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ANTICANCER ACTIVITY OF CYTOKININS: STRUCTURE-ACTIVITY RELATIONSHIP STUDIES

Ph.D. Thesis

1501 V BIOLOGY - BOTANY

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Bibliographic Identification

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Abstract:	A study of the relationship between the chemical structure of cytokinins and their cytotoxic effects against a panel of human cancer cell lines with diverse histopathological origins is presented. Test compounds included almost all known natural cytokinins (N = 42) and two groups of novel synthetic analogues: derivatives of N^6-benzyladenosine with diverse substitutions on the benzyl ring (N = 48) and their analogues where the ribose moiety is replaced by a tetrahydropyran-2-yl or tetrahydrofuran-2-yl group (N = 34). Strong cytotoxic activity was limited to certain cytokinin ribosides and their corresponding ribotides. The anticancer activity of aromatic cytokinin ribosides can be improved by fluorination or *ortho*-hydroxylation of the benzyl ring. The potent anticancer activity of the natural cytokinin *ortho*-topolin riboside (median GI_{50} = 0.65 μM) was confirmed using NCI_{60}. Its activity pattern was distinctly different from those of standard anticancer drugs, suggesting that it has a unique mechanism of action. In comparison with standard drugs, *ortho*-topolin riboside showed exceptional cytotoxic activity against NCI_{60} cell lines with a mutated p53 tumour suppressor gene. *Ortho*-topolin riboside also exhibited significant anticancer activity against several tumour models in *in vivo* hollow fibre assays.
Keywords:	SAR, cytokinins, cancer, *ortho*-topolin riboside, NCI_{60}
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1. List of Abbreviations

2OH3MeOBAR	2-hydroxy-3-methoxybenzyladenosine
ADK	adenosine kinase
BA	N^6-benzyladenine
BAR	N^6-benzyladenosine
cZ	*cis*-zeatin
cZR	*cis*-zeatin riboside
DHZ	dihydrozeatin
DTP	Developmental Therapeutics Program of National Cancer Institute (Bethesda, USA)
iP	N^6-isopentenyladenine
iPR	N^6-isopentenyladenosine
K	kinetin
KR	kinetin riboside
oT	*ortho*-topolin
oTR	*ortho*-topolin riboside
mT	*meta*-topolin
mTR	*meta*-topolin riboside
pT	*para*-topolin
pTR	*para*-topolin riboside
THF	tetrahydrofuran-2-yl (group)
THP	tetrahydropyran-2-yl (group)
SAR	structure activity relationship
tZ	*trans*-zeatin
tZR	*trans*-zeatin riboside

2. Introduction, Aims of the PhD Thesis

The plant hormones cytokinins may have various therapeutical applications in human medicine. Among other properties, promising anticancer activities have been demonstrated for several natural cytokinins. However no systematic structure-activity relationship study of naturally occurring cytokinins has been reported. While a significant effort has been used to improve the activity of inhibitors of cyclin-dependent kinases derived from N^6-benzyladenine, reports of systematic modifications of natural cytokinin ribosides with the aim of improving their anticancer activity are rather rare. Discovery of new analogues of the natural cytokinins with strong anticancer effects, together with identification of structural features associated with the cytotoxic activity, would be therefore of great interest.

The main aims of the doctoral thesis were as follows:

1. To test the cytotoxic activity of selected cytokinins and cytokinin analogues against a panel of human cancer cell lines. To analyse their structure-activity relationships.

The compounds to be tested were:

- natural cytokinins
- synthetic N^6-benzyladenosine derivatives substituted on the benzyl ring
- cytokinin analogues substituted with tetrahydropyran-2-yl and tetrahydrofuran-2-yl at the N^9-position of the purine ring

2. To analyse of NCI_{60} pattern of *ortho*-topolin riboside

3. Literature Review

3.1. Chemical structure of cytokinins and their occurrence in nature

Cytokinins are important plant hormones that are defined by their ability to promote cell division in plant tissue culture in the presence of auxin (Skoog et al., 1965). Cytokinins found in plants are adenine derivatives substituted at the N^6-position with either an isoprenoid or an aromatic side chain.

The isoprenoid *trans*-zeatin (tZ) is the most abundant naturally occurring cytokinin. The abundance of other isoprenoid cytokinins (N^6-isopentenyladenine, iP, *cis*-zeatin, cZ) and derivatives with a saturated side chain, such as dihydrozeatin (DHZ), varies between plant species. While isoprenoid cytokinins are ubiquitous in plants, aromatic cytokinins (represented by N^6-benzyladenine, BA, and its hydroxylated derivatives, the topolins) have only been identified, as yet, in a limited group of plant taxa (Horgan, 1975; Strnad et al., 1992). The most abundant appears to be *ortho*-topolin riboside (oTR), which is present at micro-molar concentrations in poplar leaves after daybreak (Hewett et al., 1973). Another aromatic cytokinin, N^6-furfuryladenine (kinetin, K), first recognized as a synthetic compound, has been reported recently to occur also naturally (for a review see Barciszewski et al., 2007). Both families of cytokinin occur in several metabolic forms: free bases, ribosides, riboside-5`-phosphates, 3-, 7-, 9- and *O*-glucosides, and amino acid conjugates. The isoprenoid cytokinin iP is an atypical base, present in the tRNA of all studied organisms including humans, which plays a role in the precise control of protein synthesis. In mammals, iP is part of $tRNA^{[Ser]Sec}$ and the cognate tRNA-isopentenyltransferase is a putative tumour suppressor (Spinola et al., 2005).

Cytokinin activity is not limited to adenine derivatives as was demonstrated by discovery of the effects of phenylurea and its derivatives in plant biotests. Compounds of this type do not occur naturally in plants and are not discussed further in this text.

3.2. Physiological roles of cytokinins in plants

Cytokinins play an important role in development and growth of both root and shoot systems. Processes regulated by cytokinins include senescence, apical dominance, branching, flowering and seed germination. Cytokinins also regulate responses to various stimuli such as water and nutrient availability, light conditions and infection (Werner and Schmülling, 2009). Plants with cytokinin depletion (Miyawaki et al., 2006; Werner et al., 2001) or blocked cytokinin signal perception (Heyl et al., 2008; Riefler et al., 2006) have similar phenotypes. This "cytokinin deficiency syndrome" includes reduced shoot systems (fewer leaves and lateral branches, stronger apical dominance), enlargement of root systems and bigger seeds.

3.3. Cytokinin signaling in plants

The cytokinin signal in plants is perceived by the His-Asp phosphorelay similar to the two-component systems of bacteria. After the recognition of the cytokinin ligand by the extracellular domain of the membrane-bound histidine kinase (AHK2, AHK3, or AHK4), the intracellular domain of the receptor phosphorylates histidine phosphotransfer proteins (AHPs). These transmit the signal to nuclear response regulators (ARRs), which can activate or repress transcription of the response genes. Although differences in substrate specifity between individual cytokinin receptors exist, cytokinin bases are consistently the most active cytokinin form in both receptor assays and cytokinin biotests (Mok and Mok, 2001; Spíchal et al., 2004).

Intensity and duration of the signalling is dependent on the receptor and response regulator composition of the given cell/tissue and on the availability of individual cytokinins. The rate-limiting step in cytokinin biosynthesis is catalyzed by isopentenyltransferases (IPTs) which synthesize either free cytokinin nucleotides (adenosine phosphate-IPTs) or modify adenosine in tRNA (tRNA-IPTs). Conversion of cytokinin 5`-monophoshates into their respective free bases is catalyzed by

phosphoribohydrolase encoded by the gene LONELY GUY (LOG) (Kurakawa et al., 2007). An alternative pathway where dephoshorylation of riboside-5`-monophosphates precedes the cleavage of the glycoside bond also exists (Chen et Kristopeit, 1981a; Chen et Kristopeit, 1981b) but the genes responsible have not yet been characterized. Cytokinins are degraded by cytokinin oxidase/dehydrogenases (CKXs) which catalyze removal of the side chain. The cytokinin signal is also attenuated by conversion of free bases into less active (ribosides, ribotides) or inactive forms (glucosides, conjugates with alanine). With the exception of N^7- and N^9-glucosides, cytokinin conjugates can be converted back into free bases and are seen as transport/storage cytokinin forms. Uptake and efflux of cytokinins by cells is facilitated by members of the purine permease family (PUPs) of transmembrane channels (Gillissen et al., 2000) and by equilibrative nucleoside transporters (ENTs) (Hirose et al., 2005; Hirose et al., 2008). Cytokinins are present in both phloem and xylem fluid and serve as both acropetal and basipetal messengers (Kudo et al., 2010).

3.4. Effects of cytokinins in animal systems

Knowledge that cytokinins play a key role in the regulation of plant growth and development led to postulation that they could also affect growth and differentiation in animals, and have potential utility for treating human diseases that involve dysfunctional cell proliferation and/or differentiation. In this chapter we review the effects of cytokinins in animals at molecular, cellular, tissue and organismal level.

3.4.1. Cytotoxic effects of cytokinins and cytokinin analogues

Natural cytokinin ribosides N^6-isopentenyladenosine (iPR), kinetin riboside (KR) and N^6-benzyladenosine (BAR) (but not their respective bases) have strong cytotoxic effects against a range of human cell lines derived from both haematological malignancies and

solid tumours. Numerous studies with various experimental designs (assay principle, endpoint, length of treatment) demonstrated that cytokinin ribosides are active at sub-micromolar (against some leukaemias) or micro-molar concentrations (against other leukaemias, adherent cells). Toxicity of *trans*-zeatin riboside (tZR), another natural cytokinin that differs from iPR by hydroxylation of isoprenoid side-chain, is very limited (Ishii et al., 2002; Rattan and Sodagam, 2005). SAR analysis of synthetic derivatives of iPR showed that both saturation of the isoprenoid side chain and replacement of ribose with acyclic analogues decreases cytotoxic activity markedly (Colombo et al., 2009; Ottria et al., 2009).

In leukaemia cell lines (HL-60) cytokinin ribosides induce rapid apoptosis (Ishii et al., 2002; Mlejnek and Kuglík, P., 2000). The cell death is preceded by ATP depletion and activation of caspase-3. It has recently been demonstrated that kinetin riboside is a potential drug for the treatment of multiple myelomas. In several models of multiple myeloma, KR has been found to induce rapid suppression of cyclin D1 and D2 transcription, followed by arrest of the cell-cycle and selective apoptosis in tumour cells (Tiedemann et al., 2008).

Cytotoxic effects of iPR and KR against mammalian cell lines derived from solid tumours were reported by Cabello et al. (2009), Cheong et al. (2009), Colombo et al. (2009), Griffaut et al. (2004), Laezza et al. (2006, 2009), Meisel et al. (1998) and Spinola et al. (2007). Depending on the cell line and cytokinin used, the treatment resulted in apoptosis, G1 or G2/M block. The spectrum of the effects induced by cytokinin ribosides in those cell lines included ATP depletion, genotoxic stress (Cabello et al., 2009), JNK activation (Laezza et al., 2009), inhibition of farnesyl-protein transferase activity (Laezza et al., 2006) and changes in the levels of mitochondrial proteins (Cheong et al., 2009). Recently a microarray analysis of the effects of iPR (100 μM) on MCF7 and A549 cell lines was published. iPR induced a set of genes involved in the stress induced cell cycle arrest like PPP1R15A, DNAJB9, DDIT3, and HBP1 (Colombo et al., 2009).

In vivo the anticancer activity of iPR, KR and BAR has been demonstrated using several animal and xenograft models of cancer (Griffaut et al., 2004; Laezza et al., 2006; Tiedemann et al., 2008). iPR and BAR also showed promising activity against a diverse range of cancers in a limited clinical trial (Mittelman et al., 1975).

Micromolar concentrations of both cytokinin ribosides and cytokinin bases can also induce cell death in plant cell cultures, with some traits typical for apoptosis (activation of caspase-like proteases and fragmentation of DNA) (Mlejnek and Procházka, 2002; Mlejnek et al., 2003; Mlejnek et al., 2005). This cell death is preceded by depletion of adenosine triphosphate and the production of reactive oxygen species. In contrast to their hormonal activity, which requires interaction with specific membrane-bound receptors, intracellular conversion of cytokinins to monophosphates is necessary for this cytotoxic effect. The concentrations of cytokinin required to produce cytotoxic effects are higher than those found endogenously in plant tissues, but they do fall within the range used in plant bioassays (Carimi et al., 2003; Mlejnek et al, 2005). Phosphorylation of cytokinin ribosides by adenosine kinase (ADK) is a requirement for cytotoxic effect of cytokinin ribosides in both animal (Mlejnek and Doležel, 2005) and plant cells (Mlejnek and Procházka, 2002). Low affinity to ADK (and possibly to other nucleoside kinases) explains the lack of activity of other cytokinin ribosides (Mlejnek and Doležel., 2005) and possibly also their analogues with ribose replaced by acyclic polyols (Colombo et al., 2009; Ottria et al., 2009). In contrast to other nucleoside analogues that are converted to nucleoside triphosphates, the dominant metabolites of cytokinin ribosides are their respective riboside monophosphates (Mlejnek and Doležel, 2005). This observation suggests that cytokinin ribosides have a different mechanism of action from classical antimetabolites that after phosporylation directly interfere with the synthesis of nucleic acids.

In cultured plant cells, the toxicities of cytokinin bases and the corresponding ribosides are comparable, because in contrast to human cell lines plant cells can convert both forms of cytokinins efficiently into riboside-5´-monophosphates (Mlejnek and Procházka, 2002; Mlejnek et al., 2005).

3.4.2. Cytotoxic effects of other synthetic compounds structurally related to cytokinins

Both isoprenoid and aromatic cytokinins are weak and non-speficic inhibitors of human protein kinases including cyclin-dependent kinases (Veselý et al., 1994). The screening

of synthetic analogues led to the discovery that BA derivatives with C2 and N9 substitutions (exemplified by olomoucine, roscovitine, olomoucine II and bohemine) are strong and specific inhibitors of important protein kinases such as CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK5/p35 and ERK1/MAP kinase.

The cytotoxic effect of IB-MECA (1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide) and other N^6-benzyladenosine-5'-N-methylcarboxamides is traditionally explained by their interaction with the adenosine A3 receptor. However, Mlejnek recently demonstrated that the ability of IB-MECA to induce apoptosis in certain leukaemia cell lines was dependent on its intracellular accumulation (Mlejnek and Doležel, 2010).

In contrast to cytotoxic cytokinin ribosides neither roscovitine-type CDK inhibitors nor N^6-benzyladenosine-5'-N-methylcarboxamides can be easily converted to the respective riboside-5´-phoshates. In the first case position 9 of the purine ring is occupied by an alkyl, in the second conversion of ribose to N-alkyl amide of riborunic acid makes 5`-hydroxyl unavailable for phosphorylation.

3.4.3. Effects of cytokinins on differentiation of animal cells

The ability of cytokinin bases to induce or promote differentiation of human cells was demonstrated in keratinocytes (Berge et al., 2006; Vičanová et al., 2006) and several leukaemia cell lines including HL-60 and K-562 (Ishii et al., 2002).

Kinetin at concentrations of 40 to 200 µM induced growth arrest and changes of several markers of differentiation (keratins K10 and K14, involucrin) in human keratinocytes in cell culture. The effect was augmented by the presence of Ca^{2+} ions. Other markers of differentiation (trans-glutaminase) were unchanged suggesting that kinetin-induced differentiation might be mediated by pathways different from those activated by other differentiation inducing agents. In a later work Berge et al. (2008) reported that treatment with kinetin modulated the sensitivity of aging keratinocytes to the differentiating effects of Ca^{2+}. The effect was accompanied by the induction of Hsp90, Hsp70 and heme-oxygenase-1 suggesting that the beneficial effects are mediated

by stress-induced hormesis. Positive effect of kinetin on the levels of filaggrin, another marker of keratinocyte differentiation, was observed in *in vitro* reconstructed skin equivalent. In contrast to 2D culture, kinetin promoted growth of keratinocytes as indicated by the increase in the number of Ki67-positive cells (Vičanová et al., 2006). The both experimental systems mentioned above used human keratinocytes from healthy donors. We note that models using keratinocytes from psoriatic lesions might be more appropriate for evaluation of utility of cytokinins in therapy of psoriasis.

Granulocytic differentiation in the leukaemia HL-60 cell line was mediated by phosphorylation of ERK1/2, expression of CEBPD and S100P (Ishii et al., 2005a; Ishii et al., 2005b). While cytokinin bases induced differentiation at rather high concentrations (25-100 μM), their ribosides caused rapid apoptosis at low micromolar levels. Treatment with caspase inhibitors shifted the activity of iPR in HL-60 from pro-apoptotic to growth inhibitory and differentiating activity (Ishii et al., 2002).

3.4.4. Cytoprotective and gerontomodulatory effects of cytokinins, cytokinins as cosmeceuticals

The interest in the „anti-aging“ activity of cytokinins started in 1994 when Rattan and Clark discovered positive effects of kinetin on several characteristics related to aging in fibroblasts during serial passage *in vitro*. The size and morphology of the fibroblast passaged in the presence of kinetin resembled those of the cells at lower passage numbers. The treatment with kinetin decreased the number of actin stress fibres and autoflourescence due to accumulated lipofuscin was also less intense (Rattan and Clark, 1994). Similar effects of *cis*-zeatin and Pyratine-6 (N^6-furanyl-9-tetrahydropyran-2-ylpurine) on *in vitro* aging of fibroblast population were reported more than 10 years later (Rattan and Sodagam, 2005; Szüčová et. al., 2008). Positive effects of cytokinins on skin composition and its structure were observed in an *in vitro* artificial skin model (Vičanová et al., 2006) and a mouse model of aging skin (Szüčová et. al., 2008). In a clinical trial evaluating effects on aging and photodamaged skin Pyratine-6 improved skin moisturization, roughness, mottled hyperpigmentation, fine wrinkles and facial

erythema within 4 weeks. With mean improvement of overall skin appearance of 28 % Pyratine-6 seems to be superior to kinetin for which an improvement of 4 % was reported in an earlier study with similar design (McCullough et al., 2008; Szüčová et. al., 2008). Both Pyratine-6 and kinetin also showed positive effect on acne rosacea (Ortiz et al., 2009; Wu et al., 2007).

It was proposed that kinetin is a product of oxidative damage of DNA with protective effects on biological macromolecules (Barciszewski et al., 1997). Such an effect was later confirmed in *in vitro* models of oxidative damage of biological macromolecules (Olsen et al., 1999; Verbeke et al., 2000). Superoxide dismutase (SOD)-like activity of Cu^{2+} complexes of kinetin and BAP were reported (Goldstein and Czapski, G., 1991). The ability of Cu^{2+} complexes of BAP derivatives with SOD-like activity to protect against oxidative damage *in vivo* (aloxan induced diabetes) was observed by (Štarha et al., 2009). Notably, kinetin effects related to aging are not limited to cells and tissues, as kinetin increased the life span *of Zaprionus* fruitflies. The effect was accompanied by enhanced catalase activity and by reduced fecundity (Sharma et al., 1997). Other dermatologic/cosmetic applications of cytokinins might include therapy of psoriasis (Berge et al., 2006) and correction of pigmentation disorders. While kinetin was reported to decrease hyperpigmentation, BA is a stimulator of melanogenesis (Kim et al., 2009).

3.4.5. Can cytokinins play a role in intercellular signaling in humans?

Although free iPR and possibly also K are endogenous molecules in humans (Barciszewski et al., 1996; Burns et al. , 1976), whether they or their putative metabolites (ribosides, ribotides) exert any physiological effects at concentrations occurring in the human body remains unclear. Because the levels of the free forms of iPR are dependent on breakdown of tRNA it is tempting to speculate that they might serve as an intracellular or even extracellular signal of states which are accompanied by increased tRNA turnover like cell growth and death. High tRNA turnover was observed in cancers and explained by rapid degradation of aberrantly modified tRNA molecules (Borek et al., 1983). Loss of

cell membrane integrity might serve as one of possible mechanisms of the release of iPR (or its metabolites) into intercellular space.

If some specific signalling pathways activated by human endogenous cytokinins exist they have to be entirely different from the cytokinin signalling pathways in plants. In plants cytokinins are recognized by receptor histidin kinases which are part of His-Asp phosphorelay resembling the two-component environmental sensors of bacteria. No signalling system with similar organization to the cytokinin pathway exists in mammals. Sequence comparison identified 2 mammalian proteins having homology with two-component histidine kinases, but none of them is a membrane protein (Besant et al., 2005). In humans, the extracellular adenosine, ADP and ATP signal through purinergic receptors which are either G-protein coupled receptors (P1 adenosine and P2Y adenylate receptor families) or ligand-gated ion channels (P2X adenylate receptor family) (Burnstock, 2007). Adenosine receptor A3 recognizes certain N^6-benzyladenosine derivatives (e.g. IB-MECA) but whether they interact with endogenous cytokinins is still unknown. Cytokinins also interact with systems regulating the availability of extracellular adenine nucleosides and nucleotides. Similarly to the situation in plants (Kudo et al., 2010), in mammalian cells cytokinin ribosides are transported by nucleoside transporters (our unpublished results) and phosphorylated by adenosine kinase. Cytokinins are also degraded by deamination, which is catalyzed by adenine deaminase in mammalian cells (Hall and Mintsioulis, 1973). Considering the existence of degradation mechanism and the abundance of adenosine (and adenylates) in the cells, if endogenous iPR was to exert any meaningful effect it would probably have to be a strong antagonist of purinergic signaling or an inhibitor of systems regulating the availability of adenosine or adenylate in the extracellular space. To conclude, rather than a system responsive to endogenous cytokinins, the purinergic signaling might present a promising target of therapeutically applied cytokinins both natural and synthetic. The activity of the enzymes limiting the availability of adenosine and adenylates may contribute to the resistance to cytokinins.

4. Material and Methods

4.1. Test compounds

The cytokinins isopentenyladenine, iPR, *trans*-zeatin, *trans*-zeatin riboside, dihydrozeatin, benzyladenine, BAR, kinetin, and KR were purchased from Sigma (St. Louis, MO). N6-benzyladenine-3-glucoside, N^6-benzyladenine-7-glucoside, N^6-benzyladenine-9-glucoside, N^6-benzyladenosine-5`-monophosphate, dihydrozeatin riboside, dihydrozeatin riboside-5`-monophosphate, dihydrozeatin-*O*-glucoside, dihydrozeatin riboside-*O*-glucoside, isopentenyladenosine-5`-monophosphate, kinetin-9-glucoside, kinetin riboside-5`-monophosphate, 2-methylthio-*trans*-zeatin riboside, 2-methylthio-N^6-isopentenyladenosine, *cis*-zeatin, *cis*-zeatin riboside, *cis*-zeatin riboside, *cis*-zeatin riboside-5`-monophosphate, *trans*-zeatin-7-glucoside, *trans*-zeatin-9-glucoside and *trans*-zeatin-*O*-acetyl were generous gifts from Olchemim Ltd. (Olomouc, Czech Republic). Synthesis of the other studied cytokinins is described in Voller et al. (Phytochemistry, in press 2010, Supplement I); Doležal et al. (2007) (Supplement II) and Szučová et al. (2009) (Supplement III).

4.2. Calcein AM cytotoxicity assay

The following cell lines obtained from the American Type Culture Collection (Manassas, VA, USA) were used for toxicity testing: RPMI 8226 (multiple myeloma), CEM (T-lymphoblastic leukaemia), K562 (chronic myelogenous leukaemia), HL-60 (promyelocytic leukaemia), MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), G361 (malignant melanoma), HOS (human osteosarcoma) and BJ (human foreskin fibroblasts). These cells were maintained in standard DMEM or RPMI medium (Sigma, MO, USA) supplemented with 10% heat-inactivated fetal calf serum, 5g/l glucose, 2mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycine under standard cell culture

conditions (37 °C, 5% CO_2 in a humid environment) and sub-cultured two or three times per week using the standard trypsinization procedure.

Approximately 10.000 cells in 80 µl of growth medium were seeded into 96-well microtitre plates. After 12 h incubation, the compounds to be tested were added in 20 µl portions. Control cultures were treated with DMSO alone. The final concentration of DMSO in the medium did not exceed 0.5%. Serial, triplicate 3-fold dilutions (six in total, highest concentration in incubations 166 µM) of each compound were tested. After 72 h incubation, Calcein AM (Molecular Probes) solution was added to a final concentration of 1 µg/ml, and the cells were incubated for a further hour. The fluorescence of free calcein was then quantified using a Fluoroscan Ascent fluorometer (Microsystems), and the percentage of surviving cells in each well was calculated by dividing the fluorescence obtained from each well with exposed cells by the mean fluorescence obtained from control wells × 100%. Finally, IC_{50} values (the concentrations causing a 50% decrease in cellular esterase activity) were calculated for each compound from the generated dose-response curves.

4.3. Analysis of NCI_{60} activity pattern

The activity pattern (GI_{50} values for individual NCI_{60} cell lines) of oTR was correlated with those of 214 antineoplastics in the "Approved Oncology Drugs" and "Standard Agents" DTP datasets and with expression patterns (gene expression measurements for individual NCI_{60} cell lines) of adenosine kinase (the probes 204119_s_at and 204120_s_at on U133A Affymetrix arrays, Shankavaram et al., 2007). If a compound had been tested over several ranges of concentration, the pattern from the greatest number of repetitions was used. Pearson correlation coefficients were calculated on a log-log scale. Resulting p-values were adjusted by Bonferroni correction for multiple testing. The list of "Standard agents" and "Approved Oncology Drugs" can be accessed at http://dtp.nci.nih.gov/docs/cancer/searches/standard_agent_table.html and http://dtp.cancer.gov/branches/dscb/oncology_drugset_explanation.html, respectively. Relevant activity patterns were extracted from DTP Cancer Screening Data May 2009

Release (http://dtp.nci.nih.gov/docs/cancer/cancer_data.html). Microarray data normalized using the RMA algorithm were downloaded from the CellMiner database (Shankavaram et al., 2009).

The comparison of the effect of p53 status on the growth inhibitory activity (GI_{50}) of oTR and 214 standard antineoplastics was carried out according to method published by Weinstein et al. (1997) based on the comparison of p-values from Wilcoxon rank sum test for the difference in the activity of individual compounds against p53wt and p53mut cells.

All calculations and manipulations of data were carried out using statistical software R (http://cran.r-project.org/).

5. Summary of Results

The first systematic analysis of the relationship between the chemical structure of cytokinins (N = 47) and their cytotoxic effects against a panel of human cancer cell lines with diverse histopathological origins (N = 8) was carried out (Voller et al, Phytochemistry, in press 2010, Supplement I). It confirms cytotoxic activity of KR, iPR, BAR and the lack of the activity of their corresponding bases reported in previous studies. Cytotoxic activity of hydroxylated aromatic cytokinins (*ortho*-, *meta*-, *para*-topolin riboside) and the isoprenoid cytokinin *cis*-zeatin is reported for the first time. Most cell lines in the panel showed greatest sensitivity to *ortho*-topolin riboside (IC_{50} = 0.5 – 11.6 μM). Cytokinin nucleotides, some synthesised for the first time in this study, were usually active over a similar concentration range to the corresponding ribosides. Cytokinin free bases, 2-methylthio derivatives and both *O*- and *N*-glucosides showed little or no toxicity.

The cytotoxic cytokinin ribosides including those previously tested in mouse xenograft experiments (iPR, BAR, KR) and clinical trials (iPR, BAR) also showed significant toxicity against BJ skin fibroblasts. Future *in vivo* experiments will be required to demonstrate whether there is a therapeutic window for novel cytotoxic cytokinins. The results of the mouse hollow fibre assay described below, demonstrating the *in vivo* anticancer activity of oTR against implanted human tumours at concentrations causing no acute toxicity, suggest that this is a possibility.

The study of 48 novel synthetic cytokinin analogues of BAR with diverse substitutions of benzyl ring (Doležal et al., 2007, Supplement II) identified several other compounds with cytotoxic effects in submicromolar or micromolar levels. In contrast to unsubstituted BAR which was active against both MCF-7 (breast adenocarcinoma, IC_{50} = 5.4 μM) and leukaemia cell lines (IC_{50} = 0.94, 1.4 and 5.5 μM for HL-60, CEM and K562 respectively), the activities of most of the synthetic analogues were limited to leukaemias. The highest activity was usually associated with fluorination and *ortho*-hydroxylation. For example, 2-hydroxy-3-methoxybenzyladenosine (2OH3MeOBAR) was about 5 times more active against CEM and HL-60 cell lines than BAR. Its isomer 2-hydroxy-4-

methoxybenzyladenosine showed only a limited activity against leukaemia cell lines but was the most active compound against MCF-7 (IC_{50} = 2.2 µM). Activity against MCF-7 comparable with BAR was also observed in the case of 2,3-dihydroxybenzyladenosine. This compound was also toxic (IC_{50} =16.9 µM) for osteosarcoma cell line HOS which is highly resistant against BAR (IC_{50} >166 µM). G-361 (melanoma), another cell line resistant to BAR (IC_{50} >166 µM), was sensitive to 4-fluorobenzyladenosine (IC_{50} =13.2 µM). The existence of such diverse activity profiles suggests that the individual aromatic cytokinin ribosides may differ in their mechanisms of action, at least partially.

Replacement of ribose of BAR derivatives (including oTR and 2OH3MeOBAR) with either tetrahydropyran-2-yl (THP) or tetrahydrofuran-2-yl (THF) group yielded novel compounds with no or marginal cytotoxicity against both cancer cell lines and skin fibroblasts BJ (Szüčová et al., 2009, Supplement III). Similar results were obtained in the case of THP and THF analogs of KR. (Szüčová et al., 2008).

Earlier studies of Mlejnek (Mlejnek and Doležel, P., 2005) demonstrated that cytotoxic effects of cytokinins are connected with their conversion to the corresponding riboside monophosphates. In contrast to plants, human cells are able to synthesize cytokinin riboside phosphates efficiently only from cytokinin ribosides but not from cytokinin bases. The results of our three SAR studies are in agreement with this hypothesis. Strong cytotoxicity activity was limited to certain cytokinin ribosides and replacement of the ribose moiety by glucose, THP and THF led to a marked decrease or loss of the activity. Whether ribose can be efficiently replaced by other sugars remains to be determined. It would also be of interest to find out whether the loss of activity observed after the substitution of cytokinin ribosides with 2-methylthio group is caused by a decreased affinity of these derivatives for ADK. The knowledge of the structural features responsible for the attenuation of the cytotoxicity might be useful for the synthesis of cytokinin analogues intended for therapeutical areas other than cancer. Finally, no relationship between the structural requirements for cytotoxic activity and those for activity in plant biotests were found.

Because of their promising biological activity and drug-like chemical structure, we sent oTR for testing on NCI_{60}, standard panel of 59 cell lines originating from 9 different tissues (Voller et al, Phytochemistry, in press 2010, Supplement I). oTR was

highly active against almost all the cell lines, with a median GI_{50} value of 0.65 μM (Supplement IV). For all except two cell lines GI_{50} values were less than 10 μM. In comparison with standard drugs, oTR showed exceptional cytotoxic activity against NCI_{60} cell lines with a mutated p53 tumour suppressor gene. Correlation analysis revealed that the activity pattern of oTR was distinctly different from those of clinically evaluated anticancer drugs and therefore oTR might have a unique mechanism of action. The observation that activity of oTR was correlated with expression levels of adenosine kinase agrees with our experiments showing that pharmacological inhibition of ADK protects cells against oTR.

Finally, the activity of oTR against tumours derived from 12 NCI_{60} cell lines was tested *in vivo* in hollow fibre assays. oTR was administered by intraperitoneal injection for four consecutive days at two dose levels, 100 and 150 mg/kg/day, which were found to be safe in a preliminary acute toxicity study. The implants were evaluated by MTT assay at the day following the last injection. oTR caused a 50% or greater reduction of tumour mass in 16 out of 24 intraperitoneal implants (ip score 32 out of 48). No tumour reduction exceeding this threshold was achieved when the drug was injected subcutaneously (sc score 0 out of 48). According to DTP methodology based on the analysis of the performance of a large set of the drugs in the hollow fibre assays and in the follow-up xenograft studies, compounds with an ip score greater than 7 or total (ip + sc) score greater than 19 are considered as suitable candidates for follow-up xenograft experiments.

6. Conclusions

The doctoral thesis relates to analysis of relationships between the chemical structure of cytokinins and their cytotoxic activity against a panel of human cancer cell lines. Test compounds included (i) almost all known natural cytokinins representing all basic structural types (N = 47), (ii) novel synthetic derivatives of BAR with substitution on benzyl ring (N = 42), and (iii) their analogues in which the ribose moiety was replaced by THF or THP group (N = 34).

The most important results of this thesis are as follows:

1) An SAR study of natural cytokinins supports the hypothesis that strong cytotoxic activity is limited to certain cytokinin ribosides and their corresponding ribotides. Absence of the ribose moiety or its replacement by glucose, THP or THF group led to a marked decrease in the activity. The anticancer activity of aromatic cytokinin ribosides can be improved by fluorination or by *ortho*-hydroxylation. Natural cytokinin oTR and several other synthetic derivatives showed higher activity against some cancer cell lines than cytokinin ribosides studied until now.

The potent anticancer activity of oTR (median GI_{50} = 0.65 μM) was confirmed using $NCI_{60.}$ The activity pattern of oTR was distinctly different from those of standard anticancer drugs, suggesting that it has a unique mechanism of action. In comparison with standard drugs, oTR showed exceptionally high cytotoxic activity against NCI_{60} cell lines with a mutated p53 tumour suppressor gene.

In *in vivo* hollow fibre assays, after intraperitoneal application oTR exhibited significant anticancer activity against most of the cell lines implanted intraperitoneally but no activity against subcutaneous implants.

7. Summary in Czech (Souhrn)

Předkládaná disertační práce se zabývá vztahem mezi strukturou a protinádorovým účinkem rostlinných hormonů cytokininů (N^6-substituované deriváty adeninu a adenosinu). Na lidských nádorových liniích byly otestovány nejen téměř všechny známé přirozeně se vyskytující cytokininy (N = 47), ale i nové syntetické deriváty odvozené substitucí benzenového jádra N^6-benzyladenosinu (N = 42) a jeho analog s tetrahydropyran-2-ylovou a tetrahydrofuran-2-ylovou skupinou namísto ribosy (N = 34). Studie potvrdila již známou cytotoxickou aktivitu přirozených cytokininových ribosidů a identifikovala několik dalších ribosidů s aktivitou srovnatelnou nebo vyšší. Například přirozený cytokinin *ortho*-topolin ribosid (oTR) vykazoval silnější účinnost na většině nádorových linii a syntetický derivát 2-hydroxy-3-methoxybenzyladenosin byl vysoce účinný na leukemické nádorové linie. Silná cytotoxická aktivita byla obecně spojena s přítomností fluoru na benzenovém jádře N^6-benzyladenosinu či jeho substitucí hydroxylovou skupinou v pozici 2. Pozorování, že silnou aktivitu mají pouze cytokininové ribosidy a jejich fosfáty, ale ne jejich analoga, kde je ribosa nahrazena glukosou, tetrahydropyranylovou nebo tetrahydrofuranylovou skupinou, je v souladu s již publikovanou hypotézou, že cytotoxický účinek cytokininů souvisí s jejich konverzí na cytokinin ribosid-5´-monofosfáty. Ztráta cytotoxické aktivity byla pozorována také po substituci methylthio skupinou v poloze 2 purinového aromatického systému. Informace o tom, které substituce způsobují snížení cytotoxicity se mohou uplatnit při vývoji léčiv s jinou indikací než léčba onemocnění spojených s poruchou proliferace.

Silná aktivita *ortho*-topolin ribosidu (mediánová GI_{50} = 0.65 μM) byla dále potvrzena testováním na NCI_{60}, standardním panelu 59 lidských nádorových linií pocházejících z 9 tkáňových typů. Korelační analýza ukázala, že profil protinádorové aktivity *ortho*-topolin ribosidu se významně liší od profilů antineoplastik, které byly hodnoceny v klinických testech, což naznačuje, že má zřejmě odlišný mechanismus účinku. Ve srovnání se standardními antineoplastiky vykazoval *ortho*-topolin ribosid vysokou aktivitu proti nádorovým liniím s mutovaným genem pro tumorový supressor

p53. *In vivo* protinádorová aktivita *ortho*-topolin ribosidu byla následně demonstrována na vybraných NCI_{60} liniích implantovaným v dutých vláknech myším.

8. List of Papers

Voller, J., Zatloukal, M., Lenobel, R., Doležal, K., Béreš, T., Kryštof, V., Spíchal, L., Niemann, P., Džubák, P., Hajdúch, M., Strnad, M. (2010) Anticancer activity of natural cytokinins: A structure-activity relationship study. Phytochemistry (in press)

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10. Supplements

Supplement I

Voller, J., Zatloukal, M., Lenobel, R., Doležal, K., Béreš, T., Kryštof, V., Spíchal, L., Niemann, P., Džubák, P., Hajdúch, M., Strnad, M. Anticancer activity of natural cytokinins: A structure-activity relationship study. Phytochemistry (accepted 2010).

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Anticancer activity of natural cytokinins: A structure–activity relationship study

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ABSTRACT

Cytokinin ribosides (N^6-substituted adenosine derivatives) have been shown to have anticancer activity both *in vitro* and *in vivo*. This study presents the first systematic analysis of the relationship between the chemical structure of cytokinins and their cytotoxic effects against a panel of human cancer cell lines with diverse histopathological origins. The results confirm the cytotoxic activity of N^6-isopentenyladenosine, kinetin riboside, and N^6-benzyladenosine and show that the spectrum of cell lines that are sensitive to these compounds and their tissues of origin are wider than previously reported. The first evidence that the hydroxylated aromatic cytokinins (*ortho*-, *meta*-, *para*-topolin riboside) and the isoprenoid cytokinin *cis*-zeatin riboside have cytotoxic activities is presented.

Most cell lines in the panel showed greatest sensitivity to *ortho*-topolin riboside (IC_{50} = 0.5–11.6 μM). Cytokinin nucleotides, some synthesized for the first time in this study, were usually active in a similar concentration range to the corresponding ribosides. However, cytokinin free bases, 2-methylthio derivatives and both *O*- and *N*-glucosides showed little or no toxicity. Overall the study shows that structural requirements for cytotoxic activity of cytokinins against human cancer cell lines differ from the requirements for their activity in plant bioassays. The potent anticancer activity of *ortho*-topolin riboside (GI_{50} = 0.07–84.60 μM, 1st quartile = 0.33 μM, median = 0.65 μM, 3rd quartile = 1.94 μM) was confirmed using NCI_{60}, a standard panel of 59 cell lines, originating from nine different tissues. Further, the activity pattern of oTR was distinctly different from those of standard anticancer drugs, suggesting that it has a unique mechanism of activity. In comparison with standard drugs, oTR showed exceptional cytotoxic activity against NCI_{60} cell lines with a mutated p53 tumour suppressor gene. oTR also exhibited significant anticancer activity against several tumour models in *in vivo* hollow fibre assays.

1. Introduction

Cytokinins are important plant hormones that are defined by their ability to promote cell division in plant tissue culture (Skoog et al., 1965). Cytokinins found in plants are adenine derivatives substituted at the N^6-position with either isoprenoid or an aromatic side chain (Table 1). Isoprenoid *trans*-zeatin (tZ) is the most abundant naturally occurring cytokinin. The abundance of other isoprenoid cytokinins (N^6-isopentenyladenine, iP, *cis*-zeatin, cZ) and derivatives with a saturated side chain, such as dihydrozeatin (DHZ), varies between plant species. While isoprenoid cytokinins are ubiquitous in plants, aromatic cytokinins (represented by N^6-benzyladenine, BA, and its hydroxylated derivatives, the topolins) have only been identified, as yet, in a limited group of plant taxa (Horgan et al., 1975; Strnad, 1997; Strnad et al., 1992, 1997). The most abundant appears to be *ortho*-topolin riboside, which is present at micro-molar concentrations in poplar leaves after daybreak (Hewett and Wareing, 1973). Another aromatic cytokinin, N^6-furfuryladenine (kinetin, K), first recognized as a synthetic compound, has been reported to occur naturally (for a review see Barciszewski et al. (2007)). Both families of cytokinin occur in several forms: free bases, ribosides, riboside-5′-phosphates, 3-, 7-, 9- and *O*-glucosides, and amino acid conjugates (Table 1). The isoprenoid cytokinin iP is an atypical base, present in the tRNA of all studied organisms, which plays a role in the precise control of protein synthesis. In mammals, iP is part of $tRNA^{[Ser]Sec}$ and the cognate tRNA-isopentenyltransferase is a putative tumour suppressor (Spinola et al., 2005). Molecules with a N^6-isopentenyladenine moiety are released into the cytosol and subsequently into the body fluids as a result of tRNA turnover (Chheda and Mittelman, 1972).

Abbreviation: PBS, phosphate buffer saline; DTP, developmental therapeutics program of National Cancer Institute (DTP, Bethesda, USA); SAR, structure–activity relationship.

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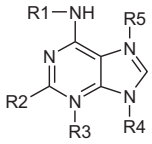
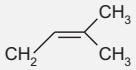
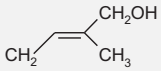
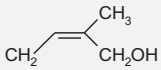
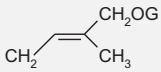
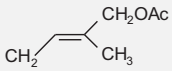
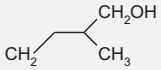
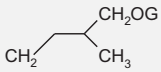
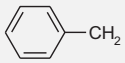
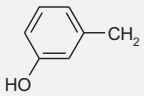
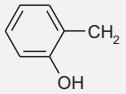
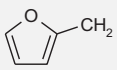
Table 1
Structures of the cytokinins used in this study.



R1	R2	R3	R4	R5	Trivial name	Abbreviation
	H	–	H	–	N^6-isopentenyladenine	iP
	H	–	Ribosyl	–	N^6-isopentenyladenosine	iPR
	CH_3S	–	Ribosyl	–	2-Methylthio-iPR	2MeSiPR
	H	–	–	Glucosyl	iP-7-glucoside	iP7G
	H	–	Glucosyl	–	iP-9-glucoside	iP9G
	H	–	Ribotide	–	iPR-5′-monophosphate	iPRMP
	H	–	Ribotide	–	iPR-5′-diphosphate	iPRDP
	H	–	Ribotide	–	iPR-5′-triphosphate	iPRTP
	H	–	H	–	*trans*-Zeatin	tZ
	H	–	Ribosyl	–	*t*-Zeatin riboside	tZR
	H	–	–	Glucosyl	*t*-Zeatin-7-glucoside	tZ7G
	H	–	Glucosyl	–	*t*-Zeatin-9-glucoside	tZ9G
	H	–	Ribotide	–	tZR-5′-monophosphate	tZRMP
	CH_3S	–	Ribosyl	–	2-Methylthio-*t*-zeatin riboside	2MeStZR
	H	–	H	–	*cis*-Zeatin	cZ
	H	–	Ribosyl	–	*cis*-Zeatin riboside	cZR
	H	–	H	–	*t*-Zeatin-*O*-glucoside	tZOG
	H	–	Ribosyl	–	*t*-Zeatin riboside-*O*-glucoside	tZROG
	H	–	H	–	t-Zeatin-O-acetyl	ActZ
	H	–	H	–	Dihydrozeatin	DHZ
	H	–	Ribosyl	–	Dihydrozeatin riboside	DHZR
	H	–	–	Glucosyl	Dihydrozeatin-7-glucoside	DHZ7G
	H	–	Glucosyl	–	Dihydrozeatin-9-glucoside	DHZ9G
	H	–	Ribotide	–	DHZR-5′-monophosphate	DHZRMP
	H	–	H	–	Dihydrozeatin-*O*-glucoside	DHZOG
	H	–	Ribosyl	–	Dihydrozeatin riboside-*O*-glucoside	DHZROG
	–	–	H	–	N^6-benzyladenine	BA
	–	–	Ribosyl	–	N^6-benzyladenosine	BAR
	–	Glucosyl	–	–	N^6-benzyladenine-3-glucoside	BA3G
	–	–	–	Glucosyl	N^6-benzyladenine-7-glucoside	BA7G
	–	–	Glucosyl	–	N^6-benzyladenine-9-glucoside	BA9G
	–	–	Ribotide	–	BAR-5′-monophosphate	BARMP
	–	–	Ribotide	–	BAR-5′-diphosphate	BARDP
	–	–	Ribotide	–	BAR-5′-triphosphate	BARTP
	–	–	H	–	*meta*-Topolin	*m*T
	–	–	Ribosyl	–	*meta*-Topolin riboside	*m*TR
	–	–	Glucosyl	–	*meta*-Topolin-9-glucoside	*m*T9G
	–	–	H	–	*ortho*-Topolin	*o*T
	–	–	Ribosyl	–	*ortho*-Topolin riboside	*o*TR
	–	–	Glucosyl	–	*ortho*-Topolin-9-glucoside	*o*T9G
	–	–	Ribotide	–	oTR -5′-monophosphate	oTRMP
	–	–	Ribotide	–	oTR -5′-diphosphate	oTRDP
	–	–	Ribotide	–	oTR -5′-triphosphate	oTRTP
	CH_3S	–	Ribosyl	–	2-Methylthio-oTR	2MeSoTR
	H	–	H		Kinetin	K
	H	–	Ribosyl		Kinetin riboside	KR
	H	–	Ribotide		KR -5′-monophosphate	KRMP

Knowledge that cytokinins play key roles in the regulation of plant growth and differentiation led to postulation that they could also affect growth and differentiation in animals, and have potential utility for treating human diseases that involve dysfunctional cell proliferation and/or differentiation. Abundant evidence supporting these hypotheses has subsequently been obtained. The ability of cytokinin bases to induce or promote the differentiation of human cells has been demonstrated in both keratinocytes (Berge et al., 2006) and several leukaemia cell lines, including HL-60 and K-562 (Ishii et al., 2003).

However, while cytokinin bases induce differentiation at relatively high concentrations (25–100 μM), their ribosides cause rapid

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apoptosis of leukaemia cell lines at low micro-molar concentrations (Mlejnek, 2001). Cell death in HL-60 is preceded by depletion of adenosine triphosphate, activation of caspases and mitochondrial depolarization (Mlejnek, 2001; Ishii et al., 2002). Intracellular conversion of ribosides to their respective monophosphates is known to be required for their action (Mlejnek and Doležel, 2005). It has recently been demonstrated that kinetin riboside (KR) is a potential drug for the treatment of multiple myelomas (Tiedemann et al., 2008). In several models of multiple myeloma, KR has been found to induce rapid suppression of cyclin D1 and D2 transcription, followed by arrest of the cell-cycle and selective apoptosis in tumour cells (Tiedemann et al., 2008). Several authors have reported cytotoxic effects of N^6-isopentenyladenosine (iPR), KR and N^6-benzyladenosine (BAR) on human cell lines derived from solid tumours (Cabello et al., 2009; Choi et al., 2008; Griffaut et al., 2004; Laezza et al., 2009; Meisel et al., 1998; Spinola et al., 2007). Whether treatments resulted in cell cycle block and/or apoptosis was dependent on the cell line and the cytokinin used. The anticancer activity of iPR, KR and BAR has been demonstrated *in vivo* using several animal and xenograft models of cancer (Choi et al., 2008; Laezza et al., 2006; Tiedemann et al., 2008). iPR and BAR have also shown promising activity against a diverse range of cancers in a limited clinical trial (Mittelman et al., 1975).

Micro-molar concentrations of both cytokinin ribosides and cytokinin bases can also induce cell death in plant cell cultures, with some identifiable characteristics of apoptosis (activation of caspase-like proteases and fragmentation of DNA) (Mlejnek and Procházka, 2002). This cell death is preceded by depletion of adenosine triphosphate and the production of reactive oxygen species. In contrast to their hormonal activity, which requires interaction with specific membrane-bound receptors, intracellular conversion of cytokinins to monophosphates is necessary for this cytotoxic effect. The concentrations of cytokinin required to produce cytotoxic effects are higher than those found endogenously in plant tissues, but they do fall within the range used in plant bioassays (Carimi et al., 2003; Mlejnek et al., 2003, 2005).

Although the cytotoxic activity of natural cytokinins and their analogues (Doležal et al., 2006, 2007) against mammalian cells has been repeatedly demonstrated, there have been no previously published systematic studies of the structure-cytotoxic activity relationship (SAR) of natural cytokinins. Available reports indicate that only KR, iPR, BAR, *trans*-zeatin riboside (tZR) and their free bases have been tested, to date. Therefore, the present study was undertaken to examine the cytotoxic effects of almost all known naturally occurring cytokinins against a panel of cancer cell lines of diverse histopathological origin and then determine the basic SAR in terms of their growth-inhibitory effects. Furthermore, the activity pattern of *ortho*-topolin riboside (oTR) against NCI_{60}, a thoroughly characterized panel of 59 human cancer cell lines (Shoemaker, 2006) was analysed. Finally, we report results of *in vivo* tests of the anticancer activity of oTR against models representative of human tumours in hollow fibre assays.

2. Results and discussion

In addition to their essential roles in the growth and development of plants, cytokinins have various effects in man and animals at both cellular and whole organism levels (Berge et al., 2006; Slaugenhaupt et al., 2004; Rattan and Sodagam, 2005; Wu et al., 2007). Hence, cytokinins and their derivatives have many potential therapeutic applications, including possible efficacy in the treatment of proliferative diseases such as cancers. The anticancerous activity of cytokinins in a variety of cultured cell lines, several xenografts and even a clinical trial has been documented (Ishii et al., 2002; Mittelman et al., 1975; Tiedemann et al., 2008). However, only a few cytokinins have been tested as yet for anticancer activity. Here, we report an analysis of the relationship between the structures of 47 cytokinins (Table 1) and their cytotoxic activity against a panel of cell lines derived from diverse malignancies (Table 2). The tested compounds include almost all known natural purine cytokinins, representing all structural variants. Several compounds were synthesized for the first time for this study, including 2-methylthio-*ortho*-topolin riboside (2MeSoTR), *ortho*-topolin riboside-5'-monophosphate (oTRMP) and selected cytokinin riboside di- and triphosphates. Cytotoxic activity was evaluated after 72-h treatments using a standard viability test, based on quantification the fluorescent product of the enzymatic hydrolysis of Calcein AM. The determined activities were expressed as IC_{50} values (concentrations leading to a 50% decrease in cellular esterase activity).

Treatment with the ribosides, oTR, iPR, BAR and KR resulted in a dose-dependent reduction in the viability (IC_{50} = 0.5–13.6 μM) of the cell lines derived from both haematological malignancies (CEM, HL60, K562 and RPMI) and solid tumours (MCF7, HeLa, and HOS). The leukaemia cell lines, HL60 and CEM, were most sensitive, with determined IC_{50} values $\leqslant$1.7 μM. Median IC_{50} values across the whole panel of cancer cell lines were 2.27, 4.15, 5.10 and 5.95 μM for oTR, BAR, KR and iPR, respectively. While the cytotoxic activity of the cytokinin ribosides iPR, BAR and KR against cancer cell lines has already been reported (Ishii et al., 2002; Laezza et al., 2009; Meisel et al., 1998; Mlejnek and Doležel, 2005; Spinola et al., 2007) this is the first report of the activity of oTR. The findings that cytokinin ribosides are active at sub-micro-molar (some leukemias) or low micro-molar concentrations (other leukemias, adherent cells) are consistent with those of previous studies, despite variations in the design of the cytotoxicity assays used in terms of principle/mechanism, definition of the endpoint and length of treatment. In contrast to oTR, the *meta*- and *para*-isomers only exhibited inhibitory activity against HL60 (with IC_{50} values of 24 and 7.5 μM, respectively). Among the cytokinin ribosides with hydroxylated isoprenoid side chains, *cis*-zeatin riboside (cZR) but not tZR or dihydrozeatin riboside (DHZR), was active against the leukaemia cell lines CEM and HL60 (with IC_{50} values of 18.8 and 7.9 μM, respectively).

Compared to the tested cytokinin ribosides, the free bases typically had much weaker effects on cell proliferation, with IC_{50} values either over the highest concentration used (>166 μM) or at least 50 times higher than the IC_{50} values determined for their respective ribosides. Similar differences between the cytotoxic activity of cytokinin bases (K, iP, and BA) and their corresponding ribosides have been reported by other authors (Ishii et al., 2002; Mlejnek and Doležel, 2005). In cultures of plant cells, the toxicities of cytokinin bases and the corresponding ribosides are comparable, because in contrast to human cells plant cells can convert both forms of cytokinin efficiently into riboside-5′-monophosphates (Mlejnek et al., 2003, 2005). Therefore, it was postulated that low toxicity of K, iP and BAP in human leukaemia HL-60 cell line is due to the low activity of human phosphoribosyl transferase towards cytokinin bases (Mlejnek and Doležel, 2005). The differences observed in the cytotoxic activities of *ortho*-topolin (oT), *para*-topolin (pT), *meta*-topolin (mT) and *cis*-zeatin (cZ) compared to their corresponding ribosides may have similar causes.

With the exception of *trans*-zeatin riboside-*O*-glucoside (tZROG), which exhibited some activity against the leukaemia cell lines CEM and HL-60 ($IC_{50} \sim 26$ μM), cytokinin *O*- and *N*-glucosides were inactive in the assay ($IC_{50} > 166$ μM). The observation that cytokinin bases and cytokinin glucosides showed limited activity, or none at all, supports the hypothesis that the presence of a ribose moiety at N9 of the purine ring is essential for potent anticancer activity in cytokinins. A decrease in the cytotoxic activity of two orders of magnitude was also observed after substitution of oTR and

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Table 2
Antiproliferative activity of cytokinins expressed as IC_{50} values in a 3-day Calcein-AM assay. Presented values are averages of at least three independent experiments, where individual replicate values fell within 25% of the average.

	CEM	HL60	K562	RPMI 8226	HOS	MCF7	G361	HELA	BJ
BA	>166	>166	140	>166	>166	>166	>166	>166	>166
BA7G	>166	>166			>166				
BA9G	>166	>166			>166				
BA3G	>166	>166			>166				
BAR	1.3	0.93	5.9	4.6	13.6	3.7	15.0	1.9	1.7
BARMP	1.1	0.95	3.7	5.3	14.4	5.0	>166	2.3	2.6
BARDP	2.0	1.3	4.0	4.5		8.0			
BARTP	2.3	4	3.9	4.7		11			
K	155	>166	>166	>166	>166	>166	>166	>166	>166
K9G	>166	>166			>166				
KR	1.6	0.8	10.5	7.4	5.9	4.3	21.9	4.3	2.1
KRMP	1.8	1	13.6	16.5	11.9	4.3	38.8	12.2	
iP	92	>166	150	>166	>166	>166	>166	>166	>166
iP9G	>166	>166			>166				
iPR	1.7	0.71	5.2	6.4	11.7	6.9	>166	5.5	2.5
iPRMP	2.4	1.2	4.3	5.3	5.1	2.8	>166	4.2	2.1
iPRDP	2.5	1.4	5.3	5.4		3.5			
iPRTP	3.4	2.8	6.6	7		5.2			
2MeSiPR	119	85.5	>166	>166	>166	>166	>166	>166	>166
oT	118	78	>166	>166	>166	>166	103	120	>166
oT9G	>166	>166			>166				
oTR	0.5	0.6	2.4	1.5	3.1	7.7	11.6	2.13	1.5
oTRMP	1.3	0.48	4.0	3.0	2.5	10.5	13.5	4.5	1.2
oTRDP	0.8	1.9	2.1	1.9	1.9	12.1	11.9		
oTRTP	1.6	1.6	3.5	4.3	2.7	14.4	17.2		
2MeSoTR	>166	>166	>166	130	>166	>166	155	>166	>166
mT	>166	>166	140	>166	>166	>166	>166	>166	>166
mTR	>166	24	>166	>166	>166	>166	>166	>166	3.9
mT9G	>166	>166			>166				
pT	>166	123	>166	>166	>166	>166	>166	>166	>166
pTR	>166	7.5	>166		>166	>166	>166	>166	15.3
pT9G	>166	>166			>166				
tZ	>166	>166	>166	>166	>166	>166	>166	>166	>166
tZ7G	>166	>166			>166				
tZ9G	>166	>166			>166				
tZOG	>166	>166			>166				
tZROG	27.5	26.3	120		166	>166	>166		
tZR	>166	95.9	>166	>166	>166	>166	>166	>166	
tZRMP	>166	78.6	>166		>166	>166	>166	>166	
ActZ	>166	>166			>166				
2MeStZR	>166	>166	>166	>166	>166	>166	>166	>166	>166
cZ	>166	>166	>166	>166	>166	>166	>166	>166	>166
cZR	18.8	7.9	>166	>166	>166	>166	>166	42.3	55.2
cZRMP	61	26.4	>166	>166	>166	>166	>166	>166	>166
2MeScZR	>166	>166	>166	>166	>166	>166	>166	>166	>166
DHZR	>166	>166	>166	>166	>166	>166	>166	>166	>166
DHZROG	151	>166	>166		>166	>166	>166	>166	
DHZMP	>166	>166	>166		>166	>166	>166		>166

iPR at C2 with a 2-methylthio group. This, along with other substitutions that also led to attenuation of the cytotoxic effects of cytokinin ribosides could be useful in programmes to develop drugs to treat conditions other than cancers. It will be of interest to establish whether these observed decreases in cytotoxic activity are the result of reduced affinity of adenosine kinase for C2-substituted cytokinin ribosides.

The present SAR study illustrates that the structural requirements for cytokinins to show potent cytotoxic activity against human cancer cell lines are different from those needed for their activity in plant bioassays. While the ribose moiety appears to be important for cytotoxic effects against a diverse range of cancer cells, conjugation of cytokinin bases with sugars, including ribose, serves to limit cytokinin signalling in plant cells. The hydroxyl position of the side chain of cytokinins had a marked effect on anticancer activity, in both aromatic (oTR >> *meta*-topolin riboside – mTR, *para*-topolin riboside – pTR) and isoprenoid (cZR >> tZR) cytokinins. In contrast, tZ and mT cytokinins are more active than their positional isomers (cZR, oTR, pTR) in plant bioassays (Holub et al., 1998; Letham and Palni, 1983; Spíchal et al., 2004).

Extracellular adenosine nucleotides play important roles in a diverse range of physiological processes, including cell death in mammals (reviewed by Burnstock (2007), for example). In addition, information is available on: the release of intracellular adenine nucleotides; their inter-conversion (cycling) in the blood and intercellular space by both membrane-bound and soluble enzymes; and specific signalling pathways (Yegutkin, 2008; Yegutkin et al., 2003). Given their importance we synthesized 5′-nucleotides of selected cytokinin ribosides and examined their activity in various tests, including the Calcein-AM viability assay.

Phosphorylation of purine ribosides also increases the solubility of nucleosides, which can lead to improvements in both drug formulation and bioavailability. The practicality of this approach has been demonstrated by the development of fludarabine (9-β-D-arabinosyl-2-fluoroadenine-5'-monophosphate), the active ingredient in the drug Fludara®, which has been approved for the treatment of certain haematological malignancies (Anderson and Perry, 2007).

Cytokinin riboside-5′-phosphates derived from oTR, iPR, KR, BAR and cZR markedly reduced the growth of cell lines that were sensitive to the parent compounds. In most cases the activity of

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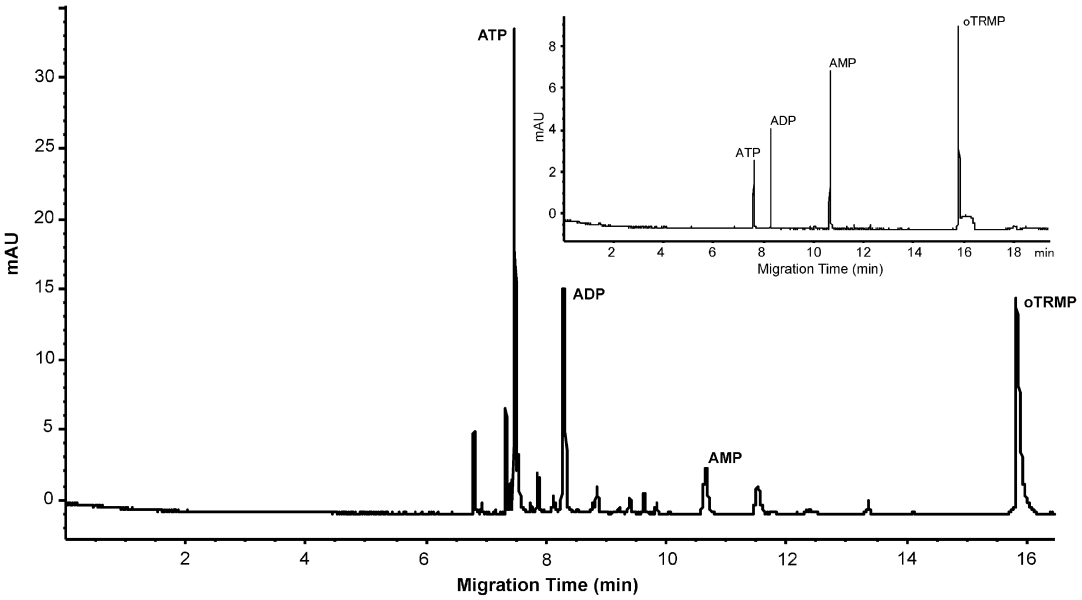

Fig. 1. Identification of intracellular oTRMP by capillary electrophoresis with UV detection (268 nm). CEM cells were treated with 25 μM oTRMP for 3 h. The inset window illustrates separation of the standards.

the ribosides and the respective riboside-5′-phosphates was either similar, or phosphorylation led to decreased activity. Simultaneous treatment with an adenosine kinase inhibitor, A-134974 (5 μM), protected CEM and K562 cells (IC_{50} > 100 μM) not only against oTR and iPR, but also against their respective mono-, di- and triphosphates. Therefore, intracellular phoshorylation appears to be an important step in the sequence of events leading to cytotoxicity after the application of not only cytokinin ribosides (Cabello et al., 2009; Mlejnek and Doležel, 2005) but also their respective riboside-5′-phosphates. These observations suggest that, similarly to other nucleotide drugs, such as fludarabine monophosphate (Malspeis et al., 1990) and triciribine monophosphate (Wotring et al., 1986), cytokinin riboside-5′-phosphates are dephosphorylated extracellularly and the resulting nucleosides are transported across the membrane and then re-phosphorylated in the cell. Intracellular accumulation of monophosphorylated oTR after the treatment of CEM cells with oTRMP (25 μM, 3 h) was demonstrated by capillary electrophoresis (see Fig. 1).

The intracellular concentration was determined to be between 0.67 and 0.92 mM (in three biological replicates), exceeding the concentration applied to cells more than 25-fold. The identity of oTRMP was confirmed by spiking the sample with a standard solution of the compound. If adenosine kinase was inhibited by A-134974 or the experiment was carried out in serum-free medium, concentrations of oTRMP were found to be below the limit of detection. The peak of oTRMP was missing when the cells were treated with DMSO vehiculum (data not shown).

In order to obtain preliminary information about the selectivity of cytokinin nucleosides and nucleotides, their cytotoxic effects against human BJ fibroblasts (as a model primary cell line) were also examined. Most of the compounds tested, including those previously used in mouse xenograft experiments (iPR, BAR, KR) and clinical trials (iPR, BAR) showed significant cytotoxicity, with IC_{50} values frequently in the low micro-molar range (Table 2). Future *in vivo* experiments will be required to demonstrate whether there is a therapeutic window for cytotoxic cytokinins. The results of the mouse hollow fibre assay described below, demonstrating the *in vivo* anticancer activity of oTR against implanted human tumours at concentrations causing no acute toxicity, indicate that this is a possibility.

Due to its drug-like properties and the promising biological activity it demonstrated, oTR was selected for further testing against the NCI_{60} cancer cell line panel at the developmental therapeutics program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (DTP, Bethesda, USA). NCI_{60} is a collection of 59 human cancer cell lines that have been extensively characterized at the DNA, RNA and protein levels and are used for routine drug screening at DTP. Comparisons of patterns of activity (GI_{50} values of a compound for individual NCI_{60} cell lines) by, for example, Pearson correlation, may be useful for identifying compounds that could have common mechanisms of action. Similarly, comparison of activity patterns and expression patterns of molecular targets provides a means to detect molecular markers that influence the cells' sensitivity to a compound (Shoemaker, 2006).

The cytotoxic activity of oTR against the NCI_{60} panel is shown in Fig. 2, in terms of GI_{50} values (concentrations causing a 50% reduction in cell growth). oTR was potently active against most of the cell lines, with a median GI_{50} value of 0.65 μM and, for all except two lines, GI_{50} values <10 μM. Similarities in the median GI_{50} values (range 0.34–1.32 μM) together with generally high degrees of variability within tissue sub-groups (max/min ratio >15 in all sub-panels except the ovarian and prostate sub-groups) suggest that factors other than the tissue origin determine sensitivity of cancer cells to oTR. Below we analyse the effects of p53 status of the cells and adenosine kinase expression on the activity of oTR and compare its activity pattern with those of 214 antineoplastics ("clinical agents") that have already been clinically evaluated.

Analysis of the influence of the mutational status of the NCI_{60} cell lines (Ikediobi et al., 2006) on their sensitivity to oTR led to the important observation that cell lines carrying the mutant p53 tumour suppressor gene (median GI_{50} = 0.60 μM) are generally more sensitive than cells with the wild type variant (median GI_{50} = 1.59 μM). TP53, the protein product of this gene, is a vital component of the regulatory system that responds to various cell stressors, including DNA damage, oncogene activation, hypoxia, and disruption of cell adhesion. Activation of TP53 can result in cell cycle arrest, senescence and apoptosis. The p53 gene is known to be either mutated or deleted in over 50% of all human cancers (Vazquez et al., 2008). Dysfunction of TP53 has also been implicated in chemo- and radio-resistance (Bossi and Sacchi, 2007;

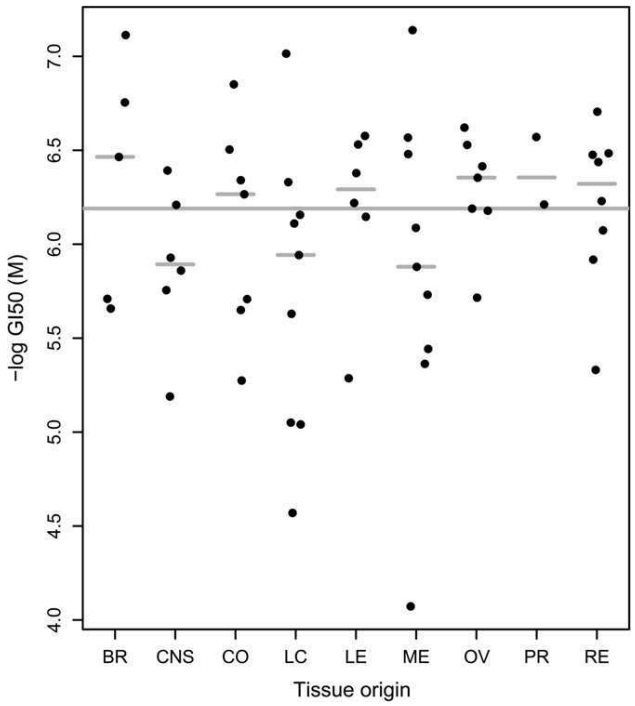

Fig. 2. Negative log GI_{50} values (M) for individual tissue types in NCI_{60}. Grey lines indicate the global and tissue-specific median. BR – breast, CNS – central nervous system cancer, CO – colon cancer, LC – non-small cell lung cancer, LE – leukaemia, ME – melanoma, OV – ovarian cancer, PR – prostate cancer and RE – renal cancer.

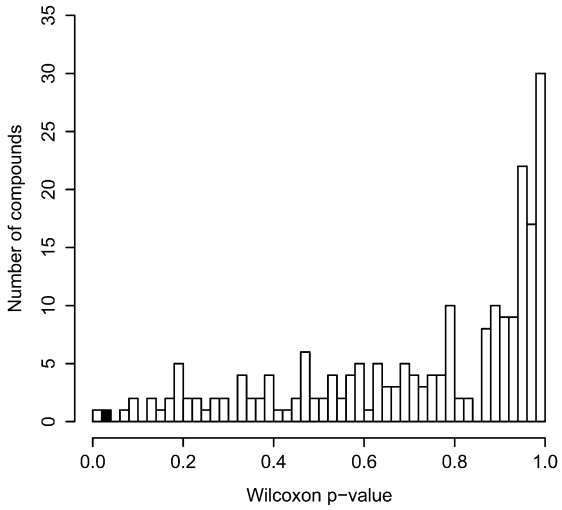

Fig. 3. Histogram showing the relationship between *p53* status and activity of oTR (black bar) and 214 clinically evaluated compounds. The strength of each relationship is expressed as a *p*-value derived from the Wilcoxon rank sum test. The alternative hypothesis was that the individual compounds are more active in cell lines with mutated p53.

Weller, 1998). The potential significance of the strong activity of oTR towards mutant p53 lines is highlighted by the report that most of nearly 90 clinically evaluated anticancer agents showed greater activity against NCI_{60} cell lines carrying wild-type p53 (Weinstein et al., 1997). We used the same approach as that applied by Weinstein et al. to compare the effect of p53 status on the growth inhibitory activity (GI_{50}) of oTR and 214 standard antineoplastics. This set of "clinical agents" was created by pooling antineoplastics from the "Approved Oncology Drugs" and "Standard Agents" DTP datasets (see Section 4 for details). *p*-Values obtained from one-sided Wilcoxon rank sum tests comparing the GI_{50} values of individual compounds on the cell lines with mutated and wild-type p53 were used as a metrics of the dependence of the activity on p53 status. The resulting distribution of *p*-values is shown in Fig. 3. While most of the compounds tested showed more activity against the cells with wild-type p53 (indicated by *p*-values > 0.5) oTR (*p*-value = 0.035, rank 2) was exceptionally active against the cells with the mutant p53 gene.

Previous studies on cytokinin ribosides have demonstrated that intracellular phosphorylation by adenosine kinase is a requirement for a cytotoxic effect (Mlejnek and Doležel, 2005) and, as mentioned above, the cytotoxic effect of oTR can be prevented by treatment with an adenosine kinase inhibitor. Therefore, it was of interest to determine whether a relationship between the activity of oTR and expression of adenosine kinase exists. The GI_{50} values of oTR were found to be negatively correlated ($r < -0.43$, $p < 0.0006$) with signals of both adenosine kinase probes on U133A Affymetrix expression arrays in the Genelogic dataset (Shankavaram et al., 2007). Significant negative correlations between the GI_{50} values of oTR and adenosine kinase expression ($r < -0.69$, $p < 0.019$) were also observed in the melanoma subgroup. Therefore, it is possible that adenosine kinase expression in cells could be used as a biomarker of the sensitivity and/or resistance of certain malignancies (for example, melanoma) to oTR. Further studies with an independent cancer cell panel are required to test this hypothesis.

In order to identify compounds that have a similar pattern of activity (and possibly, therefore, a similar mechanism of action) we calculated Pearson correlation coefficients for patterns of activity of oTR and "clinical agents". Compounds were ranked using respective *p*-values. Only an inhibitor of ribonucleotidase, caracemide (Moore and Loo, 1984) had a correlation coefficient that was higher than 0.4 ($r = 0.42$, $p = 0.0013$). Further positive correlations ($r > 0.35$, $p < 0.005$) were obtained for the purine anti-metabolites (3-deazaguanine and diglycoaldehyde), the cAMP analogue 8Cl-cAMP, and the tricyclic ribotide Akt inhibitor triciribine monophosphate (Cory et al., 1976; Kim et al., 2005; Pieper et al., 1988). None of these compounds is currently approved for use in humans. Although statistical significances (defined as $p < 0.05$) of these correlations did not survive Bonferroni correction for multiple testing, the relations reported here may have biological meaning. Correlations between the patterns of activity of oTR and the purine analogues may reflect the ability of oTR to interact with the human purinome and, more specifically, with enzymes involved in purine and nucleic acid metabolism. Triciribine monophosphate, a prodrug of triciribine, is known to be dephosphorylated extracellularly, transported into cells and then re-phosphorylated by adenosine kinase (Wotring et al., 1986). Here, we propose a similar mechanism of internalisation and metabolic activation for the cytokinin nucleotides. The correlation between the GI_{50} patterns of oTR and triciribine monophosphate might, therefore, reflect the importance of adenosine kinase in the metabolism of both drugs. We conclude that since the proportion of variance shared by the activity patterns of oTR and each individual "clinical agent" was always lower than 18% ($r^2 < 0.18$), a unique combination of biological factors is probably underlying the sensitivity of the NCI_{60} cell lines to oTR.

Finally, the activity of oTR against tumours derived from 12 NCI_{60} cell lines was tested *in vivo* in hollow fibre assays. oTR was administered by intraperitoneal injection for four consecutive days at two dose levels, 100 and 150 mg/kg/day, which were found to be safe in a preliminary acute toxicity study (data not shown). oTR caused a 50% or greater reduction (as measured by a standard MTT assay) of tumour mass in 16 out of 24 intraperitoneal implants, resulting in an ip score of 32 out of 48. No tumour

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reduction exceeding threshold assay values was achieved when the drug was injected subcutaneously (sc score 0). Ip score (but not sc score) was shown to be positively correlated with the likelihood of activity in xenograft models. Notably, the relation was stronger when both intra- and extraperitoneal grafts were considered (Johnson et al., 2001; Decker et al., 2004). According to DTP methodology, compounds with an ip score greater than 7 or total (ip + sc) score greater than 19 are considered as candidates for follow-up xenograft experiments.

While the high ip score shows promising anticancer activity of oTR at the site of application, the absence of the effect on the subcutaneous implants points to the limited systemic availability of the drug. Other routes of application might be more appropriate for the relatively polar cytokinin ribosides. In this context, it would be interesting to compare the ip and sc scores of other nucleoside analogs. In humans, significant differences in peritoneal and plasma exposure were observed after intraperitoneal application of cytarabine and gemcitabine. Both the drugs were successfully used in high dose regional therapy of intra-abdominal cancers (King et al., 1984; Kamath et al., 2009). Follow-up mouse xenograft experiments (testing various sites of implantation and modes of application) are necessary to further evaluate potential utility of oTR in cancer therapy.

3. Concluding remarks

The ability of the plant hormones cytokinins to induce apoptosis and/or block cell cycling in a wide range of cancer cells makes them potential candidates as drugs for treating a variety of cancers. This study represents the first systematic analysis of the relationship between the structure of cytokinins and their cytotoxic effects, assessed using a diverse panel of human cancer cell lines. The results confirm the anticancer activity of iPR, KR and BAR, and demonstrate that the range of cell lines sensitive to cytokinins and the tissue origin of these cells is wider than previously reported. In addition, the anticancer activity of the hydroxylated aromatic (oTR, pTR, mTR) and isoprenoid cytokinins (cZR) is reported for the first time. Against most cell lines tested, *ortho*-topolin riboside (IC_{50} = 0.5–11.6 μM) was the most active cytokinin. Cytokinin nucleotides (some synthesized for the first time in this study) were active against the same cell lines as the parent ribosides. Cytokinin free bases, including 2-methylthio derivatives as well as *O*- and *N*-glucosides, exhibited limited toxicity or none at all. It can be concluded from this study that cytokinins have different structural requirements for cytotoxic activity against human cancer cell lines than for activity in plant bioassays. The potent anticancer activity of oTR was confirmed through further testing of this compound on NCI_{60} (median GI_{50} = 0.65 μM), a standard panel of 59 cell lines originating from nine different tissues. The activity pattern determined for oTR against NCI_{60} could be clearly distinguished from the patterns of a set of standard, established anticancer drugs, suggesting that a unique combination of factors underlie its activity. Another significant finding was the high sensitivity of the NCI_{60} cell lines with mutated p53 tumour suppressor gene to oTR and significant differential toxicity of oTR in tissue origin sub-panels. oTR is toxic to rapidly dividing normal diploid fibroblasts *in vitro*, but was found to have significant anticancer activity in several tumour models *in vivo* at concentrations causing no acute toxicity.

4. Experimental

4.1. Materials

The cytokinins iP, iPR, tZ, tZR, DHZ, BA, BAR, K, and KR were purchased from Sigma (St. Louis, MO). ActZ, BA3G, BA7G, BA9G, BARMP, K9G, KRMP, iP9G, iPRMP, 2MeSiPR, cZ, tZ7G, tZ9G, tZRMP, tZOG, tZROG, 2MeStZR, cZR, cZRMP, 2MeScZR, (±)DZR, DZROG and DZMP were generous gifts from Olchemim Ltd. (Olomouc, Czech Republic). The aromatic cytokinins oT, oTR, oT9G, mT, mTR, mT9G, pT, pTR and pT9G were synthesized, according to procedures described by Holub et al. (1998). Before they were used the purity of all cytokinins was tested by HPLC (Strnad et al., 1997). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and A-134974 were purchased from Sigma (MO, USA) and Calcein AM from PAA Laboratories GmbH (Pasching, Austria). All reagents used were either of analytical grade or the highest grade available from commercial suppliers.

4.2. Cytokinin nucleotide synthesis

All N^6-substituted adenosine-5′-*O*-di- and tri-phosphates used in the present study were synthesized by treating the triethylammonium salt of the corresponding 6-chloropurine-9β-D-riboside-5′-*O*-di- or triphosphate precursors with appropriate side chain amines under aqueous, alkaline conditions. The reactions were monitored by analytical chromatography using 250 × 4.6 mm stainless steel column packed with Gemini™ C18–110, 5 μm particles (Phenomenex, Aschaffenburg, Germany) as solid phase. The mobile phase was 25% CH_3CN, 25 mM Na_2HPO_4, 4 mM tributylammonium sulphate (pH 7). The flow rate was 1.25 ml/min. The products were partially purified by preparative MPLC with DEAE-Sepharose (125 × 25 mm glass column of Q Sepharose FF, 90 μm, Amersham Biosciences, Freiburg, Germany) as the stationary phase and 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. The flow rate was 10 ml/min. The fractions containing the required product were collected, concentrated under reduced pressure, and then desalted using preparative HPLC equipped with 220 × 50 mm stainless steel column packed with ODS-AQ™ C18–100, 16 μm (YMC Europe/Sinsheim/Germany) at the flow rate of 10 ml/min. The purified compounds were eluted with 5% MeOH, concentrated at reduced pressure, and stored as 10 mM aqueous stock solutions at −70 °C.

The analytical HPLC system for the monitoring of product formation progress and its purity as well as the preparative HPLC system for product purification consisted of a L7100 pump, a L7400 variable wavelength UV-detector, and a D 7500 Integrator (Merck-Hitachi, Darmstadt, Germany). Mass spectra of the reaction products by ESI-MS were measured in isopropanol–H_2O–HCOOH (50:49.9:0.1, v/v/v). Helios β spectrometer (Spectronic Unicam, Cambridge, UK) was used to record their UV-spectra of the compounds in aqueous phosphate buffer, pH 7.

4.2.1. N^6-(Δ²-Isopentenyl)adenosine-5'-O-diphosphate (iPRDP)

iPRDP was synthesized from 6-chloropurine-9β-D-riboside-5'-*O*-diphosphate, triethylammonium salt, and 2-isopentenylamine by nucleophilic substitution with 2-isopentenylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. The formation of the product was monitored by analytical HPLC. The reaction was quenched by adding formic acid and subsequently cooling to −70 °C, then the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase and 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. Fractions containing product were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). The triethylammonium salt of iPRDP was isolated with >98% purity (yield: 32%).iPRDP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 268 (4.228) nm; ESI-MS (pos.) *m/z*: 494.1 ($[M+H]^+$); ESI-MS (neg.) *m/z*: 496.1 ($[M-H]^-$); Mr calculated for the free acid ($C_{15}H_{23}N_5O_{10}P_2$): 495.32.

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4.2.2. N^6-(Δ^2-Isopentenyl)adenosine-5'-O-triphosphate (iPRTP)

iPRTP was synthesized from 6-chloropurine-9β-D-riboside-5'-O-triphosphate, triethylammonium salt, and isopentenylamine by nucleophilic substitution with 2-isopentenylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. Again, product formation was monitored by analytical HPLC. The reaction was quenched by adding formic acid and subsequent cooling to −70 °C, the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase, using 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. Again, fractions containing product were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). The triethylammonium salt of iPRTP was isolated with a purity of >98% (yield: 42%).iPRTP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 268 (4.228) nm; ESI-MS (pos.) *m/z*: 576.1 ($[M+H]^+$); ESI-MS (neg.) *m/z*: 574.2 ($[M-H]^-$); Mr calculated for the free acid ($C_{15}H_{24}N_5O_{13}P_3$): 575.30.

4.2.3. N^6-(2-Hydroxybenzyl)aminopurine riboside-5'-O-monophosphate (oTRMP)

oTRMP was prepared from 6-chloropurine-9β-D-riboside-5'-O-monophosphate, disodium salt dihydrate, by nucleophilic substitution with 2-hydroxybenzylamine in the presence of *N*,*N*-diisopropyl-*N*-ethylamine in methanol. The reaction was carried out at 90 °C for 12 h in a nitrogen atmosphere. The solvent was removed by evaporation under vacuum and raw oTRMP was purified by *RP* C18 flash chromatography (mobile phase 15% methanol in water) followed by crystallization from propan-2-ol. The purity of the final product was 95% (HPLC) and the yield 80%.oTRMP sodium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 270 (4.312) nm; ESI-MS (pos.) *m/z*: 454.0 ($[M+H]^+$); ESI-MS (neg.) *m/z*: 452.0 ($[M-H]^-$); Mr calculated for the free acid ($C_{17}H_{20}N_5O_8P$): 453.35.

4.2.4. N^6-(2-Hydroxybenzyl)aminopurine riboside-5'-O-diphosphate (oTRDP)

oTRDP was synthesized from 6-chloropurine-9β-D-riboside-5'-O-diphosphate, triethylammonium salt, by nucleophilic substitution with 2-hydroxybenzylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. The formation of product was monitored by analytical HPLC. The reaction was, again, quenched by addition of formic acid and subsequent cooling to −70 °C, then the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase using 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. The fractions containing product were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). The triethylammonium salt of oTRDP was isolated with a purity of >99% (yield: 38%).oTRDP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 270 (4.312) nm; ESI-MS (pos.) *m/z*: 534.1 ($[M+H]^+$); ESI-MS (neg.) *m/z*: 532.0 ($[M-H]^-$); Mr calculated for the free acid ($C_{17}H_{21}N_5O_{11}P_2$): 533.32.

4.2.5. N^6-(2-Hydroxybenzyl)aminopurine riboside-5'-O-triphosphate, (oTRTP)

oTRTP was synthesized from 6-chloropurine-9β-D-riboside-5'-O-triphosphate, triethylammonium salt, by nucleophilic substitution with 2-hydroxybenzylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. Analytical HPLC was used to monitor the progress of the reaction. The reaction was quenched by the addition of formic acid and subsequent cooling to −70 °C, then the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase, using 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. The relevant fractions were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). oTRTP was isolated as the triethylammonium salt with a purity of >97% (yield: 42%).oTRTP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 270 (4.312) nm; ESI-MS (pos.) *m/z*: 614.0 ($[M+H]^+$); ESI-MS (neg.) *m/z*: 612.0 ([M−H; Mr calculated for the free acid ($C_{17}H_{22}N_5O_{14}P_3$): 613.35.

4.2.6. 2-Methylthio-6-(2-hydroxybenzyl)aminopurine riboside (2MeSoTR)

2MeSoTR was prepared from 2-methylthio-6-chloropurine-9β-D-riboside and 2-hydroxybenzylamine. The reaction was carried out, at 90 °C for 20 h, in methanol in the presence of triethylamine in nitrogen gas. The reaction mixture was then evaporated in a vacuum evaporator and residue that was insoluble in 25% methanol was re-crystallized from 70% methanol. Purity of the final product was determined to be 97% (HPLC) and the calculated yield 50.5%.

2MeSoTR: white solid; ESI-MS (pos.) *m/z*: 420.3 ($[M+H]^+$); Mr calculated for the free acid ($C_{18}H_{21}N_5O_5S$): 419.46.

4.3. Capillary electrophoresis

CEM cells were harvested by centrifugation (500*g*, 4 °C, 5 min), washed twice in an excess of ice cold phosphate buffered saline (pH 7.2) and then flash frozen in liquid nitrogen. Cell extracts were analysed using a capillary electrophoresis system supplied by Agilent Technologies (Waldbronn, Germany) equipped with an uncoated fused silica column (80.5 cm total length, 72 cm effective length, 75 μm I.D.). Parameters for sample processing and separation were adapted from those used by Friedecký et al. (2007). Briefly, the background electrolyte consisted of 40 mM citrate, 0.8 mM cetrimonium bromide (CTAB) adjusted to pH 4.3 with γ-butyric acid (GABA). Each new capillary was washed with 1 M NaOH (30 min) followed by water (30 min) and then running buffer (30 min). At the beginning of every day, the capillary was washed with solutions in the following order: 1 M NaOH (10 min), water (10 min) and the running buffer (20 min). Between each run capillary was washed with the running buffer for 2 min. Samples were injected under low pressure (50 mbar, 5 s). ATP, ADP, AMP and oTRMP were identified by spiking with standard solutions. Quantification was done using corrected peak areas at detection wavelength 254 nm. The intracellular concentration of oTR-MP was calculated using the following formula: (concentration of the analyte in the cell extract × volume of the cell extract/number of extracted cells) × (1/average cell volume). Taking CEM cells to be spherical, with a diameter of 11.2 μm (the median value as measured by cell counter Z2, Beckman), the average cell volume was calculated to be 735.2 μm^3.

4.4. Cell cultures

The following cell lines – RPMI 8226 (multiple myeloma), CEM (T-lymphoblastic leukaemia), K562 (chronic myelogenous leukaemia), HL-60 (promyelocytic leukaemia), MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), G361 (malignant melanoma), HOS (human osteosarcoma) and BJ (human foreskin fibroblasts) – were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in standard DMEM or RPMI medium (Sigma, MO, USA) supplemented with heat-inactivated fetal bovine serum (10%) 2 mM L-glutamine and penicillin–streptomycin (1%) under standard cell culture conditions (37 °C, 5% CO_2 in a humid environment) and sub-cultured two or three times per week using the standard trypsinization procedure.

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4.5. Calcein AM cytotoxicity assay

Approximately 10,000 cells in 80 μl of medium were seeded into 96-well microtitre plates. After 12 h incubation, compounds to be tested were added in 20 μl portions. Control cultures were treated with DMSO alone. The final concentration of DMSO in the medium did not exceed 0.5%. Serial, triplicate 3-fold dilutions (six in total, highest concentration in incubations 166 μM) of each compound were tested. After 72 h incubation, Calcein AM solution (Molecular Probes) was added to a final concentration of 1 μg/ml, and the cells were incubated for a further hour. The fluorescence of free calcein was then quantified using a Fluoroscan Ascent fluorometer (Microsystems), and the percentage of surviving cells in each well was calculated by dividing the OD obtained from each cell with exposed cells by the mean OD obtained from control wells × 100%. Finally, IC_{50} values (the concentrations causing a 50% decrease in cellular esterase activity) were calculated for each compound from the generated dose–response curves (Kryštof et al., 2002). The IC_{50} values presented here are averages obtained from at least three independent experiments, where individual replicate values fell within 25% of the average.

4.6. NCI_{60} cytotoxicity assay

Tests of toxicity on NCI_{60}, a set of 59 human cancer cell lines derived from nine tissue types, were performed at the developmental therapeutics program (DTP) of the National Cancer Institute (Bethesda, USA). The cytotoxicity of oTR was evaluated by measuring total cell protein using the sulforhodamine B method according to the standard DTP protocol (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html) at both time 0 and after 48 h. GI_{50} values (concentration of a drug inducing 50% reduction of growth) values were estimated from the dose response curves.

4.7. Analysis of the correlation between NCI_{60} activity and gene expression patterns

The activity pattern (GI_{50} values for individual NCI_{60} cell lines) of oTR was correlated with those of 214 antineoplastics in the "Approved Oncology Drugs" and "Standard Agents" DTP datasets and with expression patterns (gene expression measurements for individual NCI_{60} cell lines) of adenosine kinase (the probes 204119_s_at and 204120_s_at on U133A Affymetrix arrays, Shankavaram et al., 2007). If a compound had been tested over several ranges of concentration, the pattern from the greatest number of repetitions was used. Pearson correlation coefficients were calculated on a log–log scale. All calculations and manipulations of data were carried out using statistical software R. The list of "Standard Agents" and "Approved Oncology Drugs" can be accessed at http://dtp.nci.nih.gov/docs/cancer/searches/standard_agent_table.html and http://dtp.cancer.gov/branches/dscb/oncology_drugset_explanation.html, respectively. Relevant activity patterns were extracted from DTP Cancer Screening Data May 2009 Release (http://dtp.nci.nih.gov/docs/cancer/cancer_data.html). Microarray data normalized using the RMA algorithm were downloaded from the CellMiner database (Shankavaram et al., 2009).

4.8. Hollow fibre assays

The effect of two doses (100 and 150 mg/kg/day) of oTR on implanted tumours was determined in hollow fibre assays. The doses selected were derived from acute toxicity experiments that had been previously carried out (results not shown). The assays were performed and evaluated at DTP, according to a standard protocol (http://dtp.cancer.gov/branches/btb/hfa.html). The following cell lines were used: MDA-MB-231, NCI-H23, SW-620, COLO 205, LOX IMVI, OVCAR-3, NCI-H522, U251, UACC-62, MDA-MB-435, OVCAR-5 and SF-295. Each mouse was implanted with three cell lines, as both intraperitoneal (ip) implants and subcutaneous (sc) implants (six implants in total). The compound was administered intraperitoneally for 4 subsequent days. The activity against the xenografted cells was assessed by MTT assay. A 50% or greater reduction in xenograft growth was considered to be a positive result, and each positive result was given a score of two. The sum of the scores was then calculated for all implants, giving a total score, and separate scores for both intraperitoneal (ip) and subcutaneous (sc) implants for each cell line. Hence, the maximum possible score was 96 (12 cell lines × two implantation sites × two dose levels × two). A compound is categorised as active if the total score is at least 20, or the sc score is equal to or greater than eight. The DTP scoring system has been designed so that standard anticancer drugs are classified as active.

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Supplement II

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Preparation, biological activity and endogenous occurrence of N^6-benzyladenosines

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Abstract—Cytokinin activity of forty-eight 6-benzyladenosine derivatives at both the receptor and cellular levels as well as their anticancer properties were compared in various in vitro assays. The compounds were prepared by the condensation of 6-chloropurine riboside with corresponding substituted benzylamines and characterized by standard collection of physico-chemical methods. The majority of synthesized derivatives exhibited high activity in all three of the cytokinin bioassays used (tobacco callus, wheat leaf senescence and *Amaranthus* bioassay). The highest activities were observed in the senescence bioassay. For several of the compounds tested, significant differences in activity were found between the bioassays used, indicating that diverse recognition systems may operate. This suggests that it may be possible to modulate particular cytokinin-dependent processes with specific compounds. In contrast to their high activity in bioassays, the tested compounds were recognized with only very low sensitivity in both *Arabidopsis thaliana* AHK3 and AHK4 receptor assays. The prepared derivatives were also investigated for their antiproliferative properties on cancer and normal cell lines. Several of them showed very strong cytotoxic activity against various cancer cell lines. On the other hand, they were not cytotoxic for normal murine fibroblast (NIH/3T3) cell line. This anticancer activity of cytokinin ribosides may be important, given that several of them occur as endogenous compounds in different organisms.

1. Introduction

Cytokinins are an important group of plant growth regulatory substances.[1,2] These compounds are N^6-substituted adenine derivatives. They occur endogenously, mainly as free bases, nucleosides, glucosides and nucleotides, and are often present at very low concentrations (pmol/g fresh weight). In the presence of an another plant hormone, auxin, they promote cell division in plant tissue cultures and affect a wide range of other processes in plants, including seed germination, bud differentiation, branching, chlorophyll and starch production, resistance to plant-pathogens, apical dominance and leaf senescence.

Cytokinins are classified as isoprenoid or aromatic, depending on the structure of the N^6 substituent.[3] Despite the fact that 6-benzylaminopurine (BAP), one of the most effective and affordable cytokinins, has been widely used in plant biotechnology for several decades[4] and that the endogenous occurrence of its hydroxylated derivatives has been known since the isolation of N^6-(2-hydroxybenzyl)adenosine from poplar by Horgan et al.,[5] cytokinin research has typically been focused on the isoprenoid class of cytokinins, typified by zeatin, dihydrozeatin and isopentenyladenine.[3]

Recently, considerable progress has been made in elucidating the molecular mechanism of cytokinin signalling. Three cytokinin receptors, sensor histidine kinases AHK2, AHK3 and AHK4, have been described in Arabidopsis.[6–8] Subsequently, strains of *Escherichia coli* expressing AHK3 and AHK4 have been used to study the relative sensitivity of these receptors to a range of different cytokinins.[9,10] BAP and its derivatives showed only low activity. In contrast, much higher activities were found for aromatic cytokinins in a P_{ARR5}:*GUS* reporter gene assay,[9] similar to those seen for this group

Keywords: N^6-benzyladenosine; Cytokinin; Receptor; Bioassay; Antibody; Cyclin-dependent kinase; Cytotoxicity.
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of cytokinins in various bioassays. However, this assay is based on activation of the primary response gene ARR5, which already integrates the responses of several putative cytokinin pathways. What is clear is that there are two related groups of cytokinins occurring naturally in plants, which cannot be simply considered as merely alternative forms of the same signal. Nevertheless, the molecular mechanism of aromatic cytokinin action remains largely unknown and requires further investigation.

Although cytokinins regulate many cellular processes, the control of cell division is central in regulation of plant growth and development and is considered to be diagnostic for this class of plant growth regulators. They have also the ability to induce callus growth and redifferentiation into adventitious buds or roots in the presence of auxin.[11] Calli are clusters of dedifferentiated plant cells that are potentially immortal and proliferate indefinitely in a disorganized manner. This behaviour is similar to that of human cancer cells. Because of these similarities, some cytokinins have also been tested for their abilities to affect differentiation of human cancer cells.[12] In plants, cytokinin ribosides have almost the same biological effects as the free bases. Surprisingly, when the effect of various natural cytokinins and their derivatives on human myeloid leukaemia HL-60 cells was examined, it was found that both cytokinin free bases and ribosides can induce granulocytic differentiation of HL-60 cells, but cytokinin ribosides also induce apoptosis prior to the differentiation process.[13] When their mechanism of action was investigated in more detail, it was found that as in plants, cytokinin nucleosides have similar differentiation-inducing activity in several human leukaemia cell lines, as in the case of plants. However, cytokinin nucleosides induce mitochondrial disruption whereas free bases protect against mitochondrial disruption and apoptosis in leukaemia cells.[13] It has been suggested that the intracellular phosphorylation of benzylaminopurine riboside (BAPR) is necessary for the manifestation of its cytotoxicity[14] and that caspases might be critically implicated in this apoptotic process.[15] Nevertheless, the apoptosis pathways induced by cytokinin nucleotides have not yet been fully described. Moreover, we have recently shown that among 38 derivatives of 6-benzylaminopurines (belonging to the group of cytokinin free bases), some can also exhibit moderate cytotoxic activity against various human as well as murine leukaemia cell lines.[10]

Another important property of cytokinin analogues was recently demonstrated, namely that the natural isoprenoid and aromatic cytokinins were able to inhibit several human protein kinases in a non-specific manner. These kinases include CDKs, conserved regulators of the eukaryotic cell cycle, among which different family members control specific phases of the cell cycle.[16] A detailed screening of chemically synthesized cytokinin analogues revealed the fact that additional C^2 and N^9 substitutions of the BAP molecule led to a strong and specific inhibition of several important protein kinases such as CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, brain CDK5/p35 and ERK1/MAP kinase. These compounds have a strong ability to arrest cells at specific points of the cell cycle and to induce apoptosis. They are especially potent towards cancer cell lines where cell-cycle regulators are frequently mutated.[17]

Another plant growth regulator with cytokinin-like activity, diterpenoid cotylenin A, isolated from the fungus *Cladosporium* sp. has been shown to have differentiation-inducing activity in several human and murine myeloid leukaemia cell lines.[18]

It has been shown in several cases that regulators that play an important role in the differentiation and development of plants may also affect the differentiation of human leukaemia cells and thus might be clinically useful for treating acute myeloid leukaemia.[13,18]

In this study, we prepared a group of benzyladenosine derivatives and examined their receptor, cytokinin-like and cytotoxic activities. We show that the cytotoxic effects of substituted benzyladenosines are not restricted to myeloid leukaemia cells only, but can be more general.

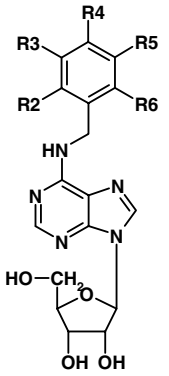
Recently, biologically effective monomethoxy derivatives of 6-benzyladenosine were isolated and identified from several different plant sources.[19] Results presented here also suggest the endogenous occurrence of two disubstituted benzyladenosines.

2. Results and discussion

2.1. Synthesis

Forty-eight derivatives of N^6-benzyladenosine with various substituents attached to the phenyl ring were synthesized (Table 1) and different aspects of their biological activities were investigated. The prepared compounds were characterized by elemental analysis, TLC, melting point, ES+ MS and by ^{1}H, ^{13}C and ^{15}N NMR (Supplementary material). To ensure accurate interpretation of data, also 2D/gs-COSY, gs-HMQC, gs-HMBC for ^{1}H and ^{13}C, $/^nJ_{H,C} = 7.5$ Hz// $/^{15}$N-GHMQS and ^{15}N-GHMBC, $/^nJ_{H,N} = 7.5$, 9.0 Hz// experiments have been made (data not shown). Compounds **4–6** as well as **14–16** have been prepared previously,[20] starting from tri-*O*-acetyl-6-chloropurine riboside. The melting points, reported for these compounds,[20] are consistent with our data, although usually somewhat lower. N^6-(methylbenzyl)adenosines were later also synthesized from 6-chloropurine riboside,[21] under the conditions described by Fleysher.[22] N^6-(3,5-difluorobenzyl)adenosine and N^6-(2,4-dimethoxybenzyl)adenosine have also been prepared recently by a different reaction, namely nucleophilic substitution starting from 6-chloropurine-β-D-1-deoxyribofuranoside[23] in the presence of Hünig's base, using a lower temperature and longer reaction time. A group of N^6-benzyladenosine derivatives, monosubstituted on

Table 1. Structures and abbreviations of prepared compounds



Compound	R_2	R_3	R_4	R_5	R_6
1	F	H	H	H	H
2	H	F	H	H	H
3	H	H	F	H	H
4	Cl	H	H	H	H
5	H	Cl	H	H	H
6	H	H	Cl	H	H
7	Br	H	H	H	H
8	H	Br	H	H	H
9	H	H	Br	H	H
10	H	I	H	H	H
11	CH_3	H	H	H	H
12	H	CH_3	H	H	H
13	H	H	CH_3	H	H
14	CH_3O	H	H	H	H
15	H	CH_3O	H	H	H
16	H	H	CH_3O	H	H
17	Cl	H	Cl	H	H
18	H	Cl	Cl	H	H
19	CH_3O	CH_3O	H	H	H
20	CH_3O	H	CH_3O	H	H
21	H	CH_3O	CH_3O	H	H
22	H	CH_3O	H	CH_3O	H
23	CH_3O	H	H	H	CH_3O
24	F	H	F	H	H
25	H	F	H	F	H
26	F	F	F	H	H
27	F	F	H	H	F
28	F	H	F	F	H
29	Cl	H	F	H	H
30	H	Cl	F	H	H
31	OH	CH_3O	H	H	H
32	OH	H	CH_3O	H	H
33	OH	H	H	CH_3O	H
34	H	OH	CH_3O	H	H
35	OH	OH	H	H	H
36	H	OH	OH	H	H
37	H	OH	H	OH	H
38	H	CH_3O	OH	CH_3O	H
39	CH_3O	H	OH	H	CH_3O
40	OH	CH_3	H	H	H
41	OH	H	H	CH_3	H
42	$OCHF_2$	H	H	H	H
43	CF_3	H	H	H	H
44	H	CF_3	H	H	H
45	H	H	CF_3	H	H
46	CF_3O	H	H	H	H
47	H	CF_3O	H	H	H
48	H	H	CF_3O	H	H

the benzyl ring with chloro, methyl or methoxy groups, has been previously prepared and tested to establish structure–activity relationship for the inhibition of *Trypanosoma brucei* glycosomal phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase.[24]

2.2. Cytokinin activity in bioassays

The majority of synthesized monosubstituted derivatives exhibited high activity in all three cytokinin bioassays employed (tobacco callus, wheat senescence and *Amaranthus* bioassays, Table 2). The results suggest that position-specific steric and hydrophobic effects of the benzyl ring participate in the variation in activity,[25] the general trend of the activity being: *meta* $\geqslant$ *ortho* > *para*. The *meta* hydroxy-substituted compounds were already previously noted to be more active than the *ortho* and *para* isomers.[26,27] Furthermore, in most cases substitution in the *para* position causes loss of activity (compare **14** with **20**, **23** with **39**, etc.). For most compounds, the highest activities were exhibited in the senescence bioassay, some compounds showing up to 220% greater activity than BAP. Almost 50% of the prepared compounds were more active than BAP in this bioassay. This is in good agreement with our earlier results describing the activ ities of the corresponding adenine derivatives.[10] On the other hand we were able to find only single substance (**14**) which exhibited significantly higher activity than BAP in the cytokinin-dependent tobacco callus bioassay. The *Amaranthus* test however revealed a number of highly active compounds mainly among the halogen derivatives. The results indicate that electron-withdrawing substituents (e.g., fluoro) enhance the activity in this group of compounds, presumably through hydrogen bond formation with electron donors of a cytokinin receptor.[28] Fluoro derivatives were recognized as the most active compounds in this series. Even the introduction of more than one fluorine atom on the benzyl ring, or its combination with a chlorine substituent, did not cause a dramatic decrease in their high cytokinin activity. The compounds **24**–**30** were therefore highly active in all three bioassays employed (Table 2). It is obvious from these results that the fluorine atom exerts a beneficial effect on activity of the benzyladenosines, as already shown for fluoro derivatives of N^6-isopetenyladenine.[29] Monomethoxy derivatives (**14**–**16**), described recently as natural cytokinins,[19] were also very active in the senescence bioassay. Among disubstituted derivatives the situation was more complex. 6-(2,4-Dichlorobenzylamino)purine riboside **17** was active in the tobacco callus bioassay but inactive in the other bioassays. The opposite was true for 3,4-dichloro derivative **18**. The same dramatic differences were present among the results of tests with disubstituted hydroxy-, as well as methoxy- and methyl-derivatives (Table 2). This indicates that among disubstituted benzyladenosine derivatives even small change in the benzyl ring substitution can lead to major changes in their profiles of cytokinin activity. This again suggests that different receptor and/or signalling systems are involved in mediating different cytokinin-dependent

Table 2. Cytokinin activity of prepared substituted 6-benzyladenosines

Compound	*Amaranthus* bioassay		Senescence bioassay		Tobacco callus bioassay	
	Optimal concentration (mol/L^{-1})	Relative activity (%)	Optimal concentration (mol/L^{-1})	Relative activity (%)	Optimal concentration (mol/L^{-1})	Relative activity (%)
1	10^{-5}	96 (±2)	10^{-4}	118 (±39)	10^{-6}	100 (±9)
2	10^{-5}	92 (±6)	10^{-4}	220 (±16)	10^{-5}	91 (±6)
3	10^{-5}	71 (±3)	10^{-4}	148 (±2)	10^{-6}	100 (±6)
4	10^{-5}	113 (±4)	10^{-4}	119 (±9)	10^{-6}	93 (±4)
5	10^{-5}	139 (±3)	10^{-4}	72 (±8)	10^{-5}	96 (±5)
6	10^{-5}	35 (±4)	10^{-4}	104 (±6)	10^{-6}	46 (±14)
7	10^{-4}	147 (±9)	10^{-5}	86 (±29)	10^{-5}	100 (±5)
8	10^{-4}	151 (±7)	10^{-4}	89 (±10)	10^{-5}	82 (±10)
9	10^{-4}	30 (±5)	10^{-4}	76 (±11)	10^{-6}	16 (±11)
10	10^{-4}	102 (±18)	10^{-4}	58 (±19)	10^{-6}	45 (±12)
11	10^{-4}	99 (±27)	10^{-4}	141 (±5)	10^{-6}	98 (±4)
12	10^{-5}	96 (±7)	10^{-4}	143 (±9)	10^{-6}	90 (±2)
13	10^{-4}	49 (±13)	10^{-4}	54,5 (±3)	10^{-6}	35 (±6)
14[19]	10^{-5}	86 (±4)	10^{-4}	198 (±12)	10^{-5}	108 (±1)
15[19]	10^{-4}	98 (±10)	10^{-4}	209 (±8)	10^{-6}	92 (±1)
16	10^{-4}	17 (±8)	10^{-4}	65,5 (±15)	10^{-6}	2 (±1)
17	10^{-4}	3 (±2)	10^{-5}	5 (±1)	10^{-5}	70 (±4)
18	10^{-4}	68 (±10)	10^{-4}	151 (±49)	10^{-6}	17 (±7)
19	10^{-4}	21 (±7)	10^{-4}	106 (±17)	10^{-6}	5 (±2)
20	10^{-4}	3 (±3)	10^{-4}	30 (±1)	10^{-5}	5 (±4)
22	10^{-4}	2 (±1)	10^{-4}	47 (±6)	10^{-6}	11 (±9)
23	10^{-4}	54 (±2)	10^{-4}	68 (±26)	nt	nt
24	10^{-4}	88 (±1)	10^{-4}	171 (±7)	10^{-6}	96 (±12)
25	10^{-4}	110 (±11)	10^{-4}	195 (±14)	10^{-6}	95 (±3)
26	10^{-4}	95 (±23)	10^{-4}	144 (±12)	10^{-5}	94 (±7)
27	10^{-4}	94 (±1)	10^{-4}	133 (±14)	10^{-6}	92 (±2)
28	10^{-4}	120 (±1)	10^{-4}	99 (±7)	10^{-5}	95 (±3)
29	10^{-4}	115 (±1)	10^{-4}	165 (±21)	10^{-5}	98 (±4)
30	10^{-4}	82 (±4)	10^{-4}	156 (±10)	10^{-6}	87 (±4)
31	10^{-4}	18 (±5)	10^{-4}	10 (±1)	nt	nt
32	10^{-4}	0	10^{-4}	22 (±5)	nt	nt
34	10^{-4}	2 (±1)	10^{-4}	19 (±1)	nt	nt
35	10^{-4}	24 (±11)	10^{-4}	7 (±5)	nt	nt
36	10^{-4}	8 (±3)	10^{-7}	17 (±14)	10^{-6}	4 (±1)
37	10^{-4}	31 (±6)	10^{-4}	96 (±16)	nt	nt
39	10^{-4}	7 (±2)	nt	nt	nt	nt
40	10^{-4}	55 (±6)	nt	nt	10^{-5}	5 (±4)
41	10^{-4}	58 (±4)	10^{-4}	164 (±8)	10^{-4}	7 (±4)
42	10^{-4}	81 (±1)	10^{-4}	180 (±36)	10^{-5}	89 (±11)
43	10^{-4}	121 (±2)	10^{-4}	40 (±6)	nt	nt
44	10^{-4}	93 (±7)	10^{-4}	95 (±7)	10^{-6}	85 (±8)
45	10^{-4}	14 (±1)	10^{-4}	52 (±10)	nt	nt
46	10^{-4}	78 (±1)	10^{-4}	61 (±5)	nt	nt
47	10^{-4}	104 (±5)	10^{-4}	90 (±19)	10^{-6}	86 (±12)
48	10^{-4}	21 (±1)	10^{-4}	3 (±2)	nt	nt

nt, not tested.

physiological responses such as senescence or cell growth and division.[10]

In contrast to these results, neither the CRE1/AHK4 nor the AHK3 cytokinin receptor was fully activated by any of our N^6-benzyladenosine derivative (Table 3). The highest level of AHK3 activation was measured for compound **3**, reaching 7.67% of *trans*-zeatin activity. All the other derivatives tested showed even much lower of AHK3 activation, ranging from 0.02% (compound **4**) up to 2.93% (compound **1**). Activation of CRE1/AHK4 was observed at even in order of magnitude lower levels, in the range 0.04 –0.39%. The negative results obtained for AHK3 are even more surprising in light of the fact that this receptor is able to recognize with high sensitivity not only isoprenoid cytokinins, but also corresponding ribosides.[8] This also supports our previous hypothesis that a different cytokinin recognition system, able to interact also with aromatic analogues, probably exists in plants.[10]

2.3. Anti-tumour activity and endogenous occurrence of studied compounds

The prepared compounds were also tested for their anti-tumour activity against cell lines derived from human T-lymphoblastic leukaemia (CEM), promyelocytic leukaemia (HL-60), human malignant melanoma (G-361), human chronic myelogenous leukaemia (K-562), human osteogenic sarcoma (HOS), breast carcinoma

Table 3. Receptor assay activity of selected substituted 6-benzyladenosines

Compound	Relative activity (%)	
	AHK3	AHK4
tZ	100.00	100.00
BAPR	2.53	0.35
1	2.91	0.12
2	0.99	0.36
3	7.67	0.39
4	0.02	0.04
5	2.63	0.15
6	0.81	0.23
14	0.04	0.12
15	1.12	0.14
16	2.45	0.22

(MCF-7) and mouse melanoma (B16). The data obtained from a Calcein AM viability/cytotoxicity assay are presented in Table 4. The IC_{50} values obtained indicate very promising cytotoxic properties of compound **31** (IC_{50} = 0.15 and 0.30 μmol/L for HL-60 and CEM, respectively), in contrast to other very similar derivatives (e.g., compounds **19**, **20** and **34**), which displayed much lower activity in this assay. The highest cytotoxicity on MCF7 and leukaemia cell lines was usually associated with *ortho*-hydroxylation (**31**, **32**, **35** and **40**) and fluorination (**1**–**3**, **24**) of benzyladenosine. The fluoro derivatives (**1**–**3**) were however the only compounds toxic (at a moderate level, IC_{50} > 44.3 μmol/L) also for normal mouse fibroblasts (NIH3T3). Compounds **13**, **16**, **38** and **39** were almost or totally inactive. On the other hand, moderate cytotoxicity of other monosubstituted halogen-, methyl- and methoxy-derivatives (**1**–**9**, **11**, **12**, **14** and **15**) to the majority of cancer cell lines used was observed

Table 4. Cytotoxicity of prepared substituted 6-benzyladenosines

Compound	Cell line/IC_{50} (μmol/L)							
	HOS	K-562	MCF7	CEM	HL-60	G-361	B16	NH 3T3
BAPR	>166.7	5.5	5.4	1.4	0.94	>166.7	>166.7	39
1	>166.7	33.2	>166.7	4.6	3.2	nt	>166.7	>166.7
2	>166.7	7.6	15.3	4.0	0.92	>166.7	nt	84
3	13.2	2.7	21	1.25	1.2	>166.7	nt	44.3
4	>166.7	64	>166.7	14.5	1.6	nt	>166.7	>166.7
5	>166.7	27.7	26.9	1.6	0.75	>166.7	nt	>166.7
6	>166.7	218	>166.7	10.2	1.7	>166.7	>166.7	>166.7
7	>166.7	10.0	>166.7	12.3	6.6	>166.7	>166.7	>166.7
8	>166.7	19.7	>166.7	5.0	8.0	>166.7	>166.7	>166.7
9	>166.7	68.2	>166.7	20.6	47.4	>166.7	>166.7	>166.7
11	>166.7	>166.7	>166.7	14	3.3	>166.7	nt	>166.7
12	>166.7	>166.7	>166.7	19.1	6.4	>166.7	>166.7	>166.7
13	>166.7	>166.7	>166.7	>166.7	>166.7	nt	nt	>166.7
14	>166.7	8.9	>166.7	3.2	2.3	>166.7	>166.7	140
15	>166.7	>166.7	>166.7	7.6	4.9	nt	>166.7	>166.7
16	>166.7	155.5	>166.7	>166.7	>166.7	114.9	19.5	nt
17	>166.7	106	126.9	86.7	96.3	128.3	>166.7	nt
18	>166.7	10.8	88.8	3.8	2.0	144.7	>166.7	nt
19	>166.7	>166.7	>166.7	>166.7	>166.7	nt	nt	nt
20	>166.7	>166.7	>166.7	>166.7	39	nt	nt	nt
22	nt	>166.7	>166.7	>166.7	20.2	nt	nt	nt
24	>166.7	9.6	>166.7	7.1	3.4	>166.7	>166.7	>166.7
25	nt	>166.7	>166.7	24.5	9.1	>166.7	>166.7	>166.7
26	>166.7	>166.7	>166.7	58.2	13	>166.7	>166.7	nt
27	nt	>166.7	>166.7	90.3	13	>166.7	>166.7	nt
29	>166.7	15.6	>166.7	20.9	9	>166.7	>166.7	>166.7
30	>166.7	5.5	>166.7	3.4	3.5	>166.7	166	>166.7
31	>166.7	27.9	20.2	0.3	0.15	148.1	>166.7	>166.7
32	>166.7	80.8	2.2	12.8	nt	nt	nt	nt
34	nt	>166.7	>166.7	>166.7	>166.7	nt	nt	>166.7
35	>166.7	25.8	5.2	nt	nt	16.9	nt	nt
37	>166.7	>166.7	>166.7	nt	nt	>166.7	nt	nt
38	>166.7	>166.7	>166.7	nt	nt	>166.7	nt	nt
39	>166.7	>166.7	>166.7	nt	nt	>166.7	nt	nt
40	>166.7	18	6.9	0.9	15	>166.7	>166.7	nt
41	>166.7	16.5	>166.7	1.9	15	>166.7	>166.7	nt
42	>166.7	>166.7	>166.7	nt	nt	>166.7	nt	nt
43	>166.7	>166.7	>166.7	nt	nt	>166.7	nt	nt
44	>166.7	>166,7	>166,7	nt	nt	>166.7	nt	nt
45	>166.7	>166,7	>166,7	nt	nt	>166.7	nt	nt
46	>166.7	81.4	>166.7	>166.7	>166.7	>166.7	>166.7	nt
47	>166.7	>166.7	>166.7	nt	nt	>166.7	nt	nt
48	>166.7	>166.7	>166.7	nt	nt	>166.7	nt	nt

nt, not tested.

(Table 4). As an exception to this, none of the tested compounds were active in HOS cells. Melanoma cell lines (G-361, B16) were also very resistant to more 90% of the compounds tested.

The ability of several natural cytokinin nucleosides to inhibit growth of HL-60 cells has been previously reported.[12] Ribosides have been shown to be potent inducers of apoptosis.[13] In contrast, cytokinin free bases effectively induced HL-60 cell differentiation and transformation into mature granulocytes.[13] Here we show that high cytotoxicity against various cancer cell lines is a commonly occurring property of substituted N^6-benzyladenosines and is not restricted only to the myeloid leukaemia as reported previously.[13]

This finding is even more important in the light of the fact that at least some of these compounds occur endogenously (Figs. 1 and 2) in different organisms.

Although originally known only as synthetic compounds[3] the endogenous occurrence of hydroxyl and methoxy BAP analogues has now been repeatedly documented.[5,30–32] Recently, a batch immunoextraction method for the rapid and effective isolation of a broad spectrum of aromatic cytokinins from biological materials was also described.[33] In the present study, this new approach has been successfully applied to isolate new cytotoxic members of the aromatic cytokinin family present endogenously in some living organisms. Several hydroxymethoxybenzyladenosines as well as dimethoxybenzyladenosines were detected and tentatively identified from their LC retention times, antibody cross-reactivities and specific MRM diagnostic transitions (Figs. 1 and 2) in *Arabidopsis thaliana* as well as *Agrobacterium tumefaciens* extracts. The presence, exact molecular mass and structure of two new disubstituted benzyladenosine derivatives in samples prepared from 10-day-old *A. thaliana* plants was confirmed by cap-LC-ESI$^+$-HR MS/MS QqTOF mass spectrometry and by comparative analysis of the chemically synthesized standards (Figs. 1 and 2). The unequivocal identification of these compounds confirms the discovery of two new plant growth substances, namely 6-(2-hydroxy-3-methoxybenzylamino)purine-9-β-D-ribofuranoside (**31**) and 6-(2,4-dimethoxybenzylamino)purine-9-β-D-ribofuranoside (**20**). Their identification in *A. thaliana* plants and *A. tumefaciens* strain *GV310* indicates that representatives of this already large group of compounds occur in a wide variety of organisms.

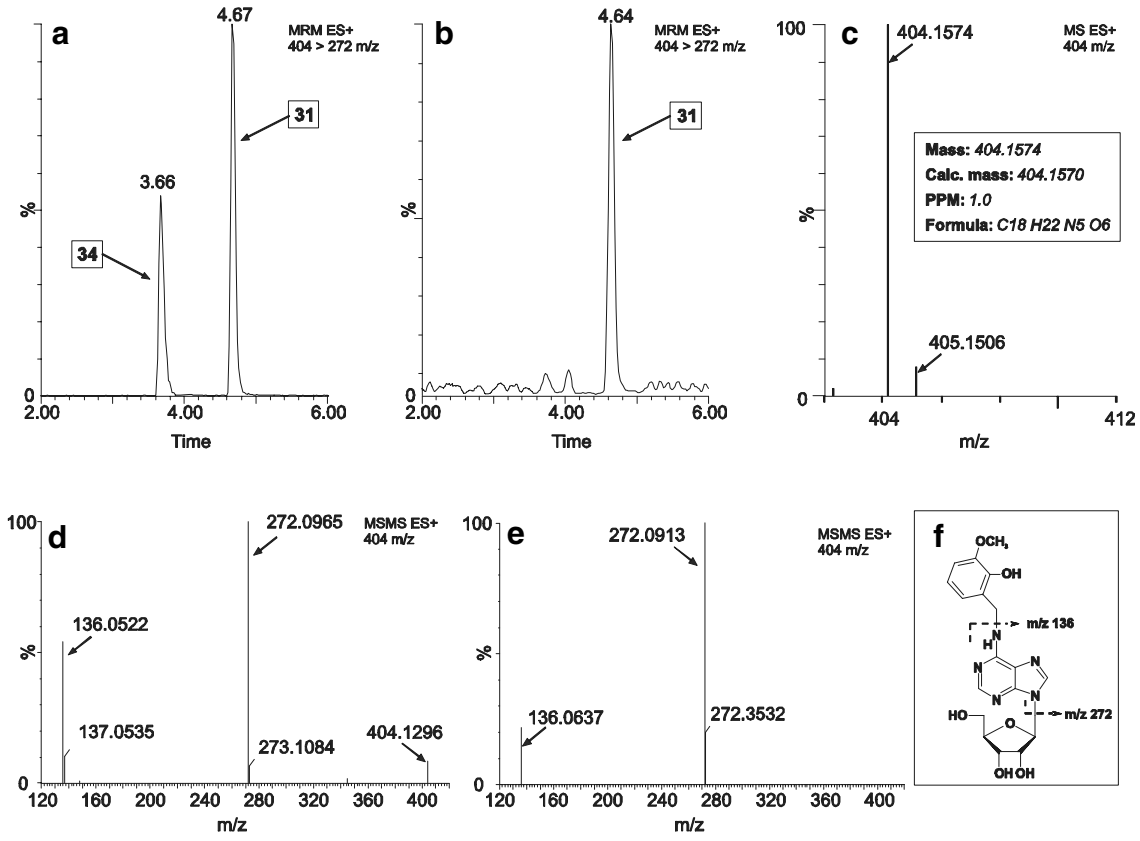


Figure 1. The identification of 6-(2-hydroxy-3-methoxybenzylamino)purine riboside (compound number **31**) in 10-day-old *A. thaliana* plants. (a) UPLC/MS (positive-ion MRM mode of *m/z* 404 > 272) chromatogram of standards number **31** and **34** (the mixture contains 10 pmol of each derivative). (b) UPLC/MS (positive-ion MRM mode of *m/z* 404 > 272) chromatogram of *A. thaliana* purified by C18 solid-phase extraction and batch immunoaffinity extraction. (c) Q-TOF mass spectrum of compound number **31** ($[M+H]^+ = 404$), isolated from *A. thaliana*, and the table of exact mass determination of 6-(2-hydroxy-3-methoxybenzylamino)purine riboside. (d) Positive-ion Q-TOF MS/MS spectrum of standard number **31** ($[M+H]^+ = 404$). (e) Positive-ion Q-TOF MS/MS spectrum of 6-(2-hydroxy-3-methoxybenzylamino)purine riboside **31** ($[M+H]^+ = 404$) isolated from *A. thaliana*. (f) The fragmentation pattern for compound number **31** in positive ion-mode ($[M+H]^+ = 404$).

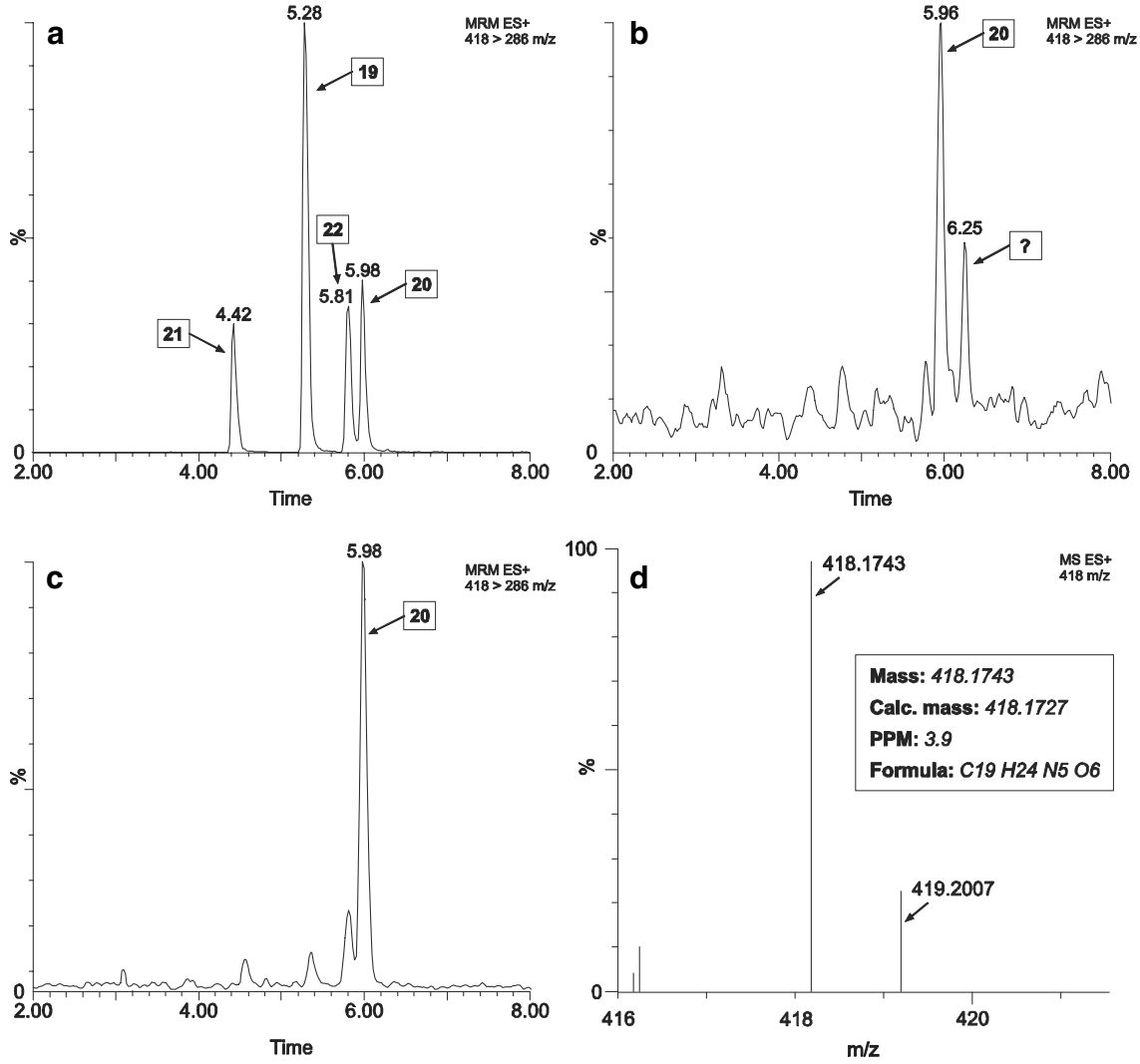


Figure 2. The identification of 6-(2,4-dimethoxybenzylamino)purine riboside (compound number **20**) in 10-day-old *A. thaliana* plants. (a) UPLC/MS (positive-ion MRM mode of *m*/*z* 418 > 286) chromatogram of standards number **19**, **20**, **21** and **22** (the mixture contains 10 pmol of each derivative). (b) UPLC/MS (positive-ion MRM mode of *m*/*z* 418 > 286) chromatogram of *A. thaliana* extract, obtained after purification by C18 solid-phase extraction. (c) UPLC/MS (positive-ion MRM mode of *m*/*z* 418 > 286) chromatogram of *A. thaliana* extract, purified by C18 solid-phase extraction and batch immunoaffinity extraction. (d) Positive-ion Q-TOF mass spectrum of compound number **20** ($[M+H]^+ = 418$), isolated from *A. thaliana*, and the table of exact mass determination of 6-(2,4-dimethoxybenzylamino)purine riboside.

Plants contain various cytokinin species, but it is still not known whether these communicate physiologically different messages or not.[34] Data from different bioassays as well as their use in tissue culture also show that plant tissues respond differently to different cytokinins. At present, the precise physiological functions of the different groups of cytokinins remain unknown.

3. Conclusions

In summary, a group of 6-benzyladenosine derivatives with different phenyl ring substituents has been prepared. Majority of them have been found to be very active in all three cytokinin bioassays used (tobacco callus, wheat leaf senescence and *Amaranthus* bioassay). In contrast, none of our N^6-benzyladenosine derivative was able to fully activate either the CRE1/AHK4 or the AHK3 cytokinin receptor. It supports our previous hypothesis that a different sensing mechanism for aromatic cytokinins may exist in plants.

On the other hand, some of the prepared derivatives displayed high cytotoxic activity against various cancer cell lines. Several compounds from this group were also isolated and unambiguously identified as endogenous in *A. thaliana* plants as well as *A. tumefaciens* extracts.

4. Materials and methods

4.1. General procedures

Elemental analyses (C, H and N) were performed on an EA1108 CHN analyser (Thermo Finnigan). The melting points were determined on Büchi Melting

Point B-540 apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck), solvent $CHCl_3$/MEOH/concd NH_4OH (8:2:0.2, v/v/v). ES+ mass spectra were recorded using direct probe on a Waters ZMD 2000 mass spectrometer. The mass monitoring interval was 50–1500 amu. The spectra were collected using 3.0 s scan time and applying a sample cone voltage 25 V at a source block temperature 80 °C, desolvation temperature 150 °C and a desolvation gas flow rate 200 L/h. The mass spectrometer was directly coupled to a MassLynx data system. NMR spectra were measured in a Bruker Avance AV 300 spectrometer operating at a temperature of 300 K (340 K for ^{15}N) and a frequency of 300.13 MHz (^{1}H), 75.48 MHz (^{13}C) and 30.42 MHz (^{15}N), respectively. Samples were prepared by dissolving the compounds in DMSO-d_6. Tetramethylsilane (TMS) was used as the internal standard.

4.2. Chemicals

6-Benzylaminopurine and DMSO were purchased from Sigma. Calcein AM was purchased from Invitrogen. 6-Chloropurine riboside, 6-(3,3-dimethylallylamino)purine (isopentenyladenine, iP), 6-((*E*)-4-hydroxy-3-methylbut-2-enylamino)purine (*trans*-zeatin, tZ), olomoucine and bohemine were obtained from Olchemim. Commercially available starting amines were purchased as listed in Supplementary material. Preparation and characterization of these not commercially available has been described previously:[10] 3,5-Dihydroxybenzylamine hydrobromide, 2-hydroxy-3-methoxybenzylamine hydrobromide, 2,3-dihydroxybenzylamine hydrobromide and 4-hydroxy-3,5-dimethoxybenzylamine hydroiodide. Milli-Q water was used throughout. The other solvents and chemicals used were all of standard pa quality. Murashige and Skoog medium including vitamins, and plant agar were obtained from Duchefa. Lach-Ner supplied sucrose, sodium dihydrogen phosphate dihydrate, sodium chloride and ethanol. Methanol, formic acid, diethyldithiocarbamic acid and water LC–MS Chromasolv were also purchased from Sigma.

4.3. Synthesis of N^6-benzyladenosines

The preparation and cytokinin activity of N^6-(2-methoxybenzyl)adenosine **14** and N^6-(3-methoxybenzyl)-adenosine **15** has been described elsewhere.[19] The general procedures for the preparation of ring substituted 6-benzyladenosines were the same as described earlier.[10,19] In brief, 6-chloropurine riboside was heated with the appropriate primary amine to 90 °C for 4 h in *n*-butanol containing an excessive amount of triethylamine. After cooling, the precipitated product was filtered off, washed with cold water and *n*-butanol and crystallized from ethanol. The identity and purity of the synthesized compounds were confirmed by elemental and melting point analysis, analytical thin layer chromatography, high performance liquid chromatography, MS and NMR (Supplementary material).

4.4. Preparation of 4-hydroxy-2,6-dimethoxybenzylamine hydroiodide

The compound was prepared from 2,4,6-trimethoxybenzylamine using a similar procedure to that previously described.[10] 2,4,6-Trimethoxybenzylamine was dissolved in 55% HI and acetanhydride was added by a syringe. The reaction mixture was subsequently magnetically stirred at room temperature for 5 h. The crude product was re-crystallized from ethanol. Elemental analysis (C, H, and N) (Found: C, 35,6; H, 4.6; N, 4.6. $C_9H_{14}NO_3I$ Calcd: C, 34.8; H, 4.5; N, 4.5%); TLC (chloroform/methanol/ammonia (8:2:0.1, v/v/v) as the mobile phase): single spot; mp 269–274 °C; ES+ MS: *m/z* 184 $[MH]^+$; ^{1}H NMR (DMSO-d_6, 300 MHz): 9.78 (s, 1H, OH), 7.69 (s, 2H, NH_2), 6.12 (s, 2H, C3,5-H), 3.85 (s, 2H, C7-H), 3.76 (s, 6H, OCH_3). ^{13}C NMR (DMSO-d_6, 75 MHz): 160.06 (C4), 159.02 (C2,6), 99.62 (C1), 91.65 (C3,5), 55.58 (–OCH_3), 31.29 (7C).

4.5. Preparation of 2-hydroxy-4-methoxybenzylamine hydroiodide

The compound was prepared from 2,4-dimethoxybenzylamine (1.5 mL; 10 mmol) using a similar procedure to that for 4-hydroxy-2, 6-dimethoxybenzylamine hydroiodide. After refluxing (107 °C) for 4 h, the reaction mixture was kept in a freezer (−20 °C) for 48 h. The colourless crystals of 2-hydroxy-4-methoxybenzylamine hydrobromide were filtered off, washed with diethyl ether, dried and re-crystallized from ethanol. Elemental analysis (C, H, and N) (Found: C, 31.5; H, 3.7; N, 5.0. $C_9H_{14}NO_3I$ Calcd: C, 31.5; H, 3.8; N, 5.2%); TLC (chloroform/methanol/ammonia (8:2:0.1, v/v/v) as the mobile phase): single spot; mp 193–194 °C; ES+ MS: *m/z* 154 $[MH]^+$; ^{1}H NMR (DMSO-d_6, 300 MHz): 9.42 (s, 1H, OH), 7.80 (s, 2H, NH_2), 7.05 (s, 1H, C6-H), 6.37 (s, 1H, C3-H), 6.23 (s, 1H, C5-H), 3.83 (s, 2H, C7-H), 3.77 (s, 3H, OCH_3). ^{13}C NMR (DMSO-d_6, 75 MHz): 158.98 (C4), 156.62 (C2), 131.38 (C6), 110.50 (C1), 106.27 (C5), 102.15 (C3), 55.35 (–OCH_3), 37.70 (7C).

4.6. Biological materials and growth conditions

Plants of *A. thaliana*, ecotype Colombia, were grown in vitro in Petri dishes containing Murashige and Skoog medium including vitamins (4.4 g MS medium, 10 g of sucrose and 10 g of plant agar/L, pH 5.7) at 23 °C in a 16 h photoperiod. Ten-day-old plants were harvested, weighed and immediately plunged into liquid nitrogen.

Agrobacterium tumefaciens, strain *GV310*, a C58 derivative cured of its Ti-plasmid,[35] was cultivated overnight in LB medium[19] (100 mL of culture) without antibiotics in a sterile box at 28–30 °C and was centrifuged the following day at 5000*g* at 4 °C for 20 min. The supernatant and sediment were analyzed separately.

4.7. Cytokinin extraction and purification

4.7.1. The *A. thaliana* samples. Samples of plant material (500–700 mg, FW) were placed individually, in 750 μL

of 70% ethanol containing diethyldithiocarbamic acid (DDC; 400 μg/g of fresh weight) in 1.5 mL Eppendorf tubes and extracted using an MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) at a frequency of 30 Hz for 3 min after adding 3-mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany) to each tube to increase the extraction efficiency. The tube contents were then stirred for 15 min at 4 °C and subsequently ultrasonicated for 5 min. After centrifugation (3 min, 15,000 rpm) the supernatants were transferred into glass tubes and stored at 4 °C. The sediments were re-extracted with 750 μL of 70% of ethanol with DDC for 1 h at 4 °C. After centrifugation (3 min, 15,000 rpm), both supernatants were combined and immediately purified.

The first purification step involved passage through a 360 mg C_{18}-Bond Elute cartridge (Varian, Palo Alto, USA), conditioned with 2× 2 mL of 80% of MeOH. Partially purified samples were evaporated and then dissolved in 50 μL of 70% ethanol and 450 μL of PBS (25 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) and purified by batch immunoaffinity extraction before LC–MS analysis as described earlier.[33]

4.7.2. The *A. tumefaciens* samples. *Agrobacterium* supernatant (30 mL) was collected and extracted by adding ethanol to 70% with 50 μL diethyldithiocarbamic acid (DDC), and incubating overnight at −20 °C. After centrifugation the sediment was mixed with 10 mL of 70% ethanol with 50 μL DDC (1 mg/10 mL) and extracted under the same conditions as the supernatant (overnight, −20 °C). Ethanolic extracts were further purified by solid-phase extraction[19] and batch immunoextraction as described above for plant material.

4.8. Chromatography and mass spectrometry conditions

All samples were first analyzed by ultra performance liquid chromatography (ACQUITY UPLC™, Waters) linked to a Quattro micro™ tandem quadrupole mass spectrometer (Waters) equipped with an electrospray interface (LC (+)ESI-MS). LC conditions were as follows: flow rate 0.25 mL/min; column temperature, 40 °C; sample heater temperature, 4 °C. MS analyses were carried out under the following conditions: source block temperature, 100 °C; desolvation temperature, 350 °C; capillary voltage, +0.60 kV cone voltage, 40 V; collision energy, 20 V. Nitrogen was used as both desolvation (550 L/h) and cone gas (2 L/h). Under these conditions, analysis was performed in multiple reaction monitoring (MRM) mode. All data acquired were processed by MassLynx 4.1 software.

The purified samples were dissolved in 15 μL MeOH/H_2O (30:70) and 10 μL of each sample was injected onto a C18 reversed-phase column (Acquity UPLC™; BEH Shield RP18; 1.7 μm; 2.1 × 150 mm; Waters). The mobile phase consisted of gradients of MeOH (solvent A) and 5 mM formic acid (solvent B). The column was eluted with a linear gradient of 30–64% solvent A (0–9 min), with retention times for the monitored compounds ranging from 2.50 to 6.50 min. To confirm the identity of the isolated compounds and their endogenous occurrence, samples were further analyzed using a CapLC™ (Waters) capillary liquid chromatograph, connected to a Q-Tof micro (Waters), a bench-top quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer. CapLC method parameters were as follows: flow rate 5 μL/min; column temperature, 35 °C; sample heater temperature, 4 °C; mobile phase A, 5 mM formic acid + 2% MeOH; mobile phase B, MeOH + 0.05% formic acid. The NanoEasy column: Symmetry C18; 5 μm; 0.3 × 150 mm (Waters) was eluted in a linear gradient of 70–0% A (1–15 min).

The electrospray ionization in the positive mode was carried out under the following conditions: source block temperature, 90 °C; desolvation temperature, 200 °C; capillary voltage, +4 kV; cone voltage, 30 V; desolvation/cone gas flow (N_2) 50/250 L/h. In the full scan mode, data were acquired in the range 50–500 Da, with a cycle time 28 μs, scan time 1.0 s and collision energy 4 V. External calibration for exact mass determination was carried out using lock spray technology with a mixture of 0.1 M NaOH, 10% formic acid and acetonitrile (1:1:8) as a reference. For the MS/MS experiments, the fragmentation was done in an argon gas-filled collision cell with collision energy 20 and 25 V. Other parameters were the same as in the simple MS experiments. The accurate masses of the parent ions and their fragments were calculated and used for the determination of the elementary composition and structure, with a fidelity of 5 ppm or better for full scan measurements.

4.9. Cytokinin activity assays

Three standard bioassays 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 were used to determine the cytokinin activity of the prepared derivatives as previously described.[26] The compounds to be tested were dissolved in DMSO and the solutions brought up to 10^{-3} M using Milli-Q water. These stock solutions were further diluted in the respective media used for each assay. The final concentration of DMSO in the media did not exceed 0.2% and did not affect biological activity in the systems used.

Cytokinin activity was tested over the concentration range 10^{-4}–10^{-8} M. Each concentration was replicated five times and the entire assay was repeated at least three times. The concentration exhibiting the highest biological response and the relative activities of each compound at this concentration were calculated (Table 2). The activity of BAP at the optimal concentration (Table 2) was set at 100 and the activities of the other tested compounds were calculated relative to that of BAP.

4.10. Bacterial cytokinin assay

The preparation of *E. coli* strain KMI001, harbouring either plasmid pIN-III-AHK4 or plasmid pSTV28-AHK3, and the bacterial cytokinin assay were

performed as previously described,[7–9] albeit with slight modifications as detailed below. The *E. coli* strains were grown overnight at 25 °C in M9 media enriched with 0.1% casamino acids to $OD_{600} \sim 1$. The preculture was diluted 1:600 in 200 μL M9 medium containing 0.1% casamino acids and 1 μL stock solution of either the tested compound or solvent control was added. The cultures were incubated for further periods at 25 °C. Incubation times of 17 and 28 h were found to be optimal for CRE1/AHK4 and AHK3, respectively. The cultures were centrifuged and 50 μL aliquots of the supernatant were transferred to microtitre plates in which each well contained 2 μL of 50 mM 4-methylumbelliferyl β-D-galactoside. The plates were subsequently incubated for 1 h at 37 °C, and the reaction was stopped by adding 100 μL of 0.2 M Na_2CO_3. Fluorescence was measured using a Fluoroskan Ascent (Labsystems, Finland) at excitation and emission wavelengths of 365 and 460 nm, respectively. The OD_{600} of the remainder of each culture was determined and β-galactosidase activity was calculated as nanomole 4-methylumbelliferone $\times$ $OD_{600}{}^{-1} \times h^{-1}$.

4.11. Anti-tumour activity testing

Human T-lymphoblastic leukaemia (CEM), human promyelocytic leukaemia (HL-60), human malignant melanoma (G-361), human chronic myelogenous leukaemia (K-562), human osteogenic sarcoma (HOS), human breast carcinoma (MCF-7), mouse melanoma (B16) and mouse normal fibroblast (NIH/3T3) cell lines (ATCC, Rockvill, Maryland, USA) were used to determine the cytotoxicity of the prepared compounds by means of a Calcein AM assay, as previously described.[10] Briefly, the cells were maintained in plastic tissue culture flasks and were grown on Dulbecco's modified Eagle's cell culture medium (DMEM) at 37 °C in a 5% CO_2 atmosphere and 100% humidity. The cells were redistributed into 96-well microtitre plates (Nunc, Denmark). After 12 h of preincubation, the tested compounds were added, to give final concentration in the range 0.5–170 μM and the cells were incubated for another 72 h. At the end of this period, the cells were incubated for 1 h with Calcein AM and the fluorescence of the living cells was measured at 485/538 nm (ex/em) with a Fluoroskan Ascent reader (Labsystems, Finland). IC_{50} values, the drug concentrations lethal to 50% of the cancer cells, were determined from the dose–response curves. All experiments were repeated in quadruplicate with a maximum deviation of 15%. Because of their limited solubility in water, all the compounds tested were dissolved in DMSO and then diluted with water to a final DMSO concentration of 0.6%.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.03.038.

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Supplement III

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Synthesis, characterization and biological activity of ring-substituted 6-benzylamino-9-tetrahydropyran-2-yl and 9-tetrahydrofuran-2-ylpurine derivatives

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ABSTRACT

In an attempt to improve specific biological functions of cytokinins routinely used in plant micropropagation, 33 6-benzylamino-9-tetrahydropyran-2-ylpurine (THPP) and 9-tetrahydrofuran-2-ylpurine (THFP) derivatives, with variously positioned hydroxy and methoxy functional groups on the benzyl ring, were prepared. The new derivatives were prepared by condensation of 6-chloropurine with 3,4-dihydro-2*H*-pyran or 2,3-dihydrofuran and then by the condensation of these intermediates with the corresponding benzylamines. The prepared compounds were characterized by elemental analyses, TLC, HPLC, melting point determinations, CI+ MS and ^{1}H NMR spectroscopy. The cytokinin activity of all the prepared derivatives was assessed in three classical cytokinin bioassays (tobacco callus, wheat leaf senescence and *Amaranthus* bioassay). The derivatives 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**3**) and 6-(3-hydroxybenzylamino)-9-tetrahydrofuran-2-ylpurine (**23**) were selected, because of the high affinity of their parent compound *meta*-topolin (*m*T, 6-(3-hydroxybenzylamino)purine) to cytokinin receptors, as model compounds for studying their perception by the receptors CRE1/AHK4 and AHK3 in a bacterial assay. Both receptors perceived these two derivatives less well than they perceived the parent compound. Subsequently, the susceptibility of several new derivatives to enzyme degradation by cytokinin oxidase/dehydrogenase was studied. Substitution of tetrahydropyran-2-yl (THP) at the N^9 position decreased the turnover rates of all new derivatives to some extent. To provide a practical perspective, the cytotoxicity of the prepared compounds against human diploid fibroblasts (BJ) and the human cancer cell lines K-562 and MCF-7 was also assayed in vitro. The prepared compounds showed none or marginal cytotoxicity compared to the corresponding N^9-ribosides. Finally, the pH stability of the two model compounds was assessed in acidic and neutral water solutions (pH 3–7) by high-performance liquid chromatography (HPLC).

1. Introduction

Cytokinins belong to a group of plant growth hormones involved in the regulation of almost all stages of plant growth and development.[1] Naturally occurring cytokinins are based structurally on N^6-substituted adenine. Members of this group are classified as isoprenoid (ISCK) and aromatic (ARCK) according to the substituent on the N^6-atom of adenine.[1,2] Although ISCKs such as isopentenyladenine (iP), *trans*-zeatin (tZ) or dihydrozeatin have attracted the most attention,[3] ARCKs may also occur naturally and have been isolated, for example, from poplar leaves.[4–7] Two representatives of ARCKs, 6-benzylaminopurine (BAP) and 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *m*T) have been increasingly used in plant micropropagation; in particular, BAP is currently regarded as one of the most effective and affordable cytokinins for routine use.[8–16] Nevertheless, the application of BAP has some disadvantages in the propagation of many crop species, including problems with heterogeneity of growth and rooting inhibition.[16,17] One possible way to eliminate the undesirable side effects of BAP whilst simultaneously retaining its beneficial organogenic activity is the development of new BAP derivatives.[16,17]

Various modifications of BAP moiety by substitution, especially at the N^9 atom of the adenine, directly influence the invigorating effects of cytokinins on plant growth and are associated with the reinforcement of targeted cytokinin effects.[17,18] The most effective N^9-substituted derivatives developed so far are 6-benzylamino-9-tetrahydropyran-2-ylpurine (PBA) and 6-benzylamino-9-tetrahydrofuran-2-ylpurine (FBA), which have both proved to be considerably more active than BAP in evoking of several growth

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responses.[18] Skoog et al. observed that PBA is exceptionally potent in chlorophyll retention, while Arena et al. described the growth-promoting effect of PBA on adventitious shoot formation in comparison to kinetin or iP.[19,20] In addition, PBA has, for a long time, been used successfully to control flowering and inflorescence development in various plants.[21–23] It has been suggested, however, that the observed increased activity of these N^9-substituted derivatives could be a consequence of their ability to release free bases, thus supplying desirable concentrations over a prolonged period.[18] Thus, susceptibility to enzymatic cleavage is probably the critical factor determining the biological activity of N^9-substituted cytokinins. Hence, less active compounds are probably not susceptible to cleavage of N^9-substituents and exhibit low or zero activity because of their stability.[18,24,27–30] These data are somewhat controversial because of the fast release of free aglycone after the application of much less active 9-methyl-BAP that was observed by Fox et al.[24] These authors studied the metabolism of 9-methyl-BAP, considered to exhibit low activity, in tobacco and soybean callus tissue and demonstrated rapid conversion to several products. The metabolites were not identified definitively, although it was proposed that conversion to free BAP occurred.[24] Pietraface and Blaydes examined the metabolism of 9-methyl-BAP in germinating lettuce seed and, based on chromatographic data, suggested that BAP riboside and nucleotide formation was occurring.[25] Nevertheless, free BAP was not detected. Although free BAP has not been found as a metabolite, the conversion to BAP 9-glucoside and 7-glucoside (inactive metabolites) has been established unequivocally by mass spectrometry.[25] The formation of these glucosides presumably requires them to be converted to BAP.

Zhang and Letham studied the metabolism of several 9-substituted BAP derivatives and suggested that another reason for their enhanced antisenescence properties might originate from differences in the catabolism of BAP,[18] PBA and possibly FBA. Their study established that PBA and FBA were subject to both N^6-debenzylation and N^9-substituent breakage in soybean leaves. However, debenzylation was more prominent in the metabolism of PBA than in the metabolism of BAP. Besides, these compounds were less likely to form inactive or toxic metabolites.[26] With respect to the different types of cytokinin derivatives mentioned here, the most remarkable seemed to be 9-tetrahydropyranyl and 9-tetrahydrofuranyl BAPs. We, therefore, prepared new series of 6-(hydroxy- and 6-(methoxy-benzylamino)purines substituted by tetrahydropyran-2-yl (THP) and tetrahydrofuran-2-yl (THF) groups at the N^9 position of purine, in order to improve their biological functions and maintain or even decrease their already low cytoxicity. Both these groups are cyclic ethers in which the ether oxygen is attached to the carbon linked to the N^9 atom of the purine moiety. THP and THF were originally described as protective groups in organic chemistry that are easily removed under acidic conditions.[31,32] Hence, we examined the stability of selected model derivatives, specifically 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**3**) and 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**23**), in acidic or neutral water solutions (pH 3–7) to be sure that breakage of THP or THF did not occur in the media used for the biological and receptor assays. Furthermore, using the model compounds in a bacterial assay, we studied the effect of the THP and THF groups on the ability of *Arabidopsis* cytokinin receptors AHK3 and CRE1/AHK4 to detect the compounds.[33]

Several papers about ARCK derivatives have mentioned the relationship between benzyl ring substitutions and cytokinin activity.[33–36] Therefore, we also studied the differences in biological activity of 6-(hydroxy- and 6-(methoxybenzylamino)-9-tetrahydropyran-2-ylpurine derivatives in classical cytokinin bioassays. Cytokinin activity was compared in three tests—the stimulation of tobacco callus growth, the retention of chlorophyll in excised wheat leaves and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons. To investigate possible differences in in vivo stability, the newly prepared derivatives were also tested as substrates for cytokinin oxidase/dehydrogenase. This enzyme catalyzes irreversible cleavage of the N^6-substituent from the cytokinin molecule, leading to the total loss of biological activity, and seems to be one of the main mechanisms by which cytokinin homeostasis is maintained in plant tissues.[3]

It has been suggested recently that cytokinin derivatives substituted by ribose in the N^9 position exhibit significant cytotoxic activity.[36] In order to exclude cytotoxicity of newly prepared compounds with potential agricultural and cosmetic use their cytotoxic effect against human diploid fibroblasts (BG) and cancer cell lines (MCF-7 and K-532) was evaluated. The cytotoxic activity of selected THP and THF derivatives was then compared to the corresponding riboside analogues.

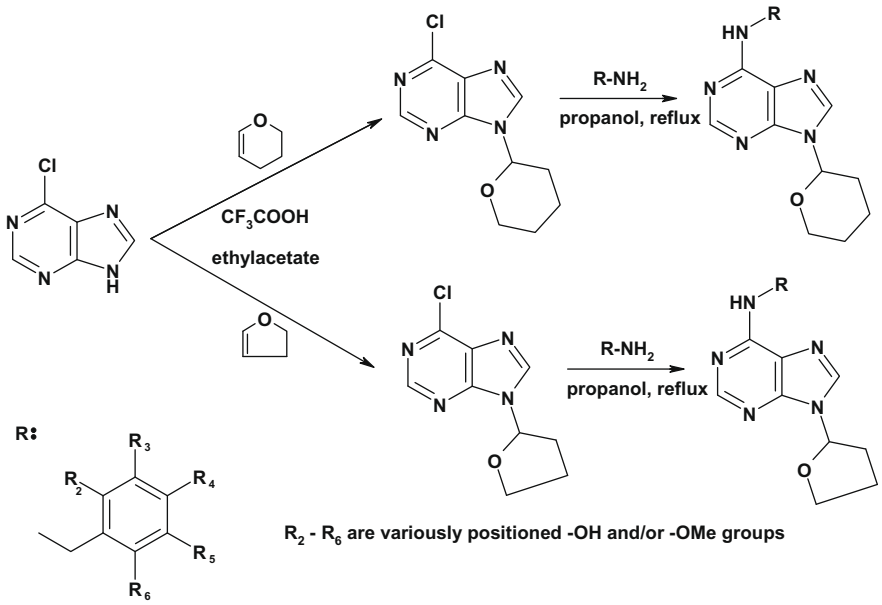
2. Results and discussion

2.1. Synthesis

Thirty-three ring-substituted derivatives of benzylamino-9-tetrahydropyran-2-ylpurine and 9-tetrahydrofuran-2-ylpurine were synthesized according to Scheme 1 (Table 1, Scheme 2). Prepared compounds were characterized by elemental analyses, thin layer chromatography (TLC), melting point determinations, CI+ MS and ^{1}H NMR spectroscopy. For better lucidity, Scheme 2 shows the schematic representation of newly prepared tetrahydropyran-2-yl and tetrahydrofuran-2-yl derivatives. The purity of prepared derivatives was confirmed by high-performance liquid chromatography (HPLC). Table 2 shows C, H, N elemental analysis data, mp, CI+ MS and HPLC purity data, whilst ^{1}H NMR spectral data are given in Supplementary data. Compounds PBA (**1**) and FBA (**21**) were prepared using a slightly modified version of a method previously published in the literature.[18] The melting points and ^{1}H NMR spectral data for compounds **1** and **21** prepared in our laboratory were consistent with the data found in the literature.[18] The preparation of new derivatives is described in greater detail in Section 4.3.

2.2. Stability in acidic solutions

Tetrahydropyran-2-yl (THP) and tetrahydrofuran-2-yl (THF) groups have been commonly used in organic chemistry as protective groups, readily removable under acidic conditions.[31] Therefore we verified that THP or THF groups did not break off under our bioassay conditions, since their removal would turn them into the corresponding free bases thereby influencing the cytokinin activity. 6-(3-Hydroxybenzylamino)-9-(tetrahydropyran-2-yl)purine (**3**) and 6-(3-hydroxybenzylamino)-9-(tetrahydrofuran-2-yl)-purine (**23**) were chosen as the model compounds. We performed HPLC stability measurements in 10^{-4} M stock solutions with pH decreasing from 7 to 3. The percentage of the THPP and THFP derivatives **3** and **23** together with the pH- and time-dependent release of the free base (*m*-topolin), as determined by HPLC, is given in Table 3. Both tested compounds were stable at pH 7 and 6 even 24 h after sample preparation. Compound **23** started to decompose after 24 h at pH 5 (9% of the free base released). A significant decomposition of **3** and **23** occurred after 24 h at pH 4 and increased significantly at pH 3 (Table 3). It can, therefore, be concluded that the prepared THPP and THFP cytokinin derivatives are not entirely stable at pH < 4 and under more acidic conditions they decompose to their free bases. Since the pH of the media in the assays used within the framework of this study varied between 6 and 7, we could be sure that the original compounds were those being tested.



Scheme 1. Schematic representation of the syntythetic pathway of THPP and THFP derivatives described in the study.

2.3. Cytokinin activity in bioassays

Cytokinin activity of all the prepared compounds was determined by three classical cytokinin bioassays (tobacco callus, wheat senescence and *Amaranthus* bioassays) and the results are presented in Table 4. The activities of the compounds were compared to those of BAP, which represents a highly active, commonly used cytokinin. Figure 1A–C compares the activity of 6-(*ortho*-, 6-(*meta*- and 6-(*para*-hydroxybenzylamino)tetrahydropyran-2-ylpurines in all three bioasays, showing a decrease in cytokinin activity between the substituents, as follows: *meta* > *ortho* ≫ *para*. These results are in agreement with those previously published in the literature related to *ortho*-, *meta*-, and *para*-topolin.[7,34,35]

For 6-(methoxybenzylamino)tetrahydropyran-2-ylpurine derivatives, the cytokinin activity decreased in the substituent order *ortho* > *meta* ≫ *para*; this is also consistent with the data found in the literature (Fig. 1D–F).[35,36] Moreover, it was found that substitution in the *para*-position caused the loss of activity in senescence as well as in tobacco callus bioassay. This finding has been reported recently for similar compounds,[36] differing in their N^9-substitution by ribose, and supported by the fact that compounds **4**, **7**, **18**, **24**, **27**, **30**, **32**, **34** and **35**, which are substituted in the *para* position by a methoxy group, were inactive in all three bioassays (Table 4, Fig. 1). We also compared the activity of THPP and THFP derivatives in tobacco callus, senescence and *Amaranthus* biotests with the corresponding free base. This test used compounds **3** and **23** and their parent compound 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *m*T). The results presented in Figure 2 show that the antisenescence activity of the THPP derivative was slightly higher than that of the free base and the THFP derivative (Fig. 2A). The activities of the THPP and THFP derivatives were comparable in the tobacco callus assay and did not differ significantly from the activity exhibited by the corresponding base (Fig. 2B). A different result was found in the *Amaranthus* bioassay, where both THPP and THFP derivatives were less active, although their activities were of a similar order to that in the senescence assay (Fig. 2C). Moreover, the results of *Amaranthus* and tobacco callus bioassay are similar to the values obtained for the corresponding ribotides.[7,34,36] However, the activity of methoxybenzyladenosines in senescence bioassay is twice higher in comparison with those for THPP and THFP derivatives. This, again, suggests that different receptor and/or signaling systems are involved in transduction of different cytokinin-dependent physiological responses.

Almost all THPP and THFP derivatives containing two or more hydro or methoxy groups on the benzyl ring were inactive in the tobacco callus bioassay; the exceptions were compounds **11**, **12** and **29**, although these exhibited only half the activity of BAP (Table 4). None of di- or tri- substituted compounds exceeded the activity of BAP either in the senescence or *Amaranthus* bioassays (Table 4). This fact supports the recently published fact that di- and trihydroxy (methoxy) BAPs are not strongly active in cytokinin assays.[35]

2.4. Recognition by cytokinin receptors

In our previous work we have shown that, in contrast to activity in the classical cytokinin bioassays, the position of ARCK benzyl ring substituents do not improve the recognition of a compound by cytokinin receptors in a bacterial assay.[35,36] Here we used a bioassay employing transformed *E. coli* strains expressing the cytokinin receptors AHK3 and CRE1/AHK4 with the cytokinin-activated reporter gene *cps::lacZ* to investigate the effect of THP and THF groups on the ability of *meta*-topolin, as a model compound, to activate the cytokinin signaling pathway through these receptors.[33,37,38] As shown in Figure 3, *m*T itself was recognized well by both receptors, although to a lesser extent than the *trans*-zeatin used as a control. In contrast, the THPP and THFP derivatives were not detected by CRE1/AHK4 at all (Fig. 4A). Partial receptor activation occurred only with AHK3 at the highest concentration, corroborating the sensitivity of AHK3 to N^9-substituted cytokinins (Fig. 3).[33] The experiment showed that THP and THF ARCK derivatives are only weak ligands of the cytokinin receptors. This fact means that, like other aromatic cytokinins, a different recognition system that is able to interact with BAP derivatives may exist in plants.[35]

2.5. Enzymatic degradation in vitro

Cytokinin oxidase/dehydrogenase in higher plants is encoded by small gene families, and the enzymes are distinguished by diverse subcellular compartmentalization as well as by different spa-

Table 1
Structures of the prepared compounds and their abbreviations

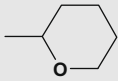
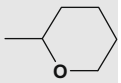
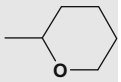
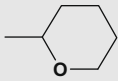
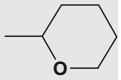
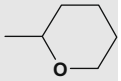
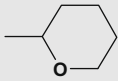
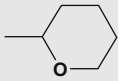
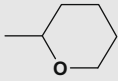
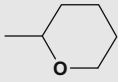
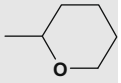
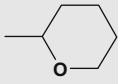
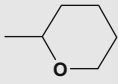
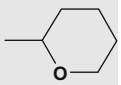
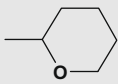
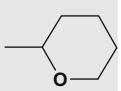
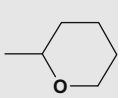
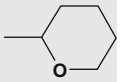
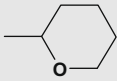
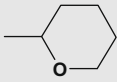
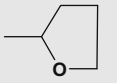
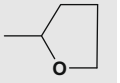
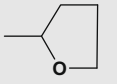
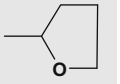
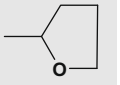
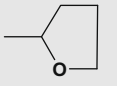
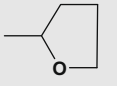
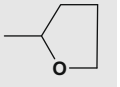
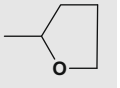
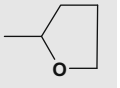
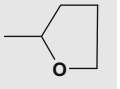
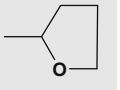
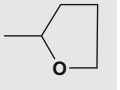
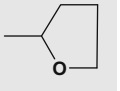
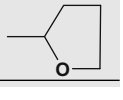
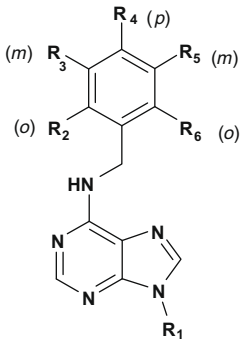
Compound	R_2 (*o*)	R_3 (*m*)	R_4 (*p*)	R_5 (*m*)	R_6 (*o*)	R_1
PBA (**1**)	H	H	H	H	H	
2	OH	H	H	H	H	
3	H	OH	H	H	H	
4	H	H	OH	H	H	
5	OCH_3	H	H	H	H	
6	H	OCH_3	H	H	H	
7	H	H	OCH_3	H	H	
8	OH	OCH_3	H	H	H	
9	H	OCH_3	OH	H	H	
10	OCH_3	H	OCH_3	H	H	
11	OCH_3	H	H	OCH_3	H	
12	OCH_3	H	H	H	OCH_3	
13	H	OCH_3	OCH_3	H	H	
14	H	OCH_3	H	OCH_3	H	
15	OH	OH	H	H	H	
16	H	OH	H	OH	H	
17	OCH_3	OCH_3	OCH_3	H	H	

Table 1 (*continued*)

Compound	R_2 (*o*)	R_3 (*m*)	R_4 (*p*)	R_5 (*m*)	R_6 (*o*)	R_1
18	OCH_3	H	OCH_3	OCH_3	H	
19	OCH_3	H	OCH_3	H	OCH_3	
20	H	OCH_3	OCH_3	OCH_3	H	
FBA (**21**)	H	H	H	H	H	
22	OH	H	H	H	H	
23	H	OH	H	H	H	
24	H	H	OH	H	H	
25	OCH_3	H	H	H	H	
26	H	OCH_3	H	H	H	
27	H	H	OCH_3	H	H	
28	H	OCH_3	OCH_3	H	H	
29	H	OCH_3	H	OCH_3	H	
30	OCH_3	H	OCH_3	H	H	
31	OCH_3	H	H	OCH_3	H	
32	H	OCH_3	OH	H	H	
33	OCH_3	OCH_3	OCH_3	H	H	
34	OCH_3	H	OCH_3	OCH_3	H	
35	H	OCH_3	OCH_3	OCH_3	H	



Scheme 2. Schematic representation of tetrahydropyran-2-yl and tetrahydrofuran-2-yl derivatives; (*o*) indicates *ortho*-, (*m*) *meta*- and (*p*) *para*- position in the benzyl ring.

tial and temporal expression patterns.[39] In this study, the recombinant maize enzyme *Zea mays* cytokinin oxidase/dehydrogenase 1 (ZmCKX1) was used to study the ability of selected compounds, especially those that bioassays showed to be more active than their corresponding free bases, to be cleaved as substrates by this enzyme. This protein has previously been localized in the apoplast of vascular bundles, and exhibits its peak expression there in germinated seeds and young roots.[40,41] Thus, it can contribute to the degradation of exogenous cytokinins in micropropagation systems where these, and other additives, are absorbed from the growing medium through the developing roots and are then distributed via the xylem bundles.

ARCKs are, in general, poor substrates for cytokinin oxidase/dehydrogenase activity; this probably increases their stability in vivo compared to isoprenoid cytokinins. None of the seven isoenzymes of cytokinin oxidase/dehydrogenase from *Arabidopsis thaliana* prefer aromatic cytokinins.[42] It has been shown that aromatic rings act as spherical obstacles to the formation of substrate/product intermediates. Thus, the catalytic reaction cannot be speeded up even in the presence of appropriate electron acceptors within ISCK, which should transfer electrons from the reduced FAD cofactor of the enzyme and thus dramatically increase the rate of degradation.[43] Kinetic constants of maize cytokinin oxidase/dehydrogenase for selected THPP derivatives compared to their free bases, BAP, BAPR and PBA are given in Table 5. N^9-substitution by THP decreases turnover rates of all aromatic cytokinins to some extent. Furthermore, the lower susceptibility to degradation by ZmCKX1 of these derivatives is sustained by higher K_m values, decreasing the total efficiency (k_{cat}/K_m) by a factor of 7–33. The higher activity of THPP derivatives in bioassays may therefore be explained by their greater resistance to enzymatic breakdown by CKX, which increases their stability and prolongs their lifespan in plant tissues. Previous studies of the metabolism of several 9-substituted BAP derivatives have suggested that the reason for their enhanced cytokinin activity might originate from differences in the catabolism of BAP, PBA and possibly FBA.[18] This attribute can even overcome the poorer recognition of THPP and THFP derivatives by the known cytokinin receptors.

Table 2
Elemental analyses, melting points and CI+ MS and HPLC purity of the prepared compounds

Compound	Elemental analyses calculated/found			Mp (°C)	CI+ MS [M+H$^+$]	HPLC (%)
	%C	%H	%N			
PBA,1	66.0/66.1	6.2/6.1	22.6/22.5	110–112	310	>98
2	62.8/62.7	5.9/5.8	21.5/21.6	172–175	326	>98
3	62.8/62.4	5.9/5.9	21.5/21.3	120–121	326	>99
4	62.8/62.8	5.9/5.9	21.5/21.3	173–174	326	>99
5	63.7/63.9	6.2/6.3	20.6/20.7	106–108	340	>98
6	63.7/63.9	6.2/6.3	20.6/20.6	134–135	340	>98
7	63.7/63.9	6.2/6.2	20.6/20.6	137–138	340	>99
8	60.8/60.7	5.9/5.9	19.7/19.8	187–188	356	>98
9	60.8/60.6	5.9/6.0	19.7/19.8	189–190	356	>98
10	61.8/61.8	6.3/6.3	18.9/18.7	479–180	370	>98
11	61.8/61.7	6.3/6.4	18.9/18.9	150–151	370	>98
12	61.8/61.8	6.3/6.3	18.9/18.9	170–171	370	>98
13	61.8/61.9	6.3/6.3	18.9/18.8	156–157	370	>98
14	61.8/61.9	6.3/6.4	18.9/18.9	145–146	370	>98
15	59.8/59.9	5.6/5.6	20.5/20.5	174–175	342	>98
16	59.8/59.9	5.6/5.7	20.5/20.4	212–214	342	>99
17	60.1/60.2	6.3/6.2	17.5/17.5	142–143	400	>98
18	60.1/60.2	6.3/6.4	17.5/17.6	128–129	400	>98
19	60.1/60.0	6.3/6.4	17.5/17.4	178–179	400	>98
20	60.1/59.9	6.3/6.3	17.5/17.8	127–128	400	>98
FBA,21	65.1/65.1	5.8/5.8	23.7/23.8	100–102	296	>98
22	61.7/61.9	5.5/5.5	22.5/22.3	135–136	312	>98
23	61.7/61.7	5.5/5.5	22.5/22.6	124–126	312	>98
24	61.7/61.8	5.5/5.6	22.5/22.5	182–183	312	>98
25	62.8/62.9	5.9/5.8	21.5/21.5	97–99	326	>98
26	62.8/62.9	5.9/6.0	21.5/21.5	87–88	326	>98
27	62.8/62.9	5.9/5.8	21.5/21.5	182–183	326	>98
28	60.8/60.9	5.9/5.9	19.7/19.8	131–132	356	>98
29	60.8/60.7	5.9/5.7	19.7/19.6	99–100	356	>98
30	60.8/60.7	5.9/5.9	19.7/19.6	125–126	356	>98
31	60.8/60.6	5.9/5.8	19.7/19.7	103–104	356	>98
32	59.8/60.0	5.6/5.7	20.5/20.5	140–142	342	>98
33	59.2/59.1	6.0/6.0	18.2/18.2	140–141	386	>98
34	59.2/59.2	6.0/5.9	18.2/18.2	99–100	386	>98
35	59.2/59.3	6.0/6.0	18.2/18.3	118–119	386	>98

2.6. Cytotoxicity

The substitution of certain BAP derivatives with ribose moiety in the N^9 position is known to significantly increase their cytotoxicity against human cells.[35,36] Therefore, we compared the cytotoxic effects of corresponding ribosyl and tetrahydropyran-2-yl derivatives in vitro against cancer human cell lines (breast carcinoma, MCF-7, and chronic myelogenous leukaemia, K-562) by Calcein AM viability assay. The results summarizing the IC_{50} values for all the tested compounds are presented in Table 6.

We discovered that the alternation of ribose by a THP or THF group in the N^9 position can markedly decrease cytotoxicity. Figure 4 shows a comparison of dose–response curves for 6-(2-hydroxy-3-methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (**8**) and 6-(2,3-dihydroxy-benzylamino)-9-tetrahydropyran-2-ylpurine (**15**) and their analogues with ribose at N^9. While cytotoxic effects of both THPP derivatives against the MCF-7 cell line were negligible, their riboside analogues 6-(2-hydroxy-3-methoxybenzylamino)-9-β-D-ribofuranosylpurine and 6-(2,3-dihydroxybenzylamino)-9-β-D-ribofuranosylpurine exhibited significant cytotoxicity with IC_{50} values of 20.2 μmol/L and 5.2 μmol/L, respectively. Similar results were obtained for K-562 cells where the replacement of ribose moiety with a THP group led to an increase in the IC_{50} value from 27.9 μmol/L to >100 μmol/L (compound **8**) and from 27.9 to 92 μmol/L (compound **15**). IC_{50} values for the corresponding free bases were above 100 μmol/L.[35]

The cytotoxicity of the prepared compounds was also evaluated in human diploid fibroblasts (BJ) by MTT viability assay. Almost all the tested THPP and THFP compounds were only marginally toxic or were non-toxic, even at the highest concentration tested (100 μmol/L or solubility limit). Their toxicity profile was comparable or better than the profile of the corresponding cytokinin bases under the assay conditions.[35] We, therefore, conclude that tetrahyropyranylation or tetrahydrofuranylation of cytokinins can lead to a general reduction in cytotoxicity, a useful property in agricultural applications and cosmetics.

3. Conclusions

We prepared and characterized thirty-three hydroxy and/or methoxy ring-substituted 6-benzylamino-9-tetrahydropyran-2-ylpurine and 6-benzylamino-9-tetrahydrofuran-2-yl derivatives. The prepared compounds were subjected to three cytokinin bioassays. The N^9 substitution by a THP or THF group either did not change or improved the biological activity of free bases recorded in classical cytokinin bioassays, although it negatively influenced the recognition of these compounds by cytokinin receptors. The improved activity of the THP cytokinins could be explained by their higher resistance to enzymatic breakdown by CKX. The resistance to degradation, and thus probably their prolonged lifespan in vivo, can be enhanced by the presence of hydroxy- or methoxy groups in the *meta* position of the benzyl ring. The specificity constant (k_{cat}/K_m) of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine toward ZmCKX1 is 25-fold and 530-fold lower than that for 6-benzylaminopurine and 6-(3-hydroxybenzylamino)purine, respectively. However, in vivo metabolic studies of the newly prepared compounds are required to explain in more detail the mode of action of THPP and THFP derivatives in plants. Such a study is underway in our laboratory.

The hydroxy- and methoxybenzyl THFPs and THPPs with high levels of cytokinin activity, along with the majority of newly prepared compounds, were found to be non-toxic in the cytotoxicity tests performed in vitro on different cancer and normal human cell lines. HPLC assaying of the stability of *meta*-topolin THPP and THFP derivatives at various pHs (3–7) showed that the compounds were stable in the pH range of the media used for the cytokinin bioassays. The fact that almost all the substances exhibited no or only slight cytotoxicity even at high concentrations is very encouraging and may mean that these cytokinins can be used not only in the

Table 3
Stability of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (3) and 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (23) in acidic solutions

pH	Compound peak area (%)			
	3	Free base	**23**	Free base
(A) 1 h After sample preparation				
7	99.9	0.1	99.7	0.3
6	99.8	0.2	99.6	0.4
5	99.5	0.5	99.5	0.5
4	99.5	0.5	97.2	2.8
3	98.8	1.2	63.8	36.2
(B) 24 h After sample preparation				
7	99.8	0.2	99.7	0.3
6	99.3	0.7	99.4	0.6
5	99.2	0.8	90.8	9.2
4	93.9	6.1	37.5	62.5
3	39.6	60.4	0.3	99.7

The pH stability of compounds **3** and **23** in 10^{-4} M water solutions with pHs decreasing from 7 to 3 measured 1 h (A) and 24 h (B) after sample preparation. The percentage of the released free base 6-(3-hydroxybenzylamino)purine (*m*T) was determined by HPLC.

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

Compound	Tobacco callus bioassay		*Amaranthus* bioassay		Senescence bioassay	
	Optimal concentration ($mol\ l^{-1}$)	Relative activity(%)	Optimal concentration ($mol\ l^{-1}$)	Relative activity (%)	Optimal concentration ($mol\ l^{-1}$)	Relative activity (%)
PBA (1)	10^{-6}	102 (±2)	10^{-4}	114 (±7)	10^{-4}	104 (±1)
2	10^{-5}	37 (±2)	10^{-4}	46 (±9)	10^{-4}	31 (±10)
3	10^{-6}	95 (±8)	10^{-4}	100 (±6)	10^{-4}	140 (±10)
4	10^{-5}	20 (±9)	10^{-4}	34 (±9)	10^{-4}	4 (±8)
5	10^{-6}	93 (±9)	10^{-4}	84 (±2)	10^{-4}	104 (±5)
6	10^{-6}	90 (±1)	10^{-4}	76 (±2)	10^{-4}	72 (±5)
7	10^{-6}	10 (±6)	10^{-4}	18 (±5)	n.a.	
8	10^{-4}	12 (±4)	10^{-4}	18 (±7)	n.a.	
9	10^{-4}	8.7 (±2)	10^{-4}	26 (±0.2)	10^{-4}	7 (±3)
10	n.a.		10^{-4}	9 (±4)	10^{-4}	25 (±1)
11	10^{-5}	42 (±10)	10^{-4}	28 (±5)	10^{-4}	19 (±3)
12	10^{-4}	57 (±5)	10^{-4}	13 (±1)	10^{-4}	14 (±3)
13	n.a.		10^{-4}	8 (±3)	10^{-4}	8 (±0.3)
14	n.a.		10^{-4}	9 (±2)	10^{-4}	7 (±4)
15	10^{-4}	3,2 (±0.1)	10^{-4}	8 (±3)	n.a.	
16	10^{-5}	36 (±1)	10^{-4}	33 (±6)	10^{-4}	15 (±8)
17	n.a.		10^{-4}	4 (±3)	10^{-4}	7 (±4)
18	10^{-4}	11 (±3)	10^{-4}	3 (±2)	n.a.	
19	10^{-4}	2 (±1)	10^{-4}	14 (±2)	10^{-4}	3 (±2)
20	10^{-4}	17 (±9)	10^{-4}	19 (±6)	10^{-4}	7 (±4)
FBA (21)	10^{-6}	95 (±3)	10^{-4}	109 (±9)	10^{-4}	107 (±8)
22	10^{-4}	58 (±8)	10^{-4}	41 (±7)	10^{-4}	25 (±8)
23	10^{-6}	87 (±11)	10^{-4}	82 (±5)	10^{-4}	131 (±7)
24	10^{-5}	19 (±1)	10^{-4}	10 (±5)	10^{-4}	8 (±6)
25	10^{-5}	91 (±7)	10^{-4}	107 (±10)	10^{-4}	72 (±1)
26	10^{-6}	96 (±2)	10^{-4}	123 (±8)	10^{-4}	82 (±11)
27	10^{-6}	7 (±3)	10^{-4}	25 (±6)	10^{-4}	16 (±4)
28	10^{-6}	3 (±2)	10^{-4}	5 (±2)	10^{-4}	7 (±1)
29	10^{-4}	14 (±6)	10^{-4}	18 (±3)	10^{-4}	8 (±0.1)
30	10^{-6}	3 (±0.1)	10^{-4}	14 (±2)	10^{-4}	18 (±9)
31	10^{-5}	61 (±7)	10^{-4}	41 (±9)	10^{-4}	41 (±2)
32	10^{-4}	13 (±2)	10^{-4}	17 (±6)	10^{-4}	4 (±5)
33	10^{-4}	7 (±1)	10^{-4}	15 (±2)	10^{-4}	9 (±4)
34	10^{-4}	14 (±4)	10^{-4}	14 (±2)	10^{-4}	9 (±1)
36	10^{-4}	4 (±2)	10^{-4}	17 (±3)	10^{-4}	10 (±4)

n.a. Means not active.

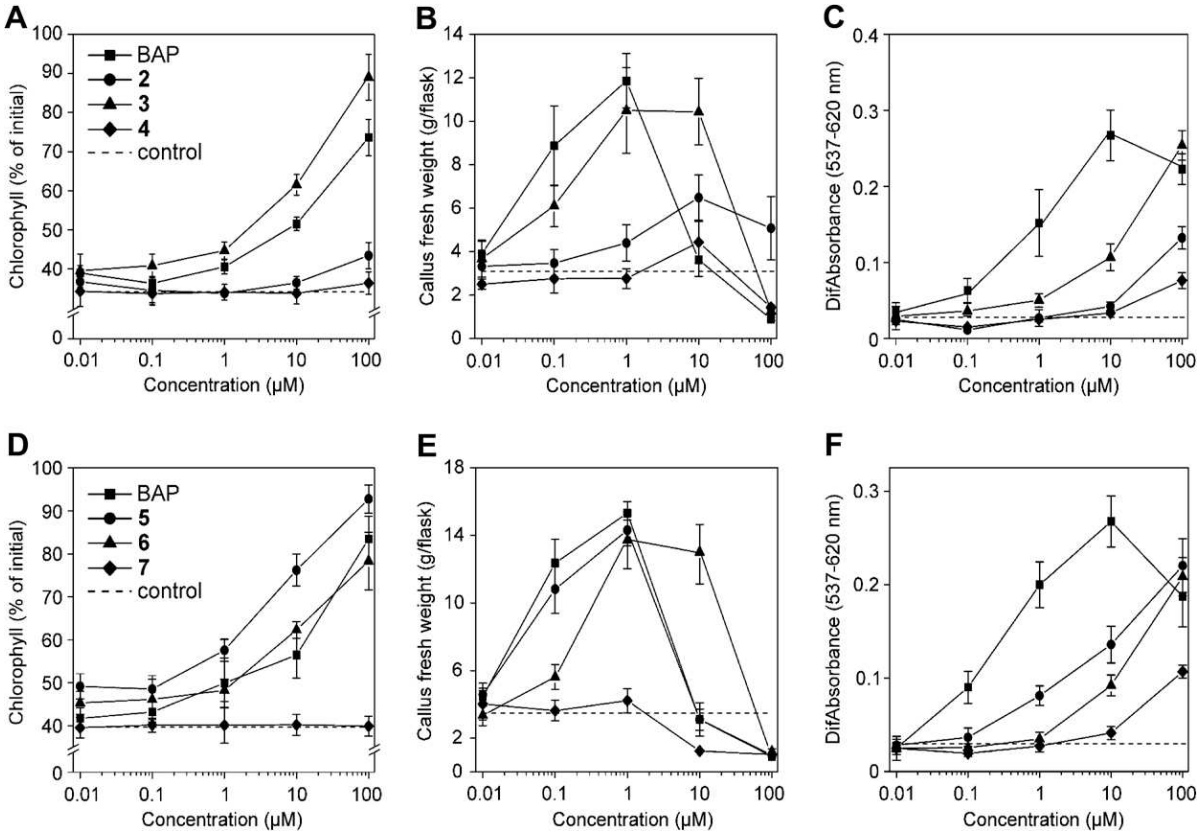


Figure 1. The influence of hydroxy or methoxy group position in the benzyl ring of 6-benzyl-9-tetrahydropyran-2-yl-purines on their biological activity in classical cytokinin bioassays. Comparison of the impact of hydroxy (A–C) and methoxy (D–F) groups substituted in *ortho*- (circles), *meta*- (triangles), and *para*- (diamonds) positions, respectively, on: the retention of chlorophyll in excised wheat leaves (A, D); the stimulation of cytokinin-dependent tobacco callus growth (B, E); and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons (C, F). The graphs show representative examples of results obtained in individual bioassays. BAP was used as a positive control (squares), the dashed line shows the solvent control (0.2% DMSO). Error bars represent SD ($n = 5$).

regulation of plant growth and development, agriculture and plant biotechnology, but also, potentially, in cosmetics, where the use of cytotoxic substances is prohibited.

4. Experimental

4.1. Chemicals

6-Chloropurine was purchased from Olchemim, triethylamine (TEA), ethyl acetate (EtOAc), *n*-propanol, *n*-butanol, isopropanol, diethyl ether, hexane, *N*,*N*′-dimethylsulfoxide and other common organic solvents used for syntheses and subsequent crystallization of the products were purchased from Sigma–Aldrich and Lachema, and used as received. 2,3-Dihydrofuran (98%) was purchased from Across Organics; 3,4-dihydro-2*H*-pyran and trifluoracetic acid were obtained from Fluka; furfurylamine, 2-methoxybenzylamine, 3-methoxybenzylamine, 4-methoxy-benzylamine, 2,4-dimethoxybenzylamine, 2,5-dimethoxybenzylamine, 2,6-dime-thoxybenzylamine, 3,4-dimethoxybenzylamine, 3,5-dimethoxybenzylamine, 2,4,6-trimethoxybenzylamine, 3,4,5-trimethoxybenzylamine, aniline, 4-methoxyaniline, 4-aminophenol were obtained from Aldrich; 2-hydroxybenzylamine, 3-hydroxybenzylamine, 4-hydroxy-

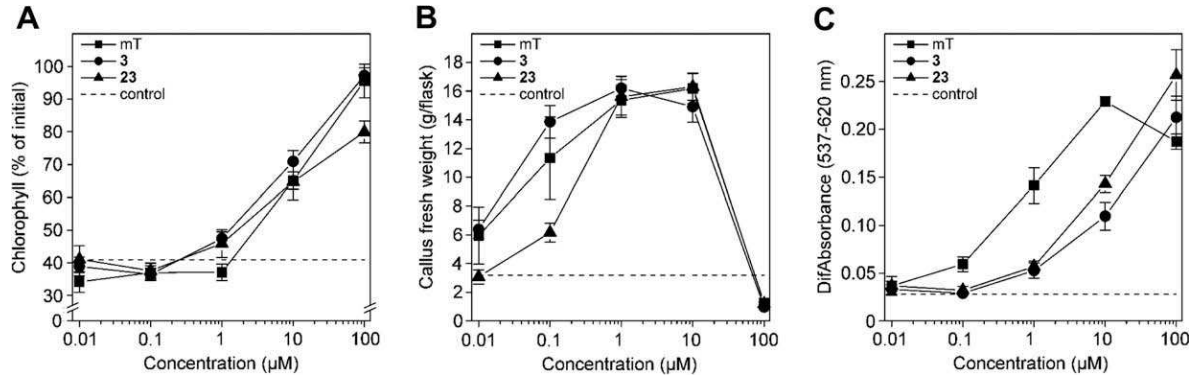


Figure 2. The influence of 9-tetrahydropyran-2-yl and 9-tetrahydrofuran-2-yl groups on the biological activity of 6-benzyl-(3-hydroxybenzylamino)purine in classical cytokinin bioassays. (A) The effect on chlorophyll retention in excised wheat leaf tips; (B) the growth of cytokinin-dependent tobacco callus; (C) the effect on dark betacyanin synthesis in *Amaranthus caudatus* cotyledon-hypocotyl explants. The dashed line shows the solvent control (0.2% DMSO). Error bars represent SD ($n = 5$).

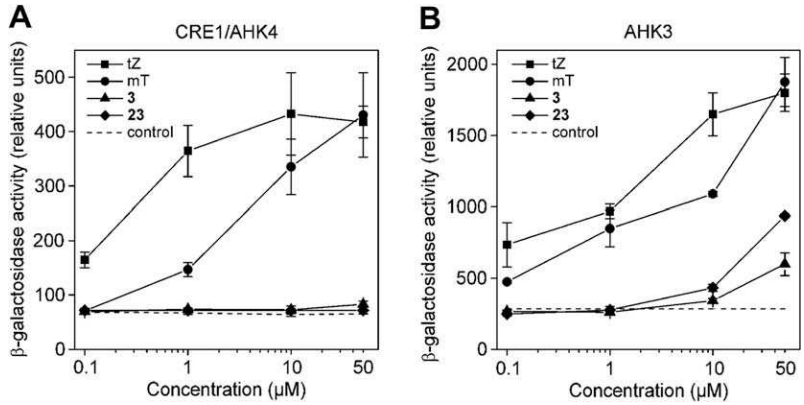


Figure 3. Comparison of the perception of THP and THF substituted 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *m*T) and *m*T by selected cytokinin receptors. The effect of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (6(3OHBA)9THPP) and 6-(3-hydroxybenzylamino)-9-tetrahydrofuran-2-ylpurine (6(3OHBA)9THFP) on the sensing of *m*T by the cytokinin receptors CRE1/AHK4 (A) and AHK3 (B). *trans*-Zeatin (tZ) was used as the positive control. The dashed line shows the solvent control (0.5% DMSO). Error bars show SD ($n = 3$).

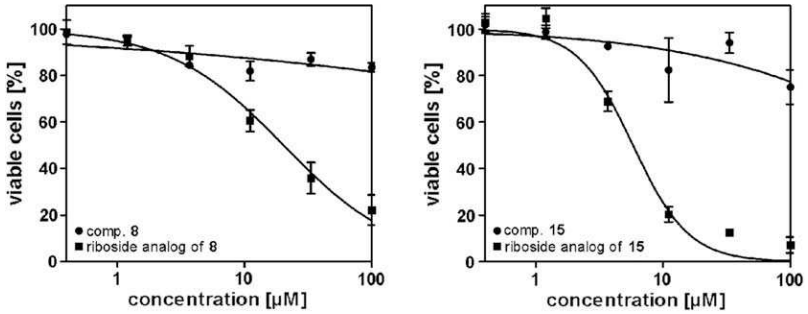


Figure 4. The effect of THP substitution on the cytotoxicity of MCF-7 cells compared to the corresponding ribosides. The compounds 6-(2-hydroxy-3-methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (**8**) and 6-(2,3-dihydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**15**) did not reduce the number of viable MCF-7 cells. The MCF-7 human breast cancer cell line was treated for 72 h with increasing concentrations of **8**, **15** or their riboside analogues. Then, the number of viable cells was determined by a Calcein AM assay. Results represent the average ± SD in three independent experiments.

benzylamine, 4-hydroxy-3-methoxybenzylamine, 2,3-dihydroxybenzylamine, 2,3,4-trimethoxybenzylamine, 2,4,5-trimethoxybenzylamine were supplied by Olchemim, The preparation and characterization of the commercially unavailable 2-hydroxy-3-methoxybenzylamine hydrobromide and 3,5-dihydroxybenzylamine hydrobromide have been described previously.[35] Mili-Q water was used throughout. The other solvents and chemicals used were all of standard *pa* quality. The substances PBA (**1**) and (**21**) were prepared according to slightly modified versions of standard methods described in the literature.[18]

Table 5
Kinetic constants of maize cytokinin oxidase/dehydrogenase for selected cytokinins and their THPP derivatives; enzyme activity was determined by measuring the amount of aldehyde produced by oxidative cleavage of the side chain of the measured cytokinins, using the 4-aminophenol assay[42]

Compound	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m
iP	0.208	1.8	0.1156
BAP	0.225	29.6	0.0076
BAPR	0.218	n.d.	n.d.
PBA, **1**	0.069	98.9	0.0007
*m*T	0.244	24.4	0.0100
3	0.032	125.0	0.0003
6-*fb*	0.145	n.d.	n.d.
6	0.006	n.d.	n.d.
4-*fb*	0.871	5.5	0.1583
4	0.195	33.8	0.0058
7-*fb*	0.476	n.d.	n.d.
7	0.256	n.d.	n.d.

All data represent mean values of at least two replicates. Deviations between replicates did not exceed 10%; n.d.—not determined.
iP: N^6-(2-isopentenyl)adenine, BAP: 6-benzylaminopurine, BAPR: 6-benzylaminopurine-9-ribosylpurine, *fb*: free base.

4.2. General procedures

Evaporations were carried out under vacuum rotary oil pump for *n*-propanol, *n*-butanol and ethyl acetate. Elemental analyses (C, H, N) were determined on an EA1112 Flash analyzer (Thermo-Finnigan). The melting points were determined on Büchi Melting Point B-540 apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck Co.). $CHCl_3$:MeOH (9:1, v:v) or ethyl acetate/toluene (8:2, v:v) were used as solvents. CI+ mass spectra were recorded using a Polaris Q (Finnigan) mass spectrometer equipped with a Direct Insertion Probe (DIP). The compounds were heated in an ion source with a 40–450 °C temperature gradient, the mass monitoring interval was 50–1000 am, and spectra were collected using 1.0 s cyclical scans, applying 70 eV electron energy. In the CI+ ionization mode, isobutane was used as a reagent gas at a flow-rate of 2 L/h. The mass spectrometer was directly coupled to an Xcalibur data system. ^1H spectra were recorded on a Bruker Avance 300 spectrometer operating at a temperature of 300 K and a frequency of

Table 6
Cytotoxicity IC_{50} values of the prepared compounds in Calcein AM (K-562 and MCF-7) and in MTT (BJ) assays

Compound	Cell line/IC_{50} (μmol/L)		
	K-562	MCF-7	BJ
1	>100	>100	>100
2	>100	>100	>100
3	81	>100	>100
4	>100	>100	>100
5	97	>100	>100
6	61	97	>100
7	>100	>100	>100
8	>100	>100	>100
9	>100	>100	>100
10	>25	>25	>25
11	16.5	25.4	>100
12	10.3	>100	>100
13	>100	>100	>100
14	>100	>100	>100
15	92	>100	63.3
16	>100	>100	>100
17	>37.5	>37.5	>37.5
18	>100	>100	>100
19	>50	>50	>50
20	>100	>100	>100
21	>100	>100	>100
22	>100	>100	>100
23	>100	>100	>100
24	>100	>100	>100
25	>100	>100	>100
26	>100	>100	>100
27	>100	>100	>100
28	>100	>100	>100
29	>100	>100	>100
30	>100	>100	>100
31	>50	>50	>50
32	>100	>100	>100
33	>100	>100	>100
34	>100	>100	>100
35	>100	>100	>100

The maximum concentration tested was 100 μmol/L, except for compounds **11** and **17,** where lower concentrations (25 and 37 μmol/L, respectively) were used due to their limited solubility in the culture media.

300.13 Hz. Samples were prepared by dissolving the substances in DMSO-d_6. Tetramethylsilane (TMS) was used as the internal reference standard.

4.3. Synthesis of 9-tetrahydropyran-2-ylpurine (2–20) and 9-tetrahydrofuran-2-ylpurine (22–35) cytokinin derivatives

The synthesis of the derivatives consisted of two independent steps, as presented in Scheme 1. In the first step, we employed a modified version of a method found in the literature.[44,45] 6-chloropurine (10 g, 64.7 mmol) was stirred with 3,4-dihydro-2*H*-pyrane (12.4 g, 147 mmol, 14 mL) for 6-chloro-9-tetrahydropyran-2-ylpurine (**1a**) or with 2,3-dihydrofurane (11.3 g, 162 mmol, 12 mL) for 6-chloro-9-tetrahydrofuran-2-ylpurine (**1b**), for 10 min in ethyl acetate (100 mL) and subsequently, trifluoroacetic acid (10.9 g, 84.2 mmol for **1a** and 9.6 g, 95.5 mmol for **1b**), was added dropwise. The reaction mixture was stirred at room temperature for 1 h and then neutralized by the appropriate amount of a mixture of ammonia and water (2:3). The ethyl acetate phase was separated and purified using charcoal and SiO_2. Yellowish waxy products were obtained after vacuum evaporation of the solvents. The products were washed with cyclohexane (**1a**) or hexane (**1b**) and dried in the air. The yields, HPLC purity, MS (CI+) and ^{1}H NMR data, along with the melting points (mp) are presented in Supplementary data section. In the second step, **1a** (1 g, 4.2 mmol) or **1b** (1 g, 4.4 mmol) was coupled with the appropriate benzylamine in 1:1.2 ratio in *n*-propanol or *n*-butanol (30 mL) in the presence of triethylamine (4 g, 39.5 mmol, 5 mL) at about 100 °C. The reaction lasted for 3 h. The reaction mixture was then evaporated to dryness and the residue was treated with 50 mL of ethyl acetate and 50 mL of distilled water. The ethyl acetate phase was separated and purified using SiO_2 and charcoal and then dried over Na_2SO_4. Final products were isolated by crystallization using diethyl ether or hexane and dried in air. C, H, N elemental analyses, CI+ MS and HPLC purity data and mp for compounds **2**–**35** are given in Table 2 while the yields and ^{1}H NMR are given in Supplementary data.

4.4. pH Stability testing of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (3) and 6-(3-hydroxybenzylamino)-9-tetrahydrofuran-2-ylpurine (23)

The pH stability of compounds **3** and **23** was analyzed by HPLC-PDA (System Gold; Beckman Instruments, Fullerton, CA, USA); analytes were monitored at 270 nm. 10^{-2} M solutions of compounds **3** or **23** in methanol were prepared and diluted to 10^{-4} M using McIlvaine buffer solution for the appropriate pH (3, 4, 5, 6 or 7).[46] One hour after incubation at 25 °C, 5 μL of the prepared 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, 10% A; 0–25 min, a linear gradient to 90% A; followed by 5 min isocratic elution of 90% A, where A was 100% methanol and B was 15 mM formic acid adjusted to pH 4 with ammonium. The HPLC measurement of the solutions was repeated after a 24 h incubation at 25 °C. The analyses were repeated at least three times.

4.5. Cytokinin bioassays

All the prepared compounds were tested in three cytokinin bioassays—the tobacco callus, *Amaranthus* and senescence bioassays—and their activity was compared with BAP. The biological activities of THP and THF derivatives of 6(3-hydroxybenzylamino)purine (**3**, **23**) and 6(3-methoxybenzylamino)purine (**6, 26**) were compared to those of free bases. Tested cytokinin derivatives were dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to 5×10^{-2} M solutions. This stock solution was further diluted in the media appropriate to each biotest to concentrations from 10^{-8} to 10^{-4} M. The final concentration of DMSO in the media did not exceed 0.2% and thus did not affect the biological activity of the substance tested in the assay. Five replicates were prepared for each compound concentration and the entire tests were repeated at least twice. Detailed descriptions of the conditions and performance of the tobacco callus bioassay, *Amaranthus* bioassay and senescence bioassay are given in Zatloukal et al.[47]

4.6. Bacterial receptor assay

Escherichia coli KMI001 strains harboring plasmids pIN-III-AHK4 and pSTV28-AHK3 (Suzuki 2001, Yamada 2001,) were provided by Dr. T. Mizuno. The strains were used in the bacterial receptor assays as described, but with a slight modification.[33] The assay was optimized to 96-well microtiter plates according to the literature.[35] The preculture was diluted 1:10 and the incubation time was 6 h. Relative activation of cytokinin receptors was determined by measuring β-galactosidase activity using the fluorescent substrate 4-methylumbelliferyl-β-D-galactoside (Sigma) and monitoring the culture density at OD_{600}. The test was performed in triplicate and the entire test was repeated at least twice.

4.7. Enzymatic cytokinin degradation assay

The enzyme, recombinant ZmCKX1 produced by the yeast *Pichia pastoris* and purified as described previously,[48] was obtained from Dr. Kristin Bilyeu from USDA/ARS Plant Genetics Research Unit, University of Missouri, Columbia, USA. The end-point assay was performed according to the literature.[49] The reaction mixture for measuring dehydrogenase activity contained 100 mM McIlvaine buffer pH 6.0, an aliquot of 10 mM cytokinin substrate stock dissolved in DMSO and 0.1 mM 2,6-dichlorophenolindophenol. The final concentration of the substrate, when the k_{cat} parameter was determined, was 0.2 mM. The Michaelis constants were estimated using a double reciprocal plot with the substrate concentration in the range 0.05–0.25 mM.

4.8. Cytotoxicity testing

4.8.1. Calcein AM assay

The cytotoxicity of the prepared compounds against human chronic myelogenous leukaemia (K-562) and human breast carcinoma (MCF-7) cell lines was determined by standard Calcein AM assay.[35] The cells were maintained in plastic tissue culture flasks and grown in Dulbecco's modified Eagle's cell culture medium (DMEM) at 37 °C in a 5% CO_2 atmosphere and 100% humidity. The cells were seeded into 96-well microtitre plates (Nunc, Denmark) and after 12 h of preincubation, the tested compounds were added to give a final concentration in the range 0–100 μM. The concentration was adjusted in the case of compounds with limited solubility. The cells were incubated for another 72 h. At the end of the incubation period Calcein AM in PBS was added to a final concentration of 1 μg/mL. After another 1 h of incubation, fluorescence at 485/538 nm (ex/em) was measured with a Fluoroskan Ascent reader (Labsystems, Finland). IC_{50} values, the drug concentration causing a 50% reduction in Calcein AM conversion, were calculated from the dose–response curves. All experiments were repeated in quadruplicate with a maximum deviation of 15%. Because of their limited solubility in water, all the compounds tested were dissolved in DMSO and then diluted with water to a final DMSO concentration of 0.6%.

4.8.2. MTT test on human fibroblasts

Human diploid fibroblasts BJ (passage 15–20) were seeded in a 96-well plate (5000 cells per well). After 6 h, the cultivation medium (DMEM containing 5 g/l glucose, 2 mM glutamin, 100 U/mL penicillin, 100 μg/ml streptomycin and 10% fetal calf serum) was removed and fresh medium containing a test compound in concentration range 0–100 μM was added. The concentration was adjusted in the case of compounds with limited solubility. Each concentration was tested five times. MTT was added to the cells after 72 h to a final concentration of 0.5 mg/ml. The incubation time was 3 h. The resulting MTT was dissolved in DMSO and absorbance at 570 nm with a reference wavelength of 650 nm was measured. The IC_{50} value, that is, the compound concentration causing a 50% reduction in mitochondrial activity, was calculated from the dose–response curves.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.01.041.

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Supplement IV

Activity of oTR expressed as GI_{50} (the concentration causing 50 % reduction of growth) against NCI_{60}.

Cell line	Malignancy type	GI_{50} (μM)
BT-549	breast cancer	0.18
HS 578T	breast cancer	1.95
MCF7	breast cancer	2.20
MDA-MB-231/ATCC	breast cancer	0.08
T-47D	breast cancer	0.34
SF-268	CNS cancer	0.62
SF-295	CNS cancer	6.46
SF-539	CNS cancer	0.40
SNB-19	CNS cancer	1.38
SNB-75	CNS cancer	1.75
U251	CNS cancer	1.18
COLO 205	colon cancer	0.54
HCC-2998	colon cancer	1.96
HCT-116	colon cancer	0.14
HCT-15	colon cancer	0.46
HT29	colon cancer	2.24
KM12	colon cancer	5.31
SW-620	colon cancer	0.31
A549/ATCC	lung cancer	26.91
EKVX	lung cancer	1.14
HOP-62	lung cancer	2.34
HOP-92	lung cancer	0.78
NCI-H226	lung cancer	9.09
NCI-H23	lung cancer	0.47
NCI-H322M	lung cancer	0.70
NCI-H460	lung cancer	8.90
NCI-H522	lung cancer	0.10
CCRF-CEM	leukemia	0.29
HL-60(TB)	leukemia	0.26
K-562	leukemia	0.71
MOLT-4	leukemia	0.60
RPMI-8226	leukemia	5.16
SR	leukemia	0.42
LOX IMVI	melanoma	0.07
M14	melanoma	0.82
MALME-3M	melanoma	3.60
MDA-MB-435	melanoma	0.27
SK-MEL-2	melanoma	0.33
SK-MEL-28	melanoma	4.33
SK-MEL-5	melanoma	1.86
UACC-257	melanoma	84.59
UACC-62	melanoma	1.32
IGROV1	ovarian cancer	0.66
NCI/ADR-RES	ovarian cancer	0.44
OVCAR-3	ovarian cancer	0.38
OVCAR-4	ovarian cancer	0.30
OVCAR-5	ovarian cancer	1.92
OVCAR-8	ovarian cancer	0.24
SK-OV-3	ovarian cancer	0.65
DU-145	prostate cancer	0.27
PC-3	prostate cancer	0.61
786-0	renal cancer	0.59
A498	renal cancer	1.21
ACHN	renal cancer	0.33
CAKI-1	renal cancer	0.36
RXF 393	renal cancer	4.66
SN12C	renal cancer	0.20
TK-10	renal cancer	0.33
UO-31	renal cancer	0.84