel column chromatography. The synthetic procedure is summarized (Fig. 9) and described in more detail in the section Unpublished results.

Synthetic procedure IV

Protection of the D-glucuronic acid carboxyl group by the allyl group and its subsequent one-step removal was performed as described in Alaoui et al. (2006). Hydroxyl group protection and further reaction with 6-chlorpurine was performed as described in Synthetic procedure III. The synthetic route is summarized (Fig. 10) and described in more detail in section Unpublished results.

pH Stability

In order to evaluate the stability of prepared compounds due to the pH lability of 9-substituted THF group, the pH stability test was slightly modified and performed according to the literature (Szűčová et al., 2009). The pH stability of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran- 2-yl)purine (**5**), 6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl) purine (**6**) and 2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine (**9**) was analysed by HPLC-PDA (System Gold; Beckman Instruments, Fullerton, CA, USA); analytes were detected at 270 nm using a PDA detector (Beckman System Gold 168). The solution of tested compound (10^{-2} M; DMSO) was prepared and diluted to 10^{-4} M using McIlvaine buffer solution for the appropriate pH (2, 3, 4, 5, 6 or 7). One hour after incubation at 25 °C, 5 μL of the solution was directly injected onto a reversed phase column (Symmetry C18; 5 μm, 150 x 2.1mm; Waters, Milford, USA). At a flow-rate of 0.3 mL/min, the following binary gradient was used: 0 min,10% B; 0-24 min; linear gradient to 90% B; 25-34 min; isocratic elution of 90% B; 35-45 min; linear gradient to 10% B, where A was 15mM formic acid adjusted to pH 4 with ammonium and B was 100% methanol The HPLC measurement of the solutions was repeated after a 24 h incubation at 25 °C.

Biological activity

Evaluation of CK activity in tobacco callus bioassay, amaranthus caudatus betacyanin bioassay and wheat leaf senescence bioassay (WLSA) was carried out according to Hönig et al. (2018, **I**). In the same study, are described the methods used for cytotoxicity measurement *via* resazurin reduction assay on human skin cells as well as methods for phototoxicity and photoprotection assessment. Oxygen radical absorbance capacity evaluation and protocols for oxidative stress bioassays in *Caenorhaditis elegans* are also presented in this study (Hönig et al., 2018; **I**).
Markers of damage caused by UVA and UVB irradiation including ROS production, GSH depletion and Caspase-3-activity were studied as described in (Hönig et al., 2017; **V**).

Survey of published results

Publication I

- Eleven Kin derivatives were prepared and properly characterized using ^{1}H NMR, mass spectrometry combined with HPLC purity determination and elemental C, H, N analyses. The biological activity of new compounds was studied in both plant and animal systems. Cytokinin-like activity was determined in three CK bioassays, such as tobacco callus, wheat leaf senescence assay and Amaranthus bioassay. It was found that despite the saturation of furfuryl substituent in 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine, this compound possessed the highest activity in WLSA. Selected compounds were subsequently tested on normal human dermal fibroblasts (NHDF) and keratinocyte cell lines (HaCaT) to exclude possible phototoxic effects and, on the other hand, to reveal possible UVA and UVB photoprotective activity. Protection against 5- hydroxy-1,4-naphthoquinone (juglone) induced oxidative stress was tested in *Caenorhabditis elegans in vivo*. Compounds 6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine, and 2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine, were found to be the most active in human skin cell protection against UVA/UVB irradiation and concurrently possessed highest activity in oxidative stress protection in *C. elegans*. Protection of *C. elegans* and NHDF from oxidative and UV stress indicate antioxidant properties. These compounds did not act as direct radical scavengers in the ORAC assay. The obtained data suggest that the mechanism of photo- and nematode protection against oxidative stress is indirect and triggers other mechanisms of oxidative protection. (Hönig et al., 2018, **I**)

Publication II

- Antisenescent activity of natural CKs and their derivatives together with antioxidant properties in plants were reviewed. The effect on chlorophyll content, photosystem II and other parts of the photosynthetic apparatus was summarized. The influence of CKs on activation of antioxidative enzymes in senescing tissues is described as well as changes in the levels of naturally occurring antioxidants, such as phenolic acids and flavonoids, in plant explants. Emphasis was placed on the structure-activity relationship, and key structural motives necessary for antisenescent activity of CKs were postulated.

The role of different CK receptors and downstream proteins that are involved in the antisenescent and antioxidant activity of CKs were discussed. (Hönig et al., 2018, **II**).

Publication III

- Several fluorescent derivatives of iP were designed and prepared and properly characterized to study the properties of the CK receptor. Several fluorescent labels were used and attached to the C2 or N9 atom of the adenine moiety *via* 2- or 6-carbon linker. All prepared compounds were screened for affinity for the *Arabidopsis thaliana* CK receptor (CRE1/AHK4). Most compounds did not interact with the receptor due to attached label. However two C2-labeled rhodamine B (with 2- and 6-carbon linker) iP and one N9- labeled 4-chloro-7- nitrobenzofurazan (NBD) iP interacted well. Arabidopsis seedlings were used for in planta staining experiments in Arabidopsis thaliana cell suspension culture using live cell confocal microscopy (Kubiasová et al., 2018, **III**).

Publication IV

- The photoprotective and antioxidant properties of CKs and other natural products was reviewed. The antioxidant properties of Kin and its THP derivative in animals especially in human and human skin cells were discussed. These two compounds are currently used in cosmetics for the treatment of photodamaged skin due to their ability to reduce symptoms of skin photoaging such as fine wrinkles, rough skin texture and mottled hyperpigmentation. (Plíhalová et al, 2018, **IV**).

Publications V and VI (patent and patent application)

- N9-THF substituted derivatives published in Hönig et al., (2018, **I**) are part of the granted Czech patent no. 307722 and PCT application WO/2017/036434 (PCT/CZ2016/050029) as substances possessing anti-senescent and UV-photoprotective properties that can be used in cosmetics preparations, plant protection preparations and in preparations for the treatement/application of tissue cultures (Hönig et al., 2017, **V**; Hönig et al., 2019, **VI**). 6-(Tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine protected NHDF cells against UVA induced ROS production as well as depletion of endogenous antioxidant GSH. Concurrently, in UVB irradiated NHDF cells treated with 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine, lower activity of protein caspase-3, which is activated during apoptosis was measured (Hönig et al., 2017, **V**; Hönig et al., 2019, **VI**).

Survey of unpublished results

1. 2-Chloro and/or -9-(tetrahydropyran-2-yl) substituted kinetin-like derivatives

A series of compounds complementary to those published in Hönig et al (2018, **I**) was prepared where THF protective group in position N9 was replaced by THP group. Compounds were characterized and tested for cytokinin-like biological activity as described in Hönig et al (2018, **I, Fig 8)**

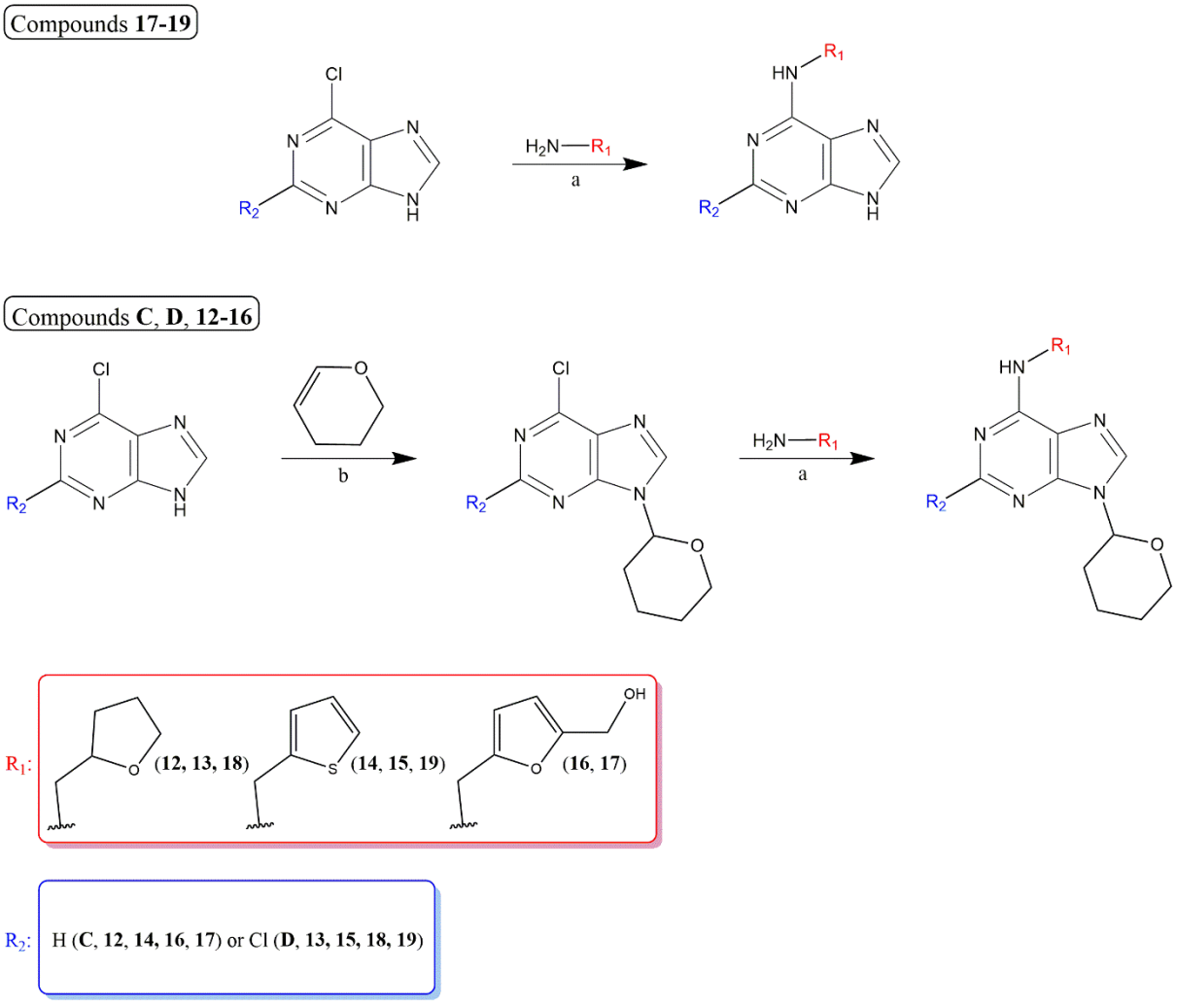


Figure 8 *Reaction scheme for the synthesis of prepared 6-substituted, 6,9-disubstituted and 2,6,9-trisubstituted purine derivatives with 9-THP protecting group. a) Et3N, propanol, 100 °C, 5 h; b) EtOAc, CF3COOH, NH3, RT, 3.5 h (**C**) overnight (**D**)*

Preparation procedure

*6-Chloro-9-(tetrahydropyran-2-yl)purine (**C**)*

6-Chloropurine (10 g; 65 mmol) and 3,4-dihydropyran (8.9 mL; 98 mmol) were dissolved in EtOAc (200 mL) and TFA (9.7 mL; 130 mmol) was added dropwise on ice. The reaction mixture was stirred at room temperature for 3.5 h and then neutralized by the appropriate amount of ammonia and water (1:1). The ethyl acetate phase was washed with water, dried over Na2SO4 and concentrated in vacuo. A yellow oily compound was obtained. The pure product was obtained after crystallization from ethanol at -20 °C overnight. Yield: 61%. HPLC purity 98.9 %. MS ESI+ m/z: 239 $[M+H]^+$.

*2,6-Dichloro-9-(tetrahydropyran-2-yl)purine (**D**)*

2,6-Dichloropurine (25 g; 132 mmol) and 3,4-dihydropyran (19 mL; 203 mmol) were dissolved in EtOAc (315 mL) and TFA (30 mL; 330 mmol) was added dropwise on ice. The reaction mixture was stirred at room temperature overnight and then neutralized by the appropriate amount of ammonia and water (1:1). The ethyl acetate phase was washed with water, dried over Na2SO4 and concentrated in vacuo. A yellow oily compound was obtained. The pure product was obtained after crystallization from diethylether. Yield: 78%. HPLC purity 99.4%. MS ESI+ m/z: 273 $[M+H]^+$.

*6-(Tetrahydrofuran-2-ylmethylamino)-9-(tetrahydropyran-2-yl)purine (**12**)*

Prepared according to Synthetic procedure II using **C** (0.5 g) as a starting material. The final product was obtained after crystallization from diethylaether. Yield: 59%. HPLC purity 99.9%. MS ESI+ m/z: 304 $[M+H]^+$. ^{1}H NMR (500 MHz, Chloroform-d) δ 8.34 (s, 1H), 7.99 (s, 1H), 6.13 (s, 1H), 5.70 (dd, J = 10.4, 2.3 Hz, 1H), 4.20 – 4.11 (m, 2H), 3.96 – 3.84 (m, 1H), 3.81 – 3.74 (m, 2H), 3.65 (s, 1H), 2.14 – 1.97 (m, 4H), 1.96 – 1.85 (m, 2H), 1.83 – 1.71 (m, 2H), 1.71 – 1.60 (m, 2H)

*2-Chloro-6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydropyran-2-yl)purine (**13**)*

Prepared according to Synthetic procedure II using **D** (0.5 g) as a starting material. The product was obtained after crystallization from diethylether. Yield: 60%. HPLC purity 99.9%. MS ESI+ m/z: 338 $[M+H]^+$. ^{1}H NMR (500 MHz, Chloroform-d) δ 8.00 (s, 1H), 6.61 (s, 1H), 5.68 (dd, J = 10.9, 2.4 Hz, 1H), 4.18 – 4.09 (m, 2H), 3.94 – 3.88 (m, 1H), 3.88 – 3.81 (m, 1H), 3.80 – 3.72 (m, 2H), 3.68 – 3.56 (m, 1H), 2.17 – 2.08 (m, 1H), 2.08 – 1.98 (m, 2H), 1.98 – 1.83 (m, 3H), 1.82 – 1.69 (m, 2H), 1.69 – 1.60 (m, 2H).

*6-(Thiophen-2-ylmethylamino)-9-(tetrahydropyran-2-yl)purine (**14**)*

Prepared according to Synthetic procedure II using **C** (0.5 g) as a starting material. The product was obtained after crystallization from ethanol. Yield: 44%. HPLC purity 99.2%. MS ESI+ m/z: 316 $[M+H]^+$. ^{1}H NMR (500 MHz, Chloroform-d) δ 8.43 (s, 1H), 8.01 (s, 1H), 7.22 (dd, J = 5.0, 1.3 Hz, 1H), 7.08 (s, 1H), 6.96 (dd, J = 5.1, 3.4 Hz, 1H), 6.17 (s, 1H), 5.72 (dd, J = 10.6, 2.4 Hz, 1H), 5.02 (s, 2H), 4.18 (dt, J = 10.1, 2.3 Hz, 1H), 3.82 – 3.75 (m, 1H), 2.16 – 1.97 (m, 3H), 1.84 – 1.71 (m, 2H), 1.68 – 1.62 (m, 1H).

*2-Chloro-6-(thiophen-2-ylmethylamino)-9-(tetrahydropyran-2-yl)purine (**15**)*

Prepared according to Synthetic procedure II using **D** (0.5 g) as a starting material. Solid yellowish product was obtained after crystallization from reaction mixture at RT overnight. Product was washed with water and propanol after filtration from reaction mixture. Yield: 73%. HPLC purity 99.9%. MS ESI+ m/z: 350 $[M+H]^+$. ^{1}H NMR (500 MHz, Chloroform-d) δ 8.00 (s, 1H), 7.23 (dd, J = 5.0, 1.2 Hz, 1H), 7.08 (d, J = 3.1 Hz, 1H), 6.96 (dd, J = 5.1, 3.5 Hz, 1H), 6.75 (s, 1H), 5.70 (dd, J = 10.8, 2.4 Hz, 1H), 4.98 (s, 2H), 4.16 (ddt, J = 11.6, 4.0, 1.8 Hz, 1H), 3.77 (td, J = 11.8, 2.7 Hz, 1H), 2.14 (d, J = 12.5, 3.1 Hz, 1H), 2.10 – 2.01 (m, 1H), 1.95 – 1.83 (m, 1H), 1.83 – 1.69 (m, 2H), 1.67 – 1.62 (m, 1H).

*6-(5-Hydroxymethylfuran-2-ylmethylamino)-9-(tetrahydropyran-2-yl)purine (**16**)*

Prepared according to Synthetic procedure II using **C** (0.25 g) as a starting material. Solid white product was obtained after crystallization from mixture of dichloromethane and petroleum ether. Yield: 58%. HPLC purity 99.8%. MS ESI+ m/z: 330 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.36 (s, 1H), 8.25 (s, 2H), 6.15 (dd, J = 13.8, 7.5 Hz, 2H), 5.63 (dd, J = 11.0, 2.1 Hz, 1H), 5.13 (t, J = 5.7 Hz, 1H), 4.66 (s, 2H), 4.32 (d, J = 5.7 Hz, 2H), 4.03 – 3.97 (m, 1H), 3.71 – 3.62 (m, 1H), 2.32 – 2.22 (m, 1H), 2.01 – 1.89 (m, 2H), 1.79 – 1.66 (m, 1H), 1.64 – 1.53 (m, 2H).

*6-(5-Hydroxymethylfuran-2-ylmethylamino)purine (**17**)*

Prepared according to Synthetic procedure II using 6-chloropurine (0.5 g) as a starting material. Solid white product was obtained after concentration *in vacuo*. Yield: 25%. HPLC purity 99.4%. MS ESI+ m/z: 246 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6) δ 12.95 (s, 1H), 8.21 (s, 1H), 8.12 (s, 1H), 8.02 (s, 1H), 6.17 (s, 2H), 5.14 (s, 1H), 4.67 (s, 2H), 4.32 (s, 2H).

*2-Chloro-6-(2-tetrahydrofuran-2-ylmethyl)aminopurine (**18**)*

Prepared according to Synthetic procedure II using 2,6-dichloropurine (0.25 g) as a starting material. Solid yellowish product was obtained after crystallization from reaction mixture at RT overnight. Product was washed with water and propanol after filtration from reaction mixture. Yield: 82%. HPLC purity 99.3%. MS ESI+ m/z: 254 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6) δ 13.07 (s, 1H), 8.11 (s, 1H), 8.06 (s, 1H), 4.06 (s, 1H), 3.78 (s, 1H), 3.63 (s, 1H), 3.51 (s, 1H), 3.37 (s, 1H), 2.01 – 1.73 (m, 3H), 1.62 (s, 1H).

*2-Chloro-6-(2-thiophen-2-ylmethyl)aminopurine (**19**)*

Prepared according to Synthetic procedure II using 2,6-dichloropurine (0.25 g) as a starting material. Solid white product was obtained after crystallization from reaction mixture at RT overnight. Product was washed with water and propanol after filtration from reaction mixture. Yield: 97%. HPLC purity 99.0%. MS ESI+ m/z: 266 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6) δ 13.09 (s, 1H), 8.74 (s, 1H), 8.10 (s, 1H), 7.32 (d, J = 5.1 Hz, 1H), 7.00 (s, 1H), 6.91 (t, J = 4.2 Hz, 1H), 4.73 (s, 2H).

Activity of THP derivatives in three CK bioassays (Tab. 3) is in accordance with their respective THF derivatives published in Hönig et al (2018, **I**) and in accordance to SAR established for the activity of CKs in WLSA as defined in Hönig et al (2018, **II**). The introduction of THP group to N9 atom of adenine in 6-(2-Tetrahydrofuran-2-ylmethyl)aminopurine (compound 1 in Hönig et al., 2018; **I**) increased its activity significantly. As compound (**12**) possessed even higher activity than 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (compound **5** in in Hönig et al., 2018; **I**) with almost 90% of chlorophyll retained in detached wheat leaves after 96 hours of cultivation in dark. For its very potent activity is this compound now tested in field trials. Comparably to THF introduction (compound **6** in in Hönig et al., 2018; **I**), compound (**14**) with N9-THP substitution possessed higher antisenescent properties than its corresponding free base (compound **2** in Hönig et al., 2018; **I**). In agreement with this

results, antisenescent properties of (**14**) were previously described in Ryšavá Bc. thesis (2019). The introduction of chlorine to the position C2 (**13**, **15**) lowered the activity of respective active derivatives (**12**, **14**) in WLSA.

Prepared compounds showed only low activity in *Amaranthus caudatus* betacyanin bioassay as described earlier for THF substituted CKs (Hönig et al., 2018; **I**, **II**) and were able to promote cell division of tobacco callus comparably or slightly less than standard BAP.

Table 3 *Relative CK bioassay activity of the prepared derivatives at the optimal concentration compared with the activity of 6-benzylaminopurine (BAP) (100% means 10-5M BAP for the Amaranthus betacyanin bioassay, 10-4M BAP in the case of the senescence bioassay and 10-5 M BAP for the tobacco callus bioassay) as described in (Hönig et al., 2018, I)*

	Tobacco callus bioassay		***Amaranthus caudatus* betacyanin bioassay**		**Wheat leaf senescence bioassay**	
Compound	**Concentration [mol.l^{-1}]**	**Relative activity [%]**	**Concentration [mol.l^{-1}]**	**Relative activity [%]**	**Concentration [mol.l^{-1}]**	**Relative activity [%]**
12	10^{-5}	90 ± (3)	10^{-5}	36 ± (0)	10^{-4}	135 ± (11)
13	10^{-5}	91 ± (9)	10^{-5}	72 ± (13)	10^{-4}	100 ± (3)
14	10^{-5}	98 ± (9)	10^{-5}	64 ± (13)	10^{-4}	103 ± (0)
15	10^{-5}	90 ± (14)	10^{-5}	73 ± (29)	10^{-4}	79 ± (1)
16	10^{-5}	91 ± (1)	10^{-5}	27 ± (5)	10^{-4}	77 ± (10)
17	10^{-5}	102 ± (2)	10^{-5}	80 ± (11)	10^{-4}	93 ± (2)
18	10^{-5}	100 ± (2)	10^{-5}	126	10^{-4}	84 ± (6)
19	10^{-5}	100 ± (8)	10^{-5}	71	10^{-4}	78 ± (9)

2. N-acetylglucosamine substituted CKs

A series of N-acetylglucosamine substituted CKs was prepared. Compounds were characterized and tested for cytokinin-like biological activity as described in Hönig et al (2018, **I, Fig 9)**

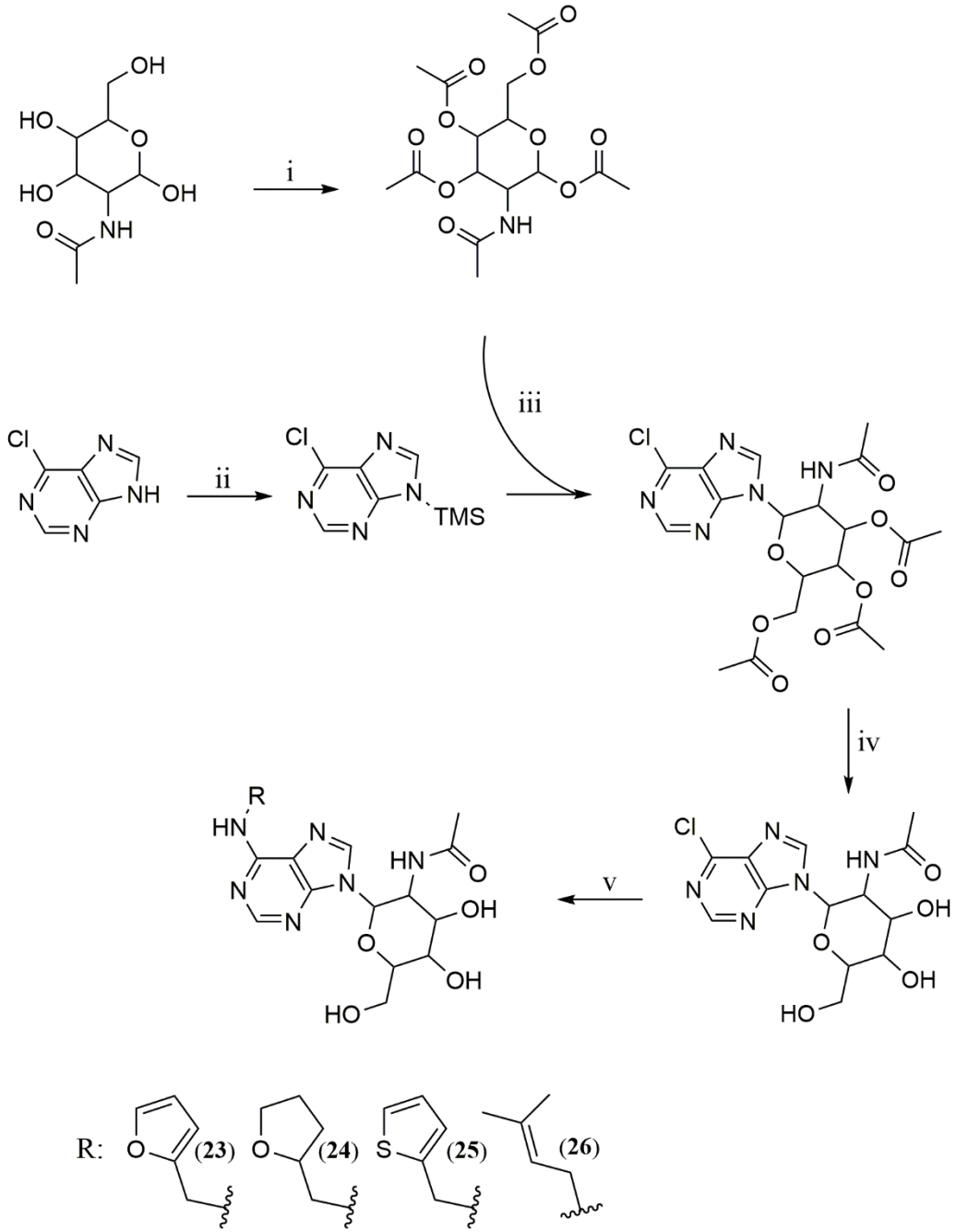


Figure 9 *Preparation of N-acetylglucosamine substituted cytokinins. i) Ac_2O, Py, 4h, rt.; ii) BSA, DCE, 0.5h, reflux; iii) TMSOTf, 5h, reflux; iv) 7N ammonia in MeOH, 3h; v) Et_3N, n-propanol, R-NH_2.*

Preparative procedure

*N-acetyl-1,3,4,6-tetra-O-acetyl-D-glucosamine (**20**)*

Prepared as described in Kong et al (2016) with few modification. N-acetyl-D-glucosamine (4 g, 18.1 mmol) was dissolved in pyridine (40 mL) and Ac_2O (20 mL) was added. Reaction mixture was stirred at room temperature for 4 hours and then concentrated in vacuo. The residue was dissolved in EtOAc and then washed with water. EtOAc phase was dried over Na2SO4 and evaporated in vacuo. Solid white product was obtained in quantitative yield.

Silylation of 6-chloropurine (E)

6-chlorpurine was silylated as described in Ando et al (2007). 6-chloropurine (500 mg, 3.2 mmol) was suspended in dry dichloroethane (DCE, 50 mL) and Bis(trimethylsilyl)acetamide (BSA, 1.2 mL, 4.8 mmol) was aded. Reaction mixture was stirred under reflux conditions for 30 min. The reaction solution was directly used for the coupling reaction with peracetylated sugar.

*6-chloro-9-(N-acetyl-1,3,4,6-tetra-O-acetyl-D-glucosamino)purine (**21**)*

Prepared as described in Ando et al (2007) with few modifications. To the solution of silylated 6-chloropurine (E) was added peracetylated sugar (02-01, 1.5 g, 4.8mmol) and Trimethylsilyl trifluoromethanesulfonate (TMSOTf, 880 µL, 4,8 mmol). Reaction mixture was stirred for 5 hours under reflux conditions. The reaction mixture was neutralized with Et3N and concentrated in vacuo. Solid product was obtained after crystallization from ethanol. Yield 26%. HPLC purity 99.1%. MS ESI+ m/z: 484 $[M+H]^+$

*6-chloro-9-(N-acetyl-D-glucosamino)purine (**22**)*

Compound (02-02, 380 mg) was deacetylated using 7N ammonia in methanol (30 mL) at 0 °C for 3 hours. The reaction mixture was then concentrated in vacuo and pale brown product was obtained after crystallization in hexan. Yield 92 %

*6-furfuryl-9-(N-acetyl-D-glucosamino)aminopurine (**23**)*

Compound (**22**, 50 mg, 0.14 mmol), furfurylamine (15 µL, 0.168 mmol) Et3N (60 µL; 0.42 mmol) were sequentially dissolved in propanol (5 mL). The mixture was refluxed for 8 h and then concentrated in vacuo. Pale brown crystal was obtained after crystallization in dichloromethane (DCM). Yield: 55 %. HPLC purity: 97.5 %. HPLC purity: 97.5 %. MS ESI-

m/z: 417 $[M-H]^-$. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.25 (s, 2H), 8.18 (s, 1H), 7.91 (d, J = 9.3 Hz, 1H), 7.53 (dd, J = 1.8, 0.8 Hz, 1H), 6.35 (dd, J = 3.1, 1.8 Hz, 1H), 6.25 – 6.22 (m, 1H), 5.58 (d, J = 10.0 Hz, 1H), 5.24 (s, 2H), 4.68 (s, 2H), 4.25 (q, J = 9.8 Hz, 1H), 3.69 (d, J = 11.7 Hz, 1H), 3.59 – 3.52 (m, 1H), 3.46 (dd, J = 12.0, 5.8 Hz, 1H), 1.55 (s, 3H).

6-(2-thiophen-2-ylmethyl) -9-(N-acetyl-D-glucosamino)aminopurine (**24**)

Compound (**22**, 50 mg, 0.14 mmol), 2-thiophenemethylamine (17 µL, 0.168 mmol) Et3N (60 µL; 0.42 mmol) were sequentially dissolved in propanol (5 mL). The mixture was refluxed for 6 h and then concentrated in vacuo. Pale brown crystal was obtained after crystallization in dichloromethane (DCM). Yield: 68 %. HPLC purity: 95 %. MS ESI- m/z: 433 $[M-H]^-$. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.41 (s, 1H), 8.28 (s, 1H), 8.18 (s, 1H), 7.91 (d, J = 9.3 Hz, 1H), 7.32 (dd, J = 5.1, 1.2 Hz, 1H), 7.03 – 7.01 (m, 1H), 6.93 (dd, J = 5.1, 3.4 Hz, 1H), 5.58 (d, J = 10.0 Hz, 1H), 5.24 (s, 2H), 4.84 (s, 1H), 4.56 (s, 1H), 4.29 – 4.21 (m, 2H), 3.69 (d, J = 11.1 Hz, 1H), 3.56 (t, J = 9.3 Hz, 1H), 3.49 – 3.43 (m, 1H), 1.55 (s, 3H).

6-(tetrahydrofuran-2-ylmethylamino)-9-(N-acetyl-D-glucosamino)aminopurine (**25**)

Compound (**22**, 50 mg, 0.14 mmol), tetrahydrofurfurylamine (17 µL, 0.168 mmol) Et3N (60 µL; 0.42 mmol) were sequentially dissolved in propanol (5 mL). The mixture was refluxed for 5 h and then concentrated in vacuo. Final product was obtained after purification via C18 SPE 1g column, 40% methanol used as eluent. Yield: 59 %. HPLC purity: 98 %. MS ESI+ m/z: 407 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.19 (s, 1H), 8.12 (s, 1H), 7.88 (d, J = 9.2 Hz, 1H), 7.66 (s, 1H), 5.52 (d, J = 10.0 Hz, 1H), 5.21 (dd, J = 10.2, 5.4 Hz, 2H), 4.55 (t, J = 5.9 Hz, 1H), 4.21 (q, J = 9.8 Hz, 1H), 4.07 – 3.99 (m, 1H), 3.73 (q, J = 7.0, 6.6 Hz, 1H), 3.68 – 3.63 (m, 1H), 3.60 – 3.48 (m, 3H), 3.46 – 3.38 (m, 2H), 3.28 – 3.21 (m, 1H), 1.90 – 1.70 (m, 3H), 1.63 – 1.54 (m, 1H), 1.52 (s, 3H).

6-(3-methylbut-2-en-1-yl)-9-(N-acetyl-D-glucosamino)aminopurine (**26**)

Compound (**22**, 50 mg, 0.14 mmol), 3-methylbut-2-en-1-amine (20.5 mg, 0.168 mmol) Et3N (60 μL; 0.42 mmol) were sequentially dissolved in propanol (5 mL). The mixture was refluxed for 6 h and then concentrated in vacuo. Final product was obtained after purification via preparative TLC, (CHCl3:MeOH; 6:1) used as mobile phase. Yield: 48 %. HPLC purity: 97.8 %. MS ESI+ m/z: 423 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.21 (s, 1H), 8.13 (s, 1H), 7.91 (d, J = 9.3 Hz, 1H), 7.81 (s, 1H), 5.56 (d, J = 10.0 Hz, 1H), 5.30 (t, J = 7.2 Hz, 1H), 5.24 (dd, J = 6.8, 5.4 Hz, 2H), 4.57 (t, J = 5.9 Hz, 1H), 4.24 (q, J = 9.8 Hz, 1H), 4.05 (s, 2H), 3.69 (dd, J = 10.5, 5.4 Hz, 1H), 3.58 – 3.52 (m, 1H), 3.49 – 3.42 (m, 1H), 3.40 – 3.34 (m, 1H), 3.31 – 3.26 (m, 1H), 1.70 (s, 3H), 1.66 (s, 3H), 1.55 (s, 3H).

Prepared N-acetylglucosamine substituted CKs showed relatively low activity in both Tobacco callus bioassay and WLSA compared to BAP (Tab. 4). However, their activity is higher than CK glucosides (Holub et al., 1998).

Table 4 *Relative CK bioassay activity of the prepared derivatives at the optimal concentration compared with the activity of 6-benzylaminopurine (BAP) (100% means 10^{-5}M BAP for the Amaranthus betacyanin bioassay, 10^{-4}M BAP in the case of the senescence bioassay and 10^{-5} M BAP for the tobacco callus bioassay) as described in (Hönig 2018,* ***I****)*

	Tobacco callus bioassay		***Amaranthus caudatus* betacyanin bioassay**		**Wheat leaf senescence bioassay**	
Compound	**Concentration [mol.l^{-1}]**	**Relative activity [%]**	**Concentration [mol.l^{-1}]**	**Relative activity [%]**	**Concentration [mol.l^{-1}]**	**Relative activity [%]**
22	10^{-5}	44 ± (12)		nt.	10^{-4}	82 ± (5)
23	10^{-5}	69 ± (2)		nt.	10^{-4}	81 ± (2)
24	10^{-5}	72 ± (3)		nt.	10^{-4}	76 ± (3)
25	10^{-5}	72 ± (13)		nt.	10^{-4}	82 ± (4)

nt.: not tested.

3. CKs derivatives made by glucuronic acid substitution

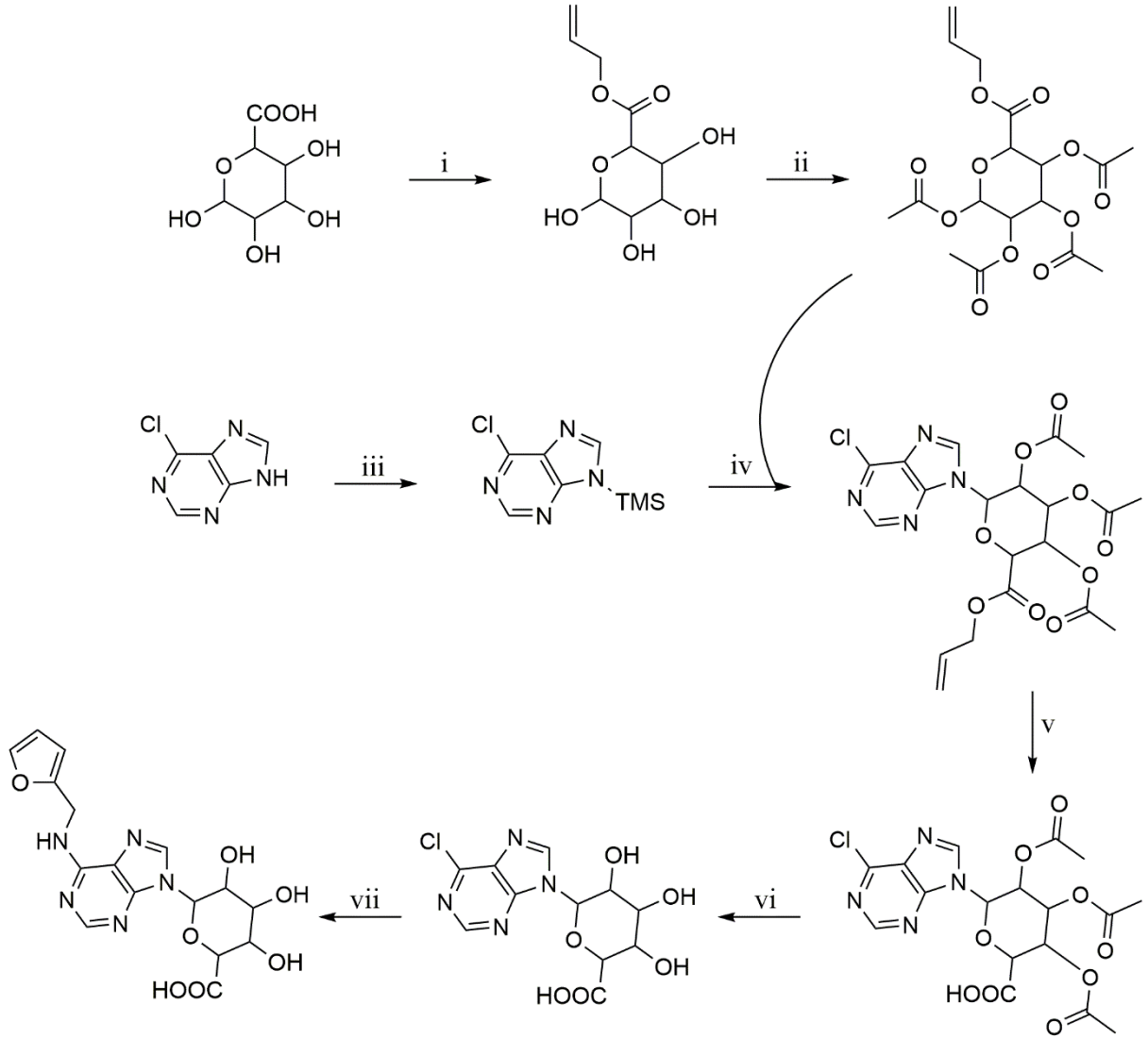


Figure 10 *Preparation of D-glucuronic acid substituted Kin. i) AllBr, DBU, DMF, overnight, rt.; ii) Ac_2O, Py, 5h, rt.; iii) BSA, DCE, 0.5h, reflux; iv) TMSOTf, 3h, reflux; v) $Pd(PPh_3)_4$, THF, 2h, ice; vi) 7N ammonia in MeOH, 3h; vii) furfurylamin, Et_3N, n-propanol, reflux. Abbreviations: AllBr, Allyl bromide; BSA, Bis(trimethylsilyl)acetamide; DCE, Dichloroethane; DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; Pd(PPh3)4, Tetrakis(triphenylphosphine)palladium(0); Py, Pyridine; TMSOTf, Trimethylsilyl trifluoromethanesulfonate*

Preparation procedure

*Allyl D-Glucuronate (**27**)*

D-glucuronid acid (6 g, 31 mmol) was dissolved in DMF (60 mL) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 5.1 mL,33 mmol) was added dropwise. Reaction mixture was stirred at room temperature for 15 min, then allyl bromide (3.3 mL, 36 mmol) was added dropwise, the mixture was stirred overnight and then concentrated in vacuo. The residue was purified over silica gel using acetone as eluent. Final product was obtained in two isomers as described in literature (Alaoui et al., 2006). Yield 78%. MS ESI+ m/z: 257 $[M+Na]^+$

*1,2,3,4-Tetra-O-acetyl-allyl-D-glucuronic Acid (**28**)*

Allyl D-glucuronate (**27**, 5 g, 51.3 mmol) was dissolved in pyridine (50 mL) and Ac_2O (30 mL) was added. Reaction mixture was stirred at room temperature for 5 hours and then concentrated in vacuo. The residue was dissolved in EtOAc and then washed with water. EtOAc phase was dried over Na_2SO_4 and evaporated in vacuo. Solid white product was obtained in quantitative yield consist of two isomers. MS ESI+ m/z: 420 $[M+NH_3]^+$

*6-chloropurine-9-(1,2,3,4-Tetra-O-acetyl-allyl-D-glucuronate) (**29**)*

To the solution of silylated 6-chloropurine (E, from 0.5 g of 6-chlorpurine) was added peracetylated sugar (**28**, 1.56 g, 3.88 mmol) and Trimethylsilyl trifluoromethanesulfonate (TMSOTf, 880 µL, 4,8 mmol). Reaction mixture was stirred for 3 hours under reflux conditions. The reaction mixture was neutralized with Et3N and concentrated in vacuo. Solid product was obtained after crystallization from methanol. Yield 26%. HPLC purity: 95.6 %. MS ESI+ m/z: 498 $[M+H]^+$

*Carboxyl group deprotection (**30**)*

Carboxyl group was deprotected as described in Alaoui et al (2006). Compound (**29**, 100 mg, 0.2 mmol) was dissolved in anhydrous THF (5 mL) then a solution of Et3N (112 µL, 0.8 mmol)/HCOOH (23 µL, 0.6 mmol) in THF (100 µL) was added at 0 °C under argon. After 10 min Tetrakis(triphenylphosphine)palladium(0) (46 mg, 0.04 mmol) was added and stirring was pursued for 30 min at 0 °C, then for 2 h at room temperature. Crude reaction mixture was concentrated in vacuo and 10 ml of water was added. Crystals of Ph_3PO were filtered of and H_2O phase was after lyophilisation used for subsequent reaction without further purification. MS ESI+ m/z: 458 $[M+H]^+$

*6-chloropurine-9-D-glucuronate (**31**)*

Compound (**30**, 100 mg) was deacetylated using 7N ammonia in methanol (10 mL) at 0 °C for 3 hours. The reaction mixture was then concentrated in vacuo and pale brown product was obtained after crystallization in aceton. Yield 60%. HPLC purity: 96 % MS ESI- m/z: 329 $[M-H]^-$

6-furfurylaminopurine-9-D-glucuronate

Compound (**31**, 150 mg, 0.45 mmol), furfurylamine (50 μL, 0.54 mmol) Et3N (240 μL; 1.35 mmol) were sequentially dissolved in propanol (10 mL). The mixture was refluxed for 8 h. This reaction unfortunately give poor conversion of starting material and final product was not isolated. However, in reaction mixture was analyzed product with corresponding molecular weight. MS ESI+ m/z: 392 $[M+H]^+$

Batter conversion was not achieved even with stronger base (DIPEA), nor with higher temperature, when reaction mixture was stirred in pressure tube in butanol.

Cyanine-5 N9-labeled benzylaminopurine

Both iP-Cy5 (Hönig 2018, **III**) and BAP-Cy5 were prepared (Fig. 11) to suit staining of *Arabidopsis* suspension cell culture with GFP-labeled CK receptor as emission maximum of Cyanine5 fluorescent label is 662 nm. Unfortunately labeled compounds did not interact with neither AHK4 nor AHK3 CK receptors.

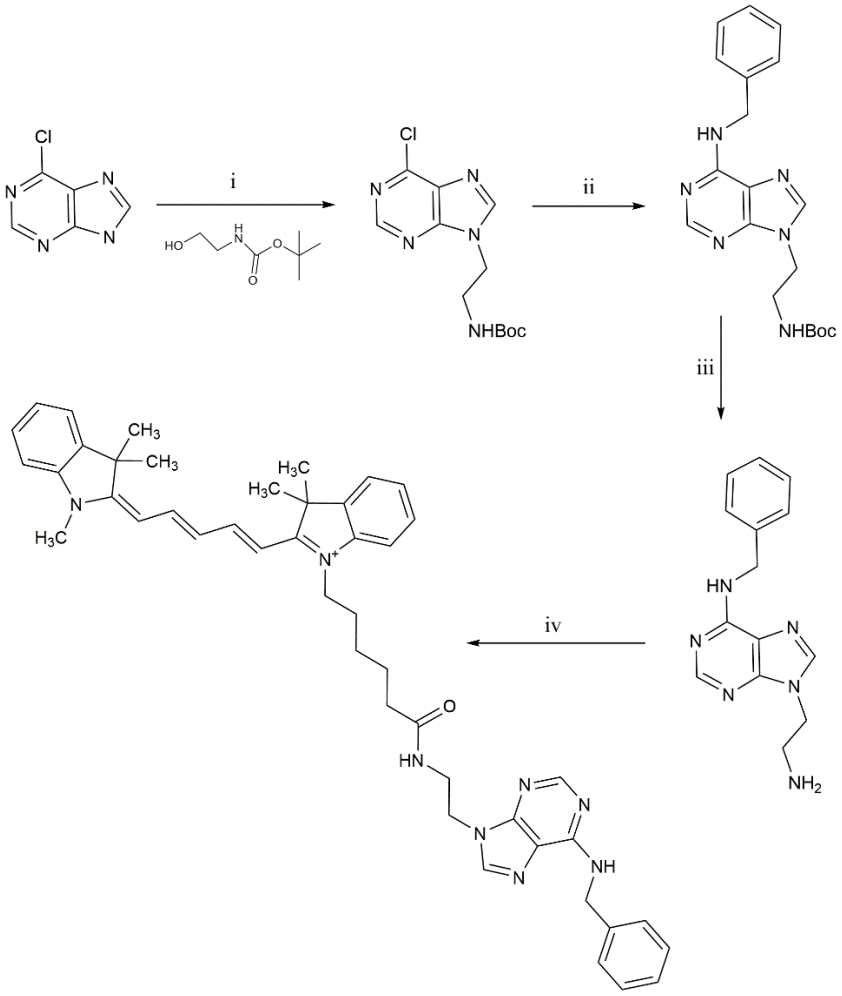


Figure 11 *Preparation of BAP-Cy5 using 6-chlorpurine as a starting material. Reaction condition i) Ph3P, DIAD, THF ii) benzylamine, Et3N, n-propanol iii) TFA, DCM iv) Cyanine 5 NHS, 0.1M NaHCO3, DMF.*

Preparation procedure

*9-{2[tert-butoxycarbonyl)amino]ethyl}-6-chloropurine (**32**)*

6-chloropurine (1 eq., 2 g, 12.9 mmol), Ph3P (2 eq., 6.77 g, 25.8 mmol) and N-Boc-ethanolamine (1.2 eq., 2.5 g, 15.5 mmol, 2.4 mL) was sequentially dissolved in cold (0 °C) THF

(10ml), and DIAD (2 eq., 5.2 g, 25.8 mmol, 5.1 mL) was added dropwise. The mixture was stirred at room temperature for 2 hours and then concentrated in vacuo. Yellowish oily residue was precipitated from toluene (50 mL) at 4 °C overnight. The resulting white solid (Ph3PO) was filtered of and washed with cold toluene. The filtrate was evaporated and the product was obtained after purification via column chromatography using CHCl3/MeOH (19:1) as eluent. Yield: 40.5%. HPLC purity: 98.3 %. MS ESI+ m/z: 298 $[M+H]^+$

*9-{2[tert-butoxycarbonyl)amino]ethyl}-6-benzylaminopurine (**33**)*

9-{2[tert-butoxycarbonyl)amino]ethyl}-6-chloropurine (1eq., 250mg, 0.84 mmol), benzylamine(1.2 eq., 104.9 mg, 1 mmol, 109µl) and Et3N (5 eq., 425 mg, 4.2 mmol, 585 µl) was dissolved in n-propanol (10 mL). Reaction mixture was stirred at reflux for 5 hours and then concentrated in vacuo. Product was obtained after crystallization from water. Yield 85%. HPLC purity: 98.8 %. MS ESI+ m/z: 369 $[M+H]^+$

*9-(2-aminoethyl) -6-benzylaminopurin (**34**)*

9-{2[tert-butoxycarbonyl)amino]ethyl}-6-benzylaminopurine (xy) (1 eq., 100 mg, 0.27 mmol) was dissolved in cold (0 °C) DCM (2.5 mL) and TFA (1.35 mL) was added dropwise. The mixture was stirred at room temoerature for 3 hour and then concentrated in vacuo. Crude reaction mixture was precipitated from diethylether (5 mL). Solid white product was obtained after filtration. Yield: 89%. HPLC purity: 99.1 %. MS ESI+ m/z: 269 $[M+H]^+$

*BAP–Cy5 (**35**)*

9-(2-aminoethyl) -6-benzylaminopurin (1 eq, 2.2 mg, 8.1 µmol) was dissolved in 0.1M sodium bicarbonate solution (4.5 mL, pH 8.5) and Cyanine 5 NHS (1 eq., 5 mg, 8.1 µmol) dissolved in amine free DMF (0.5 mL) was added. Reaction mixture was stirred at room temperature for 4 hours and then lyophilized overnight. The residue was dissolved in water (5 mL) and extracted into EtOAc (3 x 5 mL). Combined organic layers were dried (Na2SO4) evaporated in vacuo. The product was via column chromatography using CHCl3/MeOH (9:1) as eluent. Yield: 41%. MS ESI+ m/z: 734 $[M+H]^+$. 1H NMR (500 MHz, DMSO-d6) δ 8.35 – 8.21 (m, 2H), 8.16 (s, 1H), 8.04 (s, 1H), 7.96 – 7.88 (m, 1H), 7.60 (d, J = 7.4 Hz, 2H), 7.45 – 7.15 (m, 10H), 6.54 (t, J = 12.3 Hz, 1H), 6.24 (dd, J = 33.2, 13.9 Hz, 2H), 4.65 (s, 1H), 4.17 (t, J = 5.8 Hz, 2H), 4.06 – 4.00 (m, 2H), 3.58 (s, 3H), 3.57 (s, 2H), 3.55 (s, 1H), 2.00 – 1.95 (m, 2H), 1.66 (s, 12H), 1.48 – 1.41 (m, 2H), 1.32 – 1.19 (m, 4H), 0.89 – 0.82 (m, 2H).

Conclusions and perspectives

- A number of new CK derivatives were prepared, including kinetin derivatives and/or analogs with modified furfuryl ring in combination with some other substitutions such as THF or -THP groups attached to the N9 atom of the purine moiety. In addition, C2-chloro derivatives of these compounds were prepared as well. The majority of the compounds were prepared for the first time.

- New CK conjugates with N-acetylglucosamine and D-glucuronic acid were prepared for the first time.

- Fluorescently labeled iP and BAP were prepared with Cyanine5 dye attached to N9 atom by a two carbon linker.

- The prepared compound helped us to establish the SAR of CK derivatives related to their anti-senescent properties.

- Newly prepared derivatives with significant anti-senescent properties are promising for use in agriculture and tissue culture.

- Photoprotective activity against both UVA and UVB radiation of selected prepared compounds as well as lack of phototoxicity were described in human dermal fibroblasts and keratinocytes.

- The newly designed and prepared compounds 6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine and 2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine were able to protect *Caenorhabditis elegans* against juglone induced oxidative stress. Furthermore, compound 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine protected NHDF cells against UVA induced ROS production and GSH depletion. In contrast, none of the tested compounds was able to directly scavenge ROS in the ORAC assay.

In summary, standard routes of CK bases and their C2-chloro and/or N9-THF/THP derivatives were optimized to obtain compounds in decent yields and purity. New routes for CK conjugates with N-acetylglucosamine and D-glucuronic acid were implemented as well as methods for preparation of Cyanine5 labeled CKs.

Photoprotective and antioxidant activities of CK derivatives were shown on human cells and *Caenorhabditis elegans.* It was suggested that the photoprotective activity of these derivatives may be connected with their antioxidant activity, as ROS production is one of the main causes of UVA radiation damage in human skin. However, to clarify the connection between photoprotective and antioxidant properties of prepared CKs, additional molecular and gene expression studies are required and will be carried out in the near future.

List of publications

This thesis is based on the following papers, which are referred to in the text by corresponding roman numerals. The papers are appended in the Supplement section.

I. **Hönig, M.**; Plíhalová, L.; Spíchal, L.; Grúz, J.; Kadlecová, A.; Voller, J.; Rajnochová Svobodová, A.; Vostálová, J.; Ulrichová, J.; Doležal, K.; Strnad, M. New cytokinin derivatives possess UVA and UVB photoprotective effect on human skin cells and prevent oxidative stress. *Eur. J. Med. Chem.* **2018**, *150*, 946–957, doi:10.1016/J.EJMECH.2018.03.043.

II. **Hönig, M.***; Plíhalová, L.*; Husičková, A.; Nisler, J.; Doležal, K. Role of Cytokinins in Senescence, Antioxidant Defence and Photosynthesis. *Int. J. Mol. Sci.* **2018**, *19*, 4045, doi:10.3390/ijms19124045. * These authors contributed equally to this work.

III. Kubiasová, K.; Mik, V.; Nisler, J.; **Hönig, M.**; Husičková, A.; Spíchal, L.; Pěkná, Z.; Šamajová, O.; Doležal, K.; Plíhal, O.; Benková, E.; Strnad, M.; Plíhalová, L. Design, synthesis and perception of fluorescently labeled isoprenoid cytokinins. *Phytochemistry* **2018**, *150*, 1–11, doi:10.1016/j.phytochem.2018.02.015.

IV. Plíhalová, L.; **Hönig, M.**; Rajnochová Svobodová, A.; Vostálová, J. Cytokininové deriváty jako možné modulátory předčasného stárnutí kůže. *Ref. výb. dermatovenerol*. **2018**, 3, 6-16, ISSN: 1213-9106

V. **Hönig, M.**; Plíhalová, L.; Doležal, K.; Voller, J.; Strnad, M.; Spíchal L.; Vostálová, J.; Rajnochová Svobodová, A.; Ulrichová J.; Kadlecová A.; Plíhal O. Adenine derivatives and their use as uv-photoprotective agents. PCT application WO2017036434A1, **2017**

VI. **Hönig, M.**; Plíhalová, L.; Doležal, K.; Voller, J.; Strnad, M.; Spíchal L.; Vostálová, J.; Rajnochová Svobodová, A.; Ulrichová J.; Kadlecová A.; Plíhal O. Adenine derivatives and their use. Czech Republic. Patent no. 307722, **2019**

Contribution report

I. First author – synthesis, compound characterization, pH stability study, interpretation of the results, schemes, tables and figures creation (excluding Figure 1), writing the manuscript

II. Joint first author - cooperation in writing paragraphs 2, 3, 5 and 8. Table 1 creation.

III. Co-author - preparation of previously reported N9-labeled compounds (Mik, 2012) in a quantity needed for the study. Preparation and characterization of iP-Cy5.

IV. Co-author - cooperation in writing paragraphs – "UV záření a jeho působení na lidskou kůži", "Předčasné stárnutí kůže", "Použití kinetinu na živočišných a lidských buňkách", "Kinetinové deriváty v UV protekci".

V. First author – cooperation in writing the manuscript, synthesis and characterization of examples, tables creation

VI. First author - cooperation in writing the manuscript, synthesis and characterization of examples, tables creation

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Supplement I

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Research paper

New cytokinin derivatives possess UVA and UVB photoprotective effect on human skin cells and prevent oxidative stress

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ABSTRACT

Eleven 6-furfurylaminopurine (kinetin, Kin) derivatives were synthesized to obtain biologically active compounds. The prepared compounds were characterized using ^{1}H NMR, mass spectrometry combined with HPLC purity determination and elemental C, H, N analyses. The biological activity of new derivatives was tested on plant cells and tissues in cytokinin bioassays, such as tobacco callus, detached wheat leaf chlorophyll retention bioassay and *Amaranthus* bioassay. The selected compounds were subsequently tested on normal human dermal fibroblasts (NHDF) and keratinocyte cell lines (HaCaT) to exclude possible phototoxic effects and, on the other hand, to reveal possible UVA and UVB photoprotective activity. The protective antioxidant activity of the prepared cytokinin derivatives was further studied and compared to previously prepared antisenescent compound 6-furfurylamino-9-(tetrahydrofuran-2-yl) purine (Kin-THF) using induced oxidative stress (OS) on nematode *Caenorhabditis elegans* damaged by 5-hydroxy-1,4-naphthoquinone (juglone), a generator of reactive oxygen species. The observed biological activity was interpreted in relation to the structure of the prepared derivatives. The most potent oxidative stress protection of all the prepared compounds was shown by 6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**6**) and 2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine (**9**) derivatives and the results were comparable to Kin-THF. Compounds **6** and **9** were able to significantly protect human skin cells against UV radiation *in vitro*. Both the derivatives **6** and **9** showed higher protective activity in comparison to previously known structurally similar compounds Kin and Kin-THF. The obtained results are surprising due to the fact that the prepared compounds showed to be inactive in the ORAC assay which proved that the compounds did not act as direct antioxidants as they were unable to directly scavenge oxygen radicals.

Abbreviations: AAPH, 2,2′-azobis(2-amidino-propane) dihydrochloride; ArCKs, aromatic cytokinins; BAP, 6-benzylamiopurine; BJ, human foreskin fibroblast cell line; 2-DRA, 2-deoxyribose degradation assay; GPX, glutathione peroxidase; GSR, glutathione reductase; CAT, catalase; GSH, glutathione; HaCaT, human immortalized keratinocyte cell line; HL-60, human promyelocytic cell line; HPLC, high performance liquid chromatography; Kin, kinetin, 6-furfurylaminopurine; LPO, lipid peroxidase; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGM, nematode growth medium; NR, neutral red; NRK, epithelial rat kidney cell line; NHDF, normal human dermal fibroblasts; NMR, nuclear magnetic resonance; ORAC, oxygen radical absorbance capacity assay; PCR, polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

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1. Introduction

Cytokinins are phytohormones that are able to regulate cytokinesis, cell growth and differentiation, as well as leaf senescence and many other aspects of plant life [1]. Aromatic cytokinins (ArCKs) such as kinetin (Kin) and 6-benzylaminopurine (BAP) possess an aromatic substituent at the C6 atom of the purine moiety. Kin that was for the first time isolated from heated deoxyribonucleic acid preparations [2] and later on identified e.g. in the endosperm liquid of fresh young coconut fruit [3], was described as a multiactive molecule with various biological effects both in animal and plant cells [4]. In the presence of other plant hormone auxin, Kin is able to induce cell division in plant tissue culture [2]. This aside, Kin N9-substituted ArCKs such as N9-ribosides [5], N9-tetrahydropyran-2-yls (THP), N9-tetrahydrofuran-2-yls (THF) [6] and N9-chlorobutyls [7], are reported to be exceptionally potent in promoting chlorophyll retention in detached wheat leaf senescence bioassay [3]. The ability of Kin as well as N9-halogenoethyl ArCK derivatives to delay the senescence of excised wheat leaves in both dark and light conditions correlated with their ability to affect membrane lipid peroxidation which is a typical symptom of plant senescence [8] and relates to the protection of plant cells against oxidative stress (OS) [8,9]. ArCKs are increasingly becoming a subject of human interest because, in addition to the regulation of plant functions, they can also influence human and mammalian cells. ArCKs ribosides, such as Kin riboside or 6-(2-hydroxybenzylamino)purine riboside (*ortho*-topolin riboside, oTR) display high cytotoxic activity in various human cancer cell lines [5,10,11]. Trisubstituted ArCKs analogs such as 2-(hydroxyethylamino)-6-benzylamino-9-methylpurine (Olomoucine) or 6-(benzylamino)-2(*R*)-{[1-(hydroxymethyl)propyl]amino}-9-isopropylpurine (Roscovitine) were even described as cyclin-dependent kinase inhibitors and anti-tumor agents [12,13]. Although ArCK N9-ribosides are cytotoxic, THP, THF and N9-chloroalkyl derivatives showed none or only marginal cytotoxicity [21]. The antioxidant properties of Kin have been reported in both *in vitro* and *in vivo* experiments [14–19] and the ability of Kin to stimulate antioxidant enzymes catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) has been published [18]. Both Kin and BAP positively influenced glutathione (GSH) content and decreased the level of lipid membrane peroxidation in human skin fibroblasts [18]. Low Kin doses (100 nM) reduced apoptosis and protected human keratinocyte HaCaT and rat epithelial kidney NRT cells as well as peripheral lymphocytes from OS mediated cell death [19]. Kin retarded aging and prolonged the lifespan of fed fruit flies and showed stimulatory effects on the activity of CAT during the development and adult life of fruit flies [15,16]. Kin elevates the activity of antioxidant enzymes superoxide dismutase (SOD), CAT and GPX in rats [17].

Recently, a group of four CKs including Kin, BAP, 6-(4-hydroxybenzylamino)purine (*para*-topolin, pT) and 6-isopentenylaminopurine (iP) was tested for their antioxidant activity in oxygen radical absorbance capacity assay (ORAC). Among the tested CKs, Kin showed the highest activity in the concentration range used (up to 1 μM) [20].

Kin and its derivative 6-furfurylamino-N9-(tetrahydropyran-2-yl)purine (Pyratine, Pyr) prepared in 2008 [21] were reported to delay age-related characteristic of human fibroblasts *in vitro* [22–24]. Using reconstructed skin equivalent Mimeskin®, a positive effect of topical Kin treatment on both epidermis and dermis formation and development was found [25]. Pyr reduced symptoms and signs of photo damaged facial skin *in vivo* even more efficiently than Kin [21,24]. In 2012, Campos et al. [26] carried out a preclinical study on the dispersion of liposome with magnesium ascorbyl phosphate, alpha-lipoic acid and Kin and they found that the mixture showed free-radical scavenging properties but when vehiculated into a cosmetic formulation, it augmented the skin barrier function against UV damage [26]. The mechanism of action of Kin in relation to photoprotection in human skin remains unknown and few statistically significant trials have been published to date.

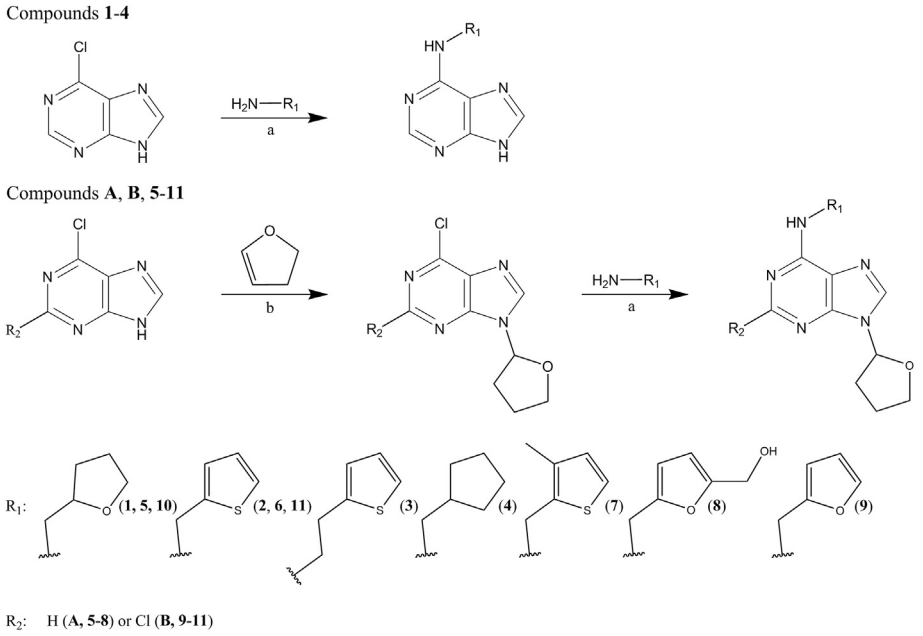
In this study, we prepared eleven Kin derivatives with modified or substituted furfuryl ring, in several cases combined with additional N9-substituent, to investigate the impact of structural changes on the protection of plant, animal and human cells and tissues. The structure activity relationship of the prepared compounds was studied using several plant bioassays including tobacco callus, chlorophyll retention (detached wheat leaves), and *Amaranthus* bioassays. The cytotoxicity of the prepared compounds against two human skin lines (fibroblasts, BJ; keratinocytes, HaCaT) was determined. The phototoxicity and UVA/UVB protective effect of selected compounds was tested on normal human dermal fibroblasts NHDF and HaCaT.

Caenorhabditis elegans is an amenable model for biomedical research, including screening of new molecules. It is easy and cheap to maintain in a laboratory while being compatible with high-throughput approaches, which allows evaluating the effect of compounds on a whole-organism level [27,28]. The protection of this nematode against OS was also studied as well as the oxygen radical absorbance capacity (ORAC) of selected active compounds.

2. Results and discussion

2.1. Organic syntheses

Eleven kinetin derivatives were prepared *via* nucleophilic substitution of 6-chloropurine, 6-chloro-9-(tetrahydrofuran-2-yl)purine- or 2,6-dichloro-9-(tetrahydrofuran-2-yl)purine with the appropriate amines (Scheme 1). The structures are shown in Table 1. The prepared compounds were characterized by C, H, and N elemental analysis, HPLC-PDA-MS, 1 H NMR and ^{13}C NMR. Elemental analysis, melting point, ESI + MS and HPLC purity data are listed in Table 2, while ^{1}H and ^{13}C NMR spectral data are provided in the Experimental part and the spectral records are provided in the Supplementary data. 6-Chloro-9-(tetrahydrofuran-2-yl)purine was prepared using a slightly modified method described in the literature [6]. 2,6-Dichloro-9-(tetrahydrofuran-2-yl)purine was prepared following the same procedure. We prepared C6-furfurylamino, C6-(thienylmethyl)amino, C6-[2-(3-thienyl)ethyl]amino, C6-(tetrahydrofuran-2-yl)methylamino- and C6-(cyklopentylmethyl)aminopurine derivatives. In addition, we modified seven compounds (**5**–**11**) with tetrahydrofuran-2-yl ring at the N9 position of purine moiety. Three compounds (**9**–**11**) were also substituted by a chlorine atom in position C2. The preparation of compound **1** has been described by De Roulet et al. (2015) [29] using similar reaction conditions (R: Et_3N, solvent: butanol, 12 h, 100 °C) but we used propanol instead of butanol and the reaction time was shortened to 4 h. Compound **2** was previously prepared *via* reaction of 6-chloropurine with 2-thiophenemethylamine in 2-methoxyethanol [30]. The approach used in this paper, allowed the use of a much lower amount of relatively expensive 2-thiophenemethylamine but it was necessary to prolong the reaction time. The preparation of compound **4** was described by De Roulet et al. (2015) [29] as a multistep reaction of adenine with cyclopentylmethanol. The utilization of cyclopentylmethylamine in reaction with 6-chloropurine as described here allowed the preparation of compound **4** in a simple one step reaction. The preparation of all derivatives is described in greater detail in the Experimental section.



Scheme 1. Reaction scheme for the synthesis of prepared 2,6- and 2,9-disubstituted and 2,6,9-trisubstituted purine derivatives. a) Et_3N, propanol, 100 °C, 4 h (**1**, **2**, **5**, **9**, **10**), 5 h (**3**, **4**, **6**, **8**) and 6 h (**7**); b) EtOAc, CF_3COOH, NH3, RT, 3.5 h.

2.2. Stability in acidic solution

The tetrahydrofuran-2-yl (THF) group has been commonly used in organic chemistry as a protective group, readily removable under acidic conditions [31]. As pH may vary in particular bioassays, we verified that THF substituted compounds are stable and do not breakdown to a corresponding free base under chosen bioassay conditions and we used a procedure described in the literature [6]. 6-(Tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl) purine (**5**) was chosen as the model compound. We performed HPLC stability measurement in 10^{-4} M stock solution with pH decreasing from 7 to 3. The results showed pH and time-dependent release of free base, as determined by HPLC. These are given in Table 3. The tested compound was stable at pH 7 and 6 even after 24 h following sample preparation. It started to decompose after 1 h at pH 3 (5.7% of the free base release) and after 24 h at pH 5 (7.4% of the free base release). A significant breakdown of tested compound occurred after 24 h at pH 4 (55.7% of the free base) and increased significantly at pH 3 (99.6% of the free base release). Since the pH of the media in the performed bioassays varied between 6 and 7, we conclude that the prepared compounds did not disintegrate in the used bioassays. We also tested the pH stability of two most biologically active compounds (**6** and **9**). Their stability in water solution in pH range 6–3 showed similar pattern to model compound **5**. These compounds were therefore stable at pH higher than 5 even after 24 h after sample preparation (Table 3).

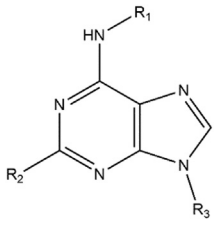
2.3. Cytokinin activity in bioassays

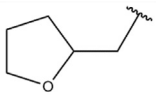
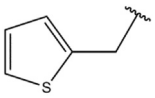
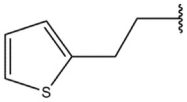
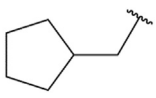
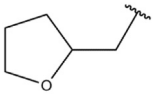
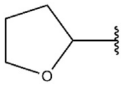
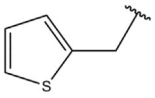
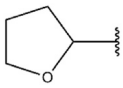
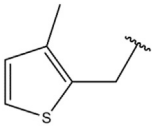
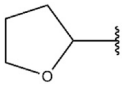
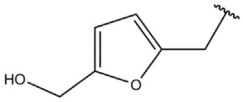
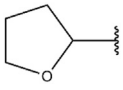
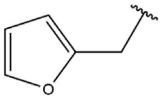
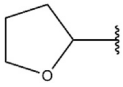
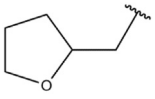
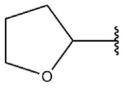
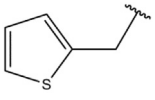
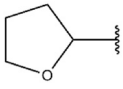
The prepared derivatives were tested in three cytokinin bioassays (tobacco callus, detached wheat leaf senescence and *Amaranthus* bioassay) and the results are presented in Table 4. The bioassays described above were used to test cytokinin activity and to evaluate the structure and activity relationships of the prepared compounds in comparison to published data.

The lowest effect of the structural changes in prepared derivatives on cytokinin activity was observed in tobacco callus bioassay. All the tested compounds showed comparable or slightly higher activity than BAP that was used as a standard. The activity of new compounds in callus bioassay is also comparable to those obtained for Kin and Kin-THP. The little effect of substituting an oxygen atom in the furfuryl ring by sulphur (**2**) in the tobacco callus bioassay has been described [32]. However, we observed no decrease in activity for C6-saturated ring derivatives (**1**, **4**) in accord with other findings [33,34]. N9-substituted derivatives were generally less active in *Amaranthus* bioassay in comparison to BAP (on average about 20% lower except for the virtually inactive compound **5**) in agreement with other reports [6,8] and observed in the case of Kin-THF and 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**5**). The biological activity of **5** in *Amaranthus* bioassay was 60% lower than the activity of its free base (**1**). On the other hand, 2-chloro-6-(thiophen-2-ylmethyl)-9-(tetrahydrofuran-2-yl)aminopurine (**11**) was active in this bioassay and this was comparable to BAP.

The presence of an oxygen atom in the furan ring showed this to be a critical structural motif for slowing the breakdown of chlorophyll in detached wheat leaf senescence bioassay. The replacement of an oxygen atom by sulphur or carbon resulted in a decrease of anti-senescent activity (**2, 4** and **11**). On the other hand, the saturation of furan ring showed no such negative impact on the anti-senescence activity – in other words, compounds with a tetrahydrofuran ring slowed the breakdown of chlorophyll (**1, 5** and **10**). High activity of N9-THF and N9-THP derivatives in promoting chlorophyll retention has been reported [7,21,32] and also observed in compounds **5** and **6**. While the replacement of an oxygen atom in the Kin molecule by sulphur in compound **2** resulted in the reduction of anti-senescent activity by 40%, following substitution by the THF group in the position N9 (**6**) increased the activity to the level of BAP. The presence of a chlorine atom in purine molecule at the C2 atom did not affect the activity in leaf senescence bioassay (**10**). On the other hand, C2-chlorine substitution lowered or completely reduced the anti-senescent activity of compounds **9**

Table 1
Structures of the prepared compounds and their abbreviations.



Compound	R_1	R_2	R_3
1		H	H
2		H	H
3		H	H
4		H	H
5		H	
6		H	
7		H	
8		H	
9		Cl	
10		Cl	
11		Cl	

and **11**, respectively. As described [32–34] the prolongation of the bridge between N^6-substituent and N^6-amino group decreased cytokinin activity (tobacco callus and *Amaranthus* bioassay) (**3**). The structural change also induced a complete loss of cytokinin activity in detached wheat leaf senescence bioassay.

2.4. Protection of Caenorhabditis elegans against induced oxidative stress

Protective activity of the compounds against chemically-induced oxidative stress was evaluated in *Caenorhabditis elegans* exposed to the lethal concentration of 5-hydroxy-1,4-naphthalenedione (juglone; 500 μM) [35]. Worms were pre-treated with test compounds (100 μM) 3 days prior to the juglone exposure. In the initial screening, the effect of the compounds on the survival of wild-type N2 worms was evaluated after 4 h. Promising results were obtained in the case of the compounds **6** and **9** (data not shown). In the follow-up experiments, those compounds were tested on larger populations and the viability was evaluated hourly for 12–14 h. This was also carried out with the BA17 (fem-1) strain with temperature inducible sterility, in order to ensure that FUDR which was used to prevent the reproduction of wild-type worms, did not interfere with the activity of compounds. The results were similar in both experimental settings - the compounds **6** and **9** protected the worms against the oxidative stress (Fig. 1, Supplementary data). In the experiment with BA17, the effect of the compounds was compared with earlier prepared compound Kin-THF as well due to certain structural similarities, e.g. structural fragment in N9 atom of purine moiety with protecting furanyl group. Kin-THF was prepared and tested as the compound that is able to delay senescence and fibroblast aging, as well as another compound similar to Kin, 6-furfurylamino-9-(tetrahydropyran-2-yl)purine (Kin-THP) but the mode of action of the compound is still unknown even when clinically tested on human skin and is currently used in cosmetic [21,24]. Kin-THF is also able to delay the senescence influence membrane lipid peroxidation in plants as we published previously [8]. Kin-THF protected the worms against the oxidative stress comparably to both active compounds **6** and **9**.

2.5. Evaluation of toxicity of prepared derivatives on human skin cells

The cytotoxicity of the prepared compounds was evaluated in human diploid fibroblasts (BJ, ATTC) and keratinocyte cell lines (HaCaT) by resazurin reduction assay after 24 h. The assay is based on reduction resofurin into fluorescent resazurin by metabolically active cells. The test compounds were only marginally toxic or non-toxic (decrease in resazurin fluorescence < 10%), even at the highest concentration tested (50 μM, data not shown).

*2.6. Phototoxicity of compounds **1**, **6**, **8**, **9** and **10** on human skin cells*

Compounds **1**, **6**, **8**, **9** and **10** were chosen out of all the prepared compounds for phototoxicity testing with the aim of covering all the modifications of Kin structure. The pre-treatment of NHDF and HaCaT with the tested compounds and the follow-up exposure of the samples to a non-toxic UVA dose did not result in any decrease in cell viability. Neutral red (NR) incorporation into both cell types and thus selected test compounds can be considered as non-phototoxic in the used concentration range (3.9–125 μM). The results using NHDF/HaCaT are shown in Supplementary data (Tables 1s and 2s). A well-known phototoxic compound, chlorpromazine (CPZ), used as a standard in the validated NRU phototoxicity test, was also used here as a positive control. CPZ treatment and following exposure to UVA radiation clearly decreased the viability of NHDF as well as HaCaT with IC50 of 25.5 ± 3.5 μM and 36.1 ± 4.7 μM, respectively.

Table 2
Elemental analyses, melting points (mp) and ESI + MS of the prepared compounds.

Compound	Elemental analysis calc./measured			mp [°C]	Yield [%]	HPLC [%]	ESI + MS [M + H$^+$]
	% C	% H	% N				
1	54.8/54.7	6.0/6.1	31.9/31.9	189–191	66	>99	220
2	51.9/51.9	3.9/4.0	30.3/30.4	251–253	88	>99	232
3	53.8/53.6	4.5/4.5	28.6/28.5	240–242	72	>97	246
4	52.2/53.3	7.7/7.9	27.7/28.2	215–219	81	>98	218
5	a	a	a	<22	76	>99	290
6	55.8/55.9	4.9/4.9	23.2/22.6	119–121	61	>98	302
7	57.1/57.0	5.4/5.4	22.2/22.0	99–104	46	>98	316
8	57.1/56.9	5.4/5.3	22.2/22.0	134–139	65	>98	316
9	52.6/51.9	4.4/4.4	21.9/20.8	144–146	57	>99	320
10	51.9/51.9	5.6/5.9	21.6/20.7	97–101	63	>97	324
11	50/50.8	4.2/4.3	20.9/20.6	158–161	56	>97	336

a: amorphous, not possible to determine.

Table 3
The pH stability of compounds **5, 6** and **9** in 10^{-4} M neutral and acidic water solutions. pH stability was measured 1 h (A) and 24 h (B) after sample preparation. The percentage of the released free base was determined by HPLC.

pH	Compound peak area [%]					
	5	Free base	6	Free base	9	Free base
(A) 1 h after sample preparation						
7	100	nd	–	–	–	–
6	100	nd	100	nd	100	nd
5	100	nd	100	nd	100	nd
4	100	nd	99.4	0.6	100	nd
3	94.3	5.7	94.6	5.4	98.8	1.2
(B) 24 h after sample preparation						
7	100	nd	–	–	–	–
6	100	nd	100	nd	100	nd
5	92.6	7.4	94.2	5.8	92.9	7.1
4	44.3	55.7	52.1	47.9	51.7	48.3
3	0.4	99.6	0.8	99.2	0.9	99.1

nd: not detected; -: not tested.

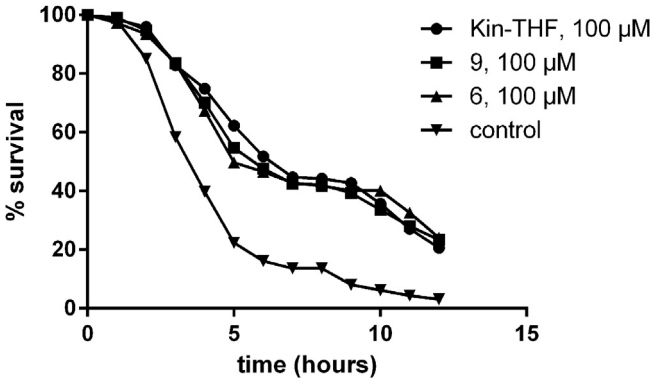


Fig. 1. Effect of compounds **6**, **9** and Kin-THF against oxidative stress in *Caenorhabditis elegans* (BA17). P-values were adjusted for multiple testing using Bonferroni method. Comparisons with p-values < 0.05 were considered as statistically significant.

Table 4
Relative cytokinin bioassay activity of the prepared derivatives at the optimal concentration compared with the activity of 6-benzylaminopurine (BAP) (100% means 10^{-5} M BAP for the Amaranthus betacyanin bioassay, 10^{-4} M BAP in the case of the senescence bioassay and 10^{-5} M BAP for the tobacco callus bioassay).

Compound	Tobacco callus bioassay		*Amaranthus caudatus* betacyanin bioassay		Wheat leaf senescence bioassay	
	Concentration [mol.l^{-1}]	Relative activity [%]	Concentration [mol.l^{-1}]	Relative activity [%]	Concentration [mol.l^{-1}]	Relative activity [%]
Kin	10^{-5}	101 ± 5	10^{-5}	68 ± 3	10^{-4}	98 ± 4
Kin-THF	10^{-5}	111 ± 7	10^{-4}	70 ± 8	10^{-4}	125 ± 11
1	10^{-5}	110 ± 8	10^{-4}	109 ± 6	10^{-4}	114 ± 8
2	10^{-5}	102 ± 2	10^{-5}	95 ± 3	10^{-4}	60 ± 12
3	10^{-5}	77 ± 12	10^{-4}	80 ± 6	–	na.
4	10^{-5}	100 ± 1	10^{-4}	97 ± 1	10^{-4}	71 ± 3
5	10^{-5}	100 ± 7	10^{-4}	38 ± 5	10^{-4}	116 ± 9
6	10^{-5}	105 ± 3	10^{-4}	81 ± 5	10^{-4}	102 ± 1
7	10^{-5}	99 ± 1	–	nt.	10^{-4}	110 ± 7
8	10^{-5}	103 ± 9	–	nt.	10^{-4}	75 ± 17
9	10^{-5}	112 ± 9	10^{-4}	88 ± 5	10^{-4}	75 ± 12
10	10^{-5}	104 ± 8	10^{-4}	83 ± 8	10^{-4}	111 ± 16
11	10^{-5}	108 ± 7	10^{-4}	101 ± 12	–	na.

nt: not tested, na:non-active, Kin-THF (6-furfurylamino-9-(tetrahydrofuran-2-yl)purin).
Kin (6-furfurylaminopurine).

*2.7. UVA and UVB photoprotection of human skin cells by compounds **1**, **6**, **8**, **9** and **10***

Derivatives **1**, **6**, **8**, **9** and **10** were chosen for photoprotectivity testing on the basis of preliminary data and due to the fact that these derivatives illustrated an extent of performed structural derivatizations of Kin molecule. The published data on Kin photoprotectivity is ambiguous and therefore we compared the photoprotective potential of our compounds with a well-known standard in the field - rosmarinic acid (RA), a naturally occurring photoprotective substance [36,37].

Firstly, NHDF and HaCaT skin cells were pre-incubated with tested compounds and exposed to a cytotoxic dose of UVA radiation. All selected compounds showed higher cell viability (amount of incorporated NR) compared to DMSO (control). The results of NHDF photoprotection are presented in Fig. 2 (A) while the

photoprotection of HaCaT results are given in the Supplementary data (Fig. 1sA, Fig. 1sB).

Prepared tested derivatives showed higher or comparable photoprotective activity to RA. Compound **6** exhibited the highest photoprotective effect in both cell models and in case of NHDF was significantly more active than RA in all tested concentrations. It was very potent even at the lowest tested concentration of 3.9 μM. At this concentration, compound **6** was more than six times and three times more effective than RA on NHDF and HaCaT, respectively. The second most effective was compound **9**, especially in HaCaT. Excluding compound **10**, all compounds were significantly more effective at the lowest tested concentration.

In the second set of experiments, cells were pre-incubated with tested compounds and exposed to a toxic dose of UVB radiation. Similarly, application of all compounds resulted in higher NR incorporation compared to DMSO (control) as shown in Fig. 2 (B). In the case of photoprotection against UVB, there was less difference between the activities of individual compounds than in the case of UVA photoprotection. Also only compounds **1** and **6** were found to be significantly more active than RA at the highest tested concentration of NHDF protection. Compound **10** that exhibited the lowest UVA photoprotective properties on HaCaT, had the most photoprotective effect on HaCaT against UVB irradiation. This phenomenon may be linked to a different cytotoxic mechanism of UVA and UVB radiation, as reviewed by Svobodová and Vostálová [38]. Compound **6** was the second most effective one. Both compounds **6** and **10** were more than twice as effective as RA at the lowest concentration tested (3.9 μM) on HaCaT. On NHDF, the protection of compounds **6** and **10** was comparable with RA. In addition to performed experiments, we compared the photoprotective activity of the active compounds **6** and **9** with their structurally similar but already described compounds Kin and 6-furfurylamino-9-(tetrahydrofuran-2-yl)purine (Kin-THF) on NHDF cells. We observed higher UVA as well as UVB photoprotective activity of both the compounds **6** and **9** compared to Kin as well as Kin-THF on NHDF. (Fig. 3).

2.8. Oxygen radical absorbance capacity (ORAC)

The radical scavenging activity of compounds **1**, **6**, **8**, **9** and **10** also tested for phototoxicity and photoprotection together with RA as a positive control, was determined by ORAC assay. The assay was recently used for the evaluation of the antioxidant activity and capacity of some natural N6-substituted adenine derivatives [20] within the frame of cytokinin group and it was stated that the antioxidant activity of Kin is the highest up to concentrations of 1 μM. The most active compound, RA, showed twelve times and thirteen times higher activity than both Kin and compound **8**, respectively. The rest of the tested compounds (**1**, **6**, **9** and **10**) had no detectable activity as shown in Table 5.

3. Conclusion

We prepared and characterized eleven ArCK derivatives. While compounds **1**, **2** and **4** were prepared according to methods described in the literature, compounds **3**, **5**–**11** were designed and prepared for the first time. We tested the cytokinin activity of the prepared compounds in three cytokinin bioassays. The changes made in Kin structure only slightly affected activity in tobacco callus bioassay in which the compounds were all comparably active to the used standard BAP. In accordance with the literature, the N9 purine atom substitution by the THF group reduced the biological activity of Kin derivatives (compounds **5**, **6**, **9** and **10**) in *Amaranthus* bioassay. The presence of an oxygen atom in the furan ring proves to be a critical structural motif for maintaining of anti-senescence activity in detached wheat leaf senescence bioassay. The replacement of an oxygen atom by a sulphur or carbon atom resulted in reduced anti-senescent activity (compounds **2**, **4**, and **11**). On the other hand, the saturation of the furan ring significantly increased the anti-senescence activity (compounds **1**, **5**, and **10**). None of the selected compounds (**1**, **6**, **8**, **9** and **10**) was phototoxic in the concentration range of 3.9–125 μM used either on the NHDF or HaCaT. Same derivatives were also used for testing UVA and UVB photoprotective properties. Compound 6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**6**) and 2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine (**9**) showed the highest photoprotectivity against UVA radiation on both NHDF and HaCaT. In the case of UVB photoprotection, compounds **6** and 6-(2-tetrahydrofuran-2-ylmethyl)aminopurine (**1**) were found to be the most effective in NHDF, while 2-chloro-6-tetrahydrofurfurylamino-9-(tetrahydrofuran-2-yl)purine (**10**) possessed high photoprotection of HaCaT. Compound **6** seems to be the most promising as it possesses both UVA and UVB photoprotective ability, even at low concentration (3.9 μM). Besides, tested compounds **6** and **9** were both able to protect *C. elegans* against OS *in vivo*. We compared the photoprotective activity of these two derivatives with Kin and Kin-THF and we found out that the protective activity of newly prepared derivatives was comparably higher. These results indicate antioxidant properties of compounds **6** and **9**. Although compounds **6** and **9** protected *C. elegans* and NHDF from oxidative and UV stress, they did not act as direct radical scavengers in ORAC assay. These data suggest that the

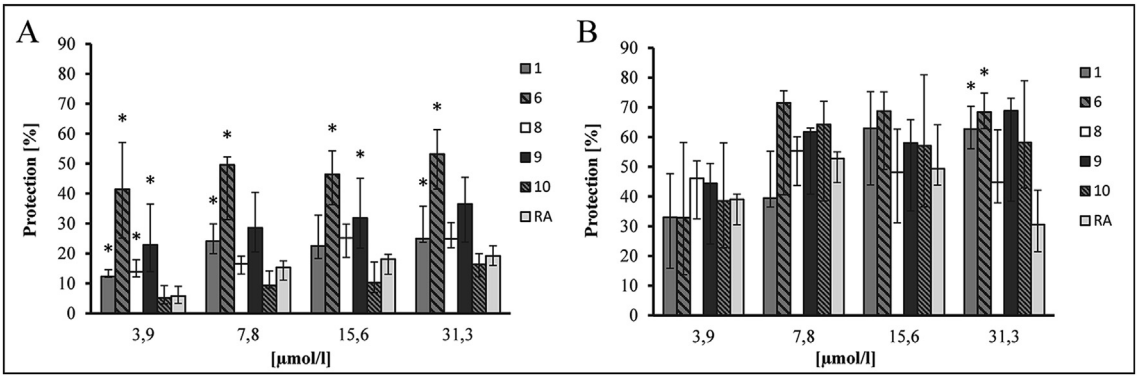


Fig. 2. Photoprotective effect of the prepared compounds **1**, **6**, **8**, **9**, **10** and positive control rosmarinic acid (RA) against UVA- (A) UVB– (B) induced damage to NHDF. The results shown in the figure represent the medians of four replicates; the error bars represent the boundaries of the first and third quartiles. To prove statistical significance the Mann-Whitney test was performed. Asterisks denote values that differ significantly from values of rosmarinic acid (Mann Whitney test; $p < 0.05$, $n = 4$).

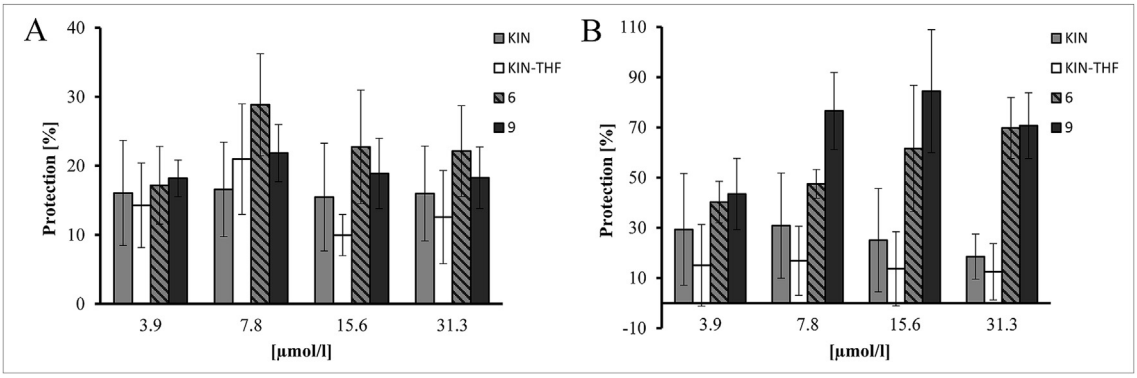


Fig. 3. Photoprotective effect of the newly prepared compounds **6**, **9**, Kin and Kin-THF against UVA- (A) UVB– (B) induced damage to NHDF. The results shown in the figure represent the average of 3 replicates; the error bars represent the standard deviation.

Table 5
Radical scavenging activity of selected prepared compounds.

Compound	ORAC (TE)
RA	9.14 ± (0.32)
Kin	0.71 ± (0.11)
1	<0.05
6	<0.05
8	0.77 ± (0.11)
9	<0.05
10	<0.05

Data are expressed as Trolox equivalents (TE) mean ± SD (n = 6).

mechanism of photo- and nematode protection against OS is indirect and triggers other mechanisms than direct interaction with ROS and therefore there must exist an alternative mode of action worth study and explaining the mode of protection caused by these compounds.

4. Experimental

4.1. General procedure

All reagents were purchased from commercial suppliers and used as received. Elemental analyses (C, H, N) were determined on an EA1112 Flash analyser (Thermo-Finnigan). The melting points (mp) were determined on SMP 30 (Stuart®) apparatus. The chromatographic purity and mass spectra of the prepared compounds were analysed using HPLC-PDA-MS method. Compounds (10 µl of 3.10^{-5} M in 1% methanol) were injected onto a reverse-phased column (Symmetry C18, 5 µm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 25 °C. Solvent (A) consisted of 15 mM ammonium formate adjusted to pH 4.0. Solvent (B) consisted of methanol. At flow-rate of 200 µl/min, following binary gradient was used: 0 min, 10% B; 0–24 min; linear gradient to 90% B; 25–34 min; isocratic elution of 90% B; 35–45 min; linear gradient to 10% B using the Waters Alliance 2695 Separations Module (Waters, Manchester, UK). The effluent was introduced then to Waters 2996 PDA detector (Waters, Manchester, UK) (scanning range 210–700 nm with 1.2 nm resolution) and a tandem mass analyser Q-Tof micro Mass Spectrometer (Waters, Manchester, UK) with an electrospray source (source temperature 120 °C, desolvation temperature 300 °C, capillary voltage 3 kV). Nitrogen was used as well as cone gas (50 l/h) and desolvation gas (500 l/h). Data acquisition was performed in the full scan mode (50–1000 Da), scan time of 0.5 s and cone voltage 20 V. Analyses were performed in positive mode (ESI+) therefore molecular ions were recorded in their protonated forms $[M+H]^+$. ^{1}H NMR spectra were measured on a Jeol 500 SS spectrometer operating at a temperature of 300 K and a frequency of 500.13 MHz The samples were prepared by dissolving the compounds in DMSO-d6. Tetramethylsilane (TMS) was used as an internal standard. Thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck). $CHCl_3$/MeOH (9:1, v/v) or EtOAc/MeOH/NH_3 (34:4:2, v/v) were used as mobile phase. Purification *via* column chromatography was carried out using silica gel Davisil R LC60A 40–63 µm.

4.2. Synthesis

4.2.1. 6-Chloro-9-(tetrahydrofuran-2-yl)purine (***A***)

The compound was prepared according to a modified procedure described in the literature [6]. 6-Chloropurine (10 g; 64.7 mmol) and 2,3-dihydrofuran (7.3 mL; 97 mmol) were dissolved in EtOAc (200 mL) and TFA (10 mL; 130 mmol) was added dropwise. The reaction mixture was stirred on ice at room temperature for 3.5 h and then neutralized by the appropriate amount of ammonia and water (1:2). The ethyl acetate phase was washed with water, dried over Na_2SO_4 and concentrated in vacuo. A yellow oily compound was obtained. The pure product was obtained after crystallization from ethanol at −20 °C overnight. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.95–2.05 (m, 1 H); 2.12–2.23 (m, 1 H); 2.37–2.44 (m, 1 H); 2.46–2.52 (m, 1 H); 3.90 (q, J = 7.54 Hz, 1 H); 4.15 (td, J = 7.72, 6.27 Hz, 1 H); 6.35 (dd, J = 6.72, 3.67 Hz, 1 H); 8.75 (s, 1 H); 8.76 (s, 1 H).

4.2.2. 2,6-Dichloro-9-(tetrahydrofuran-2-yl)purine (***B***)

2,6-Dichloropurine (10 g; 52.9 mmol) and 2,3-dihydrofuran (6 mL; 79.4 mmol) were dissolved in EtOAc (200 mL) and TFA (8.1 mL; 105.8 mmol) was added dropwise on ice. The mixture was refluxed for 3.5 h and then neutralized by the appropriate amount of ammonia and water (1:2). The ethyl acetate phase was washed with water, dried over Na_2SO_4 and concentrated in vacuo. A yellow oily compound was obtained. The pure product was obtained after crystallization from ethanol at −20 °C overnight. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.95–2.05 (m, 1 H); 2.07–2.17 (m, 1 H); 2.38–2.43 (m, 2 H); 3.90 (q, J = 7.54 Hz, 1 H); 4.14 (td, J = 7.87, 5.65 Hz, 1 H); 6.28–6.31 (m, 1 H); 8.78 (s, 1 H).

4.2.3. 6-(2-Tetrahydrofuran-2-ylmethyl)aminopurine (***1***)

6-Chloropurine (0.5 g; 3.2 mmol), tetrahydrofurfurylamine (403 µl; 3.9 mmol) and triethylamine (Et_3N) (2.5 mL; 16 mmol) were dissolved in propanol (30 mL). The reaction was refluxed for 4 h. The mixture was concentrated in vacuo. The residue was

dissolved in water and extracted into EtOAc, dried over Na_2SO_4 and evaporated in vacuo to form the white crystalline product. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.57 (br. s., 1 H); 1.69–1.92 (m, 3 H); 3.35–3.53 (m, 2 H); 3.56 (d, $J = 6.42$ Hz, 1 H); 3.73 (d, $J = 6.42$ Hz, 1 H); 4.02 (br. s., 1 H); 7.46 (br. s., 1 H); 8.04 (br. s., 1 H); 8.13 (br. s., 1 H); 12.86 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 154.93, 152.83, 149.97, 139.30, 119.26, 77.40, 67.60, 44.22, 29.08, 25.58.

4.2.4. 6-(2-Thiophen-2-ylmethyl)aminopurine (**2**)

6-Chloropurine (1 g; 6.47 mmol), 2-thiophenemethylamine (730 μl; 7.12 mmol) and Et_3N (2.24 mL; 16.175 mmol) were dissolved in propanol (65 mL). The reaction mixture was refluxed for 4 h. Solid yellowish product was obtained after crystallization from reaction mixture at 4 °C overnight. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 4.81 (br. s., 2 H) 6.90 (br. s., 1 H) 6.98 (br. s., 1 H) 7.29 (br. s., 1 H) 8.07 (br. s., 1 H) 8.19 (br. s., 2 H) 12.93 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 154.32, 152.78, 150.15, 143.77, 139.55, 127.09, 125.84, 125.23, 119.34, 38.60.

4.2.5. 6-(2-Thiophen-2-ylethyl)aminopurine (**3**)

6-Chloropurine (1 g; 6.47 mmol), 2-thiophenethylamine (910 μl; 7.76 mmol) and Et_3N (4.5 mL; 32.35 mmol) were dissolved in propanol (65 mL). The reaction mixture was refluxed for 5 h. Yellowish solid product precipitated from water and filtrated off. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 3.10 (t, $J = 7.34$ Hz, 2 H) 3.69 (br. s., 2 H) 6.89 (d, $J = 2.75$ Hz, 1 H) 6.90–6.96 (m, 1 H) 7.29 (d, $J = 5.20$ Hz, 1 H) 7.74 (br. s., 1 H) 8.05 (s, 1 H) 8.17 (br. s., 1 H) 12.89 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 154.39, 152.87, 150.43, 142.17, 139.62, 127.44, 125.62, 124.46, 118.55, 41.93, 29.81.

4.2.6. 6-Cyclopentylmethylaminopurine (**4**)

6-Chloropurine (0.325 g; 2.1 mmol), cyclopentanemethylamine (403 μl; 2.5 mmol) and Et_3N (1.5 mL; 10.5 mmol) were dissolved in propanol (20 mL). The reaction mixture was refluxed for 5 h and concentrated in vacuo. White solid was precipitated from water and filtrated off. ^{1}H NMR (500 MHz) δ 12.85 (s, 1H), 8.13 (s, 1H), 8.05 (s, 1H), 7.61 (s, 1H), 3.66 (partial overlap, 2H), 2.28–2.15 (m, 1H), 1.69–1.58 (m, 2H), 1.58–1.48 (m, 2H), 1.48–1.37 (m, 2H), 1.30–1.16 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 154.56, 152.83, 150.66, 139.43, 118.24, 45.12, 30.32, 25.30.

4.2.7. 6-(Tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**5**)

A (1 g; 4.46 mmol), tetrahydrofurfurylamine (554 μl; 5.36 mmol) and Et_3N (3.2 mL; 22.3 mmol) were sequentially dissolved in propanol (50 mL). The mixture was refluxed for 4 h and then concentrated in vacuo. The residue was dissolved in water and extracted into EtOAc using liquid-liquid continuous extractor (24 h). EtOAc solution was dried over Na_2SO_4 and evaporated in vacuo. The product was obtained after purification via column chromatography using EtOAc: MeOH: NH_3 (34:1:1; v:v) mobile phase as eluent. ^{1}H NMR (500 MHz, DMSO-d6), δ ppm: 1.53–1.64 (m, 1 H); 1.69–1.89 (m, 3 H); 1.95–2.02 (m, 1 H); 2.11–2.23 (m, 1 H); 2.30–2.44 (m, 2 H); 3.37–3.48 (m, 1 H); 3.48–3.53 (m, 1 H); 3.53–3.60 (m, 1 H); 3.70–3.76 (m, 1 H); 3.81–3.90 (m, 1 H); 3.98 (q, $J = 7.03$ Hz, 1 H); 4.09 (q, $J = 7.44$ Hz, 1 H); 6.21 (dd, $J = 6.88$, 3.82 Hz, 1 H); 7.63 (br. s., 1 H); 8.17 (br. s., 1 H); 8.21 (s, 1 H). ^{13}C NMR (126 MHz, DMSO) δ 155.02, 152.93, 148.73, 139.53, 120.01, 84.84, 77.34, 69.16, 67.59, 44.19, 31.64, 29.07, 25.55, 24.89.

4.2.8. 6-(Thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl) purine (**6**)

A (0.5 g; 2.23 mmol), 2-thiophenemethylamine (275 μl; 2.68 mmol) and Et_3N (1.6 mL; 11.15 mmol) were sequentially dissolved in propanol (25 mL). The mixture was refluxed for 3 h, then next 2-thiophenemethylamine (23 μl; 0.23 mmol) was added and the reaction mixture was further refluxed for 2 h. Reaction mixture was concentrated in vacuo, the residue was dissolved in water and extracted into EtOAc, dried over Na_2SO_4, evaporated in vacuo and mixed with diethyl ether. The product precipitated from diethyl ether. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.92–2.00 (m, 1 H); 2.12–2.22 (m, 1 H); 2.31–2.38 (m, 1 H); 2.38–2.43 (m, 1 H); 3.85 (td, $J = 7.68$, 6.34 Hz, 1 H); 4.08 (td, $J = 7.68$, 6.50 Hz, 1 H); 4.79 (br. s., 2 H); 6.21 (dd, $J = 6.88$, 3.82 Hz, 1 H); 6.88 (dd, $J = 5.04$,3.44 Hz, 1 H); 6.97 (dd, $J = 3.40$, 1.03 Hz, 1 H); 7.27 (dd, $J = 5.12$, 1.22 Hz, 1 H); 8.23 (s, 2 H); 8.36 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 154.45, 152.86, 149.00, 143.58, 139.79, 127.06, 125.85, 125.24, 120.09, 84.83, 69.16, 38.58, 31.62, 24.90.

4.2.9. 6-(5-Methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**7**)

A (0.148 g; 0.66 mmol), 3-methylthiophene-2-methylamine (0.1 g; 0.79 mmol) and Et_3N (0.46 mL; 3.3 mmol) were sequentially dissolved in propanol (10 mL). The mixture was refluxed for 6 h under argon atmosphere. The mixture was concentrated in vacuo. The residue was dissolved in water and extracted into EtOAc. The organic fraction was dried over Na_2SO_4 and evaporated in vacuo. The product was obtained after precipitation from diethyl ether. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.93–2.00 (m, 1 H) 2.13–2.19 (m, 1 H) 2.20 (s, 3 H) 2.31–2.43 (m, 2 H) 3.81–3.89 (m, 1 H) 4.05–4.11 (m, 1 H) 4.71 (br. s., 2 H) 6.21 (dd, $J = 6.88$, 3.82 Hz, 1 H) 6.75 (d, $J = 4.89$ Hz, 1 H) 7.15 (d, $J = 5.20$ Hz, 1 H) 8.21 (br. s., 1 H) 8.22 (s, 1 H) 8.31 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 154.39, 152.85, 148.94, 139.71, 136.79, 133.96, 130.22, 123.49, 120.07, 84.83, 69.17, 36.76, 31.64, 24.91, 13.98.

4.2.10. 6-(5-Hydroxymethylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**8**)

A (0.25 g; 1.1 mmol), 5-hydroxymethylfuran-2-ylmethylamine (0.169 g; 1.3 mmol) and Et_3N (0.464 mL; 3.3 mmol) were dissolved in propanol (10 mL). The reaction mixture was refluxed for 5 h. A crude, concentrated reaction mixture was mixed with water. Precipitated solid white product was filtrated off. ^{1}H NMR (500 MHz, CHLOROFORM-d) δ ppm 2.08–2.15 (m, 2 H) 2.42–2.57 (m, 2 H) 2.79 (br. s., 1 H) 4.05 (q, $J = 7.64$ Hz, 1 H) 4.26 (dt, $J = 8.48$, 6.46 Hz, 1 H) 4.55 (s, 2 H) 4.80 (br. s., 2 H) 6.18 (d, $J = 3.06$ Hz, 1 H) 6.22 (d, $J = 3.06$ Hz, 1 H) 6.27 (dd, $J = 6.27$, 3.21 Hz, 1 H) 6.36–6.63 (m, 1 H) 7.86 (s, 1 H) 8.37 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 154.83, 154.60, 152.88, 152.67, 148.95, 139.77, 120.12, 108.20, 107.72, 84.85, 69.18, 56.13, 37.10, 31.64, 24.90.

4.2.11. 2-Chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine (**9**)

B (0.5 g; 1.93 mmol), furfurylamine (204 μl; 2.31 mmol) and Et_3N (1.35 mL; 9.65 mmol) were sequentially dissolved in propanol (25 mL). The mixture was refluxed for 4 h, concentrated in vacuo and mixed with water. A yellowish solid was filtrated off. The pure product was acquired after recrystallization from $CHCl_3$ and ethanol. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.93–2.01 (m, 1 H); 2.12 (dt, $J = 12.38$, 7.41 Hz, 1 H); 2.31–2.38 (m, 2 H); 3.82–3.88 (m, 1 H); 4.07 (td, $J = 7.79$, 6.11 Hz, 1 H); 4.57 (d, $J = 5.50$ Hz, 2 H); 6.15 (t, $J = 5.20$ Hz, 1 H); 6.21 (d, $J = 2.75$ Hz, 1 H); 6.33 (br. s., 1 H); 7.51 (dd, $J = 1.83$, 0.92 Hz, 1 H); 8.25 (s, 1 H); 8.72 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 155.20, 153.49, 152.55, 149.87, 142.54, 140.21, 119.17, 111.04, 107.65, 84.98, 69.28, 37.16, 31.76, 24.66.

4.2.12. 2-Chloro-6-tetrahydrofurfurylamino-9-(tetrahydrofuran-2-yl)purine (**10**)

B (0.5 g; 1.93 mmol), tetrahydrofurfurylamine (240 μl; 2.31 mmol) and Et_3N (1.35 mL; 9.65 mmol) were sequentially

dissolved in propanol (25 mL). The mixture was stirred under reflux for 4 h, concentrated in vacuo, and mixed with water. The pure product was obtained from crude precipitate after recrystallization from petroleum ether. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.50–1.64 (m, 1 H); 1.68–1.91 (m, 4 H); 1.92–2.02 (m, 1 H); 2.07–2.19 (m, 1 H); 2.29–2.38 (m, 2 H); 3.44–3.52 (m, 1 H); 3.53–3.60 (m, 1 H); 3.65–3.74 (m, 1 H); 3.80–3.89 (m, 1 H); 3.93–4.03 (m, 1 H); 4.03–4.10 (m, 1 H); 6.06–6.19 (m, 1 H); 8.17–8.22 (m, 1 H); 8.22 (s, 1 H). ^{13}C NMR (126 MHz, DMSO) δ 155.60, 153.60, 149.64, 139.96, 119.02, 84.93, 77.02, 69.25, 67.58, 44.43, 31.74, 29.13, 25.51, 24.67.

4.2.13. 2-Chloro-6-(thiophen-2-ylmethyl)-9-(tetrahydrofuran-2-yl) aminopurine (**11**)

B (0.5 g; 1.93 mmol), 2-thiophenmethylamine (238 μl; 2.23 mmol) and Et_3N (1.35 mL; 9.65 mmol) were sequentially dissolved in propanol (25 mL). The mixture was refluxed for 5 h. Yellowish solid crystallized from the reaction mixture at 4 °C overnight. Pure product was obtained after re-crystallization from hot ethanol. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.92–2.01 (m, 1 H); 2.07–2.17 (m, 1 H); 2.29–2.39 (m, 2 H); 3.85 (q, J = 7.34 Hz, 1 H); 4.03–4.10 (m, 1 H); 4.72 (d, J = 5.50 Hz, 2 H); 6.15 (t, J = 5.04 Hz, 1 H); 6.90 (dd, J = 4.74, 3.52 Hz, 1 H); 6.98 (d, J = 2.75 Hz, 1 H); 7.31 (d, J = 4.59 Hz, 1 H); 8.24 (s, 1 H); 8.87 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO) δ 154.98, 153.48, 149.87, 142.42, 140.25, 127.13, 126.41, 125.66, 119.13, 84.99, 69.28, 38.81, 31.75, 24.66.

4.3. pH stability of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**5**)

pH stability evaluation was slightly modified and performed according to the literature [6]. The pH stability of compounds **5, 6** and **9** was analysed by HPLC-PDA (System Gold; Beckman Instruments, Fullerton, CA, USA); analytes were detected at 270 nm using PDA detector (Beckman System Gold 168). Solution of tested compound (10^{-2} M; DMSO) was prepared and diluted to 10^{-4} M using McIlvaine buffer solution for the appropriate pH (2, 3, 4, 5, 6 or 7). One hour after incubation at 25 °C, 5 μL of the solution was directly injected onto a reversed phase column (Symmetry C18; 5 μm, 150 × 2.1 mm; Waters, Milford, USA). At flow-rate of 0.3 mL/min, the following binary gradient was used: 0 min, 90% B; 0–24 min; linear gradient to 10% A; 25–34 min; isocratic elution of 10% A; 35–45 min; linear gradient to 90% A, where A was 15 mM formic acid adjusted to pH 4 with ammonium and B was 100% methanol The HPLC measurement of the solutions was repeated after a 24 h incubation at 25 °C.

4.4. Evaluation of cytokinin activity

Three standard cytokinin bioassays were performed as described previously [39,40], based on the stimulation of tobacco callus growth, the retention of chlorophyll in excised wheat leaves and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons and used to reveal cytokinin activity as described [39].

Tobacco callus bioassay was slightly modified according to Nisler et al. (2016) [40]. It was performed in 6-well microplates (3 mL of MS medium containing tested cytokinin in each well, into which 0.1 g of callus was placed). Biological activity was determined from the increase in fresh callus weight after four weeks of cultivation [39,40].

In the *Amarathus* bioassay the seeds of *Amaranthus caudatus* var. *atropurpurea* were cultivated for 3 days in darkness before the roots of the seedlings were cut off. The explants, consisting of two cotyledons and a hypocotyl, were than cultivated with the tested cytokinin in a dark room. After 48 h incubation the concentration of betacyanin was determined [39].

In the wheat leaf senescence bioassay, the tip sections of the firs leaf of *Triticum aestivum* cv. Hereward seedlings were cultivated in microtiter plates containing a cytokinin solution. After 4 days in darkness leaves were removed and the chlorophyll retention was determined [39].

Tested cytokinin derivatives were dissolved in DMSO and further diluted as required in the media used for each bioassay to the final concentration range 10^{-4} to 10^{-8} M. The final concentration of DMSO in the media did not exceed 0.5%. Five replicates were prepared for each compound concentration and the entire tests were repeated at least three times. From the data acquired in these bioassays, the concentration inducing the strongest biological response was used to calculate the relative activity. The activity of BAP (benzylaminopurine; active standard) at the optimal concentration (10^{-5} M BAP for the *Amaranthus* betacyanin bioassay, 10^{-4} M BAP in the case of the senescence bioassay and 10^{-5} M BAP for the tobacco callus bioassay) was set at 100 and the activities of the tested compounds were related to it at their optimal concentrations. Optimal concentration for BAP in *Amaranthus caudatus* bioassay is 10^{-5} M, while its activity falls down rapidly at the concentration 10^{-4} M because it becomes toxic. However the activity of prepared compounds (excluding compound **2**) grows steadily through the whole concentration range and compounds are not toxic at the highest concentration, hence their highest activity is observed at 10^{-4} M concentration. That is the reason why the activity of different compounds was compared at different (optimal) concentrations.

4.5. Resazurin reduction assay on human skin cells

Resazurin reduction assay is a standard test of toxicity based on the measurement of reduction of blue weakly fluorescent resazurin into red highly fluorescent resofurin by metabolically active cells. Using the assay, the effects of *24 h treatments with several concentrations of the compounds (six fold dilution, maximal concentration = *50 μM) on viability of human skin fibroblasts BJ and keratinocytes HaCaT were evaluated. The cell lines were obtained from the American Type Culture Collection, Manassas, VA, USA the German Cancer Research Center (*DKFZ*), Heidelberg, Germany, respectively. About 5000 cells were seeded per well of a 96-well plate 24 h before the treatment. DMSO vehiculum was used as a negative control. After 24 h, 10× concentrated solution of resazurin in DMEM medium was added to the cells into the final concentration of 100 μM. Fluorescence (ex = 570 nm, em = 610 nm) was measured after 3 h incubation.

4.6. Phototoxicity and photoprotection assessment

NHDF were isolated from skin tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. The tissue was cut into pieces of approximately 1 × 1 cm, placed in Petri dishes and cultured in the mixture of DMEM and Ham's F12 Nutrient Mixture (1:3) supplemented with FCS (10%; v/v), penicillin (100 mg/mL), streptomycin (100 U/mL), amphotericin B (0.125 mg/mL), hydrocortisone (0.8 μg/mL), adenine (24 μg/mL), insulin (0.12 U/mL), epidermal growth factor (1 ng/mL) and 3,3′,5-triiod-L-thyronin (0.136 μg/mL). The skin fragments were incubated in a humidified atmosphere with CO_2 (5%; v/v) at 37 °C. The medium was changed weekly until the fibroblasts reached confluence. After 2–3 weeks cells were

trypsinized and transferred into 75 cm^2 cultivation flasks. Fibroblasts were used between the 2nd and 4th passage. Spontaneously immortalized aneuploid human keratinocyte cell line (HaCaT) was bought from CLS (Eppeheim, Germany). Both types of cells were grown in cultivation medium consisted of DMEM supplemented with fetal calf serum (10%, v/v), penicillin (100 mg/mL) and streptomycin (100 U/mL). For all experiments NHDF and HaCaT were seeded onto 96-well plates at a density of 0.8×10^5 cells/mL or 1.6×10^5 cells/mL (0.2 mL per well), respectively.

Phototoxic potential of test compounds was determined using the validated *in vitro* NRU phototoxicity test as described by Spielmann et al. with some modification [41]. Test substances included compounds **1**, **5**, **6**, **9** and **10**. Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/mL) and streptomycin (100 U/mL)). At 24 h after seeding, the cultivation medium was changed to serum free medium containing test compound or DMSO (a negative control). The final applied concentration range of test compounds was 3.9–125 μM. DMSO was used in the concentration of 0.5%, v/v). In parallel with test compounds, CPZ a known phototoxic compound, was used as a positive control in all experiments. CPZ was used in the range of 3.9–500 μM for non-irradiated HaCaT and 0.39–100 μM for NHDF and irradiated HaCaT. The test compounds (six replicate wells per concentration) were in parallel applied on two 96-well plates with NHDF/HaCaT. After 60 min incubation with test compounds medium was discarded, cells were washed two-times with phosphate buffered saline (PBS) and then PBS supplemented with glucose (PBS-G, 1 mg/mL) was applied. Randomly, one plate was then exposed to a non-cytotoxic dose of UVA radiation (5.0 J/cm^2 for NHDF and 7.5 J/cm^2 for HaCaT)) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320–400 nm. Intensity of UVA radiation was evaluated before each irradiation by an UVA-meter (Dr. Hoenle Technology, Germany). The second (non-irradiated) plate was for the period of irradiation incubated in dark. After UVA exposure, PBS-G was discarded and serum free medium was applied. After 24 h (37 °C, 5% CO_2) cell damage was evaluated by NR incorporation into viable cells. Medium was discarded and NR solution (0.03%, w/v, PBS) was applied. After 60 min NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and $CaCl_2$ (1%, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 min of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetitions with use of cells from four donors to minimize individual sensitivity of donor cells. Phototoxic effect was evaluated as % of viability of control cells that was calculated from experimental data (absorbance) according to the following equation:

$$\text{Viability (\% of control)} = \left(\frac{(A_S - A_B)}{(A_C - A_B)}\right) \cdot 100$$

A_S … absorbance of sample (cells pre-incubated with test compound in serum free medium and irradiated)A_C … absorbance of control (cells pre-incubated with DMSO in serum free medium and irradiated)A_B … absorbance of background (extraction solution)

Photoprotective effects were tested in compounds **1**, **5**, **6**, **9** and **10**. Compounds were dissolved in DMSO and then diluted in serum free medium). At 24 h after seeding, cultivation medium was changed to serum free medium containing test compound or DMSO (a negative control). The final applied concentration range of test compounds was 3.9–31.3 μM. DMSO was used in the concentration of 0.5%, v/v. RA, a known photoprotective compound was used for comparison of test compounds photoprotective effectiveness. Each test compound was in parallel applied onto two 96-well plates with NHDF/HaCaT. After 60 min incubation, medium with test compound was discarded, cells were washed two-times with PBS and PBS-G was applied. To study UVA photoprotection, a plate was exposed to a cytotoxic dose of UVA radiation (7.5 J/cm^2 for NHDF and 10 J/cm^2 for HaCaT) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320–400 nm. To study UVB photoprotection, a plate was exposed to a cytotoxic dose of UVB radiation (150 mJ/cm^2) using the solar simulator equipped with a H2 filter transmitting wavelengths of 295–320 nm. Intensity of UVA or UVB radiation was evaluated before each irradiation by UVA- or UVB-meter (Dr. Hoenle Technology, Germany). Control (non-irradiated) plates were for the period of irradiation incubated in dark. After UVA or UVB exposure, PBS-G was discarded and serum free medium was applied. After 24 h (37 °C, 5% CO_2) cell damage was evaluated by NR incorporation into viable cells as described above. The experiments were replicated four times using the cells of four donors to minimize the extent of biological variability of donor cells. The photoprotective effect was evaluated by comparison of experimental data (absorbance) of test compounds with a positive control and a negative control (according to the following equation:

$$\text{Protection (\%)} = 100 - \left|\frac{As - Anc}{Apc - Anc}\right| \cdot 100$$

As … absorbance of sample (cells pre-incubated with test compounds in serum free medium and irradiated)Anc … absorbance of negative control (cells pre-incubated with s DMSO in serum free medium and non-irradiated = incubated in dark)Apc … absorbance of positive control (cells pre-incubated with s DMSO in serum free medium and irradiated)

4.7. Oxidative stress bioassays in Caenorhabditis elegans

Wild-type (N2) strain and BA17 (fem-1) strain obtained from Caenorhabditis Genetic Center were used in the experiments. Buffers and media used in this assay were prepared according to literature [42]. Worms were maintained at 20 °C on NGM plates seeded with *Escherichia coli* OP50 which was used as a food source. Age synchronized L4 larvae obtained by hypochlorite treatment were washed from NGM plates with S-complete medium and number of worms in 10 drops of 10 μl volume was counted. Bacterial suspension and medium was added to the worm suspension to achieve 110 worms/mL with 6 mg/mL *E. coli* OP50 as a food source. In experiments with N2 strain, suspension was supplemented with 25 μg/mL of 2′-deoxy-5-fluorouridine (FUDR) to prevent reproduction of worms. Age synchronized BA17 worms were cultivated in 25 °C which lead to feminisation of the population and addition of FUDR was not necessary. Bacterial suspension in S-complete medium was prepared thusly: overnight culture of *E. coli* OP50 in LB medium was centrifuged and bacterium pellets were washed twice with sterile distilled water. After removing the excess of water, the pellets were weighed and re-suspended in S-complete medium. The test compounds were added three days prior to the exposure to 500 μM of 5-hydroxy-1,4-naphthalenedione (juglone) that is routinely used as a ROS-generating agent in stress assays with *C. elegans* [35]. Vehicle (DMSO) was used as a negative control. The worms were counted under the inverted microscope and the animals that failed to respond to the light stimulus were scored as dead. The protective effect of the compounds was evaluated after 4 h in the initial screening on populations of approximately 110 worms per experimental condition. The experiment was repeated 3 times. Statistical significance of the difference between proportions of living worms was determined using z-test. In the follow-up experiments on a larger populations worms (approximately 250 or

190 worms on average), the activity of selected promising compounds was evaluated hourly for 12–14 h. Log-rank test was used to determine the statistical significance of differences in the survival distributions. P-values were adjusted for multiple testing using Bonferroni method. Comparisons with p-values < 0.05 were considered as statistically significant. OASIS 2 was used for the data analysis [43].

4.8. Oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) was determined as described previously [44]. Briefly, fluorescein (100 μL, 500 mM) and solutions of tested compounds diluted in phosphate buffer were added into each working well of a 96-well microplate. The reaction was started by the addition of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH; 25 μL, 250 mM) and the decrease in fluorescence (ex. 485 nm, em. 510 nm) was recorded every 3 min over 90 min by using Infinite M200 Pro (Tecan, Switzerland). The net area under the curve was used to calculate antioxidant capacity which was compared to Trolox and expressed as Trolox equivalents (TE). Thus, a value higher than 1 means that the tested compound was more active than Trolox on equimolar basis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.03.043.

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Supplement II

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Review

Role of Cytokinins in Senescence, Antioxidant Defence and Photosynthesis

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Abstract: Cytokinins modulate a number of important developmental processes, including the last phase of leaf development, known as senescence, which is associated with chlorophyll breakdown, photosynthetic apparatus disintegration and oxidative damage. There is ample evidence that cytokinins can slow down all these senescence-accompanying changes. Here, we review relationships between the various mechanisms of action of these regulatory molecules. We highlight their connection to photosynthesis, the pivotal process that generates assimilates, however may also lead to oxidative damage. Thus, we also focus on cytokinin induction of protective responses against oxidative damage. Activation of antioxidative enzymes in senescing tissues is described as well as changes in the levels of naturally occurring antioxidative compounds, such as phenolic acids and flavonoids, in plant explants. The main goal of this review is to show how the biological activities of cytokinins may be related to their chemical structure. New links between molecular aspects of natural cytokinins and their synthetic derivatives with antisenescent properties are described. Structural motifs in cytokinin molecules that may explain why these molecules play such a significant regulatory role are outlined.

Keywords: cytokinin; derivative; antisenescent; antioxidant; structure and activity relationship; genes; antioxidant enzymes; photosynthesis; plant defence

1. Introduction

Naturally occurring cytokinins (CKs) are purine based plant growth regulators that influence almost all of the developmental stages of plant life, e.g., development of vasculature, differentiation of embryonic cells, maintenance of meristematic cells, shoot formation and leaf senescence delay. CKs were first discovered as substances that promoted cell division in tissue cultures in the presence of auxin [1]. Naturally occurring CKs are purine based molecules that are substituted at the C6 atom either by an isoprene side chain (ISCKs) or aromatic core (ARCKs). ISCKs are represented by naturally occurring 6-(*E*)-4-hydroxy-3-methylbut-2-enylaminopurine (*trans*-zeatin, *t*Z), 6-(*Z*)-4-hydroxy-3-methylbut-2-enylaminopurine (*cis*-zeatin, *c*Z) and 6-(2-isopentenylamino)purine (iP). ARCKs include 6-benzylaminopurine (BAP), 6-furfurylaminopurine (kinetin, Kin) and *o*-, *m*- and

p-hydroxylated or methoxylated derivatives of BAP, called topolins [2]. Although BAP and Kin are not traditionally considered as naturally occurring CKs, exogenous treatment with them showed such strong effects on plant tissues that these molecules were often used experimentally and formed the basis of the first generation of synthetic ARCKs [3]. Nucleosides, nucleotides and other sugar conjugates of many CKs have been found in plants, and metabolic networks exist for their interconversions [1]. Moreover, the presence of a purine moiety enables a number of possible modifications, including substitution at the C2, N1, N3, C6, N7 or N9 atoms of the original purine heterocycle [3]. The family of CK compounds includes a large array of natural and synthetic purine and phenylurea derivatives. The most effective phenylurea CK is thidiazuron (TDZ) [4]. The majority of these compounds are not considered as naturally occurring, however they possess significant CK activity [1]. The action of CKs is often influenced by interaction with other hormones, e.g., auxins or ethylene [4]. One of the most valued features of CK action and the focus of this review is regulation of senescence, especially senescence delay, owing to the potential for economic benefits [5–8]. Leaf senescence is the final step in leaf development and is often accompanied by colour changes from green to yellow or brown [9]. Leaf yellowing is not only age dependent, it can also be induced by a number of other factors, including biotic stress, mechanical damage, harvesting and environmental stress [10]. Leaf senescence seems to be directly related to a decrease in CK concentration and activity. Generally, CKs are more effective when applied to detached plant organs [5,11], however they can also delay the senescence of attached leaves. CKs are also effective in delaying the breakdown of chlorophyll, suggesting that they may play a role in maintaining the photosynthetic apparatus of plants [5]. Many of the biological activities of CKs in plants can be explained by their involvement in cellular oxidative stress, and the antioxidant capacity of these molecules has already been described [12]. Leaf senescence is accompanied by a gradual decline in antioxidants and an increase in reactive oxygen species (ROS) and content of malondialdehyde (a decomposition product of lipid peroxidation) in certain plants [13,14]. Increased lipid peroxidation and H_2O_2 formation have been demonstrated along with a decline in the activities of enzymes, such as catalase (CAT) and ascorbate peroxidase (APX), as well as glutathione (GSH) content during senescence in pea and *Arabidopsis* leaves [13,15].

Regulation of the onset of senescence is important owing to its impact on dynamic nutrient relocations during the degradation of cellular components [9]. Leaf senescence can be regulated in a number of ways that include ethylene production and expression of senescence associated genes (SAGs). Important roles are played by antioxidant enzymes, such as CAT, APX and superoxide dismutase (SOD), and compounds related to oxidative stress, such as phenolic acids, flavonoids and H_2O_2. Changes in chlorophyll are inseparably linked to photosynthesis. Exogenous treatment with CKs and their derivatives or genetic engineering to create plants that overproduce CKs can lead to significant senescence delay. In this review, we focus on the main mechanisms of action by which CKs and their derivatives delay leaf senescence.

2. Antisenescent and Antioxidant Activity of Natural Cytokinins (CKs), Kinetin (Kin) and 6-Benzylaminopurine (BAP)

The first evidence for the antisenescent activity of CKs was provided by Richmond and Lang [16] in an experiment testing exogenous application of Kin on excised leaves of cocklebur plants (*Xanthium pensylvanicum*) and tobacco. The results showed that this led to re-greening in yellowing tobacco leaves, however not in *Xantium pensylvanicum* [17]. The need to measure the ability of CKs in retarding chlorophyll degradation in various plant tissues prompted the development of several different senescence bioassays. CKs can be applied as droplets on leaves or by floating leaves (or their parts) on a CK solution or by inserting the base of a detached leaf into a CK solution [18]. The latter method has been optimized with wheat leaf segments [19], known as the wheat leaf senescence assay (WLSA), and is widely used to assess the antisenescence properties of CKs. Briefly, a tip section of a seven-day-old wheat leaf is inserted into a solution of CK and is placed in the dark for five days.

Afterwards, any chlorophyll remaining in the leaf tips is extracted by 80% ethanol and its content is determined spectrophotometrically at 665 nm [19].

Comparison of the antisenescent activity of different CK free bases in the WLSA performed according to protocol described by Holub et al. showed that *t*Z clearly had the highest activity, followed by 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *m*T), of which its activity was only marginally lower than that of *t*Z [19]. BAP and Kin had similar activity in the WLSA, however both compounds were less active than *t*Z and *m*T [19]. In contrast, 6-(2-hydroxybenzylamino)purine (*ortho*-topolin, *o*T), *cis*-zeatin and iP were considered as CKs with low or no antisenescent effect [19–21]. The activities of *t*Z, *c*Z, *m*T, BAP and Kin in the WLSA are compared in Figure 1A. Holub et al. [19] further showed that 9-ribosides of *t*Z, BAP, *m*T and *o*T were more active than their free bases [19]. In the case of *t*Z and *trans*-zeatin riboside (*t*ZR), this was in accord with results of Spíchal et al. [22] and Kim et al. [23]. Both *t*Z and *t*ZR strongly activated the *Arabidopsis* CK receptor AHK3, which was shown by Kim et al. [23], to play a crucial role in CK-mediated leaf longevity through phosphorylation of the CK response regulator ARR2. Conversely, benzylaminopurine riboside (BAPR) and *m*TR did not activate the AHK3 receptor [22], however they were found to be active in the WLSA [19]. Doležal et al. [24] suggested that ARCKs, especially *o*-, *m*-methoxy and halogeno derivatives of BAP, may protect against the degradation of the photosynthetic apparatus. This was based on findings that substituted ARCKs had antisenescent activities similar to or higher than those of *t*Z yet activated *Arabidopsis* CK receptors AHK3 and CRE1/AHK4 only weakly [22,24].

Better understanding of CK involvement in plant senescence was provided by analyses of CK concentration levels through plant development and tissues. These studies revealed an inverse relationship between CK levels and the progression of senescence in a variety of tissue and plant species [5,25]. The importance of naturally occurring ISCK sugar conjugates, dihydrozeatin riboside (DHZR) and zeatin riboside (ZR), in xylem sap during plant senescence was identified in experiments on soybean [26]. CK levels in the xylem sap of soybean (*Glycine max*) decreased rapidly with the onset of leaf senescence [26]. Reduction in endogenous CKs (zeatin (Z), dihydrozeatin (DHZ), ZR, DHZR) was also observed in tobacco leaves during the progression of leaf senescence [27]. However, in exogenous CK application experiments, it was unclear how much of the hormone was actually transported to and taken up by the plant tissues. Moreover, these factors may differ in different species or under different experimental conditions [25]. Podlešáková et al. published a study on the acropetal transport of 6-(3-methoxybenzylamino)purine (*meta*-methoxytopolin, *m*MT), 9-(4-chlorobutyl) and 9-(tetrahydropyran-2-yl, THP) derivatives of *m*MT. It was shown that the derivatives allowed the gradual release of the active base and had a significant impact on the distribution and amount of endogenous ISCK in various plant tissues [28]. Although a relationship between CK levels and senescence progression was found, the regulatory role of CKs in plant senescence was clarified using transgenic plants. Cloning of the *Agrobacterium* IPT (isopentenyl transferase) gene encoding a CK producing enzyme provided a way to genetically engineer plants in which endogenous levels of these hormones could be increased [6]. The effect of CK treatment on these genetically altered plants is discussed in the text below.

Since naturally occurring CKs contain heterocycles in their structure and are able to regulate leaf senescence, questions were raised about their possible antioxidant activity. Increased levels of H_2O_2, lipid peroxidation and membrane leakiness were observed during leaf senescence in tobacco, rice, pea, sunflower and barley [13,29–33]. External application of BAP increased the activity of CAT and APX enzymes and reduced the level of H_2O_2 during dark senescence of wheat leaves. It was suggested that externally applied CKs may protect the cell membranes and photosynthesis system from oxidative damage during the delay of senescence in the dark [34,35]. However, in dexamethasone-inducible IPT transgenic tobacco plants with increased levels of endogenous CKs, higher concentrations of H_2O_2 were detected, especially in chloroplasts. Moreover, the increased levels of H_2O_2 resulted into elevated lipid peroxidation [36]. It was shown by the analysis of the end-product of lipid peroxidation,

malondialdehyde (MDA), that lipid peroxidation is not associated with leaf senescence in stay-green tobacco plants [14].

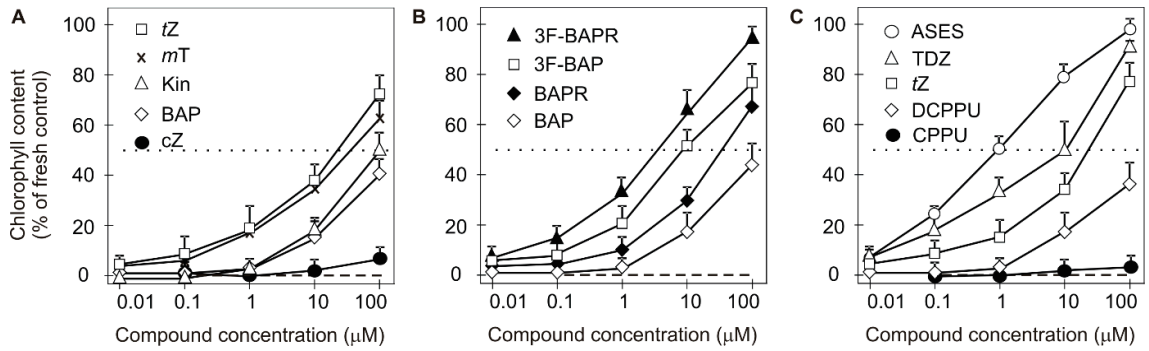

Figure 1. Evaluation of the biological activity of (**A**) classical cytokinins, (**B**) 3-fluoro derivatives of BAP and BAPR and (**C**) urea based cytokinins in the wheat leaf senescence assay performed in the dark (according to [19]). The dotted line indicates where the chlorophyll content in the leaves is 50% of that in fresh control leaves, while 100% represents the chlorophyll content in fresh control leaves. Dashed lines indicate values for the control treatment (DMSO control) with no added compound. Error bars show the S.D. of the mean for four replicate determinations.

3. Antisenescent and Antioxidant Activity of C2, C8 and N9 Purine-Based CK Derivatives

Since ISCKs and ARCKs appear to be crucial to senescence regulation, a number of their derivatives have been prepared to study the structure and activity relationship of these processes. In 1989, Zhang et al. measured the antisenescent activity of nine substituted CK derivatives using soybean leaf discs. Of the 14 BAP derivatives tested, including 9-alanine, 9-tetrahydropyranyl and 9-tetrahydrofuranyl, the last two derivatives were found to be the most active in the bioassay due to their great stability and ability to gradually release free BAP [37]. In the same year, another series of 9-substituted derivatives of four common naturally occurring CKs were tested in the soybean callus assay. Specifically, 9-(2-Carboxyethyl), 9-(2-carbo-*t*-butoxyethyl) and 9-(2-nitroethyl) derivatives of *t*Z, *c*Z, DHZ and iP all reduced the biological activity of the parent compounds [38]. Another 33 6-BAP-9-tetrahydropyranyl (THP) and 9-tetrahydrofuranyl (THF) derivatives with variously positioned hydroxyl- and methoxy-functional groups on the benzyl ring were prepared by Szüčová et al. [39]. The majority of the prepared derivatives showed higher antisenescent activity than the standard BAP or parent CKs (exceptions were derivatives substituted in *p*- positions or derivatives with a blocked N6-H atom) [39–41]. In contrast, derivatives shorter by a CH_2 group, i.e., 6-anilinoaminopurine derivatives, prepared by Zatloukal et al. were shown to be inactive in the WLSA [42]. Eight Kin derivatives were prepared by the addition of various halogenoalkyls, ethoxyethyl, carboxyl or THF groups to the N9 atom of the purine moiety. Some of these derivatives, especially those bearing THF, ethoxyethyl and chloroethyl, were highly active in the WLSA (reaching 110–131% of the original Kin) [34]. Moreover, compounds that were active in the senescence bioassay significantly reduced peroxidation of membrane lipids under dark conditions [34].

In addition to N9-substitution, a number of BAP derivatives were prepared by Doležal et al. with substitutions (halogen(s), methyl or methoxy group(s)) in various positions on the N6-benzyl ring [24]. Among them, 3-fluoro-BAP (3F-BAP) exhibited the highest antisenescent activity in the WLSA (Figure 1B). Some derivatives also possessed increased antisenescent activity compared to BAP (for details, see [24]). One year later, Doležal et al. published similar work on the preparation and testing of BAPR derivatives [43]. Unsurprisingly, BAPRs were found to be more effective than the corresponding BAPs (Figure 1B). Specifically, 3F-BAPR was the most active compound in the WLSA, with antisenescent activity up to 220% of that of BAP [43] (Figure 1B). Vylíčilová et al. [44] prepared 14 halogenated derivatives of BAPR by the addition of chlorine at the C2 atom of purine. The compounds exhibited increased antisenescence activity in the WLSA compared

to BAPs, however lower activity than BAPR derivatives. In other words, chlorine substitution at C2 decreased the antisenescent activity of BAPR and counteracted the positive activity of ribose. The antisenescent activity of 2-chloro-6-(3-fluorobenzylamino)purine-9-riboside (2cl-3F-BAPR) is thus similar to the activity of 3F-BAP. For clarity, we have again compared the antisenescence activity of BAP, BAPR, 3F-BAP a and 3F-BAPR in one WLSA according to Holub et al. [19] (Figure 1B). In addition, Vylíčilová et al. further showed that the introduction of chlorine to the C2 position of the purine moiety dramatically decreased the toxicity of several toxic previously prepared BAPs and BAPRs [24,43,44]. The antisenescent activity of 2-chloro-6-(3-fluororbenzylamino)purine-9-riboside (2Cl-3F-BAPR) was similar to the activity of 3F-BAP. However, this study of Vylíčilová et al. was the first to uncover possible targets of the BAPR derivatives in the inhibition of chlorophyll degradation [44]. Genome-wide expression profiling showed that the synthetic halogenated derivatives of BAPR affected transcription of a unique combination of genes coding for components of the photosystem II (PSII) reaction centre, light-harvesting complex II (LHCII) and oxygen-evolving complex, as well as several stress factors responsible for regulating photosynthesis and chlorophyll degradation [44].

More recently, 11 Kin derivatives were prepared and tested in the WLSA. The presence of an oxygen atom in the furan ring was shown to be a critical structural motif for slowing the breakdown of chlorophyll in the WLSA. Replacement of the oxygen atom by sulphur or carbon resulted in decreased antisenescent activity. On the other hand, saturation of the furan ring did not have such a negative impact on the antisenescent activity. Moreover, 9-THF substitution had no effect or slightly improved the antisenescent activity of 6-(tetrahydrofuran-2-ylmethyl)aminopurine, Kin and 6-(thiophen-2-ylmethyl)aminopurine (thiokinetin). The addition of chlorine at the C2 atom of purine moiety lowered or completely reduced the antisenescent activity of the prepared compounds. Furthermore, the prolongation of the C-bridge carrying the N6-substituent of 6-(2-thiophen-2-ylethyl)aminopurine led to the complete loss of the antisenescent activity in the WLSA [45].

Synthetic CK analogues, particularly 6-alkynyl and 6-alkenylaminopurines, some of which were also substituted at the N9 atom of the purine moiety, have been tested for their antioxidant activity as potential diphenylpicrylhydrazyl (DPPH) scavengers and as inhibitors of 15-lipoxygenase enzyme, together with naturally occurring CKs BAP, Kin and *t*Z [46]. Whereas naturally occurring CKs were unable to scavenge DPPH, some of the prepared compounds were significantly more active than BAP, Kin and *t*Z. The most active compounds were 6-(3-thienylethenyl)purine, with 18% scavenging activity after 15 min, however also derivatives incorporating 2-furyl in their structure [46].

Substitution of the C6 atom of the purine moiety seems to be crucial for the antisenescent properties of the prepared derivatives. A unique example of an inactive CK is isopentenylaminopurine (iP). Neither iP nor its derivatives are active in the WLSA. To test this, a series of N6-(3-methylbut-2-en-1yl)amino)purine (iP) derivatives substituted at the N9 atom of the purine moiety was prepared in 2011 [21]. As expected, none of these compounds were active in the WLSA, although the prepared compounds were evaluated as active CKs in other CK bioassays [21].

Recently, another 58 CK derivatives substituted at the C8 atom of the purine moiety, 27 of which were 9-tetrahydropyranyl precursors of CKs, were published by Zahajská et al. [47]. The introduction of C8 substitution led to a decrease or even the complete loss of antisenescent activity in the majority of compounds compared to the corresponding free bases. However, 6-benzylamino-7,9-dihydro-8H-purin-8-one (8-oxo-BAP) exhibited higher activity than BAP by 34%. Concurrently, among Kin derivatives, methoxy- and 2-hydroxyethyloxy-C8 substituents did not decrease the activity of Kin. With some exceptions, compounds with a 9-(tetrahydropyran-2-yl) protective group exhibited even lower activity in the WLSA than their THF deprotected analogues [47]. This observation is in contrast to previous studies, in which the introduction of 9-THP or 9-THF protective groups was found to not decrease the antisenescent activity of the corresponding free bases [37,40,45,48].

According to the above, the crux of the antisenescent activity of CKs appears to be appropriate substitution at the N6 atom. Concurrently, the N6-H hydrogen must stay unsubstituted. Substituents at

nearby atoms, such as N6, must contain an oxygen atom incorporated into oxo-, hydroxyl or methoxy groups and/or halogen atoms, such as fluorine or chlorine. Further, *t*Z, Kin and topolins are typical examples of CKs shown to be very active in antisenescent bioassays. Substitution at C8 drastically reduces the antisenescent properties of nearly all, even the active CKs, with one exception: compounds substituted by oxygen at C8 remain active. Substituents at the N9 atom can vary, however they do not normally affect the antisenescent properties much if the N6 substitution is "antisenescent", with the exception of ribosides, which in some cases are more active. Questions arise concerning whether other sugars at the N9-position may have a similar or even better effect. The structural trends described above show that the antisenescent effects are probably connected with the presence of electronegative atoms, oxygen and halogens, which are close to the N6 and/or N9 atoms of purine. Both the N6H and N7H hydrogen atoms are present and "free to operate" in all active compounds. The second condition is the presence of –O–, –OH, –X or $–OCH_3$ substitutions close to this area. These two factors can lead to the concentration of electron density and increase of antisenescent activity in such compounds. The electron density enhancement at these strategic atoms is most probably responsible for the antisenescent effects.

Based on the available literature and a large amount of data, it is evident that the antisenescent properties of some CK derivatives are not directly associated with their CK activity. Moreover, iP and its derivatives are active CKs, however they are inactive in the WLSA [21,47]. Conversely, some derivatives of BAP, BAPR, 9-THF (THP)—BAP and Kin exhibit low CK activity at the receptor level, lower or average activity in other CK bioassays, however increased antisenescence activity [34,39,43].

An example of such phenomena—the presence of an oxygen containing group and lack of relation of CK activity to the antisenescent activity—was provided recently by Nisler et al. [49]. They showed that urea derived compounds are extremely active in the WLSA (Figure 1C) if they contain a methoxy group (a compound called ASES), hydroxyl or other electron-rich groups. Interestingly, the compounds also needed to have a second NH not substituted (like the N6H in purines) to be active in the WLSA. It was also shown that the compounds exhibited none or very low CK activity in almost all CK assays. Analysis of chloroplast membrane proteins showed that one way in which these urea derivatives delay senescence is by inhibition of photosystem II degradation [49]. This study agrees with that of Vylíčilová [44] and, at the protein level, supports and complements results at the transcriptional level.

Finally, it is worth noting that according to the literature, CKs (as well as other compounds with antisenescent properties) inhibit chlorophyll degradation by several mechanisms that may act synergistically. Some have been identified and well described, whereas others await discovery.

4. Antisenescent Activity of Urea Based CKs and Their Derivatives

Urea based compounds represent another class of highly active synthetic CKs. The best known are TDZ, *N*-(2-chloropyridin-4-yl)-*N*′-phenylurea (phenylurea, CPPU) and *N*-(2,6-dichloro-pyridin-4-yl)-*N*′-phenylurea (DCPPU). So far, TDZ appears to be the best representative of the synthetic CKs in terms of CK receptor activation [22], promotion of CK dependent callus growth [50,51] and, most importantly, inhibition of plant senescence [52–55]. In most CK bioassays, including the WLSA, TDZ exhibits even higher activity than *t*Z [22,49]). This may be due to a number of reasons, e.g., TDZ exhibits strong CK activities in several bioassays, however in contrast to *t*Z, cannot be degraded by cytokinin oxidase dehydrogenase (CKX, a key enzyme involved in CK degradation in plants [56,57]). TDZ cannot be deactivated by *O*-glucosylation and can maintain a higher content of endogenous CKs in plant tissue by inhibiting the function of CKX [58–60]. Owing to its high CK and particularly antisenescence activity, TDZ has been extensively studied and used to prolong the life of cut flowers [55]. In this field, no other substance has received as much attention as TDZ.

Several mechanisms for explaining how TDZ delays plant senescence have been shown in studies of various plant species [61]. In *Matthiola incana* cut flowers, TDZ reduced stress responses by inhibiting abscisic acid production, resulting in a higher content of chlorophyll and carotenoids [61] in stem leaves. Like ethylene, abscisic acid promotes senescence [62], occurs as a response to stress and is considered

to be a marker of stress-induced senescence [63]. It was further shown that in *Pelargonium* cuttings, TDZ induced strong expression of PhETR1 (a negative acting ethylene receptor gene), thus decreasing the sensitivity of *Pelargonium* leaves to ethylene [64]. Ethylene is known to accelerate and accompany senescence [65,66]. In *Pelargonium zonale*, TDZ was shown to increase the levels of APX and SOD, which are enzymes of the antioxidant defence system [67]. Another complex study was carried out on stems and florets of cut *Chrysanthemum morifolium* plants. Both BAP and TDZ were found to increase the activity of antioxidant enzymes SOD and peroxidase (POD), reduce production of H_2O_2, minimize lipid peroxidation and maintain high levels of sugars in cut stems and florets. Treated plants also showed increased water uptake and prolonged the post-harvest quality of florets and leaves. In this study, TDZ again exhibited higher activity and in lower concentrations than BAP [68]. The antisenescent activity of CPPU, DCPPU, TDZ and ASES was compared in one WLSA (Figure 1C) according to Holub et al. [19].

To conclude, from the literature reviewed, it appears that TDZ delays senescence in plants by the same mechanisms as described previously for purine-derived CKs. However, its activity is higher, most probably because it cannot be enzymatically inactivated.

5. Ability of CKs and CK Derivatives to Improve the Antioxidant Capacity and Secondary Metabolite Content

CKs and their derivatives are often prepared for plant biotechnological applications and preferentially used in tissue cultures [3]. When particular CKs or their derivatives are employed in the micropropagation media of species with known antioxidant properties, plants exhibit increased antioxidant capacity through the explants grown [69]. In particular, a number of medicinal plants and often those high in antioxidants must be grown using micropropagation techniques due to their endangered status in nature. Such effects have been observed during the tissue culture and acclimatization of *Merwilla plumbea*, a plant widely used in traditional African medicine in Southern Africa and currently threatened in the wild because its medicinal use includes the bulbs [70]. Five CKs and their derivatives were evaluated during *M. plumbea* micropropagation: BAP, iP, *m*T, 3-*m*TR and 6-(3-methoxybenzyl)-9-(tetrahydropyran-2-ylamino)purine (*meta*-methoxytopolin THP, *m*MTHP) [70]. The antioxidant activity and phenolic acid content during the tissue culture and acclimatization of the plantlets were determined. The findings indicated that the phytochemical content during in vitro propagation of *M. plumbea* were influenced by the CKs and the majority of the phenolic acids were higher in the tissue culture than in the acclimatized plantlets [70]. Similar effects on the secondary metabolite content were found in a study on micropropagated "Williams" banana. However, the activity of specific compounds differed for different plant species [69]. The influence of CKs from tissue culture media on flavonoid levels of micropropagated plantlets is important as flavonoids have been identified as compounds that are able to scavenge free radicals. [71]. TDZ was found to significantly influence shoot multiplication and accumulation of secondary metabolites in *Scutellaria altissima* culture. *Scutellaria* plants express several pharmacologically and clinically important phytochemicals [72]. In particular, the flavonoid verbascoside possesses strong anti-inflammatory, antibacterial, antiviral and antioxidant activity [73]. Under the conditions used in the study, 2.5 times more flavonoids and six-times more verbascoside were accumulated in shoots that were grown on the medium supplemented with CKs than in shoots that were grown on CK free medium [74]. In further work on *Scutellaria altisima* explants, a higher level of other important flavonoids, such as baicalin and wogonoside, were observed in 12-week-old micropropagated plants. The shoot cultures were grown on MS agar medium that was supplemented with BAP [75]. Wogonoside in particular is interesting as this flavonoid is very active against lipid peroxidation [76]. The medicinally important plant *Eucomis autumnalis* has been treated with 0.01, 0.1 and 10 µM of 2-chloro-6-(3-methoxyphenylamino)purine (INCYDE) and CK antagonist 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55) alone or in combination with BAP or naphthaleneacetic acid and the antioxidant response was evaluated in 10-week-old in vitro

regenerates [77]. The levels of phytochemicals, especially those of flavonoids, were significantly affected by both tested compounds PI-55 as well as INCYDE. Besides, INCYDE application significantly increased the antioxidant activity of *E. autumnalis* in the DPPH test. On the other hand, the *beta*-carotene test was unaffected by INCYDE, however it was enhanced by BAP when it was used as a control [77]. Significantly improved antioxidant activity in the oxygen radical absorbance capacity (ORAC) assay was observed after one application of INCYDE in field-grown lettuce [78]. Both Kin and BAP enhanced the production of hypericins in *Hypericium maculatum* and hyperforin in *H. hirsutum* shoot cultures [79]. Naphthodianthrones (hypericin and pseudohypericin) and phloroglucinol hyperforin are valuable compounds that are associated with antiviral, antioxidant and other biological activities [80]. The increased flavonoid content and antioxidant activity of the common European herb sage (*Salvia officinalis* L.) has also been assessed during micropropagation [81]. The effects of four different CKs, i.e., TDZ, BAP, Z and iP, were evaluated. Levels of phytochemicals apigenin and its derivatives apigenin 7-methyl ether, scutellarein 6-methyl ether, scutellarein 6,7-dimethyl ether and luteolin were found to be comparable to those measured in plants grown on media without added CKs. On the other hand, BAP added to the media caused the production of hardened plants that successfully adapted in ex vitro conditions [81].

Aside from the above effects, CKs and their derivatives are able to influence the activity of antioxidant enzymes [35,82]. It was observed that the application of BAP prevents the degradation of chlorophyll in wheat senescent leaves and increases the activity of enzymes CAT and APX [35,83–85]. Increased SOD activity was observed after addition of *t*ZR to the grass *Agrostis palustris* [86]. BAP also increased levels of the APX enzyme after four and six days' incubation of wheat leaves [35]. Application of BAP reduced H_2O_2 accumulation and lipid peroxidation of *Litchi* [83]. Furthermore, higher activity of SOD, CAT and APX and DPPH radical scavenging capacity were found in BAP treated *Litchi*. Data showing the influence of antioxidant compounds in different plant species are summarized in Table 1. It is apparent that the activity of individual CKs varies depending on the plant species and secondary metabolite group. However, whereas H_2O_2 and lipid peroxidation levels usually decrease after CK treatment, antioxidant enzymes CAT, APX and SOD, and antioxidant secondary metabolites, such as phenolic acids and flavonoids, increase.

Table 1. Effects of cytokinins on levels of antioxidant related enzymes, secondary metabolites and antioxidant activity in different assays. Arrows show ↑ increase or ↓ decrease in concentration or activity.

Cytokinin	Plant	Concentrations of Antioxidant Related Enzymes, Secondary Metabolites and Antioxidant Activity	Reference
BAP	St John's-wort (*Hypericum hirsutum* sc.)	↑hyperforin	[79]
	St John's-wort (*Hypericum macalatum* sc.)	↑pseudohypericin, hyperforin	[79]
	Wheat (*Triticum aestivum* L.) leaves	↑CAT, ↑APX, ↓level of H_2O_2	[35]
	Litchi (*Litchi chinensis Sonn*). fruit	↑SOD, ↑CAT, ↑APX, DPPH assay ↓level of H_2O_2, ↓lipid peroxidation,	[83]
	Skullcap (*Scutellaria altisima*) explants	↑baicalin, ↑wogonoside ↓lipid peroxidation	[76]
	Eggplant (*Solanum melongena* L.) plants	↓lipid peroxidation, ↑SOD, ↑CAT, ↑POD, ↑APX	[84]
	Wheat (JM20) plants	↓lipid peroxidation, ↑SOD, ↑CAT, ↑POD, ↑APX	[85]
	Summer maize (hybrids DengHai605, Zheng-Dan958) plants	↓lipid peroxidation, ↑SOD, ↑CAT, ↑POD	[87]

Table 1. *Cont.*

Cytokinin	Plant	Concentrations of Antioxidant Related Enzymes, Secondary Metabolites and Antioxidant Activity	Reference
BAP	Rice (*Oriza sativa* cv. Taichung Native 1) leaves	↓lipid peroxidation	[88]
BAP	Maerwilla (*Merwilla plumbea*) explants	↑phenolic acids (PA, VA)	[70]
iP		↑phenolic acid (CafA)	
*m*TR		↑phenolic acids (PA, FA, 4CA), ORAC	
*m*T		ORAC	
*m*T	*Banana* (*Musa* spp. AAA cultivar 'Williams') explants	↑total phenolics, ↑proanthocyanidins	[69]
*m*MTTHP		↑total phenolics, ↑total flavonoids, ↑proanthocynidins	
*m*MTTHP	Maerwilla (*Merwilla plumbea*) explants	↑phenolic acids (4CA, FA)	[70]
*t*ZR	Creeping Bentgrass (*Agrostis palustris* L.) plants	↓lipid peroxidation, ↓electrolyte leakage, ↑SOD, ↑CAT	[86]
*t*ZR	Creeping Bentgrass (*Agrostis palustris* L.) plants	↓lipid peroxidation, ↑SOD, ↑CAT	[89]
TDZ	Skullcap (*Scutellaria alpina*) explants	↑flavonoids (BC, WO) ↑verbascoside	[75]
CPPU	Maize (*Zea mays* L.) seedlings	↓lipid peroxidation, ↓level of H_2O_2, ↑CAT	[90]
CPPU	Tomato (*Lycopersicon esculentum* Mill.) leaves	↓lipid peroxidation, ↑SOD, ↑APX	[91]
INCYDE	Lettuce (*Lactuca sativa*)	↑4CA, ↑FA, ORAC	[78]
INCYDE	*Eucomis autumnalis* explants	↑flavonoids, DPPH and β-carotene acid antioxidant assay	[77]
PI-55	*Eucomis autumnalis* explants	↑flavonoids, DPPH and β-carotene acid antioxidant assay	[77]
Kin	St John's-wort (*Hypericum hirsutum* sc.)	↑hyperforin	[79]
Kin	St John's-wort (*Hypericum macalatum* sc.)	↑pseudohypericin, hyperforin	[79]
Kin	Tomato (*Solanum lycopersicum* L.) plants	↓level of H_2O_2, ↓lipid peroxidation, ↓electrolyte leakage, ↑SOD, ↑CAT, ↑ascorbate-glutathione cycle, ↑total phenols, ↑flavonoids	[92]
Kin	Oat (*Avena sativa* L. cv. Victory) leaves	↓lipid peroxidation, ↑SOD, ↑CAT,	[29]
Kin	Anthurium (*Anthurium andraeanum* Lindl.) leaves	↑APX	[82]

4CA, 4-coumaric acid; APX, ascorbate peroxidase; BAP, 6-(benzylamino)purine; BC, baicalin; CAT, catalase; CafA, caffeic acid; CPPU, *N*-(2-chloropyridin-4-yl)-*N*′-phenylurea; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FA, ferulic acid; INCYDE, 2-chloro-6-(3-methoxyphenylamino)purine; iP, 6-(2-isopentenylamino)purine; Kin, kinetin; mMTTHP, 6-(3-hydroxybenzylamino)purine-9-THP; mT, meta-topolin, 6-(3-hydroxybenzylamino)purine; mTR, 6-(3-hydroxybenzylamino)purine riboside; ORAC, oxygen radical absorbance capacity; PA, protocatechuic acid; PI-55, 6-(2-hydroxy-3-methylbenzylamino)purine; POD, peroxidase; sc, shoot culture; SOD, superoxide dismutase; TDZ, thidiazuron; *t*ZR, *trans*-zeatin riboside; VA, vanillic acid; WO, wogonoside.

6. CKs and (A)Biotic Stress Responses

CKs play an important and complex role in abiotic stress responses [93]. Endogenous CK levels decrease when the plant is under abiotic stress, such as mineral, salt or drought stress. Transgenic plants overexpressing an IPT gene under the control of a maturation and drought-induced promoter were shown to recover from drought [94]. In addition, CKs were shown to enhance immunity to biotic stress [94]. Some pathogens are able to "use" host plants to produce high levels of CKs, such as the gram-negative bacterium *Agrobacterium tumefaciens* [95]. *A. tumefaciens* uses AHK3 and AHK4-dependent transcription reprogramming to make host cells more receptive to infection [96,97]. A similar situation occurs during infection by the gram-positive bacterium *Rhodococcus fascians*, which uses a higher CK production pool for leaf gall formation and extending the ectopic growing shoot primordia [97–99]. Some fungal pathogens also produce CKs, such as the ergot fungus *Claviceps purpurea*, which infects the ovaries of rye [100]. These pathogens reprogramme the

biosynthesis of CKs for more extensive infection. On the other hand, exogenous application of CKs affects salicylic acid mediated defence responses, e.g., in *Pseudomonas syringae* pv. tomato [97,101].

CKs have been suggested to be instrumental in mediating host susceptibility to fungal biotrophs by generating a green island around infection zones [102,103]. Although CKs are undoubtedly involved in defence mechanisms against biotic stress, their production often accompanies attack by particular phytopathogens. There is evidence that the reduction in photosynthesis in infected leaves results from increased invertase activity [103]. Paradoxically, infection seems to have a positive effect on antisenescent processes in the host plant as part of the protection of chlorophyll against disintegration (green islands). This antisenescent "improvement" is probably induced by the pathogen to gain more plant material for spreading its infection. However, although we know some of the ways by which these pathogens affect antisenescent responses, much remains unknown.

7. CKs and Photosynthesis

The main function of leaves is to provide assimilates for plant growth through photosynthesis. CKs affect the functional as well as the structural aspects of photosynthesis at several levels. CKs induce cell division and differentiation even in the early stages of leaf development. Chernyaev et al. studied and reviewed the effect of CKs at the level of the whole leaf and found that they changed the leaf structure to have a greater number of cells per leaf area [104] and a larger number of vascular bundles and xylem and phloem elements [105]. Another structure closely connected to photosynthesis at the leaf level is the stomata. CKs acting as antagonists of abscisic acid can increase stomatal conductance [61,92,106–108] and, thus, modulate leaf gas exchange and the availability of CO_2—the essential substrate for photosynthetically active tissue [6].

At the cellular level, CKs have a major effect on chloroplasts. As early as 1969, Boasson and Laetsch [109] reported that when etiolated tobacco leaves were transferred to light, the application of CKs increased the number of chloroplasts. Further research confirmed that CKs promoted the differentiation of etioplasts, their transition to chloroplasts [110–112], chloroplast division [113] and, finally, increased the number of chloroplasts [114]. In the presence of BAP, greening and plastid biogenesis in *Lupines lutus* and *Cucumis sativus* were substantially promoted [115,116]. Concurrently, a higher concentration and slower degradation of light-sensitive protochlorophyllide oxidoreductase, a key enzyme in chlorophyll biosynthesis, was observed in *Lupines lutus* and *Cucumis sativus* cotyledons [115,116].

At the level of the thylakoid membrane, it has been observed that CKs promote grana formation [110,117,118] and increase the content of photosynthetically active pigments [118–120] and starch grains [117,121–124]. CK regulation of chloroplast development, chlorophyll biosynthesis and nuclear plastid-related as well as plastid genes have been reviewed recently (for further reading, see [125]). CKs are reported to affect pigment-protein complexes involved in the light (primary) phase of photosynthesis, as well as enzymes of the dark (secondary) phase. Of the genes most upregulated by CKs, the most widely documented genes are those coding for the light-harvesting chlorophyll *a/b* binding proteins of photosystem II (*CAB*) and small and large subunits of RUBISCO (*RBCS*, *RBCL*) [112,126,127].

A close relationship between CKs and chloroplasts was confirmed by work showing that chloroplasts contain CKs of endogenous origin [128–130]. Chloroplasts of *Arabidopsis* contain four of seven IPT enzymes (AtIPT1, AtIPT3, AtIPT5 and AtIPT8) that catalyse the rate-limiting step of CK biosynthesis [129]. Senescence as the final stage in leaf development is accompanied by a decrease in CK content. Ananieva et al. [131,132] showed that progression of senescence of zucchini cotyledons correlated with a gradual decrease in the concentration of physiologically active CKs, together with an increase in the storage CK O-glucosides. Similarly, the content of active CK forms decreased in detached wheat and *Arabidopsis* leaves senescing in darkness [133,134].

As the photosynthetic apparatus is a possible source of ROS that may lead to destruction of assimilates that are intended for transport to growing or store tissue, its decomposition

during senescence is highly regulated. In this regulation, CKs again play an important part. During senescence, changes in the chloroplast ultrastructure are well-documented, such as alterations in size and shape, disorganization of the thylakoid membrane, increased number of plastoglobuli and decreased chlorophyll content and photosynthesis function (for a review, see [135]). Application of exogenous CKs usually slows down typical senescence-induced changes, e.g., chlorophyll content decrease [119,123,136–138], plastoglobuli formation [118,121,139] and drop in key photosynthetic parameters, such as the rate of CO_2 assimilation (A) [6,87,92,140,141], photochemical quenching (qP) and maximal photochemical efficiency of PSII (F_v/F_m) [87,91,92,123,142,143]. The senescence decelerating effect of CKs is not only connected to the upregulation of genes for biosynthesis and protection, however also to the downregulation of *SAGs* [144,145] and CK-mediated inhibition of degrading enzymes (e.g., activity of chlorophyllase, Mg-dechelatase, chlorophyll degrading peroxidase [146], RNase activity [147] and expression of pheophytinase [148]).

The effect of each CK ligand depends not only on its structure and concentration, however it also may be species-specific. Various CKs show modified ligand affinity for their receptors, e.g., in *A. thaliana*, *Brasica napus*, maize and potato [22,149–151]. The diverse effects of particular CKs regarding physiological responses to exogenous application have also been documented. Whereas BAP is widely known for its antisenescence effect on leaves of, e.g., broccoli, bean, barley, maize and wheat [85,87,137,152,153], after application to lettuce, no or only a weak effect was found [152,154]. Similarly, kin has repeatedly been reported as very active in senescence deceleration (e.g., [155–157]), however it showed an ambiguous effect on *Anthurium* [82]. It should be noted that the CK effect also depends on the age of cells and leaves [158] and light conditions [123,158,159].

8. Mechanisms and Genes Implicated in the Antisenescent and Antioxidant Activity of CKs

The ability of plants to perceive CKs acts through a modified bacterial two-component pathway that functions via a multi-step phosphorelay [160]. CK molecules in *Arabidopsis* are perceived by sensor histidine kinase receptors, named AHK2, AHK3 and CRE1/AHK4 [161–163]. Gene responses to CK treatment have been examined and reported a number of times [93,161,164]. The first genes to be induced by CKs (IBC genes), now known as A response regulator genes ARR5 and ARR4, were identified in 1998 [161,165]. AHK3 in particular was found to be the major *Arabidopsis* CK receptor connected with the antisenescent effect of CKs. It also plays a major role in CK-mediated leaf longevity through specific phosphorylation of a type-B response regulator, ARR2 [23,166]. Zwack et al. [167] showed that cytokinin response factor 6 (CRF6), the expression of which is downstream of the perception of CKs by AHK3, is involved in negative regulation of senescence. Recent research demonstrated that AHK2 and AHK3 are implicated in the routine repair of D1 protein, which is necessary for the functioning of photosystem II (PSII). This protective function of CKs during light stress depends on type B ARR1 and ARR12 [168]. On the other hand, the receptor CRE1/AHK4 probably affects oxidative damage during senescence. Janečková et al. [134] studied *Arabidopsis* mutants with two non-functional and only one functional CK AHK receptor and observed that the dark senescence induced increase in lipid peroxidation was retarded in the mutant with solely functional CRE1/AHK4 and also partially in the mutant with solely functional AHK2 receptor. Different patterns of hormonal regulation suggest that CKs may act transcriptionally to alter responses to ROS that are often produced during abiotic stress [93]. Over the last 10 years, using the model *Arabidopsis* plant, several transcriptomic datasets related to CKs have been generated from different technological platforms, culminating in RNA sequencing [164]. It is well-known today that both exogenous application of active CKs and an increase in their endogenous content can delay senescence [6,134,169]. Current molecular genetics strategies to manipulate leaf senescence are based either on enhancing CK production [6,7] and perception [170] or exogenous application of CKs and their derivatives [6,171]. It was shown that it is possible to genetically engineer IPT plants to overproduce CKs [6,7]. These IPT plants as well as exogenously added CKs, e.g., BAP, can be used in vegetables such as broccoli to effectively delay senescence as well as postharvest senescence owing to their antagonistic effect on ethylene [172].

Functional analysis of differentially expressed genes in BAP supplied postharvest broccoli showed regulation in the expression of genes involved in CK signalling, nutrient transport and photosynthesis. The addition of BAP caused downregulation of the gene responsible for ethylene synthesis [172]. Flowers of *Petunia x hybrida* transformed with PSAG12-IPT that overproduced endogenous CKs during the senescence period and produced the same amount of ethylene as wild-type flowers, however were less sensitive to exogenous ethylene and had a much longer lifetime [173]. This demonstrates that endogenous CKs lower the sensitivity of plants to ethylene and extend their lifetime.

Many genes associated with senescence (SAGs) have been identified [174]. SAGs are genes that are expressed during senescence [170] that encode for degradative enzymes, such as RNAses [175], proteinases [176–178] and lipases [179], and products involved in nutrient translocation processes [180]. Temporal control of senescence was achieved when the promoter of the SAG12 gene was linked to an IPT gene [94]. The SAG12::IPT autoregulatory senescence inhibition system has also been successfully implemented in a number of important crops, such as rice, ryegrass, tomato, alfalfa, cauliflower, wheat, cassava and cotton [171,181]. Currently, it is used to delay senescence in green vegetables, such as lettuce and broccoli [94]. P_{SAG12}-IPT tobacco plants that were grown in a growth-limiting nitrogen supply showed delayed senescence-associated declines in nitrogen, protein and Rubisco levels and photosynthesis rates [182]. Weaver and Gan published the expression of several *Arabidopsis thaliana* SAGs in attached and/or detached leaves and compared the response to age, dehydration, darkness, abscisic acid, cytokinin (BAP) and ethylene treatment [144]. BAP inhibited SAG expression in dark detachment experiments and the inhibition was generally greater in younger leaves. Thus, there is an indisputable connection between CK action and the light influence on senescence as the key factors in senescence regulation [134]. Lara et al. demonstrated that CKs upregulate the expression of extracellular cell wall invertase and that this enzyme is required for the delay of senescence caused by CKs [183]. This enzyme plays a crucial role in source-sink regulation, which has been shown to be a key molecular mechanism in CK senescence inhibition [183]. Vylíčilová et al. prepared a series of 2,6-disubstituted ARCK derivatives in 2016 and investigated the relation between the antisenescent effect of these compounds derived from CKs and their influence on photosystem II [44]. For most of the active compounds, regulation of gene expression in senescent *Arabidopsis* leaves was observed. In agreement with previously published data, it was shown that CK derivative treatment upregulated CK response regulators and other CK responsive genes, such as ARR15, ARR5, ARR8, ARR7, ARR4, ARR6, ARR9, CKX4, CRE1/AHK4, CRF2 and CRF5, however also genes encoding components of the photosystem II light harvesting complex (LHCII), i.e., At2g05070, At5g54270, At1g44575, At3g55330 and At2g39470, even though BAP had a negligible effect on these genes [44]. This supports the abovementioned suggestion of the need for electronegative atoms (which BAP lacks) in the vicinity of N6H and N7H.

Transgenic plants have also been used to examine the influence of endogenous CKs on antioxidant defence systems. CKs induced the activity of antioxidant enzymes in transgenic plants, e.g., during plant ontogeny of P*ssu*-ipt tobacco [184]. Whereas the control plants showed a decline in total CK content, the transgenic plants exhibited at least a 10 times higher content of CKs than the controls, especially of Z and ZR. The transgenic plants also showed elevated activity of antioxidant enzymes, such as glutathione reductase (GR), SOD and APX [184]. Old tobacco leaves of P_{SAG12}-IPT plants had a much longer lifespan and the concentrations of antioxidants ascorbate and GSH were higher than in wild-type leaves. At the same time, the chlorophyll and protein contents together with photosynthetic rate were increased. The decline in activity of antioxidative enzymes APX, GR and SOD during senescence was slowed down by CKs that were produced in P_{SAG12}-IPT plants [185]. It was shown that old leaves of P_{SAG12}-IPT plants and their chloroplasts maintained higher physiological parameters than in the control due to the extension of the period of greater antioxidant protection [186]. In a field study, stay-green cv. P3845 of *Zea mays* with enhanced levels of endogenous CKs showed higher CAT and SOD activities than earlier senescent cv. Hokku 55 [187].

The effects of exogenous CKs on photosynthetic capacity and antioxidant enzyme activities were evaluated in WN6 (a stay-green wheat cultivar) and JM 20 (control wheat, [85]). WN6 reached a higher grain mass, mainly due to a higher photosynthetic rate resulting from a maximal quantum yield of PSII photochemistry. Exogenously applied BAP enhanced antioxidant enzyme activities and decreased MDA content, as well as increased endogenous *t*Z levels [85].

9. Conclusions

Here, we review a family of important plant growth regulators—CKs—that are inseparably linked with plant senescence. The emphasis is on the interconnections between the CK influence on the photosynthetic apparatus, determinable antisenescent properties measured by several bioassays, antioxidative enzyme regulation, levels of antioxidant secondary metabolites in a number of plants or their explants and genes involved in plant senescence and its regulation. We highlight several molecular aspects that may represent new connections in the mechanism of action of these amazing small molecules that are indisputably involved in plant defence against biotic and abiotic stress. The role of systematic study and synthesis of new CK derivatives in relation to their biological functions is discussed, especially their antisenescent properties, together with their structural changes in comparison to the original molecules. Links between changes in structure and biological activities, especially effects on photosynthetic apparatus, secondary metabolite production and senescence related gene involvement, are described with regard to the overall functions of CKs within plants. Common structural motifs in CK molecules that could serve as a guide to specifically why these molecules have antisenescent properties are outlined. The involvement and ability of biotic stresses to enter the transcriptional process of CK production are also mentioned. This is especially relevant to green island formation and the influence on photosynthesis, which continue to be important issues. However, many questions remain which need further exploration of these interesting molecules with ancient perception and regulatory systems.

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Abbreviations

2Cl-3F-BAPR	2-chloro-6-(3-fluorobenzylamino)-9-β-D-ribofuranosylpurine
3F-BAP	6-(3-fluorobenzylamino)purine
3F-BAPR	6-(3-fluorobenzylamino)-9-β-D-ribofuranosylpurine
AHK	*Arabidopsis* histidine kinase
APX	ascorbate peroxidase
ARCK	aromatic cytokinins
ARR	*Arabidopsis* response regulator
BAP	6-(benzylamino)purine
CAT	catalase
CK	cytokinin
CPPU	*N*-(2-chloropyridin-4-yl)-*N*′-phenylurea
CRF	cytokinin response factor
*c*Z	6-(*Z*)-(4-hydroxy-3-methylbut-2-enylamino)purine (*cis*-zeatin)
DCPPU	*N*-(2,6-dichloro-pyridin-4-yl)-*N*′-phenylurea
DHZ	6-(4-hydroxy-3-methylbutylamino)purine (dihydrozeatin)
DHZR	6-(4-hydroxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine (dihydrozeatin-riboside)
GSH	glutathione

INCYDE	2-chloro-6-(3-methoxyphenylamino)purine
iP	6-(2-isopentenylamino)purine
IPT	isopentenyl transferase
ISCK	isoprenoid cytokinins
Kin	6-(furfurylamino)purine (kinetin)
MDA	malondialdehyde
*m*T	6-(3-hydroxybenzylamino)purine (meta-topolin)
*m*TR	6-(3-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (meta-topolin-9-riboside)
ORAC	oxygen radical absorbance capacity
*o*T	6-(2-hydroxybenzylamino)purine (ortho-topolin)
PI-55	6-(2-hydroxy-3-methylbenzylamino)purine
POD	peroxidase
ROS	reactive oxygen species
SAGs	senescence-associated genes
SOD	superoxide dismutase
TDZ	1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (thidiazuron)
THF	tetrahydrofuran-2-yl
THP	tetrahydropyran-2-yl
*t*Z	6-(*E*)-(4-hydroxy-3-methylbut-2-enylamino)purine (*trans*-zeatin)
*t*ZR	6-(*E*)-(4-hydroxy-3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (*trans*-zeatin-9-riboside)
WLSA	wheat leaf senescence assay
Z	6-(4-hydroxy-3-methylbut-2-enylamino)purine (zeatin)
ZR	6-(4-hydroxy-3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (zeatin-9-riboside)

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Supplement III

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Design, synthesis and perception of fluorescently labeled isoprenoid cytokinins

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ABSTRACT

Isoprenoid cytokinins play a number of crucial roles in the regulation of plant growth and development. To study cytokinin receptor properties in plants, we designed and prepared fluorescent derivatives of 6-[(3-methylbut-2-en-1-yl)amino]purine (N^6-isopentenyladenine, iP) with several fluorescent labels attached to the C2 or N9 atom of the purine moiety *via* a 2- or 6-carbon linker. The fluorescent labels included dansyl (DS), fluorescein (FC), 7-nitrobenzofurazan (NBD), rhodamine B (RhoB), coumarin (Cou), 7-(diethylamino)coumarin (DEAC) and cyanine 5 dye (Cy5). All prepared compounds were screened for affinity for the *Arabidopsis thaliana* cytokinin receptor (CRE1/AHK4). Although the attachment of the fluorescent labels to iP *via* the linkers mostly disrupted binding to the receptor, several fluorescent derivatives interacted well. For this reason, three derivatives, two rhodamine B and one 4-chloro-7-nitrobenzofurazan labeled iP were tested for their interaction with CRE1/AHK4 and *Zea mays* cytokinin receptors in detail. We further showed that the three derivatives were able to activate transcription of cytokinin response regulator *ARR5* in Arabidopsis seedlings. The activity of fluorescently labeled cytokinins was compared with corresponding 6-dimethylaminopurine fluorescently labeled negative controls. Selected rhodamine B C2-labeled compounds **17**, **18** and 4-chloro-7-nitrobenzofurazan N9-labeled compound **28** and their respective negative controls (**19**, **20** and **29**, respectively) were used for *in planta* staining experiments in *Arabidopsis thaliana* cell suspension culture using live cell confocal microscopy.

Abbreviations: ABA, abscisic acid; 2-AmEtAm, 2-aminoethylamino-; 6-AmHexAm, 6-aminohexylamino-; AHK, histidine-kinase receptor from *A. thaliana*; AFCS, Alexa Fluor 647 labeled castasterone; ARR5:GUS, Arabidopsis response regulator 5: β-glucuronidase; ARCKs, aromatic cytokinins; BRI1, protein brassinosteroid insensitive 1; Cou, coumarin; Cou-OH, coumarin-3-carboxylic acid; CK(s), cytokinin(s); Cy5, cyanine 5 dye; Cy5-NHS, NHS ester; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIAD, diisopropyl azodicarboxylate; DMSO, dimethylsulfoxide; DEAC, 7-(diethylamino)coumarin; DEAC-OH, 7-(diethylamino)coumarin-3-carboxylic acid; DAP, 6-dimethylaminopurine; DS, dansyl; DS-Cl, dansyl chloride; NBD, 7-nitrobenzofurazan; NBD-Cl, 7-nitrobenzofurazan chloride; ESI^+-MS, electrospray ionization mass spectrometry (positive mode); EtOAc, ethyl acetate; FC, fluorescein; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; IAA, indole-3-acetic acid; ISCK, isoprenoid cytokinins; iP, 6-[(3-methylbut-2-en-1-yl)amino]purine, N^6-isopentenyladenine; MeOH, methanol; NAA, naphthalene acetic acid; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; PrOH, *n*-propanol; RhoB, rhodamine B; RhoB-NHS, rhodamine B NHS ester; RT, room temperature; TFA, trifluoroacetic acid; TLC, thin layer chromatography; THF, tetrahydrofuran; *tZ*, *trans*-zeatin.

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1. Introduction

Naturally occurring isoprenoid cytokinins (ISCK), such as 6-[(3-methylbut-2-en-1-yl)amino]purine (iP), *trans*-zeatin (*tZ*) and *cis*-zeatin, are plant signaling molecules. For this reason, they have attracted the attention of biologists owing to their importance in numerous aspects of plant growth and development, cell division, seed germination, the formation and activity of shoot and root meristems, apical dominance, auxiliary bud release, nutrition mobilization, leaf senescence and responses to pathogens (Davies, 2007). Fluorescently labeled ISCK may be a useful alternative tool for research into cytokinin perception and signaling in plants. Although several cytokinin receptors have been already described, e.g., in species such as Arabidopsis (Inoue et al., 2001; Suzuki et al., 2001), maize (Yonekura-Sakakibara et al., 2004), legumes *Medicago truncatula* (Gonzalez-Rizzo et al., 2006), *Lotus japonicus* (Murray et al., 2007; Tirichine et al., 2007) and rice (Du et al., 2007), there remains the need for mapping the receptor domain in order to understand the relation between the chemical structure and activity of cytokinin derivatives. This approach is indispensable developing new strategies in plant biotechnology, such as plant tissue culture, modern agriculture and plant protection against stress (Plíhalová et al., 2016). Fluorescent labeling is an important tool in cell biology research, e.g., staining and immunostaining techniques (Doskočilová et al., 2013; Mason, 1999; Ovečka et al., 2014; Šamajová et al., 2014), and for visualizing of small bioactive molecules. It offers several advantages over traditional radio-ligand binding techniques, i.e., fluorescence labels are relatively safe and inexpensive compared to tritiated or iodinated compounds and a wide range of fluorophores are available to suit different experimental setups (McGrath et al., 1996; Daly and McGrath, 2003). Fluorescent ligands are continually being developed to meet the demands of the pharmacological community and are being used to study pharmacological receptor systems (Daly and McGrath, 2003). Hiratsuka and Kato used a fluorescent analogue of colcemid with 7-nitrobenzo-furazan (NBD, NBD-colcemid) to visualize tubulin (Hiratsuka and Kato, 1987). Fluorescent labeling of small active molecules has been shown to be effective for visualizing plant hormones, such as auxins, abscisic acid, jasmonates, gibberellins, brassinosteroids and even strigolactones. In one such study, abscisic acid (ABA) was coupled with fluorescein isothiocyanate (FITC) and used to study direct interaction of ABA with the plasma membrane as although ABA receptors were unknown at the time, they were predicted to lie in the membrane (Asami et al., 1997). Fluorescent brassinosteroid was prepared by labeling castasterone with Alexa Fluor 647 (AFCS) and the endocytosis of BRI1-AFCS complexes in living cells was visualized (Irani et al., 2012). Fluorescent labeling at the cellular level has also been done using gibberellins labeled with FITC (Pulici et al., 1996). 1,4-Dithiobutylene and 1,3-dithiopropylene spacers were employed between the fluorescent label and gibberellin, particularly for the compound 17-mercaptobutylthio-3α,10-dihydroxy-20-norgibberella-7,19-dioic acid-19,10-lactone. It was shown that derivatives with longer spacers between gibberellin and FITC were more active in the ability to induce α-amylase activity in the embryoless half grain, a process known to be specifically induced by active Gas synthesized by the embryo. However, an approximately 10-fold higher concentration of the fluorescent probe than GA_3 was needed to obtain a comparable biological effect (Pulici et al., 1996; Lace and Prandi, 2016). Synthesis of fluorescently labeled strigolactone analogs (DS, FC, BODIPY) has been used to search for possible strigolactone receptors *in vivo* (Prandi et al., 2013). Rhodamine and fluorescein auxin derivatives have been synthesized by direct conjugation of FITC and rhodamine B to the NH group of IAA (Sokolowska et al., 2014). Both fluoroprobes were shown to retain auxin activity in three different bioassays (Sokolowska et al., 2014). Tsuda and Hayashi introduced an NBD label into 5-hydroxy-IAA and 7-hydroxy-NAA but the prepared auxin analogs were found to be inactive toward auxin receptors (Tsuda et al., 2011; Hayashi et al., 2014; Lace and Prandi, 2016). Fluorescently labeled jasmonate has been synthesized by bonding jasmonoyl-L-isoleucine to coumarin 343 *via* the carboxyl group of isoleucine (Liu et al., 2012). The fluorescent probe was examined in cabbage using a root growth inhibition bioassay and the effect of fluorescently labeled probe on the root growth of cabbage seedlings was similar to that of the methyl jasmonate, the standard bioactive jasmonate. Like approaches to other plant growth regulators, in preparing a fluorescent probe for visualizing a cytokinin receptor, the compound has to possess cytokinin activity and high affinity for the receptor while nonspecific binding to other cellular structures needs to be minimized. When the first attempts to prepare a cytokinin fluorescent probe failed in the 1970s, a different strategy based on the construction of mimetic adenine-like molecules was developed (Skoog et al., 1975; Specker et al., 1976). Modifications of cytokinins, particularly in the purine moiety, has led to the preparation of fluorescent imidazo[4,5-*g*]- and imidazo[4,5-*f*]-quinazolines, 4-substituted 2-methylthiopyrido[2,3-*d*]pyrimidines and 7-phenylethynylimidazo[4,5-*b*]pyridines and their ribosides, which were shown to have only weak or negligible cytokinin activity in a tobacco callus bioassay (Specker et al., 1976; Hamaguchi et al., 1985; Nishikawa et al., 2000). Zawadski's group prepared synthetic cytokinin *N*-phenyl-*N*′-(4-pyridyl) urea labeled with 4-chloro-7-nitrobenzofurazan and rhodamine B fluorescent labels and detected binding of the cytokinin-specific protein VrCSBP by fluorescence correlation spectroscopy (Zawadski et al., 2010). It has been suggested that the loss of biological activity could be prevented by separation of the pharmacophore from the fluorescent moiety through the introduction of a spacer or linker (Leopoldo et al., 2009). However, so far, only a few studies have systematically evaluated spacer length for fluorescent probes and none have directly evaluated purine based cytokinins. Spacer length and position of the spacer (label) in the purine moiety can both have a large impact on the biological activity of such cytokinin derivatives. Appropriate positional attachment of fluorophores to small molecule ligands is critical for retaining both receptor binding affinity and efficacy (Leopoldo et al., 2009). In addition to standard fluorophores such as fluorescein and rhodamine, we have also endeavoured to find new efficient fluorolabels with fewer limitations for use in biological systems and during confocal microscopy imaging. For example, fluorescein is known to self-quench after bioconjugation (Lace and Prandi, 2016; Sjöback et al., 1995) as the emission properties of fluorescein greatly depend on environmental pH (Lavis et al., 2007) and it often exists as an equilibrium between lactone and quinoid forms (Lace and Prandi, 2016). Although rhodamine dyes are less sensitive to pH than fluorescein, they are poorly soluble in water (Lace and Prandi, 2016). Despite these limitations, both are widely used for labeling bioactive molecules. We have also used the small heterocyclic molecule 4-chloro-7-nitrobenzofurazan (NBD-Cl), which is a benzoxadiazole compound with low molecular weight. Derivatives of NBD-Cl have been used for the preparation of novel kinase substrates, lipid probes and fluorescent analogs of native lipids and the study of a variety of processes (Chattopadhyay, 1990; Lavis and Raines, 2008; Lace and Prandi, 2016).

In this work, we prepared fluorescently labeled iP derivatives because iP is known to bind to *Arabidopsis thaliana* CRE1/AHK4, *Zea mays* ZmHK1 and other cytokinin receptors. 6-Dimethylaminopurine (DAP) analogs with no cytokinin activity were also prepared to obtain fluorescent negative controls for receptor bioassays. The prepared compounds contained a 2- or 6-

carbon linker between the iP or DAP molecule and the fluorescent labels. The fluorescent labels used included dansyl (DS), fluorescein (FC), 7-nitrobenzofurazan (NBD), rhodamine B (RhoB) and coumarin (Cou), 7-(diethylamino)coumarin (DEAC) and cyanine 5 dye (Cy5). We selected the C2 or N9 atom of the purine moiety for linker attachment to prepare 2,6- and 6,9-disubstituted purine derivatives. The ability of all prepared compounds to bind the cytokinin receptor CRE1/AHK4 was tested. The three most successful derivatives were also tested for interaction with CRE1/AHK4 and ZmHK1 in detail as well as for their ability to activate the expression of the *ARR5* gene, which is the primary cytokinin response regulator in Arabidopsis (D'Agostino et al., 2000). Two active C2-fluorescent probes labeled with RhoB and one active N9-fluorescent probe labeled with NBD together with their fluorescent controls were used for staining of *Arabidopsis thaliana* cell suspension culture and live cell imaging using confocal microscopy.

2. Results and discussion

2.1. Synthesis

We prepared thirty new 2,6- or 6,9-disubstituted adenine derivatives with the linker attached to an appropriate fluorescent label either at the C2- or N9-atom of the purine moiety and elemental analyses, fluorescent label, length of the linker, ESI-MS and spectral data are shown in Table 1. In addition to the fluorescently labeled iP derivatives, we also prepared DAP analogs labeled with the same fluorescent labels as the iP derivatives for use as negative controls in future experiments. The syntheses of fluorescently labeled iP and DAP derivatives consisted of several steps, as described in the Experimental section and in the Supplementary data. In the first step, an appropriate intermediate substituted with a linker terminating in an amino group was prepared (Schemes 1 and 2). This derivative was labeled with an appropriate fluorescent label (Schemes 3 and 4). For substitutions at the C2 atom of the purine moiety, only fluorescent labels that could be attached to an amino group were used, e.g., NBD-Cl, NHS activated coumarin-3-carboxylic acid, DS-Cl, NHS activated DEAC, FITC and NHS activated RhoB. For the N9 position of the purine atom moiety, we used an appropriate linker terminating in an amino group and NBD-Cl, Cy5-NHS, RhoB-NHS and FITC as fluorescent labels. Since the two carbon linker has proved to be more efficient in the case of C2 derivatives, we have adopted this design strategy for N9 fluorescent probes (**25**–**28** and **30**). Compound **29**, i.e., DAP-N9-NBD, was also prepared as the only negative control (specifically for comparison with the only active compound **28**).

2.2. Live cell hormone binding assays

Cytokinin–receptor interaction is a crucial step in the initiation of cytokinin signaling in plant cells (Romanov et al., 2005). The functionality and ability of intended fluorescent probe to enter the cells can be verified by activation of the appropriate receptors. Therefore, the prepared compounds were tested in a direct binding assay with *E. coli* expressing functional CRE1/AHK4 or AHK3 receptors from *A. thaliana* to test their ability to compete with the radiolabeled natural ligand *trans*-zeatin (2-[^{3}H]*tZ*). Unlabeled *trans*-zeatin, isopentenyladenine (iP), adenine and DMSO were used as positive and negative controls, respectively (Fig. 1A and B; Spíchal et al., 2009).

We compared the activity of fluorescently labeled DAP derivatives **3**, **4**, **7**, **8**, **11**, **12**, **15**, **16**, **19**, **20** and **29** prepared as the negative controls with iP fluorescently labeled probes at CRE1/AHK4 receptor. DAP as well as iP derivatives were fluorescently labeled *via* a two or six carbon linker attached to the C2 or N9 atom of the purine moiety. DAP control derivatives were chosen given that the isopentenyl substituent is considered to be responsible for the cytokinin receptor binding properties (Hothorn et al., 2011; PDB:3T4J). As predicted, all control derivatives (black columns in Fig. 1A) were inactive.

Some of the desired fluorescently labeled derivatives of iP, namely **2**, **5**, **6**, **9**, **13**, **14**, were also found to be unable to compete with 2-[^{3}H]*tZ* at the CRE1/AHK4 receptor (Fig. 1A). Whereas DS and DEAC fluorescent labels deactivated iP probes regardless of the linker length, iP derivatives of NBD (**1, 28**), Cou (**10**), RhoB (**17**, **18**) and FC (**21** and **22**) showed at least partial affinity to the cytokinin receptor CRE1/AHK4. Although the RhoB labeled C2-derivatives **17** and **18** were active in competing with 2-[^{3}H]*tZ* at the receptor binding site, the iP RhoB labeled N9-derivative **27** was inactive. The FC labeled derivatives behaved similarly.

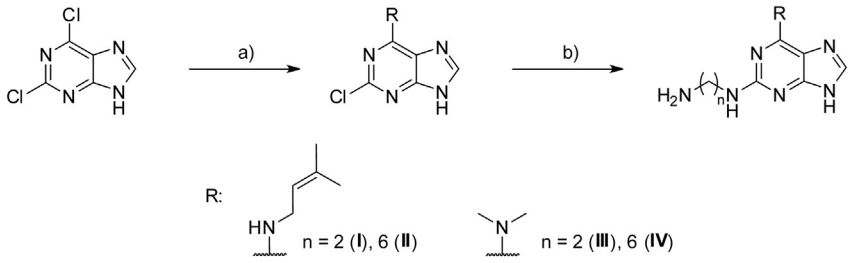
Whereas the activity of both RhoB labeled derivatives **17** and **18** was comparable, the FC labeled derivative **21** with a shorter two-carbon linker exhibited a higher affinity than the derivative **22** with a six-carbon linker. A similar trend was observed for the NBD labeled iP fluorescent probes: the two-carbon linker of **1** was found to be more efficient than the six-carbon linker of **2**, which was inactive. C2-labeled iP derivative **21** exhibited highest affinity for the receptor, displacing more than 65% of 2-[^{3}H]*tZ* from the receptor at a 20 μM concentration. The compound **21** thus appeared to be a promising candidate for fluorescent staining until we found that the substance was chemically unstable and spontaneously decomposed during storage. Another promising active compound was N9-labeled **28**. This compound (bearing the NBD label) was the only N9 derivative to exhibit affinity for the CRE1/AHK4 receptor, displacing approximately 57% of 2-[^{3}H]*tZ* from receptor binding site at 20 μM concentration. As shown in Fig. 1A, with the exception of compound **28**, the other prepared N9-substituted compounds, i.e., **25**–**27** and **30**, appeared to be non-active in displacing 2-[^{3}H]*tZ* in the competition assay. In contrast to **28**, compound **29,** (negative control), was found to be inactive.

Finally, we selected two RhoB labeled C2-derivatives **17** and **18** and one NBD labeled N9-derivative **28** to test their interaction with CRE1/AHK4 within a wider concentration range (Fig. 1B). The compounds affinity for the receptor was compared to the affinity of adenine (negative control) and iP (positive control). The affinity of compounds **17** and **18** for CRE1/AHK4 was relatively weak compared to the control, better results were achieved with compound **28**, whose affinity was approximately ten times higher than compound **18**. Compound **28** decreased the binding of 2-[^{3}H]*tZ* to the receptor to 55% at 10 μM concentration. Cytokinin iP decreased the binding of 2-[^{3}H]*tZ* to the receptor to 45% at 10 nM concentration. Compound **28** showed approximately 1000 times lower affinity to the CRE1/AHK4 receptor than iP and although the affinity of **28** appeared to be low, we showed that it was sufficient for *in planta* staining studies. Although the effective concentrations of the active fluorescent probes were high in relation to the positive standard iP, the selected micromolar concentration was necessary for effective visualization of target cellular structures during confocal or fluorescent microscopy staining. Similar approaches have been reported for other phytohormone-based fluorescent probes (Irani et al., 2012).

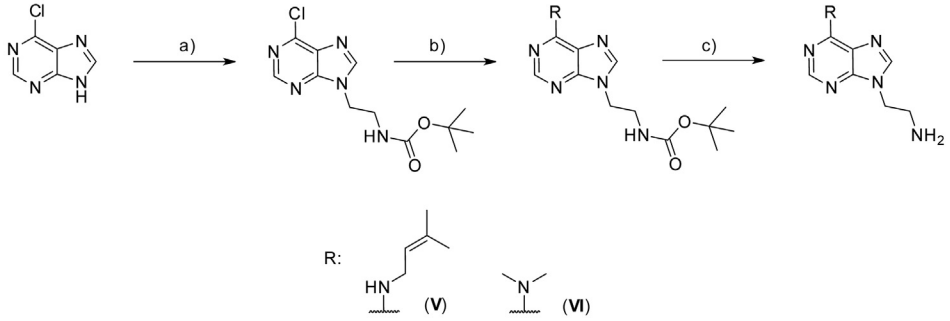
This aside, the results of the competition assay corresponded to the published structure of the CRE1/AHK4 sensor domain (Hothorn et al., 2011; PDB:3T4J). The crystal structure of the CRE1/AHK4 sensor domain in complex with iP showed four hydrogen bonds formed between the receptor cavity and the adenine moiety and these interactions appeared to be critical for receptor function. Both N6H and N7H hydrogen bonds are linked to Asp262 while N1H atom and N3H hydrogen bonds are linked to water molecules. While C2 and N9 point out of the receptor cavity, they might be

Table 1
Table of prepared fluorescently labeled purine derivatives. 2AmEtAm − 2-aminoethylamino-; 6AmHexAm − 6-aminohexylamino-; NBD − 7-nitrobenzofurazane; Cou − coumarin; DEAC − 7-(Diethylamino)coumarin; DS − dansyl, RhodB − Rhodamine B; FC − fluorescein, v.w.f.: very weak fluorescence.

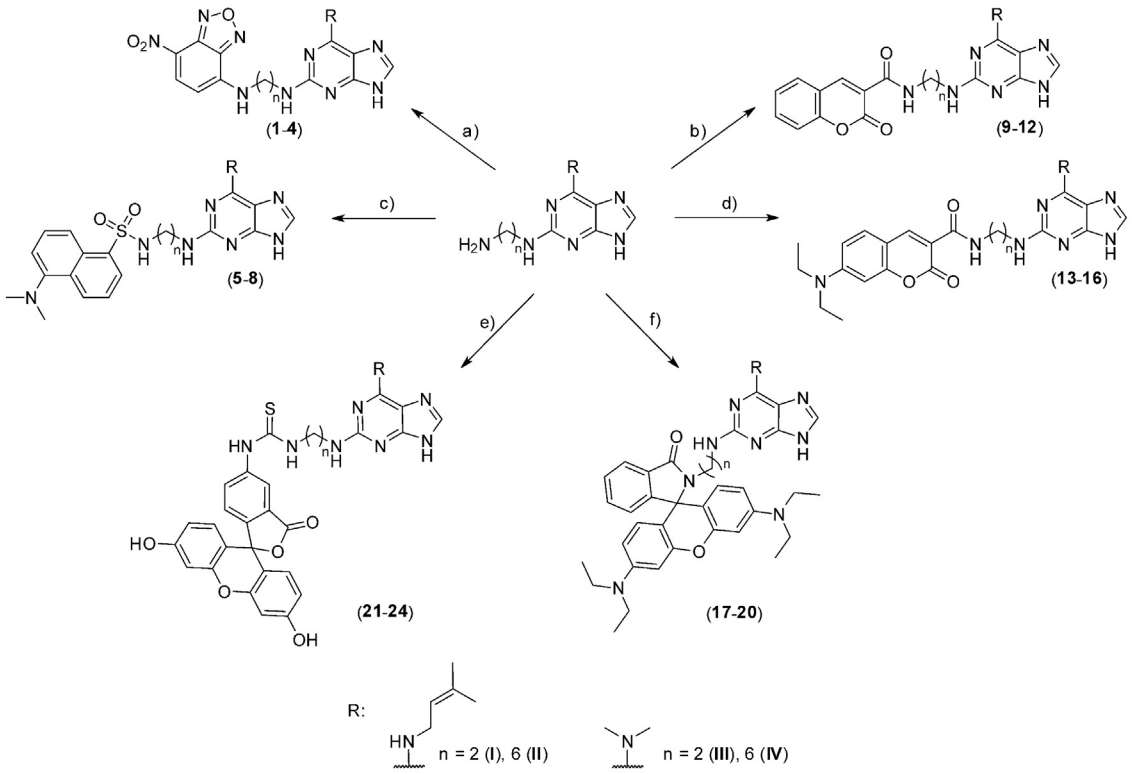
Comp.	C6	C2	N9	Fluorescent label	HPLC purity [%]	ESI-MS $[M + H]^+$	Spectral properties λex_{max} [nm]	λem_{max} [nm]
1	(3-Methylbut-2-en-1-yl)amino-	2AmEtAm	H	NBD	98.2	425	460	525
2	(3-Methylbut-2-en-1-yl)amino-	6AmHexAm	H	NBD	99.2	481	465	525
3	Dimethylamino-	2AmEtAm	H	NBD	98.4	385	v.w.f.	v.w.f.
4	Dimethylamino-	6AmHexAm	H	NBD	99.9	441	464	526
5	(3-Methylbut-2-en-1-yl)amino-	2AmEtAm	H	DS	99.9	495	257	507
6	(3-Methylbut-2-en-1-yl)amino-	6AmHexAm	H	DS	99.9	551	256	507
7	Dimethylamino-	2AmEtAm	H	DS	99.8	455	254	508
8	Dimethylamino-	6AmHexAm	H	DS	99.6	511	255	505
9	(3-Methylbut-2-en-1-yl)amino-	2AmEtAm	H	Cou	99.9	434	v.w.f.	v.w.f.
10	(3-Methylbut-2-en-1-yl)amino-	6AmHexAm	H	Cou	99.4	490	290	406
11	Dimethylamino-	2AmEtAm	H	Cou	99.9	394	v.w.f.	v.w.f.
12	Dimethylamino-	6AmHexAm	H	Cou	98.9	450	290	407
13	(3-Methylbut-2-en-1-yl)amino-	2AmEtAm	H	DEAC	99.9	505	416	464
14	(3-Methylbut-2-en-1-yl)amino-	6AmHexAm	H	DEAC	98.2	561	416	464
15	Dimethylamino-	2AmEtAm	H	DEAC	99.9	465	416	464
16	Dimethylamino-	6AmHexAm	H	DEAC	99.9	521	416	464
17	(3-Methylbut-2-en-1-yl)amino-	2AmEtAm	H	RhoB	98.0	686	544	566
18	(3-Methylbut-2-en-1-yl)amino-	6AmHexAm	H	RhoB	98.1	742	546	546
19	Dimethylamino-	2AmEtAm	H	RhoB	95.0	646	546	568
20	Dimethylamino-	6AmHexAm	H	RhoB	98.0	702	544	566
21	(3-Methylbut-2-en-1-yl)amino-	2AmEtAm	H	FC	92.4	651	544	566
22	(3-Methylbut-2-en-1-yl)amino-	6AmHexAm	H	FC	97.7	707	497	518
23	Dimethylamino-	2AmEtAm	H	FC	91.4	612.1	498	518
24	Dimethylamino-	6AmHexAm	H	FC	92.0	668.3	497	518
25	(3-Methylbut-2-en-1-yl)amino-	H	2AmEtAm	DS	98.3	481	342	500
26	(3-Methylbut-2-en-1-yl)amino-	H	2AmEtAm	FC	97.0	636	v.w.f.	v.w.f.
27	(3-Methylbut-2-en-1-yl)amino-	H	2AmEtAm	RhoB	96.5	672	554	588
28	(3-Methylbut-2-en-1-yl)amino-	H	2AmEtAm	NBD	96.8	410	475	552
29	Dimethylamino-	H	2AmEtAm	NBD	98.0	370	v.w.f.	v.w.f.
30	(3-Methylbut-2-en-1-yl)amino-	H	2AmEtAm	Cy5	97.7	713	270	641



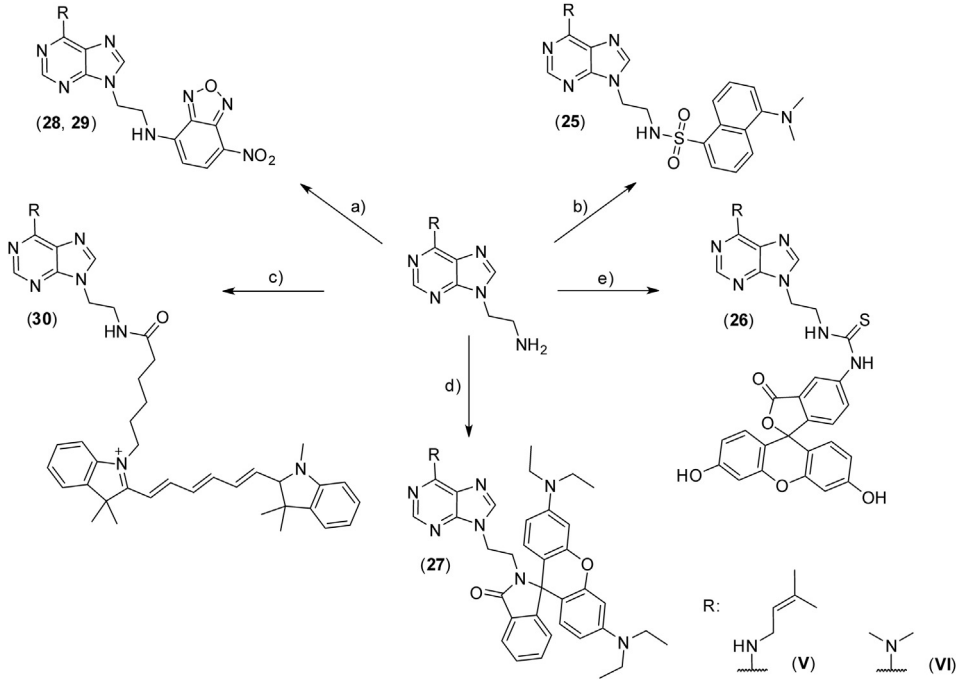
Scheme 1. Reaction scheme for the synthesis of 2,6-disubstituted purine precursors for fluorescent labeling. a) R-H, Et_3N, n-PrOH, 100 °C, 4 h; b) ethane-1,2-diamine, hexane-1,6-diamine, 165 °C, 3 h.



Scheme 2. Reaction scheme for the synthesis of 6,9-disubstituted purine precursors for fluorescent labeling. a) Boc-2-aminoethanol, PPh_3, DIAD, THF, 2 h; b) R-H, Et_3N, *n*-PrOH, 100 °C, 4 h; c) Dowex 50W X8, DCM, reflux followed by 4 M methanolic ammonia, overnight.



Scheme 3. Preparation of C2-fluorescently labeled purine derivatives. a) NBD-Cl, $NaHCO_3$, MeOH, 50 °C 1 h followed by RT overnight; b) NHS activated coumarin-3-carboxylic acid, DMSO, MeCN, carbonate buffer pH 8.6, overnight; c) dansyl chloride, Et_3N, MeOH, DCM, overnight; d) NHS activated DEAC-OH, DMSO, MeCN, carbonate buffer pH 8.6, overnight; e) FITC, Et_3N, MeOH, overnight; f) NHS activated rhodamine B, MeCN, carbonate buffer pH 8.6, overnight.



Scheme 4. Fluorescently labeled *N*9-substituted derivatives. a) 4-chloro-7-nitrobenzofurazan, $NaHCO_3$, MeOH, 50 °C, 1 h; b) dansyl chloride, 2 M Na_2CO_3, acetone, water, RT, overnight; c) cyanine 5 NHS, 0.1 M sodium bicarbonate in water, DMF; d) NHS activated rhodamine B, MeCN, carbonate buffer pH 8.6, overnight; e) FITC, MeOH, $NaHCO_3$, 50 °C, 1 h. Kation of compound **30** was compensated by $[BF_4]$ anion that was which was omitted to simplify the scheme.

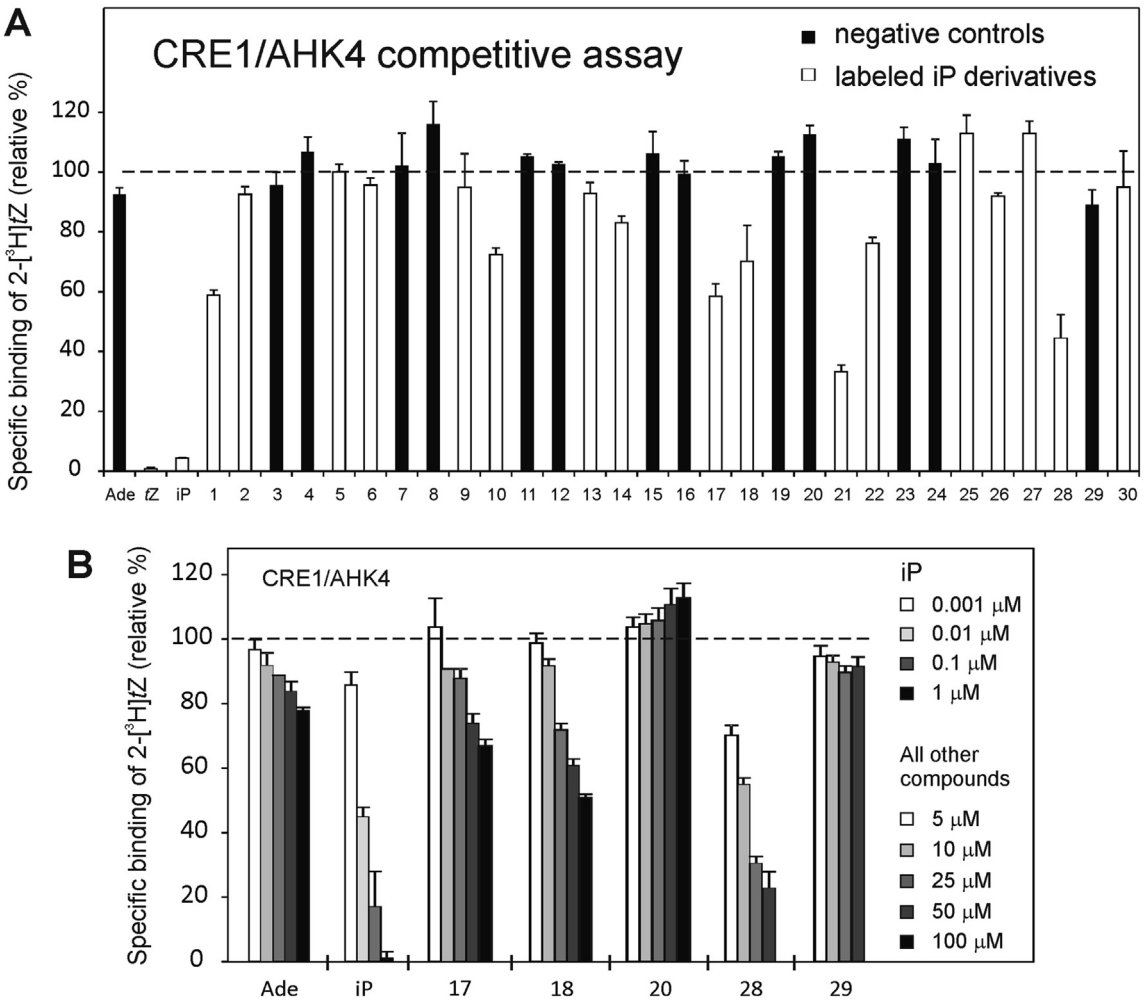


Fig. 1. The ability of the compounds to bind to the cytokinin receptor CRE1/AHK4 in live cell hormone binding assay. (A) Fluorescently labeled DAP and iP derivatives (both 20 μM) competed with 2 nM 2-[^{3}H]*tZ*. (B) Selected compounds in more detail. Negative control (**19**) for compound 17 is not shown but, exhibited the same affinity pattern as negative control **20**. Adenin (Ade) and DMSO were used as negative controls. The value obtained with DMSO was set as 100% (dashed line). The value obtained with 10 μM iP was set as 0% and was used to discriminate the non-specific binding of 2-[^{3}H]*tZ* on the bacteria. Error bars show SD values for three replicates.

accessible for linkers bearing spherically advantageous fluorescent labels in molecules **17**, **18** and **28**.

The C2-substituted derivatives were also tested with Arabidopsis cytokinin receptor AHK3, however none showed any affinity toward this receptor (data not shown).

Compounds **17**, **18** and **28** were further tested as to whether they interact with the cytokinin receptor ZmHK1, a maize orthologue of CRE1/AHK4 receptor to extend the potential use of the prepared fluorescent cytokinins to other plant species (Yonekura-Sakakibara et al., 2004). In the assay, we functionally expressed ZmHK1 in *E. coli* strain KMI001 (Podlešáková et al., 2012). Unfortunately, in the bacterial receptor competitive assay, compound **17** showed no affinity to the receptor (data not shown) while compound **18** exhibited an affinity for the ZmHK1 receptor comparable to adenine (Fig. 2A). This result contrasted with the results from CRE1/AHK4 competition assay, where both compounds showed higher affinity than adenine. However, derivative **28** decreased binding of 2-[^{3}H]*tZ* to the receptor by approximately 70% at 10 μM concentration, while adenine had the same effect at 100 μM concentration. In the higher concentration ranges, **28** was also more effective than adenine, decreasing binding of 2-[^{3}H]*tZ* to the receptor by approximately 50% at 50 μM concentration (Fig. 2A). The effect of **28** (and its negative control, **29**) at 100 μM concentration was not evaluated as the compounds were not completely soluble in the tested system at such high concentrations.

In ZmHK1 receptor activation assay, we made several unexpected observations. First, compound **18** was able to activate the receptor (Fig. 2B), even though the ligand-receptor interaction did not seem to be specific; compound **18** was a 100 times weaker activator of ZmHK1 than iP. In contrast, compound **28** was unable to activate this receptor (data not shown), although it exhibited significant affinity for this receptor. Therefore, the compound bound to the cytokinin receptor without triggering its activation in a manner we have described and characterized as anti-cytokinin behavior (Nisler et al., 2010). This may be advantageous for fluorescently labeled cytokinin probes, because it allows for specific interaction with the cytokinin receptor and at the same time limits potential constraints linked to high intracellular cytokinin concentrations.

2.3. Activation of the cytokinin primary response gene ARR5

We employed transgenic Arabidopsis (*Arabidopsis thaliana*) plants harboring the *ARR5:GUS* reporter gene (D'Agostino et al., 2000) to gain more information as to whether compounds **17**, **18** and **28** are able to trigger the cytokinin signaling pathway *in planta*.

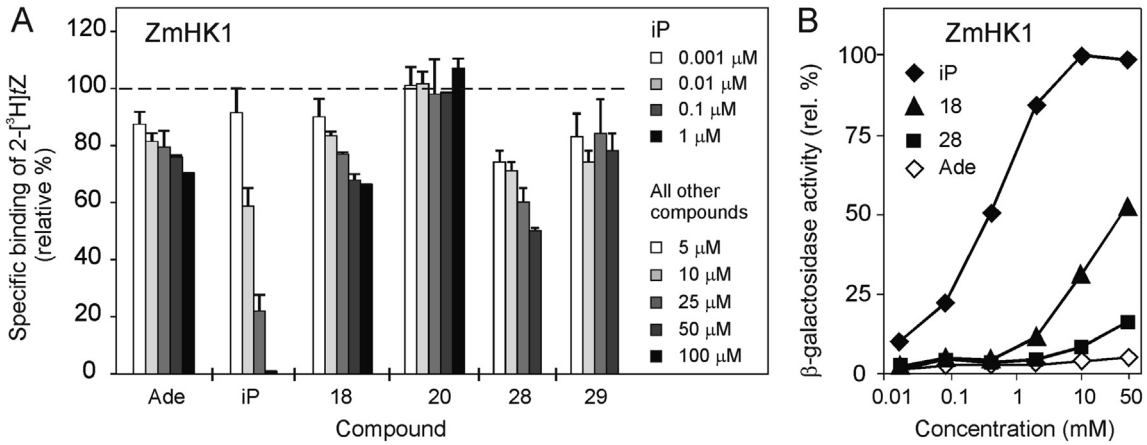


Fig. 2. Interaction of the selected compounds with the cytokinin receptor ZmHK1. Adenine (Ade) and DMSO were used as negative controls. (A) The ability of the compounds to bind to the receptor in live cell hormone binding assay. The value obtained with DMSO was set as 100% (dashed line). The value obtained with 10 μM iP was set as 0% and was used to discriminate the non-specific binding of 2-[^{3}H]*tZ* on the bacteria. Error bars show SD values for three replicates. (B) The ability of the compounds to activate the receptor. The value obtained with DMSO was set as 0% activation. The value obtained with 10 μM iP was set as 100% activation. Compound 17 activated the receptor similarly to compound 28 and for clarity is not shown. SD values did not exceed 15%.

ARR5 is a primary response gene with a cytokinin-dependent promoter, activation of which integrates the responses of several putative cytokinin signaling pathways.

As shown in Fig. 3, compounds **17**, **18** and **28** were able to activate transcription of the *ARR5:GUS* gene in Arabidopsis in a concentration dependent manner, demonstrating their ability to trigger the cytokinin response in Arabidopsis. Negative controls for the compounds were inactive in this assay. BAP and iP showed maximal activity at 1 μM concentration. None of the tested compounds was able to attain this activity even in 100 μM concentration. However, the selected compounds exhibited activity (20–30%) starting from a concentration of 10 μM. The compounds were approximately 1000 times weaker than iP, which showed activity from 10 nM concentration (Fig. 3) in this assay. Biologically insignificant activity of compound **28** (compared to **17** and **18**) is not surprising, and it is consistent with the negative results of the ZmHK1 receptor activation assay.

2.4. Visualization of cellular structures by fluorescent cytokinin derivatives

We used *Arabidopsis* suspension cell culture that allowed for fast and homogenous staining of individual cells, gave us insight into the distribution of the fluorescent signal at the subcellular level and we adopted a simple staining procedure for visualizing the subcellular structures with selected florescent probes. For staining experiments, we first observed compounds **17** and **18** and their corresponding DAP fluorescent derivatives **19** and **20**, respectively, that were used as negative controls to filter out nonspecific background signals. Arabidopsis cells were treated with 5 μM solution of fluorescent probe or negative control, immediately applied on slides and observed under confocal microscope. The internalization of RhoB-based compounds was relatively fast, reaching apparently constant pattern after approximately 15–20 min of treatment. The fluorescent signal was rather weak in all staining experiments and increased concentration of the fluorescent probe (up to 20 μM) did not significantly change the signal distribution. The signal recorded for the fluorescent probes, compounds **17** and **18,** and their respective negative controls, derivatives **19** and **20**, appeared to be similar in all cases and we observed cytoplasmic distribution of the signal combined with a characteristic patchy-pattern (Fig. 4). We hypothesize that RhoB derivatives may not represent a good candidate for synthesis of cytokinin-based fluorescent probes due to possible nonspecific interactions with cell components that prohibit efficient and specific binding to cytokinin-binding sites.

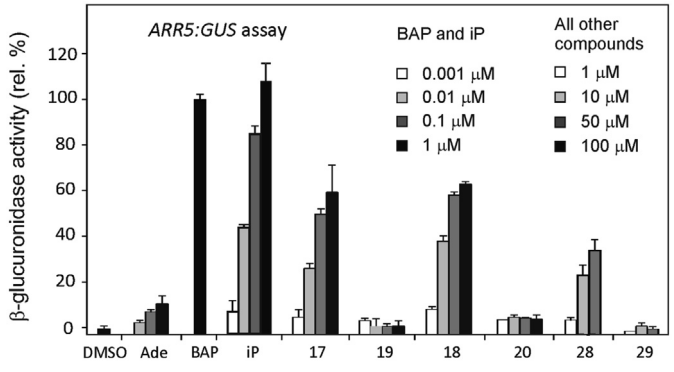


Fig. 3. The ability of the compounds to activate transcription of cytokinin primary response gene *ARR5* in Arabidopsis seedlings three days after germination. The value obtained with DMSO was set as 0% activation. The value obtained with 1 μM BAP was set as 100%. Adenine (Ade) and iP were used as negative and positive controls, respectively. Error bars show SD values for three replicates.

For this reason, we introduced derivative **28**, an NBD-based green fluorescent cytokinin probe, for *in planta* screening. Based on the results from the cytokinin CRE1/AHK4 competitive bioassay (Fig. 1A) only compound **28** proved to be significantly active in displacing radiolabeled tZ out of all synthesized C2- and N9-based NBD fluorescent probes and concurrently stable. Compound **29**, showed negligible binding to the cytokinin receptor and for this reason, it was used as a negative control. The results from live cell imaging of NBD-based probes are shown in Fig. 4. Similar to RhoB-based probes, staining of Arabidopsis cells with the NBD fluorescent probe was fast, reaching a plateau of the intracellular signal intensity after approximately 10 min of a continuous treatment with 5 μM of compound **28** (Fig. 4). While the negative control showed only weak cytoplasmic fluorescent signal, the NBD-based fluorescent probe at the same concentration showed a clear signal distribution represented by typical mesh-like structures at the cell cortex suggestive of ER structures (Fig. 4 arrows). This is in accordance with the reported localization pattern of the three known cytokinin HK receptors in Arabidopsis (Wulfetange et al.,

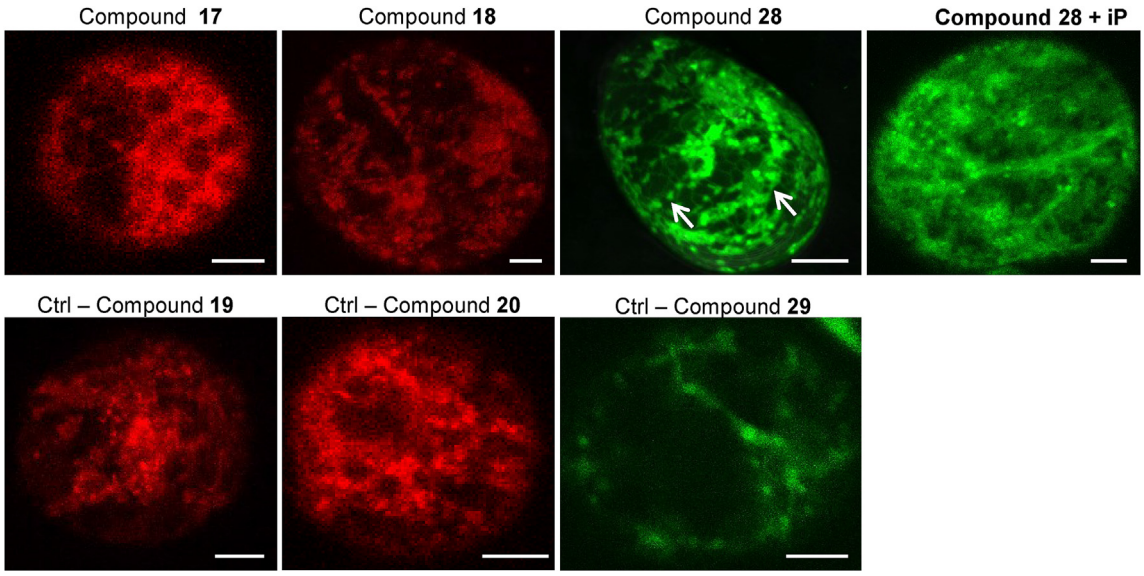


Fig. 4. Confocal fluorescence images of *Arabidopsis* suspension culture cells treated with different fluorescent probes (Compound **17**, **18** and **28**) and their controls (Ctrl - Compound **19**, **20** and **29**), all in 5 μmol concentrations and observed after 15–20 min. Rhodamine B-based compounds are shown in red, 7-nitrobenzofurazan (NBD)-based compounds are shown in green. A mesh-like localization pattern (marked by arrows) characteristic for endoplasmic reticulum was visible in the cell cortex after treatment with compound **28**, but it was lost when compound **28** was co-administered together with equimolar concentration of isopentenyladenine (iP). Scale bars, 5 μm.

2011; Caesar et al., 2011). Further, co-administration of compound **28** together with natural cytokinin iP led to moderate decrease of the fluorescent signal and the signal seemed to be less focused (Fig. 4). This suggests that compound **28** is transported to the cell even in the presence of the endogenous iP, but the competition with intracellular iP leads to the unspecific staining pattern. Therefore, it seems that some NBD-based cytokinin probes can be used for the visualization of the cytokinin HK receptors, but further experiments will be needed to confirm the specificity of the ligand-receptor interaction. N9-based cytokinin fluorescent probes may be particularly suitable for the live cell imaging as the introduction of fluorescent label into N9 position should prevent metabolic conversion of the cytokinin analogue through N-glycosylation that prevents entry into the receptor domain cavity.

3. Conclusion

Thirty new 2,6- and 6,9-disubstituted fluorescently labeled purines bearing fluorescent labels, such as dansyl (DS), fluorescein (FC), 7-nitrobenzofurazan (NBD), rhodamine B (RhoB), coumarin (Cou), and cyanine 5 dye (Cy5) were synthesized. These fluorescent derivatives and their negative controls prepared by labeling of DAP were synthesized with the aim to design probes with ligand affinity to the histidine kinase receptor domain. We prepared several derivatives that were able to bind in the active sites of two different cytokinin receptors - CRE1/AHK4 from *Arabidopsis thaliana* and ZmHK1 from *Zea mays*, and to trigger cytokinin response *in planta*.

Promising derivatives, two 2,6-iP derivatives **17**, **18** and one 6,9-iP derivative **28** underwent more precise binding study in three cytokinin receptors and were found to be active, although at higher concentration ranges than the positive iP control. Overall, our results showed that it is possible to prepare biologically active fluorescent cytokinins by the attachment of a fluorescent label to their purine moiety *via* an appropriate linker without causing extensive structural change. The activity of the prepared probes depended on the position and type of attached label on the adenine moiety. Therefore, the choice of label is also very important for maintaining cytokinin receptor affinity. We used compounds **17**, **18** and **28** and their controls for *in planta* staining but only compound **28** was an effective competitor as confirmed by radiolabeled tZ receptor binding experiments. Staining of Arabidopsis cells with compound **28** was fast, showed clear intracellular signal distribution represented by typical mesh-like structures at the cell cortex suggestive of ER after approximately 10 min of a continuous treatment with 5 μM fluorescent probe while negative fluorescent control **29** at the same concentration levels showed only weak cytoplasmic fluorescent signal. This is in agreement with reported localization pattern of the three known cytokinin HK receptors in Arabidopsis but further data are needed to confirm the ER localization of receptor-ligand complexes.

4. Experimental procedures

Chemicals and general procedures used in this manuscript are given in Supplementary material.

4.1. Syntheses of purine based intermediates and fluorescent probes

Synthesis of necessary intermediates required for fluorescent marker attachment are given in Supplementary material including their yields, m.p. HPLC purity, ESI+ MS m/z and ^{1}H and ^{13}C NMR data.

4.1.1. C2 fluorescently labeled derivatives

C2-labeled compounds were prepared according to Scheme 3. An appropriate fluorescent label was reacted with amino groups terminating the above mentioned prepared intermediates. Generally, C2-derivatives marked with NBD were prepared as follows: 0.36 mmol of the appropriate intermediate (I, II, III, IV), 1.2 equiv. of NBD-Cl (**1**–**4**) and 3 equiv. of $NaHCO_3$ were stirred in methanol at 50 °C for one h and then stirred at RT for additional 16 h. Afterwards, the solvent was evaporated under reduced pressure and the residue treated with ice cold water (5 mL). The resulting solid material was filtered, washed with ice cold water (4 x 1 mL) and then dried at 50 °C. The crude material was purified by silica column chromatography using $CHCl_3$/MeOH (4:1) as the mobile phase,

starting from pure chloroform with a methanol gradient.

1 : N^6-(3-methylbut-2-en-1-yl)-N^2-{2-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethyl}-9H-purine-2,6-diamine: Reddish solid; yield 74%.

2 : N^6-(3-methylbut-2-en-1-yl)-N^2-{6-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]hexyl}-9H-purine-2,6-diamine: Reddish solid; yield 65%.

3 : N^6,N^6-dimethyl-N^2-{2-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethyl}-9H-purine-2,6-diamine: Reddish solid; yield 81%.

4: N^6,N^6-dimethyl-N^2-{6-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]hexyl}-9H-purine-2,6-diamine (***4***): Reddish solid; yield 75%.

C2-derivatives marked with DS-Cl were prepared as follows: 0.36 mmol of I, II, III or IV and Et_3N (2 equiv.) were dissolved in dry MeOH (0.4 mL) and dry DCM (3 mL) under an argon atmosphere and added to a mixture of DS-Cl (1.5 equiv.) in DCM (2 mL). The reaction mixture was protected against light and stirred at RT for 16 h. Afterwards, the solvents were removed under reduced pressure, the residue was treated with ice cold water (5 mL) and then kept at 4 °C for 2 h. The resulting solid material was filtered, washed with 5% $NaHCO_3$ (3x 1 mL), ice cold water (5 x 1 mL) and dried in a desiccator over 4 Å molecular sieve. The crude material was purified by silica column chromatography using $CHCl_3$ - MeOH as the mobile phase with methanol gradient.

5 : 5-(dimethylamino)-N-[2-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)ethyl] naphthalene-1-sulfonamide: Pale yellow solid, yield 64%.

6 : 5-(dimethylamino)-N-[6-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)hexyl] naphthalene-1-sulfonamide: Pale yellow solid, yield 52%.

7 : 5-(dimethylamino)-N-(2-{[6-(dimethylamino)-9H-purin-2-yl]amino}ethyl)naphthalene-1-sulfonamide: Pale yellow solid.

8: 5-(dimethylamino)-N-(6-{[6-(dimethylamino)-9H-purin-2-yl]amino}hexyl)naphthalene-1-sulfonamide: Pale yellow solid, 63%.

C2- derivatives marked with coumarin was prepared as follows: 0.208 mmol)of coumarin-3-carboxylic acid and NHS (1 equiv.) were dissolved under an argon atmosphere in dry MeCN (6 mL) at 45 °C. DCC (1.16 equiv.) was added and the resulting mixture heated at 45 °C for 1 h, then stirred at RT for 20 h. Next, the solid was filtered off and washed carefully with dry MeCN (3 x 5 mL). Coumarin-3-carboxylic acid NHS ester: white solid, yield 90%.^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 2.89 (s, 4H), 7.43—7.50 (m, 2H), 7.83 (t, J = 7.2 Hz, 1H), 8.03 (d, J = 7.9 Hz, 1H), 9.13 (s, 1H). A solution of *N*-hydroxysuccinimidyl coumarin-3-carboxylic acid ester (0.208 mmol) in MeCN (4 mL) was dropwise added to **I, II, III** or **IV** (1 equiv.) dissolved in a mixture of carbonate buffer pH 8.6 (2 mL) and DMSO (2 mL). The reaction mixture was protected against light and stirred under an argon atmosphere at RT for 20 h. After dilution with cold water (5 mL) and storing at 4 °C for 2 h, a solid compound was formed. The crude material was purified by silica column chromatography using $CHCl_3$/MeOH as the mobile phase with methanol gradient.

9 : N-[2-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)ethyl]-2-oxo-2H-chromene-3-carboxamide: pale yellow solid, yield 92%.

10 : N-[6-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)hexyl]-2-oxo-2H-chromene-3-carboxamide (10): Pale yellow solid, yield 64%.

11 : N-(2-{[6-(dimethylamino)-9H-purin-2-yl]amino}ethyl)-2-oxo-2H-chromene-3-carboxamide: pale yellow solid, yield 69%.

12: N-(6-{[6-(dimethylamino)-9H-purin-2-yl]amino}hexyl)-2-oxo-2H-chromene-3-carboxamide: pale yellow solid, yield 63%.

C2-derivatives marked with DEAC were prepared as follows: DEAC-OH (0.208 mmol) and NHS (1.04 equiv.) were dissolved under an argon atmosphere in dry MeCN (6 mL) at 45 °C. DCC (1.16 equiv.) was added and the arising mixture was heated at 45 °C for 1 h and then stirred at RT for 20 h. The arising solid was filtered off and the filtrate was evaporated under reduced pressure to give the NHS ester of DEAC-OH: a yellow solid. A solution of *N*-hydroxysuccinimidyl DEAC-OH ester (1 equiv.) in MeCN (4 mL) was dropwise added to a solution of I, II, III, IV (1 equiv.) dissolved in a mixture of carbonate buffer pH 8.6 (2 mL) and DMSO (2 mL). The reaction mixture was protected against light and stirred under an argon atmosphere at RT for 20 h. After dilution with cold water (5 mL) and storing at 4 °C for 2 h, a solid was formed. The crude material was purified by silica column chromatography using $CHCl_3$/MeOH as the mobile phase with methanol gradient.

13 : 7-(diethylamino)-N-[2-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)ethyl]-2-oxo-2H-chromene-3-carboxamide: pale yellow solid, yield 61%.

14 : 7-(diethylamino)-N-[6-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)hexyl]-2-oxo-2H-chromene-3-carboxamide: pale yellow solid, yield 72%.

15 : 7-(diethylamino)-N-(2-{[6-(dimethylamino)-9H-purin-2-yl]amino}ethyl)-2-oxo-2H-chromene-3-carboxamide: pale yellow solid, yield 48%.

16: 7-(diethylamino)-N-(6-{[6-(dimethylamino)-9H-purin-2-yl]amino}hexyl)-2-oxo-2H-chromene-3-carboxamide (***16***): pale yellow solid, yield 52%.

C2-derivatives marked with rhodamine B were prepared as follows: The compound was prepared according to a slightly modified procedure described in the literature (Meng et al., 2007). Briefly, 0.209 mmol of rhodamine B and 0.109 mmol of *N*-hydroxysuccinimide were dissolved in 3 mL of dry acetonitrile at 45°C1,16 equiv. of DCC in 1 mL of acetonitrile was added and the reaction mixture heated at 45 °C for one h and then stirred at RT for 20 h. The solid was filtered off and the filtrate was evaporated under reduced pressure to give NHS rhodamine B ester: a dark green metallic solid. Afterwards, a solution of NHS rhodamine B ester in MeCN (1 mL) was dropwise added to a solution of 1 equiv. of **I, II, III** or **IV** in 1 mL of carbonate buffer (pH = 8.6). The reaction mixture was stirred at RT for 4 h and then cooled in an ice bath. The obtained solid was filtered off, washed with cold MeCN, cold water and dried at 50 °C.

17 : 3′,6′-bis(diethylamino)-2-{2-[(6-((3-methylbut-2-en-1-yl]amino}-9H-purin-2-yl)amino) ethyl)spiro[isoindoline-1,9′-xanthen]-3-one: pink solid; yield 99%.

18 : 3′,6′-bis(diethylamino)-2-{2-[(6-((3-methylbut-2-en-1-yl]amino}-9H-purin-2-yl)amino hexyl)-spiro[isoindoline-1,9′-xanthen]-3-one: pink solid; yield 99%.

19 : 3′,6′-bis(diethylamino)-2-(2-{[6-(dimethylamino)-9H-purin-2-yl)amino]ethyl}spiro-[isoindoline-1,9′-xanthen]-3-one: pink solid, yield 73%.

20: 3′,6′-bis(diethylamino)-2-(2-{[6-(dimethylamino)-9H-purin-2-yl]amino}hexyl)spiro-[isoindoline-1,9′-xanthen]-3-one: pink solid; yield 86%.

C2-derivatives marked with FITC were prepared as follows: 1 equiv. of FITC was added to a mixture of **I**, **II**, **III** or **IV** (0.136 mmol) and Et_3N (2.76 equiv.) in dry MeOH (2 mL) under an argon atmosphere. The reaction mixture was protected against light and stirred at RT for 20 h. Afterwards, the solvent was evaporated under reduced pressure, the residue was re-suspended in acetate buffer (pH 4.0, 5 mL) and then kept at 4 °C for 1 h. The resulting solid material was filtered, washed with acetate buffer pH 4.0 (5 x 1 mL) followed by water (5 x 2 mL) and dried at 50 °C.

21 : *2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-{3-[2-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)ethyl]thioureido}benzoic acid*: orange solid; yield 95%.

22 : *2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-{3-[6-({6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-2-yl}amino)hexyl]thioureido}benzoic acid*: orange solid; yield 98%.

23 : *5-[3-(2-{[6-(dimethylamino)-9H-purin-2-yl]amino}ethyl) thioureido]-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid*: orange solid; yield: 98%.

24: *5-[3-(6-{[6-(dimethylamino)-9H-purin-2-yl]amino}hexyl)thioureido]-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid*: orange solid, yield: 98%.

4.1.2. N9 fluorescently labeled derivatives

N9 labeled compounds were prepared according to Scheme 4 and given below:

*4.1.2.1. 5-(Dimethylamino)-N-{2-[6-((3-methylbut-2-en-1-yl] amino}-9H-purin-9-yl)ethyl)naphthalene-1-sulfonamide (**25**).* Compound **25** was prepared according to a procedure described in the literature (Bartzatt, 2001). Briefly, a solution of DS-Cl (1.2 equiv.) in acetone (2 mL) was added to a solution of V (0.406 mmol) dissolved in a mixture of water (5.5 mL) and 2 M Na_2CO_3 (2 mL). The flask was protected from light and the reaction mixture was stirred at RT overnight. The mixture was then extracted with diethyl ether (3 x 10 mL). The combined organic layers were washed with water (2 x 5 mL) followed by brine (2 x 5 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The product was purified by silica flash column chromatography using $CHCl_3$/MeOH (9:1) as the mobile phase. Pale yellow solid; yield 65%.

*4.1.2.2. 2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-4-[3-(2-{6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-9-yl}ethyl)thioureido]benzoic acid (**26**).* Compound **V** (0.406 mmol) was stirred in MeOH (3 mL) with FITC (1.1 equiv.) in the presence of $NaHCO_3$ (3 equiv.) at a temperature of 50 °C for 1 h. The pH of the reaction mixture was adjusted to pH 4 by adding 1 M HCl. The resulting orange solid was filtered and then washed with MeOH. The pure compound was obtained after flash chromatography using $CHCl_3$/MeOH (4:1) as the mobile phase. Orange solid: yield 65%.

*4.1.2.3. 3′,6′-Bis(diethylamino)-2-(2-(6-((3-methylbut-2-en-1-yl) amino)-9H-purin-9-yl)ethyl)spiro[isoindoline-1,9′-xanthen]-3-one (**27**).* A solution of *NHS* rhodamine B ester (1 equiv.) dissolved in MeCN (1 mL) was dropwise added to a solution of iP (0.105 mmol) dissolved in carbonate buffer pH 8.6 (1 mL). The resulting mixture was stirred at RT for 4 h and then cooled to form pink solid; yield 25%.

*4.1.2.4. N^6-(3-Methylbut-2-en-1-yl)-N^9-{2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]ethyl}-9H-purin-6-amine hydrochloride (**28**) and N^6-(dimethylamino)-N^9-{2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]ethyl}-9H-purin-6-amine (**29**).* Compounds **28** and **29** were prepared according to a protocol described in the literature (Bem et al., 2007). Briefly, **V or VI** (0.39 mmol) was dissolved in MeOH (3 mL) containing 4-chloro-7-nitrobenzofurazan (1.1 equiv.) in the presence of $NaHCO_3$ (2.5 equiv.) at a temperature of 50 °C for 1 h. The reaction mixture was cooled in an ice bath and 1 M HCl was added dropwise up to the formation of an orange solid. The resulting solid was filtered, washed with ice cold MeOH (3 x 1 mL) followed by ice cold water (4 x 1 mL) and dried at 50 °C. **28**: reddish solid; yield 52%, **29**: a reddish solid; yield 75%.

*4.1.2.5. 3,3-Dimethyl-1-{6-[(2-{6-[(3-methylbut-2-en-1-yl)amino]-9H-purin-9-yl}ethyl)amino]-6-oxohexyl)-2-[(1E,3E)-5-((E)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl]-3H-indole(**30**).* Compound **V** (16.2 μmol) was dissolved in 0.1 M sodium bicarbonate solution (4.5 mL, pH 8.5) to which was added cyanine 5 NHS (1 equiv.) dissolved in amine free DMF (0.5 mL). The reaction mixture was stirred at RT for 4 h and then lyophilized overnight. The resulting residue was dissolved in water (5 mL) and extracted using EtOAc (3 x 5 mL). The product was purified by semi-preparative HPLC. Dark blue solid; yield 50%.

4.2. Live cell hormone binding assays

Receptor direct binding assays were conducted using the *E. coli* strain KMI001 harboring the plasmid pIN-III containing a coding sequence for the cytokinin receptor CRE1/AHK4 (Yonekura-Sakakibara et al., 2004) or ZmHK1 (Podlešáková et al., 2012 or the plasmid pSTV28 containing a coding sequence for AHK3 (Suzuki et al., 2001; Yamada et al., 2001). Bacterial strains were kindly provided by Dr. T. Mizuno (Nagoya, Japan). The assays were performed according to a published procedure (Nisler et al., 2010). The competition reaction of the tested compounds was allowed to proceed with 2 nM 2-[^{3}H]tZ. Labeled *t*Z was provided by the Isotope Laboratory, Academy of Sciences, Czech Republic.

4.3. ZmHK1 receptor activation assay

The assay was performed with *E. coli* strain KMI001 harboring the plasmid pIN-III containing a coding sequence for the cytokinin receptor ZmHK1 according to a previously published protocol (Spíchal et al., 2009; Podlešáková et al., 2012).

4.4. ARR5:GUS reporter gene assay

The assay was performed according to a published protocol (Romanov et al., 2005).

4.5. Confocal laser scanning microscopy

Cell suspension culture of *Arabidopsis thaliana* ecotype Landsberg *erecta* were cultivated under continuous darkness at 23 °C on a rotary shaker with subculture intervals of 3 days in 1 x Murashige and Skoog (MS) medium (Duchefa) containing 3% (w/v) sucrose. Fluorescent probes at concentration of 5 μM were used for *in situ* staining procedure – stained cells in MS medium were immediately mounted onto microscope slides, with a cover slip, and observed with a Zeiss 710 CLSM platform (Carl Zeiss, Jena, Germany) equipped with Plan-Apochromat 40x/1.4 Oil (Carl Zeiss, Germany) objective, using excitation laser 458 nm and 514 nm and emission filters 501–573 nm and 531–703 nm for NBD-based and rhodamine B-based fluoroprobes, respectively. The post-processing of images was done using ZEN 2010 software, Photoshop 6.0/CS, and Microsoft PowerPoint.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2018.02.015.

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Supplement IV

Plíhalová, L.; **Hönig, M.**; Rajnochová Svobodová, A.; Vostálová, J. Cytokininové deriváty jako možné modulátory předčasného stárnutí kůže. *Ref. výb. dermatovenerol*. **2018**, 3, 6-16, ISSN: 1213-9106

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CYTOKININOVÉ DERIVÁTY JAKO MOŽNÉ MODULÁTORY PŘEDČASNÉHO STÁRNUTÍ KŮŽE

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SOUHRN: **UV záření (UVA a UVB), které je složkou slunečního světla dopadajícího na zemský povrch, má vysokou energii. Díky této skutečnosti může přímo reagovat s biomolekulami (DNA, lipidy nebo proteiny), případně vyvolávat tvorbu reaktivních forem kyslíku (ROS), které mohou tyto biomolekuly poškozovat, což se projeví nevratnými změnami kůže vedoucími k jejímu předčasnému stárnutí, v horším případě ke karcinogenezi. Ve snaze potlačit nežádoucí účinky slunečního záření se využívají různé přístupy ochrany kůže. Kromě anorganických a organických UV filtrů jsou zkoumány přirozeně se vyskytující nízkomolekulární látky rostlinné povahy, které mohou eliminovat nežádoucí účinky slunečního záření. Rostlinné hormony cytokininy, zejména kinetin (Kin), který je N6-substituovaným derivátem adeninu, jsou známy pro svou schopnost zpomalovat přirozené stárnutí buněk, to znamená, že vykazují tzv. antisenescenční vlastnosti. Nedávno bylo prokázáno, že některé cytokininy a jejich deriváty mají ochranné vlastnosti vůči UVA i UVB záření. Jejich využití při zpomalení předčasného stárnutí kůže působením slunečního záření je předmětem intenzivního studia.**

KLÍČOVÁ SLOVA: cytokininy – kinetin – syntetické deriváty – fotoprotekce – UV záření – předčasné stárnutí

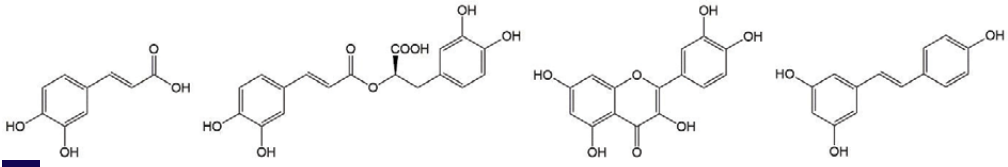
SUMMARY: UV radiation (UVA and UVB), is an integral part of the sunlight reaching the earth's surface containing high energy photons. Owning to the high energy, the photons they can directly interact with biomolecules like DNA, lipids, and proteins and/or generate reactive oxygen species (ROS) causing oxidative damage and irreversible changes in the skin. The changes can lead to premature skin ageing (photoageing) and even skin cancer. Adequate protection of the skin is hence of paramount importance. Exogenous protection approaches that are being developed include organic and inorganic sunscreens as well as various naturally occurring low molecular weight compounds of plant-origin. The plant hormones, cytokinins, especially kinetin (Kin), which is a N6-substituted adenine derivative, are known for their antisenescence and antioxidant effects. Some cytokinins and their derivatives, in addition, have been shown to have photoprotective properties against UVA and UVB radiation. Their application in the prevention of photoageing is being intensively studied.

KEY WORDS: cytokinins – kinetin – synthetic derivatives – photoprotection – antisenescence – UV radiation – photoageing

UV záření a jeho působení na lidskou kůži

Přibližně 5 % slunečního záření dopadajícího na zemský povrch je tvořeno ultrafialovým (UV) zářením o vlnových délkách od 295 do 400 nm. UV záření lze rozdělit podle vlnové délky na UVB (295–315 nm) a UVA (315–400 nm). Ačkoliv UVB reprezentuje menší část slunečního UV záření (1–5 %), je přibližně 1000krát genotoxičtější v porovnání s UVA. Většina UVB záření je absorbována v epidermální vrstvě kůže, která je tvořena zejména různě diferencovanými keratinocyty a v menší míře melanocyty a Langerhansovými buňkami. UVB fotony s vysokou energií jsou schopny vyvolat nevratné změny v základních stavebních jednotkách DNA, proteinů a lipidů (1). Báze DNA mohou přímo absorbovat energii UVB fotonů, což vede ke vzniku cyklobutanpyrimidinových dimerů a pyrimidin-pyrimidonových (6–4) fotoproduktů, které jsou odstraňovány mechanismem nukleotidové excizní reparace (NER). Při tomto druhu opravy DNA dochází k vyštěpení až 30 nukleotidů v místě poškození molekuly DNA. V následujícím kroku jsou odstraněné nukleotidy opětovně dosyntetizovány (2). UVB záření vyvolává také nepřímé poškození; při fotosensitizaci endogenních biomolekul (chromoforů) dochází k produkci reaktivních forem kyslíku (ROS), které oxidačně modifikují báze nukleových kyselin, vzniká tak například 8-hydroxydeoxyguanosin. Na odstranění oxidačně modifikovaných bází buňky využívají bázovou excizní reparaci (BER). U BER na rozdíl od NER je odstraněna a následně nahrazena pouze konkrétní modifikovaná báze (3). Největším rizikem spojeným s působením UVB záření na kůži je tedy vznik mutací, které nejsou rozpoznány a odstraněny reparačními systémy a mohou vést k vzniku rakoviny kůže včetně nejnebezpečnějšího melanomu. Světová incidence melanomu za posledních 40 let výrazně stoupla a v České republice tomu není jinak (4).

Fotony UVA tvoří dominantní část (95–99 %) UV záření dopadajícího na zemský povrch. Toto záření má menší energii než UVB záření, ale proniká do



Obr. 1 Struktura kyseliny kávové a rozmarýnové, kvercetinu a resveratrolu

hlubších vrstev kůže a zasahuje nejen epidermální, ale i dermální vrstvu tvořenou fibroblasty a bílkovinami extracelulární matrix (kolagen, elastin, aj.). Účinek UVA záření není primárně spojen s genotoxicitou; toto záření je absorbováno chromofory v biomolekulách a vede k jejich excitaci s následnou produkcí ROS. Vzniklé ROS oxidačně modifikují biomolekuly, které ztrácí nebo mění své fyziologické vlastnosti a často se akumulují v tkáni. Současně dochází k stimulaci syntézy degradačních enzymů, které štěpí komponenty extracelulární matrix, což vede k předčasnému stárnutí kůže (5).

Předčasné stárnutí kůže

Stárnutí neboli senescence je přirozený, komplexní proces probíhající zcela nezávisle, prakticky od narození až do smrti jedince. Po celý život je organismus vystaven působení stresových faktorů, které se dělí na vnitřní (metabolické) a vnější (environmentální – výživa, xenobiotika, sluneční záření, aj.). Vnitřní a vnější faktory vyvolávají postupné zpomalení buněčného metabolismu, snížení energetického potenciálu buněk (dysfunkce mitochondrií), zvýšení produkce reaktivních sloučenin, snížení antioxidační ochrany, pokles kapacity/rychlosti opravy a eliminace poškozených biomolekul vedoucí k jejich akumulaci, narušení komunikace mezi buňkami aj. Stárnutí jako takové nelze zastavit. Mluvíme-li o antisenescenčním účinku, máme na mysli zpomalení či zmírnění těchto změn. Látky zpomalující stárnutí vykazují často pleiotropní efekt. Podílí se na eliminaci nežádoucích stresových faktorů (reaktivní sloučeniny) nebo aktivaci/stimulaci ochranných, reparačních a regeneračních drah (6).

UV záření je považováno za jeden z hlavních rizikových faktorů předčasného stárnutí. S věkem klesá kapacita a účinnost reparačních mechanismů (NER, BER), což je spojeno nejen se vznikem mutací, ale i s tvorbou nefunkčních proteinů (enzymy, receptory, přenašeče aj.) a jejich akumulací, případně se změnami v diferenciaci a proliferaci buněk. Poškození DNA, které není opraveno reparačními mechanismy, vede k aktivaci tumor supresorových genů p53 a p16, které jsou nezbytné pro aktivaci apoptózy. Buňky, které nejsou eliminovány apoptoticky a současně je u nich inhibována proliferace, jsou ve stavu zvaném buněčná senescence. Tyto buňky produkují do svého okolí různé prozánětlivé mediátory (interleukin-6, interleukin-8, nádorový supresorový faktor alfa (TNFα), růstové faktory a chemokiny), které přispívají k rozvoji chronického zánětu v kůži a jejímu stárnutí (7). UV záření poškozuje také lipidy, což je spojeno se vznikem lipidových peroxidů, které mohou reagovat s okolními biomolekulami a modifikovat je. Peroxidace fosfolipidů membrán vede k zhoršení/narušení funkce membrán. Produkty oxidace lipidů se hromadí v tkáních jako tzv. lipofuscin, který je považován za marker senescence (8). Působením UV fotonů a vznikajících ROS dochází k poškození komponent extracelulární matrix (kolagen, elastin), k změně jejich vlastností (elasticita, pružnost), k snížení syntézy kolagenu a k indukci/aktivaci metaloproteinas (MMP). MMP degradují komponenty extracelulární matrix. U chronicky exponované kůže dochází k hromadění poškozených elastických vláken a vzniku tzv. elastózy (9).

Na histologické a fyziologické úrovni je možné v kožní tkáni pozorovat: atrofii stratum spinosum, zpomalení obnovy epidermálních buněk, úbytek fibroblastů, mastocytů a melanocytů, snížení schopnosti produkovat melanin, vyhlazení dermo-epidermálního spojení, úbytek kolagenních vláken, změny morfologie elastických vláken, vznik amorfní změti elastických vláken, změny ve stavbě kožních cév a atrofii vaziva v podkožní tkáni spojené s tvorbou hlubokých vrásek, snížení bariérové funkce stratum corneum a snížení hydratace kůže (10).

Na kůži opakovaně vystavené slunečnímu záření (nejčastěji v oblasti obličeje, krku a rukou) jsou pozorovány makroskopické změny spojené s předčasným stárnutím, které jsou odlišné od kůže chráněné před působením slunečního záření. Kůže je sušší, hrubší a méně elastická, s větším počtem vrásek, které jsou hlubší, melanin je nepravidelně distribuován a dochází k vzniku hyperpigmentačních skvrn a solárního lentiga. V konečném důsledku dochází k vzniku kožních novotvarů: aktinická keratóza, bazaliomy, spinalinomy, různé typy melanomu, ale i jiných benigních útvarů (11).

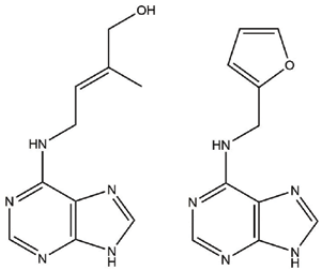
Protekce kůže proti UV záření

Kůže si vyvinula vůči nežádoucím účinkům slunečního záření různé způsoby ochrany jako je zesílení zrohovatělé vrstvy epidermis stratum corneum, syntéza pigmentu melaninu, antioxidační systém tvořený nízkomolekulárními a vysokomolekulárními molekulami aj. Tyto jednotlivé složky jsou spolu vzájemně propojeny a současně spolupracují s reparačními systémy. Při intenzivní a dlouhodobé expozici kůže slunečnímu záření dochází k překročení jejich ochranných schopností, což vede k vzniku erytému, případně k spálení kůže. Proto je snaha posílit vlastní ochranné mechanismy kůže. Jednou z možností je použití přípravků obsahujících látky, které snižují nebo eliminují účinky slunečních fotonů. Jedná se o tzv. UV filtry.

UV filtry se dělí podle povahy na anorganické a organické. Anorganické UV filtry, většinou ve formě mikro nebo nanočástic oxidů kovů, například oxidu titaničitého nebo zinečnatého, odrážejí fotony UV záření, zatímco organické látky, zpravidla aromatické povahy, absorbují energii UV fotonů a tu uvolňují ve formě tepla. Bohužel, některé studie prokázaly nestabilitu některých organických filtrů používaných v ochranných přípravcích. Po jejich aplikaci a expozici slunečnímu záření může docházet k fotoaktivaci spojené s fototoxickou a/nebo fotoalergickou reakcí (5). Proto je snaha používat v kosmetických a dermatologických přípravcích kombinace anorganických a organických filtrů s antioxidanty. Ochrana kůže se může zvýšit také použitím látek, jejichž aplikace vede k aktivaci reparačních mechanismů, např. aplikací fragmentů DNA či vyizolovaných reparačních enzymů v liposomech. Také u perorálního antidiabetika acetohexamidu, inhibitoru DOPA-dekarboxylasy, byla zjištěna aktivace BER (12, 13). Extrakt z rostliny řimbaby obecné (Chrysanthemum parthenium) snižoval poškození DNA vyvolané UV zářením aktivací primární antioxidační dráhy Nrf2-Keap1-ARE (14). Další strategie je založena na aplikaci látek inhibujících MMP, které hrají významnou roli při regeneraci tkání, hojení, tvorbě jizev či zánětu, čímž dochází k snížení poškození bazální membrány, epidermální hyperplazie, degradace kolagenu a tvorby vrásek (15). Další možností je inhibice produkce mediátorů, které stimulují senescenci. Například simvastatin, kortikosteron či

Obr. 2

Struktura zeatinu a kinetinu

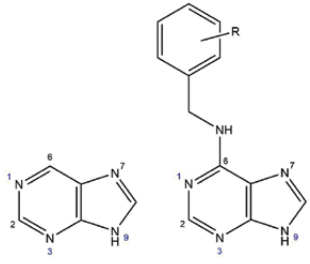


kortizol inhibují tvorbu prosenescenčních faktorů včetně prozánětlivých cytokinů (16, 17). Jiné přístupy se zaměřují na specifické odstranění nebo zpomalení kumulace senescenčních buněk či neutralizaci prozánětlivých cytokinů (18).

Ovlivnění předčasného stárnutí kůže přírodními látkami

Celá řada přirozeně se vyskytujících nízkomolekulárních látek byla studována ve vztahu k ochraně kůže před účinky UV záření. Kromě schopnosti absorbovat UV záření měla většina z těchto sloučenin i antioxidační, protizánětlivé a/nebo imunomodulační účinky. Významnou skupinu těchto přírodních látek tvoří polyfenoly. Tyto sloučeniny jsou schopné stimulovat některé cytoprotektivní signální dráhy, např. Nrf2-Keap1-ARE, což je dráha reagující na oxidoredukční statut buňky a řídící nejen syntézu antioxidačních enzymů a bílkovin, ale i proteinů, které se podílí na opravě biomolekul a na biotransformaci a exkreci látek. Polyfenoly jsou také schopny přímo absorbovat energii UV fotonů (5). Při topické aplikaci se využívají jak čisté polyfenoly (kyselina ferulová, rozmarýnová či kávová, rutin, kvercetin, resvertrol, katechin, epigalokatechin galát aj.), tak směsi či extrakty z rostlin (čajovník čínský (Camelia sinensis), rozmarýn lékařský (Rosmarinus officinalis), šalvěj lékařská (Salvia officinalis), granátové jablko (Punica granatum), tečkovka zlatá (Polypodium leucotomos), borovice přímořská (Pinus pinaster) a další. Při použití směsí či extraktů byl pozorován synergický efekt jednotlivých obsahových složek, které se zde nachází v nízkých koncentracích. Tyto obsahové látky díky svým různorodým vlastnostem ovlivňují větší počet molekulárních cílů, což se odráží v účinnější ochraně kůže před působením vnějších faktorů a zpomalením procesu stárnutí (19). Zajímavou přírodní látkou, která má výrazné protektivní účinky, je derivát stilbenu resveratrol, který se vyskytuje například v jadérkách révy vinné (Vitis vinifera), v černém rybízu, borůvkách, moruších nebo v grapefruitu. Resveratrol má silné antioxidační účinky, potlačuje lipidovou peroxidaci a snižuje poškození retinálních pigmentových epiteliálních buněk (19). Jiné práce zase popisují schopnost resveratrolu nejen bránit karcinogenezi, ale dokonce snižovat incidenci kožních tumorů (19). Pokud se prokáží ochranné vlastnosti přírodní látky, jsou často připraveny její nové syntetické deriváty s cílem zvýšení účinnosti, případně biodostupnosti a stability.

Obr. 3 Struktura purinu a 6-benzylaminopurinu s vyznačenými polohami substituce substitučních poloh



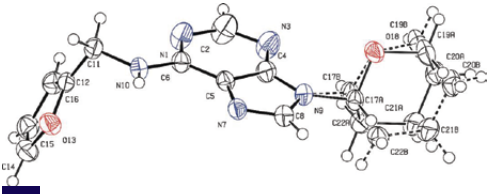
Cytokininy

Cytokininy představují důležitou skupinu rostlinných hormonů, které modulují celou řadu procesů v rostlinách. Poprvé byly popsány v roce 1955. Přirozeně se vyskytující cytokininy jsou adeninové deriváty, které jsou v rostlinách obsaženy v nepatrných koncentracích. Ovlivňují například buněčné dělení, stimulují diferenciaci chloroplastů a zejména inhibují listovou senescenci (konkrétně se jedná o prevenci degradace chlorofylu, která je průvodním jevem stárnutí listu) (20). Cytokininy a jejich synteticky připravené deriváty se používají ke stimulaci růstu rostlinného kalusu a dělení rostlinných pletiv, aklimatizaci vegetativně vypěstovaných rostlin a v dalších biotechnologických oblastech souvisejících s tkáňovými kulturami. (E)-2-Methyl-4-(7H-purin-6-ylamino)but-2-en-1-ol (zeatin, obr. 2), isoprenoidní cytokinin, byl pojmenován podle svého výskytu v kukuřici (Zea mays). Dnes se cytokininové deriváty první generace, tzn. ty prvně objevené nebo připravené, jako jsou Kin, benzylaminoadenin nebo zeatin, používají například

v tzv. mikropropagaci banánů, jahod, jablek, růží, melounů, léčivých rostlin, aj. (21, 22). Zeatin a Kin našly své uplatnění také v dermatologii. Při dlouhodobé kultivaci lidských kožních fibroblastů se zeatinem byly potlačeny typické morfologické změny provázející stárnutí buněk (velikost buněk, množství odpadních vakuol, polymerizace aktinu, odolnost vůči oxidačnímu stresu) (23). Zeatin inhiboval expresi MMP-1 stimulovanou UVB zářením v lidských kožních fibroblastech, což může být spojeno se schopností zeatinu modulovat mitogenem aktivované proteinkinázy (24). Zeatin aplikovaný na lidské keratinocyty zvyšoval expresi akvaporinu-3, proteinu regulujícího přenos vody přes membránu a současně potlačoval jeho snížení vyvolané UV zářením, čímž příznivě ovlivnil proces hojení v UV ozářených buňkách (25). V klinické studii byl zeatin (0,1%) aplikován spolu s UV filtrem (SPF 30) dobrovolníkům; po 12 týdnech bylo pozorováno zlepšení vzhledu kůže (snížení drsnosti a hrubosti kůže a výskytu jemných vrásek) (26).

Použití kinetinu na živočišných a lidských buňkách

Kin byl objeven v padesátých letech minulého století při experimentech Folke Skooga, Carlose Millera a jejich spolupracovníků (27). Carlos Miller experimentoval s kokosovým mlékem a zjistil, že obsahuje puriny, a že koncentráty z kokosového mléka jsou aktivní ve tkáňové kultuře tabákové dřeně, která nebývale rostla. Později tito vědci izolovali z autoklávované DNA bílou krystalickou látku, Kin, a zjistili, že podporuje dělení buněk, cytokinezi, proto pro danou skupinu látek dnes používáme termín cytokininy (27). Po objevení Kin byla identifikována a v laboratoři připravena celá řada cytokininových derivátů a popsány jejich biologické účinky na rostlinné i živočišné buňky (28). Kin v celé řadě in vitro a in vivo experimentů prokázal výrazné antioxidační vlastnosti. Kin chránil izolovanou DNA před poškozením vysoce aktivními a toxickými hydroxylovými radikály, vyvolaným přítomností tranzitními kovů (Fe2+, Cu+, Fentonova reakce) (29, 30). Kin také inhiboval oxidační a glykooxidační poškození proteinů (30). Ochranné vlastnosti Kin vůči oxidačnímu poškození souvisí pravděpodobně s jeho schopností stimulovat tvorbu, případně aktivitu antioxidačních enzymů. U octomilek, do jejichž stravy byl přidán Kin, došlo ke zvýšení aktivity enzymu katalasy (KAT) (31, 32). Po inkubaci lidských fibroblastů s Kin došlo k zvýšení aktivity KAT spolu s dalšími antioxidačními enzymy jako glutathionperoxidasa (GPX) či glutathionreduktasa (GSR) (33). V takto ošetřených fibroblastech došlo k zvýšení hladiny endogenního glutathionu a k snížení peroxidace lipidů, což se projevilo poklesem hladiny malondialdehydu (MDA) (33). Kin zvyšoval aktivitu enzymu superoxiddismutasy (SOD), KAT i GPX u laboratorních krys. Současně došlo k snížení peroxidace lipidů a hladiny MDA (34). Přidání Kin k stárnoucím lidským fibroblastům způsobilo oddálení senescence a snížení charakteristik spojených se stárnutím buněk. Oproti kontrole



Obr. 4 Stuktura pyratinu (47)

došlo ke snížení tvorby pigmentu lipofuscinu, avšak jiné výrazné morfologické změny buněk nebyly pozorovány (35). U stárnoucích lidských keratinocytů byla po aplikaci Kin v kombinaci s vápníkem zaznamenána výrazná stimulace jejich diferenciace, jež byla prokázána měřením obsahu specifických proteinů keratinu 10 a 14 a involukrinu (36). Výsledky další studie na rekonstruované lidské kůži ukázaly, že Kin ovlivňuje jak pokožku, tak škáru. Kin zvyšoval množství elastinu a fibrillinu I v dermis a mírně zvýšil množství laminu 5. Tyto změny měly pozitivní vliv na tvorbu bazální membrány a síť elastinu ve vrchních vrstvách škáry. V epidermis ovlivnilo ošetření Kin proliferaci keratinocytů a jejich diferenciaci (37). Kůže dobrovolníků ošetřená Kin vykazovala nižší míru poškození po působení slunečního záření, jako jsou jemné vrásky, hrubost kůže a výskyt pigmentačních skvrn. V další klinické studii byl dobrovolníkům aplikován přípravek obsahující 0,1% Kin dvakrát denně po dobu 24 týdnů. Již po 12 týdnech bylo pozorováno zlepšení kvality kůže, včetně zlepšení její bariérové funkce (38). Chiu a kol. studovali schopnost Kin (0,03%) v kombinaci s niacinamidem (vitamin B3; 4%) potlačit známky předčasného stárnutí kůže. Pomocí neinvazivních biometrologických metod zjistili zmenšení výskytu pigmentových skvrn, pórů, vrásek a taktéž zvýšení hydratace kůže (39). Topická aplikace Kin (0,1%) po dobu 12 týdnů způsobila zmírnění zarudnutí a dalších projevů mírné formy růžovky (rosacea). Přípravek byl dobře tolerován (40). Účinky a bezpečnost dlouhodobé aplikace Kin byly testovány v 100denním experimentu na bezsrstém plemeni psa (mexický naháč). Po aplikaci Kin došlo k zlepšení kvality pokožky, úbytku pigmentových skvrn a vyhlazení jemných vrásek. Zároveň v pokožce ani ve škáře nebyly histologicky prokázány změny, tedy dlouhodobá aplikace Kin na kůži se jeví jako bezpečná (41).

Cytokininové deriváty a jejich použití na lidských buňkách

Molekulu purinu, která tvoří strukturní základ přirozeně se vyskytujících cytokininů, lze substituovat v pozicích N1, C2, N3, C6, N7 a N9. U adeninového derivátu 6-benzylaminopurinu je kromě výše uvedených pozic možné připravit deriváty se substituenty na benzylovém kruhu, viz obr. 3. Díky této skutečnosti je možné připravit velké množství derivátů, které se liší nejen svými fyzikálně-chemickými, ale i svými biologickými vlastnostmi. Některé deriváty si zachovávají původní cytokininovou aktivitu, jiné tuto aktivitu ztrácí, avšak mohou substitucí získat nové vlastnosti. Při jejich přípravě je tedy vhodné postupovat systematicky a následně provádět pečlivé testování jejich biologických vlastností.

Například 2, 6, 9-trisubstituované deriváty cytokininů byly popsány jako účinné protinádorové látky. Mechanismus účinku těchto derivátů s nádory je založen na inhibici cyklin-dependentních kinas, klíčových enzymů buněčného cyklu. Jedna z těchto látek, roskovitin, byla patentována, licencována firmě Cyclacel Pharmaceuticals Ltd. a pod komerčním názvem Seliciclib zařazena do multicentrické fáze IIB klinického zkoušení léčby nádorů plic (42, 43). Mezi další látky obdobných vlastností patří patentované látky olomoucin a olomoucin II.

Další Kin derivát 6-(furfurylamino)-9-(tetrahydropyran-2-yl)purin (pyratin, Pyr,obr. 4), byl připraven a patentován Ústavem experimentální botaniky AV ČR v roce 2007. Tento nový derivát byl klinicky studován pro své antisenescenční účinky na lidskou kůži v roce 2008 (44). Topická aplikace Pyr (0,1%) vedla k celkovému zlepšení stavu pokožky (44). Patent byl licencován a od roku 2009 je Pyr součástí kosmetických přípravků (45).

Kinetinové deriváty v UV protekci

U Kin a jeho derivátů byl nedávno popsán UVA a UVB ochranný efekt na lidských kožních buňkách (46). Deriváty strukturně napodobující Kin, které byly připraveny v Centru Regionu Haná pro Biotechnologický a Zemědělský Výzkum Univerzity Palackého v Olomouci ve spolupráci s odborníky z Ústavu Experimentální Botaniky AV ČR, byly účinnější než samotný Kin. Účinnost připravených derivátů Kin byla testována na normálních lidských kožních fibroblastech a na linii lidských keratinocytů ozářených UVA nebo UVB zářením. Tyto deriváty měly vyšší ochrannou aktivitu vůči UVA i UVB záření než kyselina rozmarýnová, která byla použita jako standard. Dva deriváty Kin, konkrétně 6-(thiofen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin a 2-chloro-6-furfurylamino-9-(tetrahydro-furan-2-yl)purin) navíc chránily háďátko obecné (Caenorhabditis elegans) vůči oxidačnímu stresu. I když byly látky účinné na těchto modelech, v in vitro podmínkách v tzv. ORAC testu nereagovaly s uměle generovaným volným radikálem. Mechanismus jejich účinku tedy není spojen s přímou interakcí s radikály, respektive ROS a je předmětem dalšího studia. Příznivým zjištěním je, že látky byly účinné ve velmi nízkých koncentracích a žádná z nich nebyla fototoxická, tzn., že v kombinaci s UVA zářením se nezvyšuje toxicita studované látky (46).

ZÁVĚR

Dnešní způsob života je i přes preventivní opatření a kvalitní lékařskou péči spojen s nárůstem civilizačních chorob včetně rakoviny kůže, která nepřímo souvisí s předčasným stárnutím kožní tkáně. Tato skutečnost podněcuje úsilí vědeckých týmů nalézt látky, které by tyto nežádoucí procesy potlačily. Současná cílená organická syntéza umožňuje připravit nové deriváty inspirované látkami obsaženými v rostlinách, které vykazují nejrůznější spektrum biologických aktivit. Tato kombinatorní preparativní chemie, která vedla k přípravě výše uvedených látek aktivních na lidské buňky, je v souladu se strategií současné cílené organické syntézy cytokininů přinášet nové biologicky aktivní deriváty. Hlavním cílem tohoto přístupu je připravit účinnější deriváty než byly původní, přirozeně se vyskytující látky, které by byly dostupnější, stabilnější nebo méně toxické. Dalším důležitým faktorem je studium interakcí látky s živou buňkou nebo tkání, určení specifického buněčného cíle, na který látka působí, a odhalení mechanismu jejího účinku. Tento kombinovaný přístup přináší nová zjištění o provázanosti fungování molekulárních mechanismů při protekci kožních buněk.

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Supplement V

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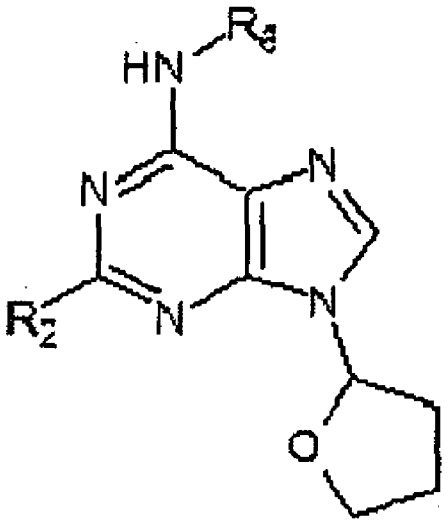

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(57) Abstract: The present invention provides adenine derivatives substituted at the C2, N6, and N9 purine positions having antisenescent and combined photoprotective UVA / UVB effects. These substances are particularly suitable as anti-senescent and UV-photoprotective component in cosmetic preparations, plant protection preparations and in preparations for the treatment/application of tissue cultures.

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ADENINE DERIVATIVES AND THEIR USE AS UV-PHOTOPROTECTIVE AGENTS

Field of invention

The invention relates to adenine derivatives with combined anti-senescent and UV-photoprotective effects against UVA and UVB radiation. Further it relates to their use thereof.

Background art

6-furfurylaminopurine (kinetin) is a compound that belongs to plant hormone group called cytokinins. Cytokinins are structurally N^6-substituted adenine derivatives. Kinetin was discovered in 1950s and considered to be a growth regulator because it positively influenced the growth of tobacco callus cells. Exogenous application of kinetin induces cell differentiation and morphogenesis of the cells of plant callus and postpones the senescence of leaves. Except for the influence on plant cells, it shows also effects on animal cells. Kinetin possess antioxidant properties and is able to protect against oxidative stress – it is able to inhibit oxidation and damage of proteins, to influence the growth of keratinocytes and to delay aging of human skin fibroblasts in vivo although the compound does not influence proliferation of these fibroblasts. Kinetin derivative 6-furfurylamino-9-(2-tetrahydropyran-2-yl)purine (trade name Pyratine) that is currently commercially used in cosmetic preparations, was prepred by merging protective tetrahydropyranyl group with the kinetin molecule. This structural modification led to the improvement of anti-senescent and antioxidant effects on plant and animal cells including the tests performed on human skin or human skin models.

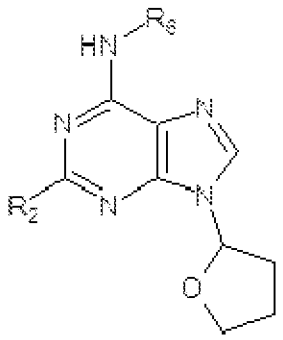
In recent years, increasing amounts of UV radiation (particularly the secondary (UVB) and long (UVA) wavelengths) reach the Earth's surface. This is a new phenomenon that contributes to the development of a number of skin diseases and disorders in humans. UVB radiation forms about 4-5 % of the total radiation and is able to penetrate the skin and the epidermis, where it causes direct and indirect adverse biological effects. UVA accounts for 90 % of the total proportion of radiation and penetrates deeper into the papillary dermis and partially into the hypodermis (10%), which causes the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Chronic skin exposure to UVA radiation can lead to premature aging of the skin, which is associated with structural damage of the dermis, resulting in the formation of wrinkles, moles and other signs of skin aging. Natural endogenous photoprotective agent is melanin, but it is not formed in sufficient amounts in human skin, particularly in relation to increasing intensity of UV radiation reaching the Earth due to ozone depletion in the atmosphere and lifestyle modifications (more

outdoor activities, clothing that covers smaller part of the body surface). If the skin is treated with a substance which prevents penetration of UV rays in particular, it can protect against premature aging but also against short term adverse effects of UV radiation. Majority of products currently used in cosmetics to protect against solar radiation are so-called sunscreens (UV filters). These sunscreens were developed to protect skin primarily against "harmful" UVB radiation which may give rise to malignant melanokarcinoma. Sunscreens are divided into preparations with physical mechanism of action (inorganic minerals that create a physical barrier to radiation on the skin, such as TiO2 or ZnO) and preparations with chemical mechanism of action (organic substances capable to absorb the radiation by changing the distribution of electrons - for example benzophenones, cinnamate, salicylate). For some existing sunscreens, adverse reactions associated mainly with photoallergic or fotoirritating reactions have been reported when using these products. A common problem of these substances is also photo-instability.

The present inventors found a compound which unexpectedly combines antisenescence effects and UV-photoprotective effects (against both UVB and UVA radiation). These substances are very stable, they are not phototoxic and they do not irritate treated skin.

Disclosure of the Invention

The invention relates to adenine derivatives of general formula I



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and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein

R2 is hydrogen or halogen;

R6 is selected from a group containing

- heteroaryl with 5- to 6-membered aromatic ring containing at least one heteroatom selected from O, S whereas other ring atoms are carbon atoms, while heteroaryl is unsubstituted or

substituted by at least one substituent selected from the group consisting of C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- heteroarylalkyl with 5- to 6-membered aromatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, wherein the alkyl contains 1 to 4 carbon atoms, whereas the heteroarylalkyl is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- heterocyclyl with 5- to 6- membered aliphatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, wherein the heterocycle is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, merkapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- heterocyclylalkyl with 5- to 6- membered aliphatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, the alkyl contains 1 to 4 carbon atoms, whereas the heterocyclylalkyl is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;- cycloalkyl with ring containing 5 to 6 carbon atoms, unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- cycloalkylalkyl with ring containing 5 to 6 carbon atoms, wherein the alkyl contains 1 to 4 carbon atoms, whereas the cycloalkylalkyl is unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- isoalkyl containing 3 to 7 carbon atoms, unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl.

Heteroalkyl preferably comprises a 5-membered ring, more preferably it contains one heteroatom in the 5-membered ring, said heteroatom being O or S. Most preferably, the heteroalkyl is furan-2-yl or thiophen-2-yl.

Heteroarylalkyl preferably comprises a 5-membered ring and a C1-C2 alkyl, more preferably it contains one heteroatom in the 5-membered ring, said heteroatom being O or S. Most preferably, the heteroarylalkyl is selected from furan-2-ylmethyl (furfuryl) and thiophen-2-ylmethyl.

Heterocyclyl preferably comprises a 5-membered ring, more preferably it contains one heteroatom in the 5-membered ring, said heteroatom being O or S. Most preferably, the heterocyclyl is selected from tetrahydrofuran-2-yl and tetrahydrothiophen-2-yl.

Heterocyclylalkyl preferably comprises a 5-membered ring and a C1-C2 alkyl, more preferably it contains one heteroatom, said heteroatom being O or S. Most preferably, the heterocyclylalkyl is selected from tetrahydrofuran-2-ylmethyl and tetrahydrothiophen-2-ylmethyl.

Cycloalkyl is preferably cyclopentyl. Cycloalkylalkyl is preferably cyclopentylmethyl.

Isoalkyl is preferably selected from isopropyl, isobutyl, isopentyl, isohexyl and isoheptyl.

Halogen is selected from the group comprising fluorine, chlorine, bromine and iodine, the most preferred halogen is chlorine.

Particularly preferred compounds of the invention are the compounds of formula I selected from the group consisting of

6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-methylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-hydroxymethylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-formylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(1-furan-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-methyltetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(1-tetrahydrofuran-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(cyclopentylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(3-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-chlorothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-bromothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(1-thiophen-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-methylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-hydroxymethylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-formylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(1-furan-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-methyltetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(1-tetrahydrofuran-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(thiophen-2-ylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(3-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-chlorothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-bromothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(1-thiophen-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(cyclopentylmethylamino)-9-(tetrahydrofuran-2-yl)purine

More preferably, the compounds of general formula I are selected from the group consisting of:
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine.

The invention further encompasses the use of adenine derivatives of general formula I as UV-photoprotective agents in cosmetic compositions, preparations for plant protection and/or in preparations for tissue culture application. Preferred use of the compounds of the invention is the use as agents having a combined anti-senescent and UV-photoprotective effect.

The compounds of the invention show combined anti-senescent and UV-photoprotective effects. Their UV-photoprotective effect was observed against UVA as well as against UVB radiation. They are suitable as components of cosmetic preparations, preparations for plant protection, preparations for tissue culture application. Cosmetic preparations comprising the compounds of the present invention are suitable for the treatment of skin, fur and hair of mammals. The preparations for tissue

culture application are suitable for the treatment of plant and mammal cell cultures, wherein the cells are e.g. keratinocytes or fibroblasts.

Object of the invention are further cosmetic preparations, preparations for plant protection, preparations for tissue culture applications, containing compounds according to general formula I.

The preparations for tissue culture can be utilized in biotechnologies, especially in tissue cultures for plant micropropagation.

The compounds of the present invention further show immunosuppressive activity through downregulation of tyrosine-protein kinase JAK3 and innate-immunity-related tyrosine-protein kinase HCK and toll-like receptor TLR2. The immunosuppressive activity may be exploited in the cosmetic use of the present compounds for preventing hypersensitive skin reactions, or in medical preparations for treatment of hypersensitive immune response or transplant rejection.

Preparations (Compositions)

Suitable administration for cosmetic application is local, topical. The cosmetic composition typically contains from 0.1 to 95 wt. % of the active ingredient, whereas single-dose forms contain preferably 10 to 90 wt. % of the active ingredient and administration forms which are not single-dose preferably comprise 1 wt. % to 10wt. % of the active ingredient. The application forms include, e.g., ointments, creams, pastes, foams, tinctures, lipsticks, drops, sprays, dispersions and the like. The compositions are prepared in a known manner, for example by means of conventional mixing, dissolving or lyophilizing processes.

Solutions of the active ingredients, suspensions or dispersions, especially isotonic aqueous solutions, dispersions and suspensions, can be prepared before use, for example in the case of lyophilised compositions which comprise the active substance alone or together with a carrier, for example mannitol.

Suspensions in oil comprise, as the oily component, vegetable, synthetic or semi-synthetic oils. Oils which may be mentioned are, in particular, liquid fatty acid esters which contain, as the acid component, a long-chain fatty acid having 8-22, in particular 12-22, carbon atoms, for example lauric acid, tridecylic acid, myristic acid, pentadecylic acid, palmitic acid, margaric acid, stearic acid, arachidonic acid, behenic acid or corresponding unsaturated acids, for example oleic acid, elaidic acid, erucic acid, brasidic acid or linoleic acid, if appropriate with the addition of antioxidants, for example vitamin E, β-carotene or 3,5-di-*tert*-butyl-4-hydroxytoluene. The alcohol component of these fatty acid esters has not more than 6 carbon atoms and is mono- or polyhydric, for example mono-, di- or trihydric alcohol, for example methanol, ethanol, propanol, butanol, or

pentanol, or isomers thereof, but in particular glycol and glycerol. Fatty acid esters are, for example: ethyl oleate, isopropyl myristate, isopropyl palmitate, "Labrafil M 2375" (polyoxyethylene glycerol trioleate from Gattefoseé, Paris), "Labrafil M 1944 CS" (unsaturated polyglycolated glycerides prepared by an alcoholysis of apricot kernel oil and composed of glycerides and polyethylene glycol esters; from Gattefoseé, Paris), "Labrasol" (saturated polyglycolated glycerides prepared by an alcoholysis of TCM and composed of glycerides and polyethylene glycol esters; from Gattefoseé, Paris) and/or "Miglyol 812" (triglyceride of saturated fatty acids of chain length C_8 to C_{12} from Hüls AG, Germany), and in particular vegetable oils, such as cottonseed oil, almond oil, olive oil, castor oil, sesame oil, soybean oil and, in particular, groundnut oil.

Ointments are oil-in-water emulsions which comprise not more than 70 %, preferably 20 to 50 % of water or aqueous phase. The fatty phase consists, in particular, of hydrocarbons, for example vaseline, paraffin oil or hard paraffins, which preferably comprise suitable hydroxy compounds, such as fatty alcohols or esters thereof, for example cetyl alcohol, or wool wax alcohols, such as wool wax, to improve the water-binding capacity. Emulsifiers are corresponding lipophilic substances, such as sorbitan fatty acid esters (Spans), for example sorbitan oleate and/or sorbitan isostearate. Additives to the aqueous phase are, for example, humectants, such as polyalcohols, for example glycerol, propylene glycol, sorbitol and/or polyethylene glycol, or preservatives and odoriferous substances.

Fatty ointments are non-aqueous and are in particular hydrocarbon-based, e.g. paraffin, vaseline or paraffin oil, and natural or semi-synthetic lipids, such as hydrogenated coconut fatty acid triglycerides or hydrogenated oils, such as hydrogenated castor or groundnut oil, and partially fatty acid glycerol esters, e.g. glycerol mono- and distearate. They further contain, e.g., fatty alcohols, emulsifiers and additives mentioned above in connection with ointments which increase water binding.

Creams are oil-in-water emulsions containing more than 50 % of water. The oil bases used include fatty alcohols, e.g., isopropyl myristate, lanolin, bees wax or hydrocarbons, preferably vaseline (petrolatum) and paraffine oil. Emulsifiers are surface active compounds with predominantly hydrophilic characteristics, such as corresponding non-ionic emulsifiers, e.g., fatty acid polyalcohol esters or ethyleneoxy adducts thereof, e.g., polyglyceridic fatty acids or polyethylene sorbitan esters or acidic polyflyceridic fatty acid esters (Tween), polyoxyethylene fatty acid ethers or polyoxyethylene fatty acid esters; or corresponding ionic emulsifiers, such as alkali sulfate salts of fatty alcohols, such as sodium laurylsulfate, sodium cetylsulfate, or sodium stearylsulfate, which are typically used in the presence of fatty alcohols, e.g., cetyl stearyl alcohol or stearyl alcohol. The aqueous phase additives include agents preventing drying out of the creams, e.g., polyalcohols such as glycerol, sorbitol, propylene glycol and polyethylene glycol, and preservatives and fragrances.

Pastes are creams or ointments containing powdered secretion-absorbing components such as metal oxides, e.g., titanium oxides or zinc oxide, further talc or aluminium silicates for binding humidity or secretion.

Foams are applied from pressurized containers and include liquid oil-in-water emulsions in aerosol form, whereas the propellant gases include halogenated hydrocarbons such as chloro-fluoro-lower alkanes, e.g., dichlorofluoromethane and dichlorotetrafluoroethane, or preferably non-halogenated gaseous hydrocarbons, air, N_2O or carbon dioxide. The oily phases used are the same as for ointments and the additives mentioned for ointments are used.

Tinctures and solutions usually comprise an aqueous-ethanolic base, to which humectants for reducing evaporation, such as polyalcohols, for example glycerol, glycols and/or polyethylene glycol, and re-oiling substances, such as fatty acid esters with lower polyethylene glycols, i.e. lipophilic substances soluble in the aqueous mixture to substitute the fatty substances removed from the skin with ethanol, and, if necessary, other excipients and additives, are admixed.

The invention is further illustrated by the following examples which should not be construed as further limiting.

Examples of carrying out the Invention

Example 1:

Preparation of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**1**)

6-Chloro-9-(tetrahydrofuran-2-yl)purine (1g; 4.46 mmol), tetrahydrofurfurylamine (554 µl; 5.36 mmol) and triethylamine (Et_3N) (3.2 ml; 22.3 mmol) were sequentially dissolved in propanol (50 ml). The mixture was stirred under reflux for 4 hours and then concentrated in vacuo. The residue was dissolved in water and extracted into EtOAC using liquid-liquid continuous extractor (24 h). Organic fraction was dried (Na2SO4) and evaporated in vacuo. The product was obtained after purification via column chromatography using (EtOAc:MeOH:NH_3; 34:1:1; v:v) as eluent. Yield: 76 %. ^{1}H NMR (500 MHz, DMSO-d_6), ppm: 1.53 - 1.64 (m, 1 H); 1.69 - 1.89 (m, 3 H); 1.95 - 2.02 (m, 1 H); 2.11 - 2.23 (m, 1 H); 2.30 - 2.44 (m, 2 H); 3.37 - 3.48 (m, 1 H); 3.48 - 3.53 (m, 1 H); 3.53 - 3.60 (m, 1 H); 3.70 - 3.76 (m, 1 H); 3.81 - 3.90 (m, 1 H); 3.98 (q, *J*=7.03 Hz, 1 H); 4.09 (q, *J*=7.44 Hz, 1 H); 6.21 (dd, *J*=6.88, 3.82 Hz, 1 H); 7.63 (br. s., 1 H); 8.17 (br. s., 1 H); 8.21 (s, 1 H).

Table 1: Compounds prepared according to example 1

N.	R_6	R_2	Elemental analysis calculated/found			
			%C	%H	%N	ES MS $[M+H]^+$
1	tetrahydrofuran-2-ylmethyl	H	58.1/58.0	6.6/6.6	24.2/24.3	290.3
2	5-methylfuran-2-ylmethyl	H	60.2/60.1	5.7/5.7	23.4/23.5	300.3
3	5-hydroxymethylfuran-2-ylmethyl	H	57.1/57.2	5.4/5.4	22.2/22.5	316.3
4	5-formylfuran-2-ylmethyl	H	57.5/57.6	4.8/4.8	22.4/22.2	314.3
5	1-furan-2-ylethyl	H	60.2/60.1	5.7/5.8	23.4/23.5	300.3
6	5-methyltetrahydrofuran-2-ylmethyl	H	59.4/59.3	7.0/7.0	23.1/23.2	304.4
7	1-tetrahydrofuran-2-ylethyl	H	59.4/59.2	7.0/7.1	23.1/23.2	304.4
8	cyklopentylmethyl	H	62.7/62.9	7.4/7.5	24.4/24.5	288.4

Example 2: Preparation of 6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (**9**)

6-Chloro-9-(tetrahydrofuran-2-yl)purine (0,5 g; 2.23 mmol), 2-thiophenemethylamine (275 µl; 2.68 mmol) and triethylamine (Et_3N) (1.6 ml; 11.15 mmol) were sequentially dissolved in propanol (25 ml) The mixture was stirred under reflux for 3 hours then 2-thiophenemethylamine (23 µl; 0.23 mmol) was added and reaction mixture was stirred under reflux for an additional 1.5 hours. The mixture was concentrated in vacuo. The residue was dissolved in water and extracted into EtOAc. Organic fraction was dried (Na_2SO_4) and evaporated in vacuo. Product was obtained after precipitation in diethylether. Yiel: 61 %. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.92 - 2.00 (m, 1 H); 2.12 - 2.22 (m, 1 H); 2.31 - 2.38 (m, 1 H); 2.38 - 2.43 (m, 1 H); 3.85 (td, *J*=7.68, 6.34 Hz, 1 H); 4.08 (td, *J*=7.68, 6.50 Hz, 1 H); 4.79 (br. s., 2 H); 6.21 (dd, *J*=6.88, 3.82 Hz, 1 H); 6.88 (dd, *J*=5.04,3.44 Hz, 1 H); 6.97 (dd, *J*=3.40, 1.03 Hz, 1 H); 7.27 (dd, *J*=5.12, 1.22 Hz, 1 H); 8.23 (s, 2 H); 8.36 (br. s., 1 H).

Table 2: Compounds prepared according to example 2

N.	R_6	R_2	Elemental analysis calculated/found			
			%C	%H	%N	ES MS $[M+H]^+$
9	thiophen-2-ylmethyl	H	55.8/55.6	5.0/5.4	23.2/23.3	302.4

N.	R_6	R_2	Elemental analysis calculated/found			
			%C	%H	%N	ES MS $[M+H]^+$
10	3-methylthiophen-2-ylmethyl	H	57.1/57.2	5.4/5.5	22.2/22.3	316.4
11	5-methylthiophen-2-ylmethyl	H	57.1/57.0	5.4/5.3	22.2/22.1	316.4
12	5-chlorothiophen-2-ylmethyl	H	50.0/50.1	4.2/4.3	20.9/20.8	336.8
13	5-bromothiophen-2-ylmethyl	H	44.2/44.1	3.7/3.8	18.4/18.5	381.3
14	1-thiophen-2-ylethyl	H	57.1/57.0	5.4/5.5	22.2/22.0	316.4

Example 3: Preparation of 2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine (**15**)

2,6-Dichloro-9-(tetrahydrofuran-2-yl)purine (0.5g; 1.93 mmol), furfurylamine (204 µl; 2.31 mmol) and triethylamine (Et_3N) (1.32 ml; 9.65 mmol) were sequentially dissolved in propanol (25 ml). The mixture was stirred under reflux for 5 hours and then concentrated in vacuo. If product did not cristallize from reaction mixture it was evaporated in vacuo. Crude reaction mixture was precipitated from ($CHCl_3$:EtOH; 1:8; v:v) or ($CHCl_3$:Ether; 1:7; v:v) and filtrated. Solid product was washed with cold water and recrystallized from EtOH

Table 3: Compounds prepared according to example 3

N.	R_6	R_2	Elemental analysis calculated/found			
			%C	%H	%N	ES MS $[M+H]^+$
15	furfuryl	Cl	52.6/52.5	4.4/4.3	21.9/21.7	320.7
16	5-methylfuran-2-ylmethyl	Cl	54.0/54.1	4.8/4.7	21.0/20.9	334.8
17	5-hydroxymethylfuran-2-ylmethyl	Cl	51.5/51.6	4.6/4.7	20.0/20.1	350.8
18	5-formylfuran-2-ylmethyl	Cl	51.8/51.9	4.1/4.2	20.1/20.3	348.8
19	1-furan-2-ylethyl	Cl	54.0/54.1	4.8/4.9	21.0/21.3	334.8
20	tetrahydrofuran-2-ylmethyl	Cl	51.9/51.8	5.6/5.7	21.6/21.5	323.8
21	5-methyltetrahydrofuran-2-ylmethyl	Cl	53.3/53.4	6.0/6.0	20.7/20.5	338.8
22	1-tetrahydrofuran-2-ylethyl	Cl	53.3/53.4	6.0/6.1	20.7/20.6	338.8

N.	R_6	R_2	Elemental analysis calculated/found			
			%C	%H	%N	ES MS $[M+H]^+$
23	thiophen-2-ylmethyl	Cl	50.1/50.1	4.2/4.3	20.9/20.8	336.8
24	3-methylthiophen-2-ylmethyl	Cl	51.5/51.6	4.6/4.7	20.0/20.2	350.8
25	5-methylthiophen-2-ylmethyl	Cl	51.5/51.6	4.6/4.7	20.0/20.1	350.8
26	5-chlorthiophen-2-ylmethyl	Cl	45.4/45.6	3.5/3.7	18.9/18.8	371.3
27	5-bromthiophen-2-ylmethyl	Cl	40.6/40.5	3.2/3.1	16.9/16.8	415.7
28	1-thiophen-2-ylethyl	Cl	51.5/51.6	4.6/4.7	20.0/20.1	350.8
29	cyklopentylmethyl	Cl	56.0/56.1	6.3/6.4	21.8/21.9	322.8

Example 4: Evaluation of cytotoxicity of novel derivatives for skin cell by MTT in vitro test

MTT assay is a standard test of toxicity based on photometric measurement of the ability of metabolically active cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Using the assay, the effects of 72 hour treatments with several concentrations of the compounds (sixfold dilution, maximal concentration = 50 microM) on viability of skin fibroblasts BJ and keratinocytes HaCaT were evaluated. About 5,000 cells were seeded per well of a 96-well plate 24 hours before the treatment. DMSO vehiculum was used as a negative control. After 72 hour treatment, new medium with MTT (Sigma, M2128) was added to a final concentration of 0.5 mg/ml. After 3 hours, medium was removed and resulting formazan in the cells was dissolved in DMSO. The absorbance was measured at 570 nm (640 nm reference wavelength). The IC50 values were calculated from the dose-response curves. 6-furfurylaminopurine riboside was used as positive controls. The following results were obtained.

Table 4: Cytotoxicity of prepared compounds in MTT in vitro assay.

Compound	IC_{50} (μM)
dimethylsulfoxid	>100
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	>100
6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)aminopurine	>100
2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine	>100
6-furfurylamino-9-ribosylpurine (comperative example)	≤ 3

Example 5: Anti-senescent activity of novel compounds tested in senescent bioassay on wheat leaf segments

Seeds of winter wheat, *Triticum aestivum* cv. Hereward, were washed under running water for 24 hours and then sown on vermiculite soaked with Knop´s solution. They were placed in the growth chamber at 25°C with a 16/8 h light period at 50 $\mu mol.m^{-2}.s^{-1}$. After 7 days, the first leaf was fully developed and the second leaf had started to grow. A tip section of the first leaf, approximately 35 mm long, was removed from 5 seedlings and trimmed slightly to a combined weight of 100 mg. The basal ends of the five leaf tips were placed in the wells of a microtiter polystyrene plate containing 150 µL of the tested derivative solution each. The entire plate was inserted into a plastic box lined with paper tissues soaked in distilled water to prevent leaf sections from drying out. After 96 h incubation in the dark at 25°C, the leaves were removed and chlorophyll extracted by heating at 80°C for 10 min in 5 mL of 80% ethanol (v/v). The sample volume was then restored to 5 mL by the addition of 80% ethanol (v/v). The absorbance of the extract was recorded at 665 nm. In addition, chlorophyll extracts from fresh leaves and leaf tips incubated in deionised water were measured. The results are means of five replicates and the entire test was repeated twice. In each experiment activities of the novel compounds were tested and compared with activity of BAP, which is known to be highly active cytokinin.

The compounds to be tested were dissolved in dimethylsulfoxide (DMSO) and the solution brought up to 10^{-3} M with distilled water. This stock solution was further diluted with the respective media used for the biotest to a concentration ranging from 10^{-8} M to 10^{-4} M. The final concentration of DMSO did not exceed 0.2 % and therefore did not affect the biological activity in the assay system used. The activity obtained for 10^{-4} M of BAP was postulated as 100 %. Kinetin was used as the second standard. Newly prepared compounds generally exceeded the efficiency of standard (BAP) by 10 % of its activity (Tab. 5).

Table 5: Relative biological activity in detached wheat leaf senescence (chlorophyll retention) biotest compared with activity of 6-benzylaminopurine (BAP) standard (100% means activity of BAP in concentration $10^{-4}mol.l^{-1}$)

Compound	maximum effective concentration ($mol.l^{-1}$)	activity (%) [$10^{-4}mol.l^{-1}$ BAP = 100%]

BAP (comperative example)	10^{-4}	100±1
kinetin (comperative example)	10^{-4}	98±4
6-furfurylamino-9-(tetrahydrofuran-2-yl)purin	10^{-4}	114±3
6-(3-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	10^{-4}	110±7
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	10^{-4}	125±9
6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	10^{-4}	112±5
2-chloro-6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	10^{-4}	127±5

Example 6: *In vitro* cytotoxic activity of new derivatives on cancer cell lines

One of the parameters used as the base for cytotoxic analysis is metabolic activity of viable cells. Microtiter assay, which uses the Calcein AM, is now widely used to quantify cell proliferation and cytotoxicity. The quantity of reduced Calcein AM corresponds to the number of viable cells in culture. The cell lines of breast cancer (MCF-7), human erythromleukemia (K562), BJ human fibroblast cells (BJ) and human keratinocyte cell line (HaCaT) were used for routine screening of cytotoxicity of the compounds. The cells were maintened in Nunc/Corning 80 cm^2 plastic bottles and grown in media for cell culture (DMEM containing 5g/l of glucose, 2mM of glutamin, 100 U/ml of penicilin, 100 µg/ml of streptomycin, 10% of fetal bovine serum and sodium hydrogencarbonate). Cell suspensions were diluted according to cell types and according to expected final cell density (10^4 of cells per well according to characteristics of cell growth), pippeted 80 µl of cell suspension on 96-well microtiter plates.. Innoculates were stabilized by 24 hrs preincubation at 37°C in CO_2. Particular concentrations of tested compounds were added in time zero as 20 µl aliquotto wells of microtiter plates.Usually, the compounds were diluted into six concentrations in four-fold dilution series. In routine testing, the highest well concentration was 166.7 µM, of change dependent on the substance. All drug concentrations were examined in duplicates .The incubation of cells with tested derivatives lasted 72 hrs at 37°C, 100 % humidity and in the atmosphere of CO_2. At the end of the incubation period, the cells were tested and analysed according to the addition of Calcein AM (Molecular probes) solution and the incubation lasted for next 1 hour. Fluorescence (FD) was measured using Labsystem FIA reader Fluorskan Ascent (Microsystems). The survival of tumor cells (The tumor cell survival-TCS) was counted according to equation: $GI_{50}=(FD_{well\ with\ derivative}/FD_{control\ well})$ x 100 %. The value of GI_{50}, that is equal to the concentration of compound at which 50 % of tumour cells are terminated.To evaluate the antitumor activity was tested toxicity of new derivatives on panel of cell lines of different

histogenetic and species origin (Tab. 6 , GI50 concentration given in μM) . It turned out that new compounds showed to be non toxic for neither of all tested tumor lines nor for nonmalignant cell lines BJ and HaCaT. Effective derivatives killed tumor cells in concentrations close to 0.1 to 50. None of the newly prepared compounds only reached the value.

Table 6: Cytotoxicity of newly prepared compouds for various cell lines

Compound	MCF-7	K562	BJ	HaCaT
6-furfurylamino-9-(tetrahydrofuran-2-yl)purine	>100	>100	>100	>100
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	>100	>100	>100	>100
6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	>100	>100	>100	>100
2-chloro-6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine	>100	>100	>100	>100

Example 7: *In vitro* test of phototoxic effects of test compounds on normal human dermal fibroblasts.

Phototoxic potential of test compound was determined by modified *in vitro* test validated phototoxicity evaluation (Spielmann H, Balls M, Dupuis J, Pape WJ, Pechovitch G, de Silva O, Holzhütter HG, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell WW, Maurer T, Pfannenbecker U, Potthast JM, Csato M, Sladowski D, Steiling W, Brantom P. The International EU/COLIPA In Vitro Phototoxicity Validation Study: Results of Phase II (Blind Trial). Part 1: The 3T3 NRU Phototoxicity Test. Toxicol In Vitro. 1998;12:305-27). Normal human dermal fibroblasts (NHDF) were used as an *in vitro* model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. Fibroblasts were used between the 2nd and 4th passage. For all experiments the fibroblasts were seeded onto 96-well plates at a density of $0.8x10^5$ cells/ml (0.2 ml per well) of cultivation medium (DMEM supplemented with fetal calf serum (10%, v/v), penicillin (100 mg/ml) and streptomycin (100 U/ml)). Test substances included compounds number 1, 3, 9, 15 and 20. Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml)). After 24h incubation was cultivation medium changed to serum free medium containing test compound or DMSO (negative control). The final applied concentrations range 3.9 - 125 μmol/l. As a control, serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. In parallel with test compound, chlorpromazine (CPZ; 0.8 - 50 μmol/l) was used as a known

phototoxic compound. The test compound was in parallel applied on two 96-well plates with NHDF. After 60 minutes incubation with test compound medium was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. A plate was then exposed to a non-cytotoxic dose of UVA radiation (5.0 J/cm^2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. Intensity of UVA radiation was evaluated before each irradiation by UVA-meter. A control (non-irradiated) plate was for the period of irradiation incubated in dark. After UVA exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % CO_2) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and $CaCl_2$ (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetitions with use of cells from four donors to minimize individual sensitivity of donor cells. Phototoxic effect was evaluated as % of viability of control cells that was calculated from experimental data (absorbance) according to the following equation:

$$\text{Viability (\% of control)} = \left(\frac{(A_S - A_B)}{(A_C - A_B)} \right) \cdot 100$$

A_S … absorbance of sample (cells pre-incubated with test compound in serum free medium and irradiated)

A_C … absorbance of control (cells pre-incubated with DMSO in serum free medium and irradiated)

A_B … absorbance of background (extraction solution)

Result: Treatment with test compounds and following exposure to non-toxic UVA dose did not cause decrease in cell viability ~ incorporation of NR and thus test compound can be considered as non-phototoxic in the used concentration range (3.9-125 µmol/l). Results are given in Tab. 7. A well-known phototoxic compound chlorpromazine, which can be used for comparison, decreases the viability of NHDF cells: on exposure to UVA radiation (UVA+), the viability decreases below 80 % of control in the presence of 6.3 µmol/l of chlorpromazine, while the viability of unirradiated cells (UVA-) decreases below 80 % in the presence of 25 µmol/l of chlorpromazine. A Above data indicate that test compounds are safe for cosmetic and dermatological application including use with following exposure of treated skin with solar radiation.

Table 7: UVA-induced effects of test compounds on NHDF viability.

1 (µmol/l)		3.9	7.8	15.6	31.3	62.5	125
-UVA	% control	96.6	106.5	99.6	102.4	102.3	109.7
	SMODCH	7.4	9.2	3.7	2.1	5.8	10.5
+UVA	% control	105.1	110.6	106.8	109.5	103.3	107.5
	SMODCH	10.3	10.7	13.4	10	8.9	8.9

3 (µmol/l)		3.9	7.8	15.6	31.3	62.5	125
-UVA	% control	103	104.9	104.2	107.7	106.6	110.4
	SMODCH	4.2	5.4	5	8	3.2	4
+UVA	% control	101.04	106.46	102.28	107.4	110.49	111.8
	SMODCH	1.9	7.8	2.9	8.2	8.3	7.1

9 (µmol/l)		3.9	7.8	15.6	31.3	62.5	125
-UVA	% control	100	103	100.5	106.7	107.9	108.4
	SMODCH	0.8	3.4	2	0.3	3.2	0.2
+UVA	% control	97.8	102.3	100.8	105.9	102.8	106.3
	SMODCH	1.5	4.4	1.4	4.3	3.7	1.2

15 (µmol/l)		3.9	7.8	15.6	31.3	62.5	125
-UVA	% control	100.3	103.5	104.3	105.4	106.3	101.2
	SMODCH	1.6	4.3	3.2	4.7	3.9	1.8
+UVA	% control	100.1	101.5	102.6	102.9	103.3	97.3
	SMODCH	0.2	2.1	0.8	2.7	2.1	4

20 (µmol/l)		3.9	7.8	15.6	31.3	62.5	125
-UVA	% control	103.1	102.9	100.6	105.6	102	104
	SMODCH	1.7	5.2	1.3	5.4	1.1	1.8
+UVA	% control	100.1	102.9	100.4	102.7	105.1	104.8
	SMODCH	3.5	4	3.7	3.9	6.1	5.2

Example 8: *In vitro* test of photoprotective effects of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine on dermal fibroblasts

Normal human dermal fibroblasts (NHDF) were used as an *in vitro* model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. Fibroblasts were used between the 2nd and 4th passage. For all experiments the fibroblasts were seeded onto 96-well plates at a density of $0.8x10^5$ cells/ml (0.2 ml per well) of cultivation medium

(DMEM supplemented with foetal calf serum (10%, v/v), penicillin (100 mg/ml) and streptomycin (100 U/ml)). Test compounds included 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (1). Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml)). After 24h incubation was cultivation medium changed to serum free medium containing test compound or DMSO (negative control). The final applied concentrations range of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine was 3.9-500 µmol/ll. As a control serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. Each test compound was in parallel applied on two 96-well plates with NHDF. After 60 minutes incubation medium with test compound was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. To study UVA photoprotection, a plate was exposed to a cytotoxic dose of UVA radiation (7.5 J/cm^2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. To study UVB photoprotection, a plate was exposed to a cytotoxic dose of UVB radiation (400 mJ/cm^2) using the solar simulator equipped with a H2 filter transmitting wavelengths of 295-320 nm. Intensity of UVA or UVB radiation was evaluated before each irradiation by UVA- or UVB-meter. Control (non-irradiated) plates were for the period of irradiation incubated in dark. After UVA or UVB exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % CO_2) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and $CaCl_2$ (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetitions with use of cells from four donors to minimize individual sensitivity of donor cells. Photoprotective effect was evaluated by comparison of experimental data (absorbance) of test compounds with a positive control and a negative control (according to the following equation:

$$\text{Protection (\%)} = 100 - \left| \frac{As - Anc}{Apc - Anc} \right| \cdot 100$$

As … absorbance of sample (cells pre-incubated with test compounds in serum free medium and irradiated)

Anc … absorbance of negative control (cells pre-incubated with s DMSO in serum free medium and non-irradiated = incubated in dark)

Apc … absorbance of positive control (cells pre-incubated with s DMSO in serum free medium and irradiated)

Results: Cells pre-incubated with test compound and exposed to UVA or UVB radiation showed higher viability (ability to incorporate NR) compared to those pre-incubated with DMSO (control) and UVA or UVB irradiated (Tab. 8 and 9).

Table 8: Photoprotective effect of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (1) on UVA-induced damage to NHDF.

UVA photoprotection	
Concentration (µmol/l)	**1**
	protection (%)
3.9	27.7 ± (6.9)
7.8	57.4 ± (14.4)
15.6	43.2 ± (10.8)
31.3	47.5 ± (11.9)
62.5	41.3 ± (10.3)
125	49 ± (12.3)
250	45.3 ± (11.3)
500	18.5 ± (4.6)

Table 9: Photoprotective effect of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (1) on UVB-induced damage to NHDF.

UVB photoprotection	
Concentration (µmol/l)	**1**
	protection (%)
3.9	4.9 ± (1.2)
7.8	44.1 ± (11)
15.6	35 ± (8.7)
31.3	38.3 ± (9.6)
62.5	44.4 ± (11.1)
125	42.7 ± (10.7)
250	46.6 ± (11.6)
500	26.3 ± (6.6)

Example 9: *In vitro* test of photoprotective effects of test compounds on dermal fibroblasts

Normal human dermal fibroblasts (NHDF) were used as an *in vitro* model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in

accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. Fibroblasts were used between the 2nd and 4th passage. For all experiments the fibroblasts were seeded onto 96-well plates at a density of $0.8x10^5$ cells/ml (0.2 ml per well) of cultivation medium (DMEM supplemented with fetal calf serum (10%, v/v), penicillin (100 mg/ml) and streptomycin (100 U/ml)). Test substances included compounds number 1, 3, 9, 15 and 20. Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml)). After 24h incubation was cultivation medium changed to serum free medium containing test compound or DMSO (negative control). The final applied concentrations range was 3.9-31.3 µmol/ll. As a control serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. Rosmarinic acid was used as positive control. Each test compound was in parallel applied on two 96-well plates with NHDF. After 60 minutes incubation medium with test compound was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. To study UVA photoprotection, a plate was exposed to a cytotoxic dose of UVA radiation (7.5 J/cm^2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. To study UVB photoprotection, a plate was exposed to a cytotoxic dose of UVB radiation (150 mJ/cm^2) using the solar simulator equipped with a H2 filter transmitting wavelengths of 295-320 nm. Intensity of UVA or UVB radiation was evaluated before each irradiation by UVA- or UVB-meter. Control (non-irradiated) plates were for the period of irradiation incubated in dark. After UVA or UVB exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % CO_2) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and $CaCl_2$ (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetitions with use of cells from four donors to minimize individual sensitivity of donor cells. Photoprotective effect was evaluated by comparison of experimental data (absorbance) of test compounds with a positive control and a negative control (according to the following equation:

$$\text{Protection (\%)} = 100 - \left| \frac{As - Anc}{Apc - Anc} \right| \cdot 100$$

As … absorbance of sample (cells pre-incubated with test compounds in serum free medium and irradiated)

Anc … absorbance of negative control (cells pre-incubated with s DMSO in serum free medium and non-irradiated = incubated in dark)

Apc … absorbance of positive control (cells pre-incubated with s DMSO in serum free medium and irradiated)

Results: Cells pre-incubated with test compounds and exposed to UVA or UVB radiation showed higher viability (ability to incorporate NR) compared to those pre-incubated with DMSO (control) and UVA or UVB irradiated (Tab. 10 and 11). All test compounds showed higher or comparable photoprotective activity with rosmarinic acid used as positive control. Therefore test compounds has high photoprotective potential.

Table 10: Photoprotective effect of test compounds and rosmarinic acid (RA, positive control) on UVA-induced damage to NHDF.

3 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	14.7	16.3	24.6	25.8
SMODCH	3.2	3.3	5.8	4.5
9 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	41.3	44.4	45.7	52.0
SMODCH	18.0	12.6	9.5	10.8
15 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	24.5	29.9	33.0	35.3
SMODCH	11.9	10.8	12.1	11.5
20 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	5.9	10.7	11.5	16.2
SMODCH	3.2	3.5	5.4	4.1
RA	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	6.1	14.7	17.0	19.3
SMODCH	3.0	3.5	3.8	3.4

Table 11: Photoprotective effect of test compounds and rosmarinic acid (RA, positive control) on UVB-induced damage to NHDF.

3 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	43.6	53.1	47.4	48.4
SMODCH	10.7	9.1	16.9	13.9
9 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**

Protection (%)	34.9	62.6	64.4	68.7
SMODCH	23.7	21.4	14.6	6.2
15 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	39.9	55.2	53	60.2
SMODCH	15.1	14.2	17.1	21
20(µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	39.8	58.3	58.2	60
SMODCH	18.3	19.3	23.2	18.8
RA	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	36.8	50.9	52.9	67.7
SMODCH	5.9	5.8	11.8	5.1

Example 10: *In vitro* test of phototoxic effects of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine on normal human epidermal keratinocytes

Normal Human Epidermal Keratinocytes (NHEK) were used as an in vitro model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. NHEK were used between the 3rd and 4th passage. For all experiments the keratinocytes were seeded onto 96-well plates at a density of 1x104 cells/ml (0.2 ml per well) of growth medium for keratinocytes (EpiLife®) supplemented with Human Keratinocyte Growth Supplement Kit and antibiotics (penicillin (100 mg/ml), streptomycin (100 mg/ml) and ampicillin (250 µg/ml)).

Test compounds included 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (1). Compound was dissolved in DMSO and then diluted in serum free medium (EpiLife® supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml) and ampicillin (250 µg/ml)). After 24h incubation was growth medium changed to serum free medium containing test compound or DMSO (negative control). The final applied concentrations range was 3.9 - 500 µmol/l. As a control, serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. In parallel with test compound, chlorpromazine (CPZ; 0.8 - 50 µmol/l) was used as a known phototoxic compound. The test compound was in parallel applied on two 96-well plates with NHEK. After 60 minutes incubation with test compound medium was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. A

plate was then exposed to a non-cytotoxic dose of UVA radiation (5.0 J/cm2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. Intensity of UVA radiation was evaluated before each irradiation by UVA-meter. A control (non-irradiated) plate was for the period of irradiation incubated in dark. After UVA exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % CO2) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and CaCl2 (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetition with use of cells from four donors to minimize individual sensitivity of donor cells. Phototoxic effect was evaluated as % of viability of control cells that was calculated from experimental data (absorbance) according to the following equation:

$$\text{Viability (\% of control)} = \left(\frac{(A_S - A_B)}{(A_C - A_B)}\right) \cdot 100$$

AS … absorbance of sample (cells pre-incubated with test compound in serum free medium and irradiated)

AC … absorbance of control (cells pre-incubated with DMSO in serum free medium and irradiated)

AB … absorbance of background (extraction solution)

Result: Treatment with test compound and following exposure to non-toxic UVA dose did not cause decrease in cell viability ~ incorporation of NR and thus test compound can be considered as non-phototoxic in the used concentration range (3.9-500 µmol/l). Results are given in Tab. 12. A well-known phototoxic compound chlorpromazine, which can be used for comparison, decreases the viability of NHEK cells: on exposure to UVA radiation (UVA+), the viability decreases below 80 % of control in the presence of 0.6 µmol/l of chlorpromazine, while the viability of unirradiated cells (UVA-) decreases below 50 % in the presence of 12.5 µmol/l of chlorpromazine. A. Above data indicate that test compounds are safe for cosmetic and dermatological application including use with following exposure of treated skin with solar radiation.

Table 12: UVA-induced effects of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine on NHEK viability

	1	
Concentration (µmol/l)	% of control	
	- UVA	+UVA

3.9	112.2 ± (11)	109.1 ± (2.3)
7.8	135.5 ± (8.2)	120.6 ± (1.6)
15.6	116.5 ± (2.8)	110 ± (2.9)
31.3	119.1 ± (8.7)	115.1 ± (2.8)
62.5	121 ± (8.7)	105.9 ± (8.7)
125	119 ± (2.8)	111.7 ± (6.8)
250	110.8 ± (12.4)	112.1 ± (3.7)
500	105.7 ± (7.1)	101.2 ± (3.3)

Example 11: Example 8: *In vitro* test of photoprotective effects of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine on normal human epidermal keratinocytes

Normal Human Epidermal Keratinocytes (NHEK) were used as an *in vitro* model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. NHEK were used between the 3rd and 4th passage. For all experiments the keratinocytes were seeded onto 96-well plates at a density of $1x10^4$ cells/ml (0.2 ml per well) of growth medium for keratinocytes (EpiLife®) supplemented with Human Keratinocyte Growth Supplement Kit and antibiotics (penicillin (100 mg/ml), streptomycin (100 mg/ml) and ampicillin (250 µg/ml)).

Test compounds included 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (1). Compound was dissolved in DMSO and then diluted in serum free medium (EpiLife® supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml) and ampicillin (250 µg/ml)). After 24h incubation was growth medium changed to serum free medium containing test compound. The final applied concentration range was 3.9 - 500 µmol/l. As a control, serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. After 60 minutes incubation with test compound medium was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. To study UVA photoprotection, a plate was exposed to a cytotoxic dose of UVA radiation (7.5 J/cm2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. To study UVB photoprotection, a plate was exposed to a cytotoxic dose of UVB radiation (200 mJ/cm2) using the solar simulator equipped with a H2 filter transmitting wavelengths of 295-320 nm. Intensity of UVA or UVB radiation was evaluated before each irradiation by UVA- or UVB-meter. Control (non-irradiated) plates were for the period of irradiation incubated in dark.

After UVA or UVB exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % CO2) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and CaCl2 (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetitions with use of cells from four donors to minimize individual sensitivity of donor cells. Photoprotective effect was evaluated by comparison of experimental data (absorbance) of test compounds with a positive control and a negative control (according to the following equation:

$$\text{Protection (\%)} = 100 - \left| \frac{As - Anc}{Apc - Anc} \right| \cdot 100$$

As … absorbance of sample (cells pre-incubated with test compounds in serum free medium and irradiated)

Anc … absorbance of negative control (cells pre-incubated with s DMSO in serum free medium and non-irradiated = incubated in dark)

Apc … absorbance of positive control (cells pre-incubated with s DMSO in serum free medium and irradiated)

Results: Cells pre-incubated with test compound and exposed to UVA or UVB radiation showed higher viability (ability to incorporate NR) compared to those pre-incubated with DMSO (control) and UVA or UVB irradiated (Tab. 13 and 14). Therefore test compound has high photoprotective potential.

Table 13: Photoprotective effect of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (1) on UVA-induced damage to NHEK

UVA photoprotection	
Concentration (µmol/l)	**1**
	protection (%)
3.9	39.7 ± (12.9)
7.8	60.2 ± (16.1)
15.6	53.5 ± (12.4)
31.3	46.8 ± (11.8)
62.5	47.9 ± (8.9)
125	51.7 ± (4.8)
250	48 ± (3.2)

500	30 ± (13.5)

Table 14: Photoprotective effect of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine (1) on UVb-induced damage to NHEK

UVB photoprotection	
Concentration (µmol/l)	**1**
	% protection (%)
3.9	53.5 ± (7.6)
7.8	72 ± (6)
15.6	69.8 ± (8.6)
31.3	65.9 ± (9)
62.5	65.9 ± (5)
125	72.9 ± (6.9)
250	65.7 ± (3.6)
500	28 ± (3.2)

Example 12: The effect of compounds on lifespan of *Caenorhabditis elegans*

Caenorhabditis elegans is a model organism used for identification of compounds with possible beneficial effect on human aging and age-related diseases. The list of compounds that prolong the lifespan of C. elegans and are known to have beneficial effect on human health includes resveratrol, curcumin and many others. Some substances that prolong the lifespan of worms are also used in skin rejuvenating and anti-aging cosmetics, for example vitamin E, coenzyme Q10, green tea or pomegranade extracts and cytokinin kinetin. *The Caenorhabditis elegans* strain used in this experiment was fem-1/HT17 . This strain has a heat-inducible mutation which causes all worms to develop into females when cultivated in 25°C (13). That prevents further reproduction and contamination of the experiment with progeny.Compounds dissolved in DMSO (100 mM stock solutions) were added into fresh NGM (nematode growth medium) to the final concentration of 10 and 50 or 100 µM and pipetted onto a Petri dish. Medium with DMSO vehiculum alone and non-treated medium were used as negative control. After solidification of NGM, the plates were seeded with 100 µl of 20x concentrated overnight suspension of *Escherichia coli* strain OP50 in LB medium. Bacteria on plates were allowed to grow overnight in 37°C. Age synchronized young adults (obtained by hypochlorite treatment) were then pipetted onto plates. Plates were kept in 25°C. In regular time intervals (1-3 days), the plates were scanned on an Epson perfection V700 photo flatbed scanner. The number of surviving worms was established by image analysis based on

comparison of several subsequent photographs and identification of moving objects. Pictures were analyzed in Fiji similarly as described here. Scripts from the original publication were slightly modified and the parameters adjusted to better suit our photo resolution and lighting. Three subsequent pictures of a plate were compared with each other. The average of the 3 resulting numbers was used to reduce the possibility of error. The overall results were then analyzed in programs OASIS and ED50v10. The statistical significance was evaluated by Log-Rank test and P-values were corrected by Bonferroni correction. The results are shown in table 15. Compounds 1, 15 and 20 significantly prolonged the lifespan of worms.

Table 15: The effect of compounds on lifespan of Caenorhabditis elegans (in days)

	DMSO	**15** 10 µM	**15** 100 µM	**20** 10 µM	**20** 100 µM	**1** 10 µM	**1** 100 µM
median	8.7	9.4	10.3	11.2	10.2	10.3	10.2
average	10.9	11.3	13.8	12.4	11.2	11.9	12.6

Example 13: *In vitro* test of phototoxic effects of test compounds on HaCaT

Spontaneously Immortalized Aneuploid Human Keratinocyte Cell Line (HaCaT) was bought from CLS (Eppeheim, Germany) and used as an in vitro model. For all experiments the keratinocytes were seeded onto 96-well plates at a density of $1{,}6x10^5$ cells/ml (0.2 ml per well) of growth medium (DMEM supplemented with fetal bovine serum (10%), penicillin (100 mg/ml) and streptomycin (100 U/ml)).

Test substances included compounds number 3, 9, 15 and 20. Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented penicillin (100 mg/ml) and streptomycin (100 U/ml)). After 24h incubation was growth medium changed to serum free medium containing test compound or DMSO (negative control). The final applied concentrations range was 3.9 - 125 µmol/l. As a control, serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. In parallel with test compound, chlorpromazine (CPZ; 0.8 - 50 µmol/l) was used as a known phototoxic compound. The test compound was in parallel applied on two 96-well plates with HaCaT. After 60 minutes incubation with test compound medium was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. A plate was then exposed to a non-cytotoxic dose of UVA radiation (5.0 J/cm2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. Intensity of UVA radiation was evaluated before each irradiation by UVA-meter. A control (non-irradiated) plate was for the period of irradiation

incubated in dark. After UVA exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % CO2) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and CaCl2 (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetition with use of cells from four donors to minimize individual sensitivity of donor cells. Phototoxic effect was evaluated as % of viability of control cells that was calculated from experimental data (absorbance) according to the following equation:

$$\text{Viability (\% of control)} = \left(\frac{(A_S - A_B)}{(A_C - A_B)}\right) \cdot 100$$

AS … absorbance of sample (cells pre-incubated with test compound in serum free medium and irradiated)

AC … absorbance of control (cells pre-incubated with DMSO in serum free medium and irradiated)

AB … absorbance of background (extraction solution)

Result: Treatment with test compounds and following exposure to non-toxic UVA dose did not cause decrease in cell viability ~ incorporation of NR and thus test compound can be considered as non-phototoxic in the used concentration range (3.9-125 µmol/l). Results are given in Tab. 16. A well-known phototoxic compound chlorpromazine, which can be used for comparison, decreases the viability of HaCaT cells: on exposure to UVA radiation (UVA+), the viability decreases below 80 % of control in the presence of 3.1 µmol/l of chlorpromazine, while the viability of unirradiated cells (UVA-) decreases below 70 % in the presence of 25 µmol/l of chlorpromazine. Above data indicate that test compounds are safe for cosmetic and dermatological application including use with following exposure of treated skin with solar radiation.

Table 16: UVA-induced effects of test compounds on HaCaT viability

3 (µmol/l)		**3.9**	**7.8**	**15.6**	**31.3**	**62.5**	**125**
-UVA	% control	100.1	100.4	102.1	107.8	106.4	109.5
	SMODCH	1.2	1.1	2.7	9.3	9.5	7.2
+UVA	% control	103.88	111.08	107.93	118.43	120.78	124.66
	SMODCH	1.7	1.6	1.4	3.7	3.2	4.2

9 (µmol/l)		**3.9**	**7.8**	**15.6**	**31.3**	**62.5**	**125**
-UVA	% control	101	101.1	100.4	101.8	103.9	103.4

	SMODCH	2.3	1.8	1	3.8	4.8	5.1
+UVA	% control	122.7	124.2	120.5	128.1	129.9	126.6
	SMODCH	3.5	4.6	5.3	4.4	3.2	3.1
15 (µmol/l)		**3.9**	**7.8**	**15.6**	**31.3**	**62.5**	**125**
-UVA	% control	106.5	107.4	108.6	108.6	109.2	105
	SMODCH	9.7	9.5	10.6	9.5	12.6	8.9
+UVA	% control	116.4	119.9	119.9	118.8	115.7	104
	SMODCH	0.7	4.9	6.3	7.7	9	8.8
20 (µmol/l)		**3.9**	**7.8**	**15.6**	**31.3**	**62.5**	**125**
-UVA	% control	103.5	102.9	104.1	108.6	110.7	110.5
	SMODCH	6.1	3.1	5.3	2.4	1.7	1.2
+UVA	% control	104.6	112.1	110.1	121.4	125.8	125.9
	SMODCH	0.7	3.3	5.6	2.2	5.9	4.2

Example 14: *In vitro* test of photoprotective effects of test compounds on HaCaT

Spontaneously Immortalized Aneuploid Human Keratinocyte Cell Line (HaCaT) was bought from CLS (Eppeheim, Germany) and used as an in vitro model. For all experiments the keratinocytes were seeded onto 96-well plates at a density of $1{,}6x10^5$ cells/ml (0.2 ml per well) of growth medium (DMEM supplemented with fetal bovine serum (10%), penicillin (100 mg/ml) and streptomycin (100 U/ml)).

Test substances included compounds number 3, 9, 15 and 20. Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented penicillin (100 mg/ml) and streptomycin (100 U/ml)). After 24h incubation was growth medium changed to serum free medium containing test compounds. The final applied concentration range was 3.9 – 31,25 µmol/l. As a control, serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. Rosmarinic acid was used as positive control. After 60 minutes incubation with test compounds medium was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. To study UVA photoprotection, a plate was exposed to a cytotoxic dose of UVA radiation (10 J/cm2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. To study UVB photoprotection, a plate was exposed to a cytotoxic dose of UVB radiation (150 mJ/cm2) using the solar simulator equipped with a H2 filter transmitting wavelengths of 295-320 nm. Intensity of UVA or UVB radiation was evaluated before each irradiation by UVA- or UVB-meter. Control (non-irradiated) plates were for the period of irradiation incubated in dark. After UVA or

UVB exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % CO2) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and CaCl2 (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetitions with use of cells from four donors to minimize individual sensitivity of donor cells. Photoprotective effect was evaluated by comparison of experimental data (absorbance) of test compounds with a positive control and a negative control (according to the following equation:

$$\text{Protection (\%)} = 100 - \left| \frac{As - Anc}{Apc - Anc} \right| \cdot 100$$

As … absorbance of sample (cells pre-incubated with test compounds in serum free medium and irradiated)

Anc … absorbance of negative control (cells pre-incubated with s DMSO in serum free medium and non-irradiated = incubated in dark)

Apc … absorbance of positive control (cells pre-incubated with s DMSO in serum free medium and irradiated)

Results: Cells pre-incubated with test compound and exposed to UVA or UVB radiation showed higher viability (ability to incorporate NR) compared to those pre-incubated with DMSO (control) and UVA or UVB irradiated (Tab. 17 and 18). All test compounds showed higher or comparable photoprotective activity with rosmarinic acid used as positive control. Therefore test compound has high photoprotective potential.

Table 17: Photoprotective effects of test compounds and rozmarinic acid (positive control) on UVA-induced damage to HaCaT

3 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	26.8	34.3	46.0	50.7
SMODCH	6.9	9.7	6.9	6.1
9 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	72.1	70.3	66.8	61.4
SMODCH	4.4	4.9	8.1	10.0
15 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**

Protection (%)	41.2	56.9	55.3	76.7
SMODCH	5.5	7.8	1.7	9.7
20 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	12.3	25.0	28.9	31.6
SMODCH	5.2	7.7	10.2	9.1
RA	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	23.6	42.3	36.2	44.0
SMODCH	7.2	7.4	12.0	16.0

Table 18: Photoprotective effects of test compounds and rozmarinic acid (positive control) on UVA-induced damage to HaCaT

3 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	27.3	46.5	47.8	53.8
SMODCH	4.4	11.6	14.4	14.4
9 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	48.8	56	55.7	56.5
SMODCH	1.9	12.6	7.7	6.3
15 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	12.3	35.3	37.8	51.3
SMODCH	7.7	12.6	7.5	3.5
20 (µmol/l)	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	45.7	66.2	64	75.2
SMODCH	7.1	1.6	3	10.3
RA	**3.9**	**7.8**	**15.6**	**31.3**
Protection (%)	22	48.5	55.7	54.3
SMODCH	0.4	1.2	2.2	13.1

Example 15: Markers of UVA protection

Normal human dermal fibroblasts (NHDF) were used as an in vitro model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in

accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. Fibroblasts were used between the 2nd and 4th passage. For all experiments the fibroblasts were seeded onto 6-well plates at a density of $0.5x10^5$ cells/cm^2 of cultivation medium (DMEM supplemented with fetal calf serum (10%, v/v), penicillin (100 mg/ml) and streptomycin (100 U/ml)).

Test compounds included 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine and positive control (rosmarinic acid) . Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml)). The final applied concentrations range was 2.5-20 µmol/l. As a control serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. Each test compound was in parallel applied on two 6-well plates with NHDF ($3,15x10^5$ cells/cm^2). After 60 minutes incubation medium with test compound was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. To study UVA photoprotection, a plate was exposed to a cytotoxic dose of UVA radiation (10 J/cm2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H1 filter transmitting wavelengths of 320-400 nm. Intensity of UVA radiation was evaluated before each irradiation by UVA- meter. Control (non-irradiated) plates were for the period of irradiation incubated in dark. After UVA exposure PBS with glucose was discarded and serum free medium was applied. After 1 hour (37 °C, 5 % CO2) cell damage was evaluated by analysis of reactive oxygen species (ROS) production. In parallel, intracellular levels of glutathion (GSH) were measured 4 hours (37 °C, 5 % CO2) after UVA application.

ROS production

ROS production was evaluated by 2,7-Dichlorodihydrofluorescein diacetate (H_2DCFDA). NHDF were incubated with (H_2DCFDA) (5 nmol/l, 20 min) 1 hour after UVA exposure. Subsequently, cells were washed two-times with PBS, scraped into PBS a sonicated. Samples were applied on 96-well plate and fluorescence was measured (488/525 nm) (INFINITE M200, Tecan, Switzerland) after centrifugation (10 000 rpm, 4°C, 10 min). Protein content was analyzed spectrophotometrically by bicinchoninic acid at 562 nm (INFINITE M200, Tecan, Switzerland).

GSH depletion

GSH levels in NHDF were evaluated by reaction with 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). Cells were washed two-times with PBS, scraped into acetic acid (1 %, v/v) and sonicated. Samples were applied on 96-well plate after centrifugation (10 000 rpm, 4°C, 10 min) and reaction mixture

was added (0,8 mol/l Tris/HCl, 20 mmol/l EDTA, pH 8,2; 20 mg/ml DTNB). Absorbance was measured at 412 nm. Protein content was analyzed spectrophotometrically by Lowry's method at 680 nm.

Activity of test compounds (not irradiated) in analyzed parameters were evaluated by comparison of experimental data according to the following equation:

$$\textbf{\% of control} = 100 \cdot \left(\frac{(A_V - A_P)}{(A_K - A_P)} \right)$$

A_P … background value

A_V … sample value (cells pre-incubated with test compounds in serum free medium)

A_K … control value (cells pre-incubated with s DMSO in serum free medium)

Photoprotective effect was evaluated by comparison of experimental data (absorbance, fluorescence) of test compounds with a positive control (cells pre-incubated with DMSO in serum free medium and irradiated) and a negative control (cells pre-incubated with DMSO in serum free medium and non-irradiated = incubated in dark) according to the following equation:

$$\textbf{Protection (\%)} = 100 - \left\{ \left(\frac{(A_V - A_K)}{(A_{UV} - A_K)} \right) * 100 \right\}$$

A_K … negative control value (DMSO in serum free medium and non-irradiated)

A_{UV} …positive control value (DMSO in serum free medium and irradiated)

A_V …sample value (cells pre-incubated with test compounds in serum free medium and irradiated)

Results: 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine did not showed negative effect on tested parameters (on non-irradiated cells) in test concentrations (2.5-20 µmol/l). Test compound protected cells against UVA-induced production of ROS as well as depletion of GSH (endogenous antioxidant) (Tab. 19 and 20) in a concentration-dependent manner. Compound 1 is more effective in protection against production of ROS but less effective in protection against GSH depletion in comparison with rosmarinic acid.

Table 19: Protective effect of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine and rozmarinic acid (positive control) on UVA-induced ROS production

ROS production		
Concentration (µmol/l)	1	RA
	Protection (%)	

2.5	21.3 ± (6.9)	20.6 ± (8.4)
5	21.3 ± (5.2)	24.1 ± (1)
10	29.6 ± (3.3)	24.1 ± (2.4)
20	48.3 ± (0.5)	30.3 ± (6.8)

Table 20: Protective effect of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine and rozmarinic acid (positive control) on UVA-induced GSH depletion

GSH depletion		
Concentration (µmol/l)	1	RA
	Protection (%)	
2.5	25.5 ± (6.8)	53.9 ± (7.1)
5	27.9 ± (7.2)	42.8 ± (2.4)
10	61.6 ± (15.7)	68.6 ± (4.3)
20	35.5 ± (14.1)	50.5 ± (9)

Example 16: Markers of UVB protection

Normal human dermal fibroblasts (NHDF) were used as an in vitro model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacký University, Olomouc and all patients signed written informed consent. Fibroblasts were used between the 2nd and 4th passage. For all experiments the fibroblasts were seeded onto 6-well plates at a density of $0.5x10^5$ cells/cm^2 of cultivation medium (DMEM supplemented with fetal calf serum (10%, v/v), penicillin (100 mg/ml) and streptomycin (100 U/ml)).

Test compounds included 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine and positive control (rosmarinic acid). Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml)). The final applied concentrations range was 2.5-20 µmol/l. As a control serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. Each test compound was in parallel applied on two 6-well plates with NHDF ($3,15x10^5$ cells/cm^2). After 60 minutes incubation medium with test compound was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. To study UVB photoprotection, a plate was exposed to a cytotoxic dose of UVB radiation (150 mJ/cm2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H2 filter transmitting wavelengths of 295-320 nm. Intensity of UVB radiation was evaluated before each irradiation by UVB- meter. Control (non-irradiated) plated were for the period of irradiation incubated in dark. After UVB exposure PBS

with glucose was discarded and serum free medium was applied. After 4 hours (37 °C, 5 % CO2) cell damage was evaluated by analysis of caspase-3 activity.

Caspase-3 ctivity

Caspase-3 activity was evaluated by specific substrate (Ac-DEVD-AMC). NHDF were washed two-times with PBS, scraped into lysis buffer (50 mmol/l HEPES, pH 7.4; TritonX-100 (0,5%; v/v), protease inhibitor, 5 mmol/l DDT). Samples were applied on 96-well plate after centrifugation (10 000 rpm, 4°C, 10 min) and reaction buffer was added (20 mmol/l HEPES, pH 7.1, 2 mmol/l EDTA, protease inhibitor, 5 mmol/l DDT) cantaining specific substrate or inhibitors. Fluorescence was measured at (400/505 nm) after 1 hour incubation (37°C, dark). Protein content was analyzed spectrophotometrically by Bradford protein assay (INFINITE M200, Tecan, Switzerland).

$$\textbf{\% of control} = 100 \cdot \left(\frac{(A_V - A_P)}{(A_K - A_P)} \right)$$

A_P … background value

A_V … sample value (cells pre-incubated with test compounds in serum free medium)

A_K … control value (cells pre-incubated with s DMSO in serum free medium)

Photoprotective effect was evaluated by comparison of experimental data (absorbance, fluorescence) of test compounds with a positive control (cells pre-incubated with DMSO in serum free medium and irradiated) and a negative control (cells pre-incubated with DMSO in serum free medium and non-irradiated = incubated in dark) according to the following equation:

$$\textbf{Protection (\%)} = 100 - \left\{ \left(\frac{(A_V - A_K)}{(A_{UV} - A_K)} \right) * 100 \right\}$$

A_K … negative control value (DMSO in serum free medium and non-irradiated)

A_{UV} …positive control value (DMSO in serum free medium and irradiated)

A_V …sample value (cells pre-incubated with test compounds in serum free medium and irradiated)

Results: 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine did not showed negative effect on tested parameters (on non-irradiated cells) in test concentrations (2.5-20 µmol/l). Compound 1 decreased UVB-induced activity of caspase-3 (Tab. 21).

Table 21: Protective effect of 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine and rozmarinic acid (positive control) on UVB-induced caspase-3 activity

Caspase-3 activity

Concentration (μmol/l)	1	RA
	Protection (%)	
2.5	41.2 ± (8.2)	60.6 ± (3.1)
5	84.4 ± (27.2)	39.7 ± (13.1)
10	71.1 ± (30.3)	38.4 ± (5.9)
20	45.9 ± (4.7)	28.7 ± (3.8)

Example 15: Comparative gene expression analysis

Comparative gene expression analysis in human dermal fibroblast (HDF) was performed to gain insight into role of compound **1** in the photoprotection against UV-induced damage. Three independent HDF lines form three patients were treated with 5 μM **1** for 24 h or mock-treated with DMSO. Alternatively, the above three biological samples (without **1**) were irradiated with UV light (5 J/cm2). 1.5-3.0 x 10^6 cells from each treatment or control was used for isolation of total RNA using trizol. cDNA sequencing libraries were prepared with the Illumina TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA) according to standard Illumina's protocols and sequenced on HiSeq 2500 apparatus (50 bp single-end reads).

Data were subjected to differential transcriptomic analysis with the aim to characterize significantly regulated genes and their expression levels. To reveal the molecular mechanism of the action of **1**, we compared data from mock (DMSO)-treated HDF or UV-treated HDF with those obtained after 24 h treatment with **1**. For data analysis, we performed *ab initio* method where sequencing reads were mapped to the reference genome. Comparison of the control group vs samples treated with **1** did not show any significantly regulated genes indicating **1** had low effect on the gene expression under normal conditions. Interestingly, when **1**-treated group of data was compared with UV-treated group we could detect 1306 differentially regulated genes ($P \leq 0.05$). 865 of those were upregulated and 441 genes were downregulated. To limit the number of genes that respond most significantly to **1** treatment we set relatively stringent conditions – we selected genes which $\log_2FC > 1.5$, or those with $\log_2FC < -1.5$. Further inspection of the subgroups (41 upregulated genes and 41 downregulated, see Tab. 22 and Tab. 23, respectively) revealed major differences among those. In the group of the upregulated genes, we could observe a range of genes with regulatory, developmental or receptor/signaling function, such as the calcium sensing. These included calcium-activated potassium channel KCNN4, calcium sensor DYSF or calcium-dependent phospholipid-binding protein CPNE7. In addition, we noted increased expression of the negative regulator of reactive oxygen species NRROS and the scavenger cysteine-rich type 1 receptor CD163 that protects again oxidative damage suggesting that **1** facilitates a mechanism leading to the

protection against damage caused by oxygen radicals. Thus, **1** seems to protect cells against oxidative damage caused primarily by UV-light or other stress conditions.

In contrast, in the group of the downregulated genes we found regulatory genes with a large group of genes that may be related to immune response. We observed upregulation of two chemokines, CCL8 and CXCL9, and cytokines TNFSF13B and TNFSF10. In addition, several protein kinases, such as HCK and JAK3, and innate immune response-related proteins TLR2 and GBP2 were found to be downregulated by **1**. Hence, the immunosuppression mediated by **1** may contribute to the *in vivo* function of the compound.

Table 22: Genes upregulated in response to the treatment with the compound **1** ($P \leq 0.05$ and $\log_2FC > 1.5$).

Gene	Description	logFC
KIF1A	kinesin family member 1A	3.05
ANKRD33	ankyrin repeat domain-containing protein 33	2.69
HS3ST2	heparan sulfate-glucosamine 3-sulfotransferase 2	2.50
CHRNA9	neuronal acetylcholine receptor subunit alpha-9	2.21
TMEM233	interferon-induced transmembrane domain-containing protein D2	2.12
TM4SF1	transmembrane 4 L6 family member 1	2.08
SCG2	secretogranin-2	2.00
MYEOV	myeloma-overexpressed gene protein	1.98
PPP2R2C	serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B	1.95
KCNN4	Intermediate conductance calcium-activated potassium channel 4	1.85
RP11-184E9.1	non-coding RNA	1.78
ITGA10	integrin alpha-10	1.77
CCL26	C-C motif chemokine 26	1.75
NRROS	negative regulator of reactive oxygen species	1.74
DYSF	dysferlin	1.73
ANGPTL4	angiopoietin-related protein 4	1.73
FAM81A	non-coding RNA	1.71
RP11-541M12.6	non-coding RNA	1.70
RP11-367F23.2	non-coding RNA	1.70
LINC00704	non-coding RNA	1.69
FOLR3	folate receptor gamma	1.68
CD163	scavenger receptor cysteine-rich type 1 protein M130	1.67
ARHGAP22	rho GTPase-activating protein 22	1.66
CYP51A1	lanosterol 14-alpha demethylase	1.65
LINC01204	non-coding RNA	1.64
CITED4	cbp/p300-interacting transactivator 4	1.63
RP11-54A9.1	non-coding RNA	1.63
MET	MET proto-oncogene, receptor tyrosine kinase	1.63
EBF2	transcription factor COE2	1.62
CPNE7	copine-7	1.61
LINC00702	non-coding RNA	1.60
TGM2	protein-glutamine gamma-glutamyltransferase 2	1.58
AC002456.2	non-coding RNA	1.58

TMEM154	transmembrane protein 154	1.57
NUDT8	nucleoside diphosphate-linked moiety X motif 8	1.56
SLC20A1	sodium-dependent phosphate transporter 1	1.56
B4GALNT1	beta-1,4-N-acetyl-galactosaminyltransferase 1	1.55
STX1A	syntaxin-1A	1.55
CTD-2587H24.5	non-coding RNA	1.54
ADGRG1	adhesion G-protein coupled receptor G1	1.52
CCDC107	coiled-coil domain-containing protein 107	1.50

Table 23: Genes downregulated in response to the treatment with the compound **1** ($P \leq 0.05$ and $\log_2FC < -1.5$).

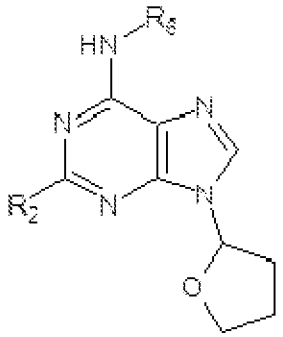
Gene	Description	logFC
RP11-383F6.1	non-coding RNA	-1.50
APOL6	apolipoprotein L6	-1.51
RP11-400K9.4	non-coding RNA	-1.51
TMEM178A	transmembrane protein 178A	-1.52
HAUS1P2	non-coding RNA	-1.54
RORB	nuclear receptor ROR-beta	-1.57
RP11-363H12.1	non-coding RNA	-1.58
SERPINB9	serpin B9	-1.59
DHRS3	short-chain dehydrogenase/reductase 3	-1.60
TP63	tumor protein 63	-1.61
GBP2	guanylate-binding protein 2	-1.61
TNFSF13B	tumor necrosis factor ligand superfamily member 13B	-1.61
ADAMTS9-AS2	non-coding RNA	-1.63
TLR2	toll-like receptor 2	-1.63
AC003986.6	non-coding RNA	-1.65
HCK	tyrosine-protein kinase HCK	-1.66
GPR37L1	G protein-coupled receptor 37 like 1	-1.66
JAK3	tyrosine-protein kinase JAK3	-1.68
TNFRSF9	tumor necrosis factor receptor superfamily member 9	-1.68
RP11-1100L3.8	non-coding RNA	-1.70
MOB3B	MOB kinase activator 3B	-1.70
RP11-379B8.1	non-coding RNA	-1.72
BRINP2	BMP/retinoic acid-inducible neural-specific protein 2	-1.77
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	-1.77
CXCL9	C-X-C motif chemokine 9	-1.79
CLNS1AP1	non-coding RNA	-1.80
RNF150	RING finger protein 150	-1.80
BTC	probetacellulin	-1.84
FAM20A	pseudokinase FAM20A	-1.86
RPL34P31	non-coding RNA	-1.89
BHLHE22	class E basic helix-loop-helix protein 22	-1.90
FGD3	FYVE, RhoGEF and PH domain-containing protein 3	-1.93
RP11-21C4.1	non-coding RNA	-1.95
CFB	complement factor B	-1.96
RARRES3	retinoic acid receptor responder protein 3	-2.09
TNFSF10	tumor necrosis factor ligand superfamily member 10	-2.14

PSAT1P3	non-coding RNA	-2.19
KCNT2	potassium channel subfamily T member 2	-2.21
RP1-181J22.1	non-coding RNA	-2.53
ABCG1	ATP-binding cassette sub-family G member 1	-2.63
CCL8	C-C motif chemokine 8	-2.80

5

CLAIMS

1. Adenine derivatives of general formula I



I

and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein

R2 is hydrogen or halogen;

R6 is selected from a group containing

- heteroaryl with 5- to 6-membered aromatic ring containing at least one heteroatom selected from O, S whereas other ring atoms are carbon atoms, whereas the heteroaryl is unsubstituted or substituted by at least one substituent selected from the group consisting of C1-C4 alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;
- heteroarylalkyl with 6-membered aromatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, wherein the alkyl contains 1 to 4 carbon atoms, whereas the heteroarylalkyl is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;
- heteroarylalkyl with 5-membered aromatic ring containing at least one heteroatom S whereas other atoms of the ring are carbon atoms, wherein the alkyl contains 1 to 4 carbon atoms, whereas the heteroarylalkyl is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;
- heterocyclyl with 5- to 6- membered aliphatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, wherein the heterocycle is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl,

merkapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- heterocyclylalkyl with 5- to 6- membered aliphatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, the alkyl contains 1 to 4 carbon atoms, whereas the heterocyclylalkyl is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;- cycloalkyl with ring containing 5 to 6 carbon atoms, unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- cycloalkylalkyl with ring containing 5 to 6 carbon atoms, wherein the alkyl contains 1 to 4 carbon atoms, whereas the cycloalkylalkyl is unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- isoalkyl containing 3 to 7 carbon atoms, unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl.

2. Adenine derivatives according to claim 1, selected from the group comprising

6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-methyltetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(1-tetrahydrofuran-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(cyclopentylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(3-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-chlorothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(5-bromothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

6-(1-thiophen-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(5-methyltetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(1-tetrahydrofuran-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(thiophen-2-ylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(3-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(5-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

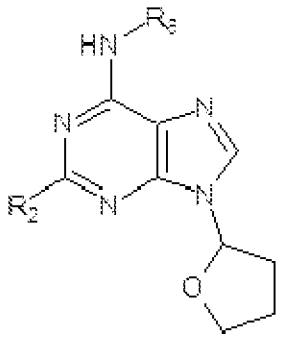
2-chloro-6-(5-chlorothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(5-bromothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(1-thiophen-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(cyclopentylmethylamino)-9-(tetrahydrofuran-2-yl)purine.

3. Use of adenine derivatives of general formula Ia



Ia

and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein

R2 is hydrogen or halogen;

R6 is selected from a group containing

- heteroaryl with 5- to 6-membered aromatic ring containing at least one heteroatom selected from O, S whereas other ring atoms are carbon atoms, whereas the heteroaryl is unsubstituted or substituted by at least one substituent selected from the group consisting of C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- heteroarylalkyl with 5- to 6-membered aromatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, wherein the alkyl contains 1 to 4 carbon atoms, whereas the heteroarylalkyl is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- heterocyclyl with 5- to 6- membered aliphatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, wherein the heterocycle is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl,

hydroxy(C1-C4)alkyl, merkapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- heterocyclylalkyl with 5- to 6- membered aliphatic ring containing at least one heteroatom selected from O, S whereas other atoms of the ring are carbon atoms, the alkyl contains 1 to 4 carbon atoms, whereas the heterocyclylalkyl is unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;- cycloalkyl with ring containing 5 to 6 carbon atoms, unsubstituted or substituted by at least one substituent selected from the group C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- cycloalkylalkyl with ring containing 5 to 6 carbon atoms, wherein the alkyl contains 1 to 4 carbon atoms, whereas the cycloalkylalkyl is unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl;

- isoalkyl containing 3 to 7 carbon atoms, unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl,

as UV-photoprotective ingredients in cosmetic preparations, in preparations for protection of plants and/or in preparations for tissue culture treatment.

4. Use according to claim 3, wherein the substituent **R6** is heteroaryl which contains a 5-membered ring containing one heteroatom selected from O and S, whereas the heteroaryl is unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl.

5. Use according to claim 3, wherein the substituent R6 is heteroaryalkyl which contains a 5-membered ring and C1-C2 alkyl, whereas the 5-membered ring contains one heteroatom S, while the heteroarylalkyl is unsubstituted or substituted by at least one substituent selected from the group containing C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl.

6. Use according to claim 3, wherein the R6 substituent is heterocyclyl containing a 5-membered ring and the said 5-membered ring contains one heteroatom selected from O and S, while the heterocyclyl is unsubstituted or substituted by at least one substituent selected from the group

comprising C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogene, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl.

7. Use according to claim 3, wherein the substituent R6 is heterocyklylalkyl containing a 5-membered ring and C1-C2 alkyl, and the said 5-membered ring contains one heteroatom selected from O and S, whereas the heterocyclylalkyl is unsubstituted or substituted by at least one substituent selected from the group comprising C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogen, carboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl.

8. Use according to claim 3, wherein the substituent R6 is selected from the group comprising cyclopentyl, cyclopentylmethyl, isopropyl, isobutyl, isopentyl, isohexyl, isoheptyl, wherein each of said substituent groups is unsubstituted or substituted by at least one substituent selected from the group comprising C1-C4 alkyl, hydroxy(C1-C4)alkyl, mercapto(C1-C4)alkyl, formyl, acetyl, halogene, arboxyl, amino, di(C1-C4)alkylamino, amino(C1-C4)alkyl.

9. Use according to claim 1, wherein the adenine derivatives are selected from the group comprising
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(5-methylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(5-hydroxymethylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(5-formylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(1-furan-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(5-methyltetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(1-tetrahydrofuran-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(cyclopentylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(3-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(5-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(5-chlorothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(5-bromothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
6-(1-thiophen-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-furfurylamino-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-methylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-hydroxymethylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-formylfuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(1-furan-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine

2-chloro-6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-methyltetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(1-tetrahydrofuran-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(thiophen-2-ylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(thiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(3-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-methylthiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-chlorothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(5-bromothiophen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(1-thiophen-2-ylethylamino)-9-(tetrahydrofuran-2-yl)purine
2-chloro-6-(cyclopentylmethylamino)-9-(tetrahydrofuran-2-yl)purine.

10. The use according to any one of the claims 3 to 9 wherein at least one adenine derivative of general formula I is used as an ingredient with a combined anti-senescent and UV-photoprotective effect.

11. The use according to any one of the claims 3 to 9 wherein at least one adenine derivative of general formula I is used as an ingredient with a combined anti-senesent, UV-photoprotective and anti-skin-hypersensitivity effect.

12. At least one adinine derivative of general formula I according to any one of the claims 3 to 9 for use in a method of treatment of an immune disorder, such as hypersensitive immune response or transplant rejection.

13. Cosmetic and/or therapeutic preparations, preparations for the protection of plants and/or preparations for the application to tissue cultures, which contain at least one compound od the general formula I according any one of claims 1 -2.

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2016/050029

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D473/00 C07D473/40 A01N1/00 A01N43/54 A61K8/49
A61Q19/00 A01P21/00 A61P37/00 A61P17/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D A01N A61K A61Q A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VÁCLAV MIK ET AL: "N9-substituted derivatives of kinetin: Effective anti-senescence agents", PHYTOCHEMISTRY, vol. 72, no. 8, 25 February 2011 (2011-02-25), pages 821-831, XP055016127, ISSN: 0031-9422, DOI: 10.1016/j.phytochem.2011.02.002 abstract page 822; figure 1; compound 8 page 823; tables 1-2 page 825; table 3 ----- -/--	1-13

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 2016	12/10/2016

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/CZ2016/050029

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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Supplement VI

Hönig, M.; Plíhalová, L.; Doležal, K.; Voller, J.; Strnad, M.; Spíchal L.; Vostálová, J.; Rajnochová Svobodová, A.; Ulrichová J.; Kadlecová A.; Plíhal O. Adenine derivatives and their use. Czech Republic. Patent no. 307722, **2019**

PATENTOVÝ SPIS

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(13) Druh dokumentu: **B6**

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A61Q 19/08	(2006.01)
A01N 43/90	(2006.01)

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(Věstník č. 13/2019)

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(73) Majitel patentu:
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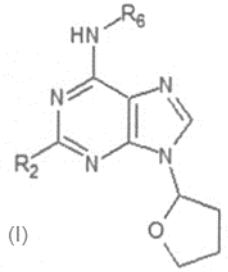
HARBER IP s.r.o., Dukelských hrdinů 567/52, 170 00 Praha 7, Holešovice

(54) Název vynálezu:
Adeninové deriváty a jejich použití

(57) Anotace:
Předkládané řešení poskytuje adeninové deriváty obecného vzorce I substituované v polohách C2, N9 a na aminoskupině, které mají antisenescenční a kombinované fotoprotektivní UVA/UVB účinky. Tyto látky jsou vhodné zejména jako antisenescenční a UV-fotoprotektivní složky v kosmetických přípravcích, v přípravcích pro ochranu rostlin a v přípravcích pro ošetření tkáňových kultur.



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CZ 307722 B6

Adeninové deriváty a jejich použití

Oblast techniky

Vynález se týká adeninových derivátů majících kombinované antisenescenční a UV–fotoprotektivní účinky proti záření UVA i UVB. Dále se týká jejich použití a přípravků je obsahujících.

Dosavadní stav techniky

6–Furfurylaminopurin (kinetin) je látka ze skupiny rostlinných hormonů cytokininů, jež jsou N^6–substituovanými deriváty adeninu. Kinetin byl objeven v padesátých letech minulého století a byl považován za tzv. růstový faktor, protože pozitivně ovlivňoval dělení buněk tabákového kalusu. Exogenní aplikace kinetinu indukuje buněčné dělení a morfogenezi buněk rostlinného kalusu, anebo například oddaluje senescenci listů. Kromě vlivu na rostlinné buňky vykazuje kinetin také účinky na buňky živočišné. Má antioxidační vlastnosti a brání oxidačnímu poškození DNA, inhibuje oxidační a glykační poškození proteinů, dokáže ovlivňovat růst keratinocytů a oddaluje stárnutí lidských kožních fibroblastů *in vitro,* aniž by látka měla efekt na proliferaci těchto fibroblastů. Derivát kinetinu 6–furfurylamino–9–(2–tetrahydropyran–2–yl)purin (obchodní název Pyratine), který je v současné době komerčně využíván v kosmetických přípravcích, byl připraven spojením protektivní tetrahydropyranylové skupiny s molekulou kinetinu. Tato strukturní modifikace vedla ke zlepšení antisenescenčních a antioxidačních účinků na rostlinné i živočišné buňky, a to i při testech prováděných na lidské kůži nebo jejích modelech.

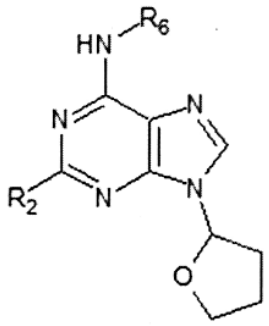
V posledních letech narůstá množství UV záření, zejména jeho středních (UVB) a dlouhých (UVA) vlnových délek, které dopadá na povrch Země, a tento nový fenomén přispívá u lidí k rozvoji řady kožních onemocnění a poruch. UVB záření představuje asi 4 až 5 % celkového objemu záření a proniká do kožní epidermis, kde způsobuje přímé i nepřímé nežádoucí biologické účinky. UVA záření tvoří 90 % celkového podílu záření a proniká hlouběji do papilární dermis a částečně i do hypodermis (10 %), kde způsobuje vznik tzv. reaktivních sloučenin kyslíku (ROS) a dusíku (RNS). Chronické vystavení kůže UVA záření může vést k tzv. předčasnému stárnutí kůže, které je spojeno s poškozením struktury dermis a jehož důsledkem je vznik vrásek, pigmentových skvrn, aj. Přirozenou endogenním fotoprotektivní látkou je melanin, ale v lidské kůži se ho netvoří dostatek, zejména v souvislosti se zvyšující se intenzitou UV záření dopadajícího na Zemi, vlivem úbytku ozonu v atmosféře, a změnou životního stylu (více outdoorových aktivit, oděv zakrývající menší část povrchu těla). Pokud je kůže ošetřena látkou, která zamezuje pronikání zejména UV paprsků, může ji chránit před předčasným stárnutím ale i krátkodobým nežádoucím účinkům UV záření.

Většina přípravků, které dnes využívají kosmetické přípravky k ochraně před slunečním zářením, tzv. sunscreens (UV filtry), byly vyvinuty, aby chránily především před "škodlivějším" UVB zářením, které může vyvolat vznik zhoubného melanokarcinomu. Sunscreens se dělí na fyzikální (anorganické minerální látky, které vytvářejí fyzikální překážku záření na kůži, jako například TiO_2 nebo ZnO) a chemické (organické látky schopné absorbovat radiaci změnou distribuce elektronů – například benzofenony, cinamáty, salicyláty). U některých stávajících sunscreens byly popsány nežádoucí účinky, spojené zejména s fotoalergickou nebo fotoiritující reakcí při používání těchto přípravků. Častým problémem těchto látek je také fotonestabilita.

Původci předkládaného vynálezu nalezli látky, které neočekávaně jeví kombinované antisenescenční účinky a UV–fotoprotektivní účinky (UVB i UVA). Tyto látky jsou velmi stabilní, a zároveň nejsou foto toxické a ani neiritují ošetřenou tkáň.

Podstata vynálezu

Předmětem vynálezu je použití adeninových derivátů obecného vzorce I



(I)

a jejich farmaceuticky přijatelných solí s alkalickými kovy, amoniakem, aminy, nebo adičních solí s kyselinami, kde

R2 je vodík nebo halogen;

R6 je vybrán ze skupiny zahrnující
– heteroaryl s 5– až 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heteroaryl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heteroarylalkyl s 5– až 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heteroarylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heterocyklyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklylalkyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heterocyklylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– cykloalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1 –C4)alkyl;
– cykloalkylalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž cykloalkylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;

– isoalkyl obsahující 3 až 7 atomů uhlíku, nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl,

jako UV–fotoprotektivních složek v kosmetických přípravcích, v přípravcích pro ochranu rostlin a/nebo v přípravcích pro ošetření tkáňových kultur.

Heteroaryl s výhodou obsahuje 5–členný kruh, výhodněji obsahuje v 5–členném kruhu jeden heteroatom, kterým je O nebo S. Nejvýhodněji je heteroaryl vybrán z furan–2–ylu a thiofen–2–ylu.

Heteroarylalkyl s výhodou obsahuje 5–členný kruh a C1–C2 alkyl, výhodněji obsahuje v 5–členném kruhu jeden heteroatom, kterým je O nebo S. Nejvýhodněji je heteroarylalkyl vybrán z furan–2–ylmethylu (furfurylu) a thiofen–2–ylmethylu.

Heterocyklyl s výhodou obsahuje 5–členný kruh, výhodněji obsahuje v 5–členném kruhu jeden heteroatom, kterým je O nebo S. Nejvýhodněji je heterocyklyl vybrán ze skupiny zahrnující tetrahydrofuran–2–yl a tetrahydrothiofen–2–yl.

Heterocyklylalkyl s výhodou obsahuje 5–členný kruh a C1–C2 alkyl, výhodněji obsahuje v 5—členném kruhu jeden heteroatom, kterým je O nebo S. Nejvýhodněji je heterocyklylalkyl vybrán ze skupiny zahrnující tetrahydrofuran–2–ylmethyl (tetrahydrofurfuryl) a tetrahydrothiofen–2–ylmethyl.

Cykloalkylem je s výhodou cyklopentyl. Cykloalkylalkylem je s výhodou cyklopentylmethyl.

Isoalkylem je s výhodou isopropyl, isobutyl, isopentyl, isohexyl, isoheptyl.

Halogen je vybrán ze skupiny zahrnující fluor, chlor, brom, jod. S výhodou je halogenem chlor.

V jednom výhodném provedení jsou látky obecného vzorce I vybrány ze skupiny zahrnující:
6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–hydroxymethylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–formylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–furan–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1 –thiofen–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–furfurylamino–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–hydroxymethylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–formylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–furan–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(thiofen–2–ylamino)–9–(tetrahydrofuran–2–yl)purin

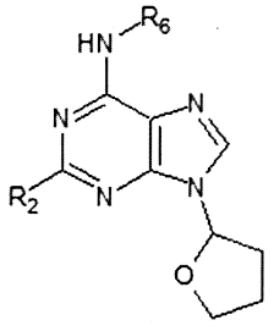
2–chlor–6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–thiofen–2–ylethylamino)–9–(tetrahydro–2–chlor–6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin.

Výhodněji jsou látky obecného vzorce I vybrány ze skupiny zahrnující:
6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–furfurylamino–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin.

Ve výhodném provedení se uvedené látky použijí jako složky s kombinovaným antisenescenčním a UV–fotoprotektivním účinkem.

Látky podle předkládaného vynálezu jeví kombinované antisenescenční a UV–fotoprotektivní účinek. UV–fotoprotektivní účinek byl pozorován pro záření UVA i UVB. Jsou tedy vhodné jako složky kosmetických přípravků, přípravků na ochranu rostlin a přípravků pro ošetření tkáňových kultur. Kosmetické přípravky s těmito složkami jsou zejména vhodné pro aplikaci na pokožku, srst či vlasy savců. Přípravky pro ošetření tkáňových kultur jsou vhodné zejména pro ošetření kultur buněk rostlin i savců, například keratinocytů a fibroblastů.

Předmětem vynálezu jsou dále adeninové deriváty obecného vzorce I *per se*



(I)

a jejich farmaceuticky přijatelné soli s alkalickými kovy, amoniakem, aminy, nebo adiční soli s kyselinami, kde

R2 je vodík nebo halogen;

R6 je vybrán ze skupiny zahrnující
– heteroaryl s 5– až 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heteroaryl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heteroarylalkyl s 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heteroarylalkyl je nesubstituovaný nebo substituovaný alespoň jedním

substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heteroarylalkyl s 5–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heteroarylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heterocyklyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklylalkyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heterocyklylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– cykloalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– cykloalkylalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž cykloalkylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl.

Předmětem vynálezu jsou ve výhodném provedení adeninové deriváty obecného vzorce I *per se*, vybrané z:
6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–thiofen–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(thiofen–2–ylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–thiofen–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin.

Přípravky

Vhodné podání pro kosmetickou aplikaci je lokální, topické. Přípravky zpravidla obsahují od 0,1 do 95 hmotn. % aktivní látky, přičemž jednorázové dávky obsahují přednostně od 10 do 90 hmotn. % aktivní látky a při způsobech aplikace, které nejsou jednorázové, obsahují přednostně

od 1 do 10 hmotn. % aktivní látky. Formy aplikace jsou např. masti, krémy, pasty, pěny, tinktury, rtěnky, kapky, spreje, disperze atd. Přípravky pro ochranu rostlin a pro ošetření tkáňových kultur jsou nejčastěji ve formě roztoku, spreje, disperze nebo suspenze. Přípravky jsou připravovány známým způsobem, např. běžným mícháním, rozpouštěcími nebo lyofilizačními procesy.

Roztoky aktivních látek, suspenze nebo disperze, obzvláště izotonické vodné roztoky, suspenze a disperze, mohou být připraveny před použitím, např. v případě lyofilizovaných preparátů obsahujících aktivní látku samotnou nebo s nosičem jako je například mannitol.

Olejové suspenze obsahují jako olejovou složku rostlinné, syntetické nebo semisyntetické oleje. Oleje, které zde mohou být zmíněny, jsou obzvláště kapalné estery mastných kyselin, které obsahují jako kyselou složku mastnou kyselinu s dlouhým řetězcem majícím 8 až 22, s výhodou pak 12 až 22 uhlíkových atomů, např. kyselinu laurovou, tridekanovou, myristovou, pentadekanovou, palmitovou, margarovou, stearovou, arachidonovou a behenovou, nebo odpovídající nenasycené kyseliny, např. kyselinu olejovou, alaidikovou, eurikovou, brasidovou a linoleovou, případně s přídavkem antioxidantů, např. vitaminu E, β–karotenu nebo 3,5–di–*tert*–butyl–4–hydroxytoluenu. Alkoholová složka těchto esterů mastných kyselin nemá více než 6 uhlíkových atomů a je mono– nebo polyhydrická, např. mono–, di– nebo trihydrické alkoholy jako methanol, ethanol, propanol, butanol nebo pentanol a jejich isomery, ale hlavně glykol a glycerol. Estery mastných kyselin jsou s výhodou např. ethyl oleát, isopropyl myristát, isopropyl palmitát, "Labrafil M 2375" (polyoxyethylen glycerol trioleát, Gattefoseé, Paříž), "Labrafil M 1944 CS" (nenasycené polyglykolované glyceridy připravené alkoholýzou oleje z meruňkových jader a složený z glyceridů a esterů polyethylen glykolu; Gattefoseé, Paříž), "Labrasol" (nasycené polyglykolované glyceridy připravené alkoholýzou TCM a složené z glyceridů a esterů polyethylen glykolu; Gattefoseé, Paříž), "Miglyol 812" (triglycerid nasycených mastných kyselin s délkou řetězce C_8 až C_{12} od Hüls AG, Německo) a zvláště rostlinné oleje jako bavlníkový olej, mandlový olej, olivový olej, ricinový olej, sezamový olej, sójový olej a olej z podzemnice olejné.

Masti jsou emulze oleje ve vodě, které obsahují ne více než 70 %, ale přednostně 20 až 50 % vody nebo vodné fáze. Tukovou fázi tvoří uhlovodíky, např. vazelína, parafínový olej nebo tvrdé parafiny, které přednostně obsahují vhodné hydroxysloučeniny jako mastné alkoholy a jejich estery, např. cetyl alkohol, nebo alkoholy lanolinu, s výhodou lanolin pro zlepšení kapacity pro vázání vody. Emulgátory jsou odpovídající lipofilní sloučeniny jako sorbitanové estery mastných kyselin (Spaný), s výhodou sorbitan oleát nebo sorbitan isostearát. Aditiva k vodné fázi jsou např. smáčedla jako polyalkoholy, např. glycerol, propylen glykol, sorbitol a polyethylen glykol, nebo konzervační prostředky či příjemně vonící látky.

Mastné masti jsou nevodné a obsahují jako bázi hlavně uhlovodíky, např. parafin, vazelínu nebo parafínový olej, a dále přírodní nebo semisyntetické tuky, např. hydrogenované kokosové triglyceridy mastných kyselin nebo hydrogenované oleje, např. hydrogenovaný ricínový olej nebo z podzemnice olejné, a dále částečné glycerolové estery mastných kyselin, např. glycerol mono– a distearát. Dále obsahují např. mastné alkoholy, emulgátory a aditiva zmíněná v souvislosti s mastmi, která zvyšují příjem vody.

Krémy jsou emulze oleje ve vodě, které obsahují více než 50 % vody. Používané olejové báze jsou mastné alkoholy, např. isopropyl myristát, lanolin, včelí vosk, nebo uhlovodíky, s výhodou vazelína (petrolátum) a parafínový olej. Emulgátory jsou povrchově aktivní sloučeniny s převážně hydrofilními vlastnostmi, jako jsou odpovídající neiontové emulgátory, např. estery mastných kyselin polyalkoholů nebo jejich ethylenoxy adukty, např. polyglycerických mastných kyselin nebo polyethylen sorbitanové estery či kyselé estery polyglycerických mastných kyselin (Tween), dále polyoxyethylenové étery mastných alkoholů nebo polyoxyethylenové estery mastných kyselin, nebo odpovídající iontové emulgátory, jako alkalické soli sulfátů mastných alkoholů, s výhodou laurylsulfát sodný, cetylsulfát sodný nebo stearylsulfát sodný, které jsou obvykle používány v přítomnosti mastných alkoholů, např. cetyl stearyl alkoholu nebo stearyl alkoholu. Aditiva k vodné fázi jsou mimo jiné činidla, která chrání krémy před vyschnutím, např.

polyalkoholy jako glycerol, sorbitol, propylen glykol a polyethylen glykol, a dále konzervační činidla a příjemně vonící látky.

Pasty jsou krémy nebo masti obsahující práškové složky absorbující sekreci jako jsou oxidy kovů, např. oxidy titanu nebo oxid zinečnatý, a dále talek či silikáty hliníku, které mají za úkol vázat přítomnou vlhkost nebo sekreci.

Pěny jsou aplikovány z tlakových nádob a jsou to kapalné emulze oleje ve vodě v aerosolové formě, přičemž jako hnací plyny jsou používány halogenované uhlovodíky, jako chlor–fluor deriváty nižších alkanů, např. dichlorfluormethan a dichlortetrafluorethan, nebo přednostně nehalogenované plynné uhlovodíky, vzduch, N_2O či oxid uhličitý. Používané olejové fáze jsou stejné jako pro masti a také jsou používána aditiva tam zmíněná.

Tinktury a roztoky obvykle obsahují vodně–ethanolickou bázi, ke které jsou přimíchány zvlhčovadla pro snížení odpařování, jako jsou polyalkoholy, např. glycerol, glykoly a polyethylen glykol, dále promazávadla jako estery mastných kyselin a nižších polyethylen glykolu, tj. lipofilní látky rozpustné ve vodné směsi nahrazující tukové látky odstraněné z kůže ethanolem, a pokud je to nutné i ostatní neutrální látky a aditiva.

Vynález je dále objasněn s pomocí příkladů, které ale jeho rozsah dále neomezují.

Příklady uskutečnění vynálezu

Příklad 1: Příprava 6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purinu (**1**)

6–Chlor–9–(tetrahydrofuran–2–yl)purin (1 g; 4,46 mmol) byl umístěn do varné baňky spolu s n–propanolem (50 ml). Ke vzniklé suspenzi byl přidán tetrahydrofurfurylamin (554 µl; 5,36 mmol) spolu s triethylaminem (Et_3N) (3,2 ml; 22,3 mmol). Reakční směs byla pod zpětným chladičem zahřívána na teplotu 100 °C. Doba reakce byla 4 h. Po zahuštění byla reakční směs extrahována pomocí kontinuálního extraktoru (EtOAc:H_2O; 24 h). Ethylacetátová (EtOAc) frakce byla následně vysušena pomocí Na_2SO_4. Takto předčištěná reakční směs byla rozdělena pomocí sloupcové chromatografie s mobilní fází (EtOAc:MeOH:NH_3; 34:1:1; v:v).Výtěžek: 74,6 %. ^{1}H NMR (500 MHz, DMSO–d_6), ppm: 1,53 – 1,64 (m, 1 H); 1,69 – 1,89 (m, 3 H); 1,95 – 2,02 (m, 1 H); 2,11 – 2,23 (m, 1 H); 2,30 – 2,44 (m, 2 H); 3,37 – 3,48 (m, 1 H); 3,48 – 3,53 (m, 1 H); 3,53 – 3,60 (m, 1 H); 3,70 – 3,76 (m, 1 H); 3,81 – 3,90 (m, 1 H); 3,98 (q, *J* = 7,03 Hz, 1 H); 4,09 (q, *J* = 7,44 Hz, 1 H); 6,21 (dd, *J* = 6,88, 3,82 Hz, 1 H); 7,63 (br. s., 1 H); 8,17 (br. s., 1 H); 8,21 (s, 1 H).

Tabulka 1: Látky připravené způsobem podle příkladu 1

Č.	R_6	R_2	Elementární analýza vypočteno/nalezeno			
			%C	%H	%N	ES MS $[M+H]^+$
1	tetrahydrofuran-2-ylmethyl	H	58,1/58,0	6,6/6,6	24,2/24,3	290,3
2	5-methylfuran-2-ylmethyl	H	60,2/60,1	5,7/5,7	23,4/23,5	300,3
3	5-hydroxymethylfuran-2-ylmethyl	H	57,1/57,2	5,4/5,4	22,2/22,5	316,3
4	5-formylfuran-2-ylmethyl	H	57,5/57,6	4,8/4,8	22,4/22,2	314,3
5	1-furan-2-ylethyl	H	60,2/60,1	5,7/5,8	23,4/23,5	300,3
6	5-methyltetrahydrofuran-2-ylmethyl	H	59,4/59,3	7,0/7,0	23,1/23,2	304,4

Č.	R_6	R_2	Elementární analýza vypočteno/nalezeno			
			%C	%H	%N	ES MS $[M+H]^+$
7	1-tetrahydrofuran-2-ylethyl	H	59,4/59,2	7,0/7,1	23,1/23,2	304,4
8	cyklopentylmethyl	H	62,7/62,9	7,4/7,5	24,4/24,5	288,4

Příklad 2: Příprava 6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purinu (**9**)

6–Chlor–9–(tetrahydrofuran–2–yl)purin (0,5 g; 2,23 mmol) byl umístěn do varné baňky spolu s n–propanolem (25 ml). Ke vzniklé směsi byl přidán 2–thiofenmethylamin (275 µl; 2,68 mmol) spolu s Et_3N (1,6 ml; 11,15 mmol). Vzniklá směs byla pod zpětným chladičem za stálého míchání zahřívána na 100 °C. Po 3 hodinách bylo přidáno (23 µl; 0,23 mmol) 2–thiofenmethylaminu. Reakce byla následně za 1,5 h ukončena. Reakční směs byla poté odpařena a přečištěna extrakcí kapalina–kapalina (EtOAc:H_2O). EtOAc frakce byla vysušena pomocí Na_2SO_4 a odpařena. Odparek byl přes noc krystalizován z diehyletheru při 4 °C.
Výtěžek: 61 %. ^{1}H NMR (500 MHz, DMSO–d_6) δ ppm 1,92 – 2,00 (m, 1 H); 2,12 – 2,22 (m, 1 H); 2,31 – 2,38 (m, 1 H); 2,38 – 2,43 (m, 1 H); 3,85 (td, *J* = 7,68, 6,34 Hz, 1 H); 4,08 (td, *J* = 7,68. 6,50 Hz, 1 H); 4,79 (br. s., 2 H); 6,21 (dd, *J* = 6,88, 3,82 Hz, 1 H); 6,88 (dd, *J* = 5,04, 3,44 Hz, 1 H); 6,97 (dd, *J* = 3,40, 1,03 Hz, 1 H); 7,27 (dd, *J* = 5,12, 1,22 Hz, 1 H); 8,23 (s, 2 H); 8,36 (br. s., 1 H).

Tabulka 2: Látky připravené způsobem podle příkladu 2

Č.	R_6	R_2	Elementární analýza vypočteno/nalezeno			
			%C	%H	%N	ES MS $[M+H]^+$
9	thiofen-2-ylmethyl	H	55,8/55,6	5,0/5,4	23,2/23,3	302,4
10	3-methylthiofen-2-ylmethyl	H	57,1/57,2	5,4/5,5	22,2/22,3	316,4
11	5-methylthiofen-2-ylmethyl	H	57,1/57,0	5,4/5,3	22,2/22,1	316,4
12	5-chlorothiofen-2-ylmethyl	H	50,0/50,1	4,2/4,3	20,9/20,8	336,8
13	5-bromothiofen-2-ylmethyl	H	44,2/44,1	3,7/3,8	18,4/18,5	381,3
14	1-thiofen-2-ylethyl	H	57,1/57,0	5,4/5,5	22,2/22,0	316,4

Příklad 3: Příprava 2–chloro–6–furfurylamino–9–(tetrahydrofuran–2–yl)purinu (**15**)

2,6–Dichlor–9–(tetrahydrofuran–2–yl)purin (0,5 g; 1,93 mmol) byl umístěn do varné baňky spolu s n–propanolem (25 ml). Ke vzniklé suspenzi byl přidán furfurylamin (204 µl; 2,31 mmol) spolu s Et_3N (1,35 ml; 9,65 mmol). Reakční směs byla pod zpětným chladičem za stálého míchání zahřívána na teplotu 100 °C. Po 5 h byla reakce ukončena. Jestliže látka nevykrystalizovala z reakční směsi, byla reakční směs po zahuštění za horka rozpuštěna ve směsi ($CHCl_3$:EtOH; 1:8; v:v), nebo za pokojové teploty v směsi ($CHCl_3$:Ether; 1:7; v:v). Po ochlazení byla vzniklá suspenze přefiltrována přes fritu a filtrační koláč byl promyt destilovanou vodou. Po vysušení byl

filtrát za horka rozpuštěn v ethanolu (EtOH), petroletheru, karbofiltrován a krystalizován při 4 °C.

Tabulka 3: Látky připravené způsobem podle Příkladu 3

Č.	R_6	R_2	Elementární analýza vypočteno/nalezeno			
			%C	%H	%N	ES MS $[M+H]^+$
15	furfuryl	Cl	52,6/52,5	4,4/4,3	21,9/21,7	320,7
16	5-methylfuran-2-ylmethyl	Cl	54,0/54,1	4,8/4,7	21,0/20,9	334,8
17	5-hydroxymethylfuran-2-ylmethyl	Cl	51,5/51,6	4,6/4,7	20,0/20,1	350,8
18	5-formylfuran-2-ylmethyl	Cl	51,8/51,9	4,1/4,2	20,1/20,3	348,8
19	1-furan-2-ylethyl	Cl	54,0/54,1	4,8/4,9	21,0/21,3	334,8
20	tetrahydrofuran-2-ylmethyl	Cl	51,9/51,8	5,6/5,7	21,6/21,5	233,8
21	5-methyltetrahydrofuran-2-ylmethyl	Cl	53,3/53,4	6,0/6,0	20,7/20,5	338,8
22	1-tetrahydrofuran-2-ylethyl	Cl	53,3/53,4	6,0/6,1	20,7/20,6	338,8
23	thiofen-2-ylmethyl	Cl	50,1/50,1	4,2/4,3	20,9/20,8	336,8
24	3-methylthiofen-2-ylmethyl	Cl	51,5/51,6	4,6/4,7	20,0/20,2	350,8
25	5-methylthiofen-2-ylmethyl	Cl	51,5/51,6	4,6/4,7	20,0/20,1	350,8
26	5-chlorthiofen-2-ylmethyl	Cl	45,4/45,6	3,5/3,7	18,9/18,8	371,3
27	5-bromthiofen-2-ylmethyl	Cl	40,6/40,5	3,2/3,1	16,9/16,8	415,7
28	1-thiofen-2-ylethyl	Cl	51,5/51,6	4,6/4,7	20,0/20,1	350,8
29	cyklopentylmethyl	Cl	56,0/56,1	6,3/6,4	21,8/21,9	322,8

Příklad 4: Cytotoxicita nových derivátů pro kožní buňky stanovená MTT in vitro testem

MTT test je standardní test toxicity založený na fotometrickém měření schopnosti metabolicky aktivních buněk redukovat MTT (3–(4,5–dimethylthiazol–2–yl)–2,5–difenyltetrazolium bromid) na formazan. V testu byl sledován vliv 72 hodinového působení několika koncentrací látek (šestinásobná ředicí řada, maximální koncentrace = 50 μM) na viabilitu kožních fibroblastů BJ a keratinocytů HaCaT. 5000 buněk bylo vyseto do jednotlivých jamek 96 jamkové mikrotitrační destičky 24 hodin před přidáním testovaných látek. Jako negativní kontrola bylo použito DMSO vehikulum. Po 72 hodinovém působení bylo přidáno nové médium s MTT (Sigma, M2128) do konečné koncentrace 0,5 mg/ml. Po třech hodinách bylo médium odstraněno a formazan

obsažený v buňkách rozpuštěn v DMSO. Absorbance byla změřena při 570 nm (640 nm referenční vlnová délka). Hodnoty IC_{50} byly vypočteny z dávkových křivek. 6–Furfurylaminopurin ribosid byl použit jako pozitivní kontrola. Byly obdrženy následující výsledky:

Tabulka 4: Cytotoxicita stanovená MTT in vitro testem

Látka	IC_{50} (μM)
dimethylsulfoxid	>100
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	>100
6-(thiofen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)aminopurin	>100
2-chlor - 6-furfurylamino-9-(tetrahydrofuran-2-yl)purin	>100
6-furfurylamino-9-ribosylpurin (srovnávací látka)	≤ 3

Příklad 5: Zpomalení stárnutí listů pšenice a zpomalení degradace chlorofylu při testování nových derivátů v senescenčním biotestu

Nádoby se semeny ozimé pšenice *Triticum aestivum* cv Hereward vyseté do vermikulitu nasyceného Knopovým živným roztokem byly umístěny do klimatizované růstové komory s 16/8 hodinovou světelnou periodou (světelná intenzita 50 $mmol.m^{-2}.s^{-1}$) a teplotou 15 °C. Po 7 dnech měly semenáčky vyvinutý první praporcový list a druhý list začínal prorůstat. Z prvních listů vždy od 10 rostlin byly odebrány vrcholové sekce dlouhé přibližně 35 mm, které byly zkráceny tak, aby jejich váha byla přesně 100 mg. Bazální konce těchto 10 listových segmentů byly umístěny do jamek mikrotitračních polystyrénových destiček obsahujících 150 ml roztoku testovaného derivátu. Destičky byly umístěny do plastického boxu vystlaného filtračním papírem, který byl nasycen vodou za účelem maximální vzdušné vlhkosti. Po 96 hodinách inkubace ve tmě při 25 °C byly listové sekce vyjmuty a chlorfyl extrahován v 5 ml 80% ethanolu zahřátím při 80 °C po dobu 10 min. Objem vzorku byl poté doplněn na 5 ml přidáním 80% ethanolu. Absorbance extraktů byla měřena při 665 nm. Jako kontroly byly měřeny rovněž chlorofylové extrakty z listů a listových vrcholů inkubované v deionizované vodě. Vypočtené hodnoty jsou průměrem z 10 opakování a celý experiment byl zopakován minimálně 3krát. Výsledky těchto experimentů byly vyneseny ve formě grafů závislosti obsahu chlorfylu v senescenčních listech na koncentraci aplikované látky. V každém experimentu byla použita jako pozitivní kontrolní látka 6–benzylaminopurin, který je znám velmi vysokou cytokininovou aktivitou. Testované cytokininy byly rozpuštěny v dimethylsulfoxidu (DMSO) a zásobní roztok doplněn vodou na výslednou koncentraci 10^{-3} M. Tento zásobní roztok byl dále ředěn testovacím médiem v koncentračním rozsahu 10^{-8} až 10^{-4} M. Finální koncentrace DMSO v médiu nepřevýšila 0,2 % a v této koncentraci neovlivňovala biologickou aktivitu testu. Ze získaných dat byla vypočítána maximální účinná koncentrace testované látky a její relativní účinnost v této koncentraci (Tab. 5). 10^{-4} M koncentrace BAP byla postulována jako 100 % biologické aktivity. Jako další srovnávací látka byl použit kinetin. Nově připravené látky převyšují zpravidla účinnost BAP standardu v rozmezí o 10 %. Nově vyvinuté látky mají tedy výraznější antisenescenční účinky než známé látky.

Tabulka 5: Účinek nových cytokinů na retenci chlorofylu v extirpovaných listových segmentech pšenice. Hodnoty v tabulce jsou vyjádřeny v % výchozího obsahu chlorofylu v čerstvých listech před inkubací.

Látka	maximální účinná koncentrace ($mol.l^{-1}$)	účinnost (%) [$10^{-4}mol.l^{-1}$ BAP = 100%]
BAP (srovnávací látka)	10^{-4}	100±1
kinetin (srovnávací látka)	10^{-4}	98±4
6-furfurylamino-9-(tetrahydrofuran-2-yl)purin	10^{-4}	114±3
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	10^{-4}	122±2
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	10^{-4}	125±9
6-(thiofen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	10^{-4}	112±5
2-chlor - 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	10^{-4}	127±5

Příklad 6: *In vitro* cytotoxická aktivita nových derivátů na nádorových buněčných liniích

Jedním z parametrů používaných jako základ pro cytotoxickou analyzuje metabolická aktivita životaschopných buněk. Například mikrotitrační analýza, kde se používá Calcein AM, je dnes rozšířena jako metoda kvantifikace buněčné proliferace a cytotoxicity. Množství zredukovaného Calceinu AM odpovídá počtu životaschopných buněk v kultuře.

Buněčné linie prsního karcinomu MCF–7, lidské erythroleukemie K562, lidské diploidní fibroblasty BJ a lidské keratinocyty HaCaT byly použity pro screening cytotoxicity sloučenin. Buňky byly udržovány v Nunc/Corning 80 cm^2 plastových lahvích a pěstovány v médiu pro buněčné kultury (DMEM obsahující 5 g/l glukózy, 2 mM glutaminu, 100 U/ml penicilinu, 100 μg/ml streptomycinu, 10% fetálního telecího séra a hydrogenuhličitan sodný). Buněčné suspenze byly připraveny a naředěny podle typu buněk a podle očekávané konečné hustoty buněk (10^4 buněk na jamku na základě charakteristik buněčného růstu), bylo pipetováno 80 μl buněčné suspenze na 96–jamkové mikrotitrační destičky. Inokuláty byly stabilizovány 24 hodinovou preinkubací při 37 °C v atmosféře CO_2. Jednotlivé koncentrace testovaných látek byly přidány v čase nula jako 20 μl alikvotní podíl do jamek mikrotitračních destiček. Obvykle byly sloučeniny testovány v šesti koncentracích v čtyřnásobné ředicí řadě. Při rutinním testování byla nejvyšší koncentrace v jamce 166,7 μM, změny této koncentrace závisí na dané látce. Všechny koncentrace byly testovány v dubletu. Inkubace buněk s testovanými deriváty trvala 72 hodin při 37 °C, 100% vlhkosti a v atmosféře obohacené CO_2. Na konci inkubační periody byly buňky analyzovány po přidání roztoku Calceinu AM (Molecular Probes) a inkubace probíhala další 1 hodinu. Fluorescence (FD) byla měřena pomocí Labsystem FIA readeru Fluorskan Ascent (Microsystems). Přežití buněk (The cell survival–TCS) bylo spočítáno podle následujícího vztahu: $GI_{50}=(FD_{jamka\ s\ derivátem}/FD_{kontrolní\ jamka})$ x 100 %. Hodnota GI_{50}, která odpovídá koncentraci látky, kdy je usmrceno 50 % buněk, byla vypočtena ze získaných dávkových křivek.

Pro vyhodnocení protinádorové aktivity byla testována toxicita nových derivátů na panelech obsahujících buněčné linie rozdílného histogenetického a druhového původu (Tab. 6, GI_{50} koncentrace uváděny v μM). Ukázalo se, že pro všechny testované nádorové linie i pro nemaligní buněčné linie BJ a HaCaT bylo působení nových sloučenin netoxické. Účinné deriváty zabíjí nádorové buňky při koncentracích blízkých 0,1 až 50 μM.

Tabulka 6: Cytotoxicita látek podle vynálezu pro různé buněčné linie

Látka	MCF-7	K562	BJ	HaCaT
6-furfurylamino-9-(tetrahydrofuran-2-yl)purin	>100	>100	>100	>100
6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	>100	>100	>100	>100
6-(thiofen-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	>100	>100	>100	>100
2-chlor - 6-(tetrahydrofuran-2-ylmethylamino)-9-(tetrahydrofuran-2-yl)purin	>100	>100	>100	>100

Příklad 7: *In vitro* test fotoxických účinků na lidské kožní fibroblasty

Možné fototoxické účinky látek byly stanoveny podle modifikovaného *in vitro* testu validovaného pro stanovení fototoxicity látek (Spielmann a kol., 1998). Jako *in vitro* model byly použity normální lidské kožní fibroblasty (NHDF). Buňky byly izolovány z fragmentů kůže od zdravých dárců, kteří podstoupili chirurgický zákrok na Oddělení plastické a estetické chirurgie FN Olomouc. Odběr tkáně a izolace buněk proběhla podle postupu a podmínek schválených Etickou komisí FN Olomouc a Lékařské fakulty UP Olomouc a po podepsání informovaného souhlasu s odběrem tkáně jednotlivými dárci. Pro experimenty byly NHDF použity mezi 2. a 4. pasáží. NHDF byly vysety na 96–jamkové desky v koncentraci $0{,}8.10^5$ buněk/ml kultivačního média (DMEM doplněné o fetální telecí sérum (10%), penicilin (100 mg/ml) a streptomycin (100 U/ml)) v množství 0,2 ml na jamku.

Testovanou látkou byl 6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin **1**. Látka byla rozpuštěna v DMSO a poté ředěna do média bez séra (DMEM s penicilinem (100 mg/ml) a streptomycinem (100 U/ml)). Výsledné aplikované koncentrace byly 3,9 až 500 □mol/1. Jako kontrola bylo použito médium bez séra s DMSO (0,5 % (v/v)), což odpovídá jeho výsledné koncentraci při experimentu s testovanými látkami. Pro srovnání s tetovanými látkami byl použit chlorpromazin (CPZ; 0,8 až 50 μmol/l) jako pozitivní fototoxická látka. Po 24 hodinách od vysetí NHDF bylo růstové médium vyměněno za médium bez séra obsahující testované látky nebo DMSO (rozpouštědlová kontrola). Roztoky testovaných látek byly paralelně aplikovány na dvě 96–jamkové desky s NHDF. Po 1 hodinové preinkubaci s látkami bylo médium odstraněno, buňky byly dvakrát opláchnuty roztokem PBS a poté bylo aplikováno PBS s glukózou (1 mg/ml). Následně byla jedna deska ozářena netoxickou dávkou (5 J/cm^2) UVA záření. Druhá deska byla po dobu ozařování inkubována v temnu. K ozáření byl použit solární simulátor *SOL 500* vybavený *H1 filtrem* (Dr. Höenle Technology, Německo), který propouští záření v rozsahu vlnových délek 320 až 400 nm. Intenzita UVA záření byla při každém ozařování stanovena pomocí UVA–metru (Dr. Höenle Technology, Německo). Po UVA expozici bylo PBS s glukózou odstraněno a k buňkám bylo aplikováno médium bez séra. Po 24 hodinové inkubaci (37 °C, 5 % CO_2) byla sledována životnost buněk (respektive buněčné poškození) stanovením inkorporace neutrální červeně (NČ) do buněk. Po odstranění média bez séra byl k buňkám aplikován roztok NČ (0,03 %; PBS). Po 1 hodinové inkubaci (37 °C, 5 % CO_2) byl roztok s NČ odstraněn, buňky byly fixovány směsí formaldehydu (0,5 %, v/v) a $CaCl_2$ (1 %, m/v) v poměru 1:1 a barvivo bylo rozpuštěno extrakčním roztokem (methanol (50 %, v/v), CH_3COOH (1 %, v/v). Po 5 minutovém intenzivním třepání byla změřena absorbance při 540 nm. Experimenty byly provedeny ve 4 nezávislých opakováních s použitím buněk od 4 dárců kvůli minimalizaci individuálních rozdílů v citlivosti buněk. Fototoxický účinek látek byl hodnocen určením procent životnosti kontrolních buněk (% životnosti kontroly) z hodnot příslušných absorbancí podle následujícího vztahu:

$$\text{Životnost (\% kontroly)} = \left(\frac{(A_V - A_P)}{(A_K - A_P)}\right) \cdot 100$$

A_V... absorbance vzorku (buňky pre–inkubované s testovanými látkami v médiu bez séra a následně ozářené)
A_K ... absorbance kontroly (buňky inkubované s DMSO v médiu bez séra a ozářené)
A_P ... absorbance pozadí (extrakční roztok)

Výsledek: Testovaná látka nejevila v použitém koncentračním rozmezí, tj. 3,9 až 500 μmol/l, fototoxické účinky (nedošlo ke snížení životnosti buněk ~ schopnosti inkorporovat NČ ve srovnání s kontrolními buňkami). Výsledky jsou znázorněny v tabulce (Tab. 7). Současně byl jako pozitivní kontrola použit CPZ, který je doporučován validovaným postupem (Spielmann H, Balls M, Dupuis J, Pape WJ, Pechovitch G, de Silva O, Holzhütter HG, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell WW, Maurer T, Pfannenbecker U, Potthast JM, Csato M, Sladowski D, Steiling W, Brantom P., Toxicol In Vitro. **1998** Jun 1;12(3):305–27) jako známá fototoxická sloučenina, viz Tab. 8. Z výše uvedených výsledků vyplývá, že testovaná látka je bezpečná při použití do kosmetických a dermatologických přípravků, i ve spojení s následnou expozicí jí ošetřené pokožky slunečnímu záření.

*Tabulka 7: Účinky látky **1** na životnost NHDF stimulovaných UVA zářením (+UVA) ve srovnání s kontrolou inkubovanou v temnu (–UVA).*

	1	
Koncentrace (μmol/l)	% kontroly	
	- UVA	+UVA
3,9	96,6 ± (7,4)	105,1 ± (10,3)
7,8	106,5 ± (9,2)	110,6 ± (10,7)
15,6	99,6 ± (3,7)	106,8 ± (13,4)
31,3	102,4 ± (2,1)	109,5 ± (10)
62,5	102,3 ± (5,8)	103,3 ± (8,9)
125	109,5 ± (10,5)	107,5 ± (8,9)
250	109,1 ± (6,7)	106,7 ± (7,8)
500	105,3 ± (11,1)	98,9 ± (4,2)

Tabulka 8: Pro srovnání: účinky fototoxického chlorpromazinu na životnost NHDF stimulovaných UVA zářením (+UVA) ve srovnání s kontrolou inkubovanou v temnu (–UVA).

	CPZ	
Koncentrace (μmol/l)	% kontroly	
	- UVA	+UVA
0,8	102,6 ± (5,8)	99,2 ± (7,1)
1,6	101,5 ± (1)	104,6 ± (7,7)
3,1	105,6 ± (6)	87,3 ± (8,6)
6,3	107,4 ± (4,6)	82,8 ± (15,4)
12,5	104 ± (3,8)	49,6 ± (17,6)
25	77 ± (7,8)	19,8 ± (14,2)
50	4,7 ± (4,2)	9,8 ± (7,8)

Příklad 8. *In vitro* test fotoprotektivních účinků na normální lidské kožní fibroblasty

Jako *in vitro* model byly použity normální lidské kožní fibroblasty (NHDF). Buňky byly izolovány z fragmentů kůže od zdravých dárců, kteří podstoupili chirurgický zákrok na Oddělení plastické a estetické chirurgie FN Olomouc. Odběr tkáně a izolace buněk proběhla podle postupu a podmínek schválených Etickou komisí FN Olomouc a Lékařské fakulty UP Olomouc a po podepsání informovaného souhlasu s odběrem tkáně jednotlivými dárci. Pro experimenty byly NHDF použity mezi 2. a 4. pasáží. NHDF byly vysety na 96–jamkové desky v koncentraci $0{,}8.10^5$ buněk/ml kultivačního média (DMEM doplněné o fetální telecí sérum (10%), penicilin (100 mg/ml) a streptomycin (100 U/ml)) v množství 0,2 ml na jamku.

Testovanou látkou byl 6–tetrahydrofurfurylamino–9–(tetrahydrofuran–2–yl)purin **1**. Látka byla rozpuštěna v DMSO a poté ředěna do média bez séra (DMEM s penicilinem (100 mg/ml) a streptomycinem (100 U/ml)). Jako kontrola bylo použito médium bez séra s DMSO (0,5 % (v/v)), což odpovídá jeho výsledné koncentraci při experimentu s testovanými látkami. Po 24 hodinách od vysetí NHDF bylo růstové médium vyměněno za médium bez séra obsahující testované látky nebo DMSO (rozpouštědlová kontrola). Testovaná látka byla paralelně aplikována na dvě 96–jamkové desky s NHDF. Po 60 min inkubaci bylo médium s látkami odstraněno, buňky byly dvakrát opláchnuty roztokem PBS a bylo aplikováno PBS s glukózou (1 mg/ml). V případě sledování ochrany vůči UVA záření byla následně jedna deska ozářena cytotoxickou dávkou UVA záření (7,5 J/cm^2) s využitím solárního simulátoru SOL 500 (Dr. Höenle Technology, Německo) vybaveným H1 filtrem, který propouští záření v rozsahu vlnových délek 320 až 400 nm. Při sledování ochrany vůči UVB záření byly buňky ozářeny cytotoxickou dávkou UVB záření (400 mJ/cm^2). K ozáření byl použit solární simulátor SOL 500 vybavený H2 filtrem, který propouští záření v rozsahu vlnových délek 295 až 320 nm. Intenzita UVA nebo UVB záření byla při každém ozařování stanovena pomocí UVA– nebo UVB–metru (Dr. Höenle Technology, Německo). Kontrolní buňky byly v obou případech po dobu ozařování inkubovány v temnu. Po UVA/UVB expozici bylo PBS s glukózou odstraněno a k buňkám bylo aplikováno médium bez séra. Po 24 hodinové inkubaci (37 °C, 5 % CO_2) bylo sledováno buněčné poškození stanovením inkorporace neutrální červeně (NČ) do buněk. Po odstranění média bez séra byl k buňkám aplikován roztok NČ (0,03 %; PBS). Po 1 hodinové inkubaci (37 °C, 5 % CO_2) byl roztok s NČ odsán, buňky byly fixovány směsí formaldehydu (0,5 %, v/v) a $CaCl_2$; (1 %, m/v) v poměru 1:1 a barvivo bylo rozpuštěno extrakčním roztokem (methanol (50 %, v/v), CH_3COOH (1 %, v/v). Po 5 min intenzivního třepání byla změřena absorbance při 540 nm. Experimenty byly provedeny ve 4 nezávislých opakováních s použitím buněk od 4 dárců kvůli minimalizaci individuálních rozdílů v citlivosti buněk.

Ochranný účinek látky na poškození vyvolané UVA nebo UVB zářením byl vypočítán z experimentálních dat (absorbancí) testovaných látek s pozitivní a negativní kontrolou podle následujícího vztahu:

$$\%\ \text{ochrany} = 100 - \left|\frac{Avz - Ank}{Apk - Ank}\right| \cdot 100$$

Avz ... absorbance vzorku (buňky pre–inkubované s testovanými látkami v médiu bez séra a následně ozářené)
Ank ... absorbance negativní kontroly (buňky inkubované s DMSO v médiu bez séra a neozářené = inkubované v temnu)
Apk ... absorbance pozitivní kontroly (buňky inkubované s DMSO v médiu bez séra a ozářené)

Výsledek: U buněk inkubovaných s testovanou látkou před UVA nebo UVB ozářením byla nalezena vyšší životnost buněk (schopnost inkorporace NČ) ve srovnání s buňkami inkubovanými s DMSO (kontrola) a ozářenými UVA nebo UVB ozářením (Tab. 9 a 10). Látka tedy vykazuje současně UVA i UVB fotoprotektivní efekt.

*Tabulka 9: Stanovení ochranných účinků látky **1** vůči poškození UVA zářením. Výsledky reprezentují průměr ± SD ze 4 experimentů provedených na NHDF izolovaných od různých pacientů.*

UVA fotoprotekce	
Koncentrace (μmol/l)	**1**
	% ochrany
3,9	27,7 ± (6,9)
7,8	57,4 ± (14,4)
15,6	43,2 ± (10,8)
31,3	47,5 ± (11,9)
62,5	41,3 ± (10,3)
125	49 ± (12,3)
250	45,3 ± (11,3)
500	18,5 ± (4,6)

*Tabulka 10: Stanovení ochranných účinků látky **1** vůči poškození UVB zářením. Výsledky reprezentují průměr ± SD ze 4 experimentů provedených na NHDF izolovaných od různých pacientů.*

UVB fotoprotekce	
Koncentrace (μmol/l)	**1**
	% ochrany
3,9	4,9 ± (1,2)
7,8	44,1 ± (11)
15,6	35 ± (8,7)
31,3	38,3 ± (9,6)
62,5	44,4 ± (11,1)
125	42,7 ± (10,7)
250	46,6 ± (11,6)
500	26,3 ± (6,6)

Příklad 9. *In vitro* test fototoxicity na normální lidské kožní keratinocyty

Jako *in vitro* model byly použity normální lidské kožní keratinocyty (NHEK). Buňky byly izolovány z fragmentů kůže od zdravých dárců, kteří podstoupili chirurgický zákrok na Oddělení plastické a estetické chirurgie FN Olomouc. Odběr tkáně a izolace buněk proběhla podle postupu a podmínek schválených Etickou komisí FN Olomouc a Lékařské fakulty UP Olomouc. Všichni dárci podepsali informovaný souhlas s odběrem tkáně. Pro experiment byly buňky použity ve 3. až 4. pasáži. NHEK byly vysety na 96–jamkové desky v koncentraci 1.10^4 buněk/jamku v růstové médium pro keratinocyty (EpiLife®) doplněné o komerční sadu růstových faktorů (Human Keratinocyte Growth Supplement Kit) a antibiotika (penicilin (100 mg/ml), streptomycin (100 mg/l) a ampicilin (250 μg/ml).

Testovanou látkou byl 6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin **1**. Látka **1** byla rozpuštěna v DMSO a poté naředěna do média bez séra (EpiLife® s penicilinem (100 mg/ml) a streptomycinem (100 U/ml) a ampicilinem (250 μg/ml)) na koncentrační rozmezí (3,9 až 500 μmol/l) tak, aby výsledná koncentrace DMSO v médiu byla 0,5 % (v/v). Kontrolní médium obsahovalo příslušný objem DMSO v médiu bez séra. Po 24 hodinové inkubaci NHEK

bylo růstové médium vyměněno za médium bez séra obsahující testované látky a DMSO (negativní kontrola). Jako pozitivní kontrola byla použita známá fototoxická sloučenina chlorpromazin (CPZ; 0,8 až 50 μmol/l). Roztoky testovaných látek byly paralelně aplikovány na dvě 96–jamkové desky s buňkami. Po 1 hodinové preinkubaci bylo médium odstraněno, buňky byly dvakrát opláchnuty roztokem PBS a poté bylo aplikováno PBS s glukózou (1 mg/ml). Následně byla jedna deska ozářena netoxickou dávkou (5 J/cm^2) UVA záření. Druhá deska byla po dobu ozařování inkubována v temnu. K ozáření byl použit solární simulátor SOL 500 vybavený H1 filtrem (Dr. Höenle Technology, Německo), který propouští záření v rozsahu vlnových délek 320 až 400 nm. Intenzita UVA záření byla při každém ozařování stanovena pomocí UVA–metru (Dr. Höenle Technology, Německo). Po UVA expozici bylo PBS s glukózou odstraněno a k buňkám bylo aplikováno bezsérové médium. Po 24 hodinové inkubaci (37 °C, 5 % CO_2) bylo sledováno buněčné poškození stanovením inkorporace NČ do buněk. Po odstranění média bez séra byl k buňkám aplikován roztok NČ (0,03 %; PBS). Po 1 h inkubaci (37 °C, 5 % CO_2) byl roztok s NČ odstraněn, buňky byly fixovány směsí formaldehydu (0,5 %, v/v) a $CaCl_2$ (1 %, m/v) v poměru 1:1a barvivo bylo rozpuštěno extrakčním roztokem (methanol (50 %, v/v), CH_3COOH (1 %, v/v). Po 5 minutách intenzivního třepání byla změřena absorbance při 540 nm. Experimenty byly provedeny ve 4 nezávislých opakováních s použitím buněk od 4 dárců kvůli minimalizaci individuálních rozdílů v citlivosti buněk. Fototoxický účinek látek byl hodnocen určením procent životnosti kontrolních buněk (% životnosti kontroly) z hodnot příslušných absorbancí podle následujícího vztahu:

$$\text{Životnost (\% kontroly)} = \left(\frac{(A_V - A_P)}{(A_K - A_P)} \right) \cdot 100$$

A_V... absorbance vzorku (buňky pre–inkubované s testovanými látkami v médiu bez séra a následně ozářené)

A_K ... absorbance kontroly (buňky inkubované s DMSO v médiu bez séra a ozářené)

A_P ... absorbance pozadí (extrakční roztok) naměřených hodnot absorbance byla pro každou koncentraci látky vypočtena viabilita buněk jako % kontroly.

Výsledek: Látka **1** nejevila v použitém koncentračním rozmezí 3,9 až 500 μmol/l fototoxické účinky, (nedošlo ke snížení životnosti buněk ~ schopnosti inkorporovat NČ ve srovnání s kontrolními buňkami), viz Tab. 11. Současně s látkou **1** byl jako pozitivní kontrola použit CPZ, který je doporučován validovaným postupem (Spielmann H, Balls M, Dupuis J, Pape WJ, Pechovitch G, de Silva O, Holzhütter HG, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell WW, Maurer T, Pfannenbecker U, Potthast JM, Csato M, Sladowski D, Steiling W, Brantom P., Toxicol In Vitro. **1998** Jun 1;12(3):305–27) jako známá fototoxická sloučenina, viz Tab. 12. Z výše uvedených výsledků vyplývá, že testovaná látka je bezpečná při použití do kosmetických a dermatologických přípravků, i ve spojení s následnou expozicí jí ošetřené pokožky slunečnímu záření.

*Tabulka 11: Účinky látky **1** na životnost NHEK stimulovaných UVA zářením (+UVA) ve srovnání s kontrolou inkubovanou po dobu ozařování v temnu (–UVA).*

	1	
Koncentrace (μmol/l)	% kontroly	
	- UVA	+UVA
3,9	112,2 ± (11)	109,1 ± (2,3)
7,8	135,5 ± (8,2)	120,6 ± (1,6)
15,6	116,5 ± (2,8)	110 ± (2,9)
31,3	119,1 ± (8,7)	115,1 ± (2,8)
62,5	121 ± (8,7)	105,9 ± (8,7)
125	119 ± (2,8)	111,7 ± (6,8)
250	110,8 ± (12,4)	112,1 ± (3,7)
500	105,7 ± (7,1)	101,2 ± (3,3)

Tabulka 12: Pro srovnání: účinky fototoxického chlorpromazinu na životnost NHEK stimulovaných UVA zářením (+UVA) ve srovnání s kontrolou inkubovanou po dobu ozařování v temnu (–UVA).

	CPZ	
Koncentrace (μmol/l)	% kontroly	
	- UVA	+UVA
0,8	103,8 ± (4,4)	121,7 ± (13)
1,6	102 ± (4,6)	77,6 ± (0,9)
3,1	97,6 ± (13,1)	70,4 ± (5,5)
6,3	99,9 ± (7)	47,1 ± (5,2)
12,5	45,4 ± (24,6)	34,7 ± (15,4)
25	29,1 ± (14,5)	26,3 ± (19)
50	17,3 ± (2,2)	21,7 ± (19)

Příklad 10. *In vitro* test fotoprotektivních účinků na normální lidské kožní keratinocyty (NHEK)

Jako *in vitro* model byly použity normální lidské kožní keratinocyty (NHEK). Buňky byly izolovány z fragmentů kůže od zdravých dárců, kteří podstoupili chirurgický zákrok na Oddělení plastické a estetické chirurgie FN Olomouc. Odběr tkáně a izolace buněk proběhla podle postupu a podmínek schválených Etickou komisí FN Olomouc a Lékařské fakulty UP Olomouc. Všichni dárci podepsali informovaný souhlas s odběrem tkáně. Pro experiment byly buňky použity ve 3. až 4. pasáži. NHEK byly vysety na 96–jamkové desky v koncentraci 1.10^4 buněk/jamku v růstové médium pro keratinocyty (EpiLife®) doplněné o komerční sadu růstových faktorů (Human Keratinocyte Growth Supplement Kit) a antibiotika (penicilin (100 mg/ml), streptomycin (100 mg/l) a ampicilin (250 μg/ml).

Testovanou látkou byl 6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin **1.** Látka **1** byla rozpuštěna v DMSO a poté naředěna do média bez séra (EpiLife® s penicilinem (100 mg/ml) a streptomycinem (100 U/ml) a ampicilinem (250 μg/ml)) na koncentrační rozmezí (3,9 až 500 μmol/l) tak, aby výsledná koncentrace DMSO v médiu byla 0,5 % (v/v). Po 24 hodinové inkubaci NHEK bylo růstové médium vyměněno za médium bez séra obsahující testované látky a DMSO (negativní kontrola). Kontrolní médium obsahovalo příslušný objem

DMSO v médiu bez séra. Jako kontrola bylo použito médium bez séra s DMSO (0,5 % (v/v)), což odpovídá jeho výsledné koncentraci při experimentu s testovanými látkami. Testovaná látka byla paralelně aplikována na dvě 96–jamkové desky s NHEK. Po 60 minutové inkubaci bylo médium s látkami odstraněno, buňky byly dvakrát opláchnuty roztokem PBS a bylo aplikováno PBS s glukózou (1 mg/ml). V případě sledování ochrany vůči UVA záření byla následně jedna deska ozářena cytotoxickou dávkou UVA záření (7,5 J/cm) s využitím solárního simulátoru SOL 500 (Dr. Hoenle Technology, Německo) vybaveným H1 filtrem, který propouští záření v rozsahu vlnových délek 320 až 400 nm. Při sledování ochrany vůči UVB záření byly buňky ozářeny cytotoxickou dávkou UVB záření (400 mJ/cm^2). K ozáření byl použit solární simulátor SOL 500 vybavený H2 filtrem, který propouští záření v rozsahu vlnových délek 295 až 320 nm. Intenzita UVA nebo UVB záření byla při každém ozařování stanovena pomocí UVA– nebo UVB–metru (Dr. Hoenle Technology, Německo). Kontrolní buňky byly v obou případech po dobu ozařování inkubovány v temnu. Po UVA/UVB expozici bylo PBS s glukózou odstraněno a k buňkám bylo aplikováno médium bez séra. Po 24 hodinové inkubaci (37 °C, 5 % CO_2) bylo sledováno buněčné poškození stanovením inkorporace neutrální červeně (NČ) do buněk. Po odstranění média bez séra byl k buňkám aplikován roztok NČ (0,03 %; PBS). Po 1 hodinové inkubaci (37 °C, 5 % CO_2) byl roztok s NČ odsán, buňky byly fixovány směsí formaldehydu (0,5 %, v/v) a $CaCl_2$ (1 %, m/v) v poměru 1:1 a barvivo bylo rozpuštěno extrakčním roztokem methanol (50 %, v/v), CH_3COOH (1 %, v/v). Po 5 minutách intenzivního třepání byla změřena absorbance při 540 nm. Experimenty byly provedeny ve 4 nezávislých opakováních s použitím buněk od 4 dárců kvůli minimalizaci individuálních rozdílů v citlivosti buněk.

Ochranný účinek látek na poškození vyvolané UVA nebo UVB zářením byl vypočítán z experimentálních dat (absorbancí) testovaných látek s pozitivní a negativní kontrolou podle následujícího vztahu:

$$\%\ \text{ochrany} = 100 - \left|\frac{Avz - Ank}{Apk - Ank}\right| \cdot 100$$

Avz ... absorbance vzorku (buňky pre–inkubované s testovanými látkami v médiu bez séra a následně ozářené)
Ank ... absorbance negativní kontroly (buňky inkubované s DMSO v médiu bez séra a neozářené = inkubované v temnu)
Apk ... absorbance pozitivní kontroly (buňky inkubované s DMSO v médiu bez séra a ozářené)

Výsledek: U buněk inkubovaných s látkou **1** před UVA nebo UVB ozářením byla nalezena vyšší životnost buněk (schopnost inkorporace NČ) ve srovnání s buňkami inkubovanými s DMSO (kontrola) a ozářenými UVA nebo UVB ozářením (Tab 13 a 14). Látka **1** tedy vykazuje současně UVA i UVB fotoprotektivní efekt.

*Tabulka 13: Stanovení ochranných účinků látky **1** vůči poškození UVA zářením. Výsledky reprezentují průměr ± SD ze 4 experimentů provedených na NHEK izolovaných od různých pacientů*

UVA fotoprotekce	
Koncentrace (μmol/l)	1
	% ochrany
3,9	39,7 ± (12,9)
7,8	60,2 ± (16,1)
15,6	53,5 ± (12,4)
31,3	46,8 ± (11,8)
62,5	47,9 ± (8,9)
125	51,7 ± (4,8)
250	48 ± (3,2)
500	30 ± (13,5)

Tabulka 14: Stanovení ochranných účinků látky ***1*** *vůči poškození UVB zářením. Výsledky reprezentují průměr ± SD ze 4 experimentů provedených na NHEK izolovaných od různých pacientů*

UVB fotoprotekce	
Koncentrace (μmol/l)	1
	% ochrany
3,9	53,5 ± (7,6)
7,8	72 ± (6)
15,6	69,8 ± (8,6)
31,3	65,9 ± (9)
62,5	65,9 ± (5)
125	72,9 ± (6,9)
250	65,7 ± (3,6)
500	28 ± (3,2)

Příklad 11: Vliv testovaných látek na délku života *Caenorhabditis elegans*

Caenorhabditis elegans se používá jako modelový organismus pro identifikaci látek schopných příznivě modulovat některé aspekty stárnutí savců včetně člověka. Rada látek prodlužujících život *C. elegans* má příznivý efekt na patologické projevy některých nemocí spojených se stářím. Příkladem takových látek může být resveratrol, kurkumin a řada dalších. Délku života *C. elegans* prodlužuje i řada látek používaných v kosmetice pro regeneraci pleti a zpomalení jejího stárnutí, jako je například výše zmíněný resveratrol, vitamin E, koenzym Q10, extrakty ze zeleného čaje nebo granátového jablka nebo cytokinin kinetin. V tomto experimentu byla použita standardní linie *Caenorhabditis elegans fem–1/*HC17 nesoucí teplotou indukovatelnou mutaci, která způsobí, že se všichni jedinci při kultivaci ve 25 °C vyvinou v samice, což zabrání další reprodukci a potenciálnímu smíchání původní experimentální populace s potomstvem. Testované látky rozpuštěné v DMSO (100 mM zásobní roztoky) byly přidány do čerstvého NGM (nematode growth medium) do finální koncentrace 10 a 100 μM. Médium bylo následně rozpipetováno do Petriho misek. Jako negativní kontrola bylo použito čisté NGM a NGM s DMSO vehikulem. Po ztuhnutí média bylo na misky rozetřeno 100 μl suspenze bakterií *Escherichia coli* OP50 v LB médiu (suspenze kultivována přes noc ve 37 °C a 20krát zkoncentrována), které slouží jako zdroj potravy pro háďátka. Misky byl ponechány přes noc v 37 °C, aby se bakterie mohly rozrůst. Na takto připravené misky byla následně nasazena populace věkově synchronizovaných mladých dospělců *C. elegans.* Misky byly uchovávány v inkubátoru ve 25 °C a ve vybraných časových intervalech (každé 1 až 3 dny) skenovány na skeneru (Epson perfection V700 photo). Počet

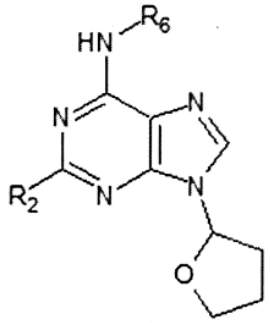
přežívajících jedinců byl určen pomocí analýzy obrazu v programu Fiji. Skript vycházející z identifikuje pohybující se objekty srovnáním série fotografií misky pořízených s malým časovým odstupem (přibližně 30 s). Byly srovnávány tři po sobě pořízené fotografie každé misky a napočtené hodnoty byly zprůměrovány, aby se snížila možnost chyby. Výsledky byly následně analyzovány pomocí programů OASIS a ED50v10. Statistická signifikance byla vyhodnocena pomocí Log–Rank testu a získané p–hodnoty byly korigovány podle Bonferroniho. Výsledky shrnuje tabulka 15. Látky 1, 15 a 20 statisticky významně prodlužovaly život *C. elegans.*

Tabulka 15: Vliv testovaných látek na délku života C. elegans (ve dnech)

	DMSO	**15** 10 μM	**15** 100 μM	**20** 10 μM	**20** 100 μM	**1** 10 μM	**1** 100 μM
medián	8,7	9,4	10,3	11,2	10,2	10,3	10,2
průměr	10,9	11,3	13,8	12,4	11,2	11,9	12,6

PATENTOVÉ NÁROKY

1. Použití alespoň jednoho adeninového derivátu obecného vzorce I



(I)

a jejich farmaceuticky přijatelné soli s alkalickými kovy, amoniakem, aminy, nebo adiční soli s kyselinami, kde

R2 je vodík nebo halogen;

R6 je vybrán ze skupiny zahrnující
– heteroaryl s 5– až 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heteroaryl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heteroarylalkyl s 5– až 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heteroarylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl,

merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heterocyklyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklylalkyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heterocyklylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– cykloalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1 –C4)alkyl;
– cykloalkylalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž cykloalkylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– isoalkyl obsahující 3 až 7 atomů uhlíku, nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl,
jako UV–fotoprotektivních složek v kosmetických přípravcích, v přípravcích pro ochranu rostlin a/nebo v přípravcích pro ošetření tkáňových kultur.

2. Použití podle nároku 1, kde v substituentu **R6** heteroaryl obsahuje 5–členný kruh, a v tomto 5–atomovém kruhu jeden heteroatom, kterým je O nebo S, přičemž heteroaryl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl.

3. Použití podle nároku 1, kde v substituentu **R6** heteroarylalkyl obsahuje 5–členný kruh a C1–C2 alkyl, a v 5–atomovém kruhu jeden heteroatom, kterým je O nebo S, přičemž heteroarylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl.

4. Použití podle nároku 1, kde v substituentu **R6** heterocyklyl obsahuje 5–členný kruh, a v 5–atomovém kruhu jeden heteroatom, kterým je O nebo S, přičemž heterocyklyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl.

5. Použití podle nároku 1, kde v substituentu **R6** heterocyklylalkyl obsahuje 5–členný kruh a C1–C2 alkyl, a v 5–atomovém kruhu jeden heteroatom, kterým je O nebo S, přičemž heterocyklylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl.

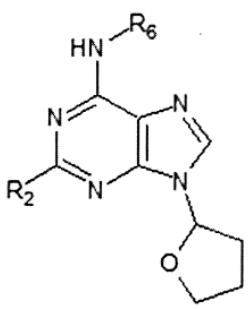
6. Použití podle nároku 1, kde substituent **R6** je vybrán ze skupiny zahrnující cyklopentyl, cyklopentylmethyl, isopropyl, isobutyl, isopentyl, isohexyl, isoheptyl, přičemž každá z

uvedených skupin je nesubstituovaná nebo substituovaná alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, hydroxy(C1–C4)alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl.

7. Použití podle nároku 1, kde adeninové deriváty jsou vybrané ze skupiny zahrnující:
6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–hydroxymethylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–formylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–furan–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–thiofen–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–furfurylamino–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–hydroxymethylmran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–formylfuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–furan–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(thiofen–2–ylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–thiofen–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin.

8. Použití podle kteréhokoliv z předcházejících nároků, kde se alespoň jeden adeninový derivát obecného vzorce I použije jako složka s kombinovaným antisenescenčním a UV–fotoprotektivním účinkem.

9. Adeninové deriváty obecného vzorce I



(I)

a jejich farmaceuticky přijatelné soli s alkalickými kovy, amoniakem, aminy, nebo adiční soli s kyselinami, kde

R2 je vodík nebo halogen;

R6 je vybrán ze skupiny zahrnující
– heteroaryl s 5– až 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heteroaryl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heteroarylalkyl s 6–členným aromatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heteroarylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heteroarylalkyl s 5– členným aromatickým kruhem obsahujícím alespoň jeden heteroatom S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heteroarylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, přičemž heterocyklyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– heterocyklylalkyl s 5– až 6–členným alifatickým kruhem obsahujícím alespoň jeden heteroatom vybraný z O, S a ostatní atomy kruhu jsou atomy uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž heterocyklylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– cykloalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;
– cykloalkylalkyl s kruhem obsahujícím 5 až 6 atomů uhlíku, kde alkyl obsahuje 1 až 4 atomy uhlíku, přičemž cykloalkylalkyl je nesubstituovaný nebo substituovaný alespoň jedním substituentem vybraným ze skupiny zahrnující C1–C4 alkyl, merkapto(C1–C4)alkyl, formyl, acetyl, halogen, karboxyl, amino, di(C1–C4)alkylamino, amino(C1–C4)alkyl;

10. Adeninové deriváty podle nároku 9, vybrané ze skupiny zahrnující:
6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
6–(1–thiofen–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(tetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methyltetrahydrofuran–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–tetrahydrofuran–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(thiofen–2–ylamino)–9–(tetrahydrofuran–2–yl)purin

2–chlor–6–(thiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(3–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–methylthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–chlorthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(5–bromthiofen–2–ylmethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(1–thiofen–2–ylethylamino)–9–(tetrahydrofuran–2–yl)purin
2–chlor–6–(cyklopentylmethylamino)–9–(tetrahydrofuran–2–yl)purin.