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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

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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.

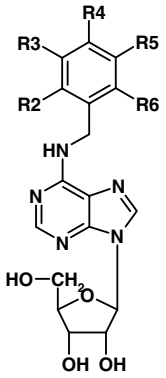
Recently, biologically effective monomethoxy derivatives of 6-benzyladenosine were isolated and identified from several different plant sources.[19] Results presented here also suggest the endogenous occurrence of two disubstituted benzyladenosines.

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 ^1H, ^{13}C and ^{15}N NMR (Supplementary material). To ensure accurate interpretation of data, also 2D/gs-COSY, gs-HMQC, gs-HMBC for ^1H 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.

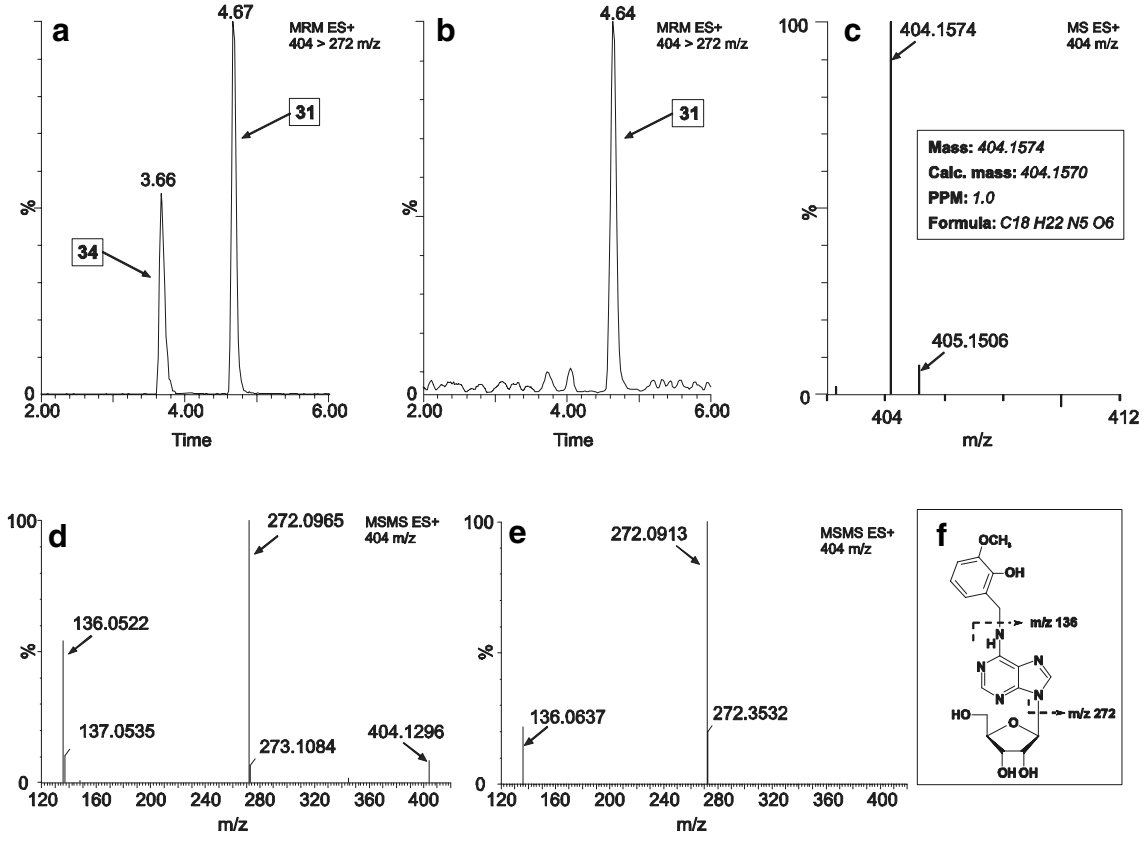


Figure 1. The identification of 6-(2-hydroxy-3-methoxybenzylamino)purine riboside (compound number **31**) in 10-day-old *A. thaliana* plants. (a) UPLC/MS (positive-ion MRM mode of *m/z* 404 > 272) chromatogram of standards number **31** and **34** (the mixture contains 10 pmol of each derivative). (b) 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 ($[M+H]^+ = 404$).

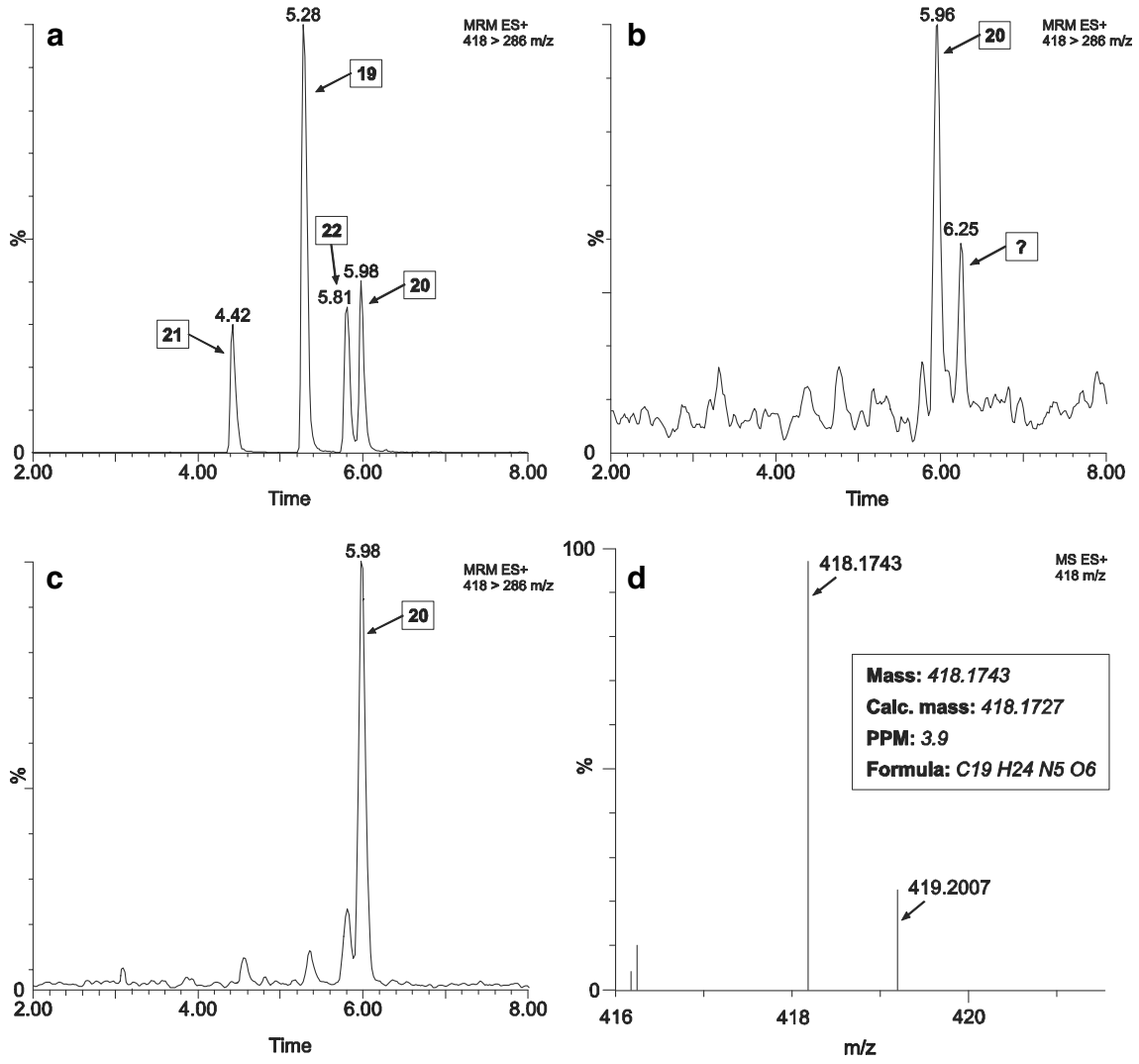


Figure 2. The identification of 6-(2,4-dimethoxybenzylamino)purine riboside (compound number **20**) in 10-day-old *A. thaliana* plants. (a) UPLC/MS (positive-ion MRM mode of *m*/*z* 418 > 286) chromatogram of standards number **19**, **20**, **21** and **22** (the mixture contains 10 pmol of each derivative). (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 $[MH]^+$; ^1H 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 $[MH]^+$; ^1H 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 × $OD_{600}{}^{-1}$ × 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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