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Preparation and biological activity of 6-benzylaminopurine derivatives in plants and human cancer cells

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Abstract—To study the structure–activity relationships of aromatic cytokinins, the cytokinin activity at both the receptor and cellular levels, as well as CDK inhibitory and anticancer properties of 38 6-benzylaminopurine (BAP) derivatives were compared in various in vitro assays. The compounds were prepared by the condensation of 6-chloropurine with corresponding substituted benzylamines. The majority of synthesised derivatives exhibited high activity in all three of the cytokinin bioassays employed (tobacco callus, wheat senescence and *Amaranthus* bioassay). The highest activities were obtained in the senescence bioassay. For some compounds tested, significant differences of activity were found in the bioassays used, indicating that diverse recognition systems may operate and suggesting that it may be possible to modulate particular cytokinin-dependent processes with specific compounds. Position-specific steric and hydrophobic effects of different phenyl ring substituents on the variation of biological activity were confirmed. In contrast to their high activity in bioassays, the BAP derivatives were recognised with much lower sensitivity than *trans*-zeatin in both *Arabidopsis thaliana* AHK3 and AHK4 receptor assays. The compounds were also investigated for their effects on cyclin-dependent kinase 2 (CDK2) and for antiproliferative properties on cancer and normal cell lines. Several of the tested compounds showed stronger inhibitory activity and cytotoxicity than BAP. There was also a significant positive correlation of the inhibitory effects on human and plant CDKs with cell proliferation of cancer and cytokinin-dependent tobacco cells, respectively. This suggests that at least a part of the antiproliferative effect of the new cytokinins was due to the inhibition of CDK activity.

1. Introduction

6-Benzylaminopurine (BAP) and its derivatives are active and easily obtainable plant growth promoting substances. They have long been thought to be purely synthetic compounds. However, some of them have also been detected and identified in different plant tissues (for details, see[1]).

BAP is also one of the most effective and affordable cytokinins used in many micropropagation systems. Nevertheless, it has disadvantages in some crops, including acclimatization problems, heterogenity in growth and rooting inhibition.[2] One way to eliminate the side effects might be based on the development and examination of BAP derivatives that do not exhibit unwanted side effects. For example, the high biological activity of 6-(3-hydroxybenzylamino)purine (*meta*-topolin, mT) in different bioassays has been described by several authors,[3,4] The positive effect of mT versus BAP was later shown in the in vitro shoot and root production and post vitro rooting of *Spathiphyllum floribundum*. Plants grown on media containing 10 μM of mT rooted better during acclimatization compared with those developed on an equimolar concentration of BAP. This fact can be explained by differences in the metabolism of BAP and mT.[2] In addition, it was shown that the endogenous occurrence of hydroxylated BAP analogues in *Zantedeschia aethiopica* fruits is correlated with the self-inhibition of fruit senescence.[5] The aim of this study was to prepare a large family of other BAP derivatives, substituted on the phenyl ring, and to compare the cytokinin activity of prepared compounds in three different bioassays, based on the stimulation of tobacco callus growth, the

Keywords: 6-Benzylaminopurine; Cytokinin; Receptor; Bioassay; Cyclin-dependent kinase; Cytotoxicity.
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retention of chlorophyll in excised wheat leaves and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons. The new cytokinin derivatives have also been screened for their capacity to be recognised by the recently discovered cytokinin receptor proteins AHK3 and AHK4.[6–8]

Although cytokinins regulate many cellular processes, the control of cell division is crucial and is considered diagnostic for this class of phytohormones; hence, the generic name is 'cytokinins'.[9] Cytokinins are involved in the regulation of both G1/S and G2/M transitions of the cell cycle. They elevate the expression of the *CYCD3* gene, which encodes a D-type cyclin.[10,11] In animal cells, D-cyclins are regulated by a wide variety of growth factors and play a key role in regulating the passage through the cell cycle restriction point in G1. It has also been shown that the constitutive expression of *CYCD3* can bypass the cytokinin requirement for cell proliferation, thus causing the cytokinin-independent growth of *Arabidopsis thaliana* calli.[10]

Cytokinins are also important for the regulation of the G2/M transition. In tobacco BY-2 cells, which are cytokinin-autonomous, endogenous zeatin cytokinins peak around S and M phases.[12] The application of lovastatin, an inhibitor of mevalonic acid synthesis, inhibits cytokinin accumulation and mitosis, respectively. Applied *trans*-zeatin (tZ) overrides the lovastatin block. This indicates that tZ is indispensable for the G2/M transition.[13] The regulation of the G2/M transition is mediated by activated cyclin-dependent kinases (CDK). In pea root tissues, mRNA of mitotic CDK was induced within 10 min by auxin application. High levels of an inactive CDC2-like protein were also found in tobacco pith cultured on auxin medium.[14] The cytokinin effect has been linked to the activation of a Cdc25-like phosphatase which dephosphorylates plant homologue of CDK1 on Thr 14/Tyr15 residues.[15] This process provides one of the potential links between cytokinin and auxin effects on the regulation of the cell cycle.

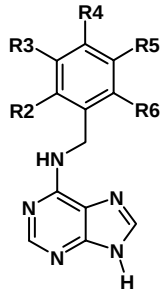
Recently, another important property of cytokinin analogues has been proved. The natural isoprenoid and aromatic cytokinins were found to inhibit several human protein kinases in a non-specific manner, including CDKs. On the other hand, the screening of chemically synthesised cytokinin analogues revealed selective CDK inhibitor olomoucine, that is, the BAP derivative 6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine. Small modifications of the BAP molecule led to the specific inhibition of several important protein kinases like CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, brain CDK5/p35 and ERK1/MAP kinase.[16] Because of the strong anticancer properties of these compounds (olomoucine, roscovitine, etc.),[17,18] the newly prepared BAP analogues have also been tested in an in vitro CDK2/cyclin E kinase inhibition assay and also for their possible cytotoxic activity against selected cancer cell line lines and normal mouse fibroblasts (NIH/3T3). In this report, we are also trying, for the first time, to correlate the relationships between the molecular mode of action with appropriate cellular effects.

2. Results and discussion

2.1. Synthesis

Thirty-eight analogues of BAP with various substituents attached to the phenyl ring had been synthesised (Table 1) in order to determine their structure–activity relationships. The prepared compounds were characterised by elemental analysis, TLC, melting point, ES + MS (Table 2) as well as ^{1}H and ^{13}C NMR (Supplementary data). The previously published syntheses of BAPs, monosubstituted on the benzyl ring with CH_3, NH_2, NO_2, OH,

Table 1. Structures 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	H	NO_2	H	H	H
18	H	H	NO_2	H	H
19	Cl	H	Cl	H	H
20	H	Cl	Cl	H	H
21	CH_3O	CH_3O	H	H	H
22	CH_3O	H	CH_3O	H	H
23	H	CH_3O	CH_3O	H	H
24	H	CH_3O	H	CH_3O	H
25	CH_3O	H	CH_3O	H	CH_3O
26	H	CH_3O	CH_3O	CH_3O	H
27	F	H	F	H	H
28	H	F	H	F	H
29	F	F	F	H	H
30	F	F	H	H	F
31	F	H	F	F	H
32	Cl	H	F	H	H
33	H	Cl	F	H	H
34	OH	CH_3O	H	H	H
35	OH	H	CH_3O	H	H
36	OH	OH	H	H	H
37	H	OH	H	OH	H
38	H	CH_3O	OH	MeO	H

Table 2. Elemental analyses, ES+ mass spectrometry analyses and melting points of prepared substituted 6-benzylaminopurines

Compound	Elemental analysis calculated/found			Mp (°C)	ES–MS [M+H$^+$]
	%C	%H	%N		
1	59.3/59.1	4.1/3.8	28.8/27.8	245–246	244
2	59.3/59.1	4.1/3.9	28.8/27.7	247–248	244
3	59.3/59.2	4.1/4.0	28.8/27.9	267–268	244
4	55.5/55.7	3.8/4.2	27.0/26.0	227–228	260
5	55.5/55.2	3.8/3.9	27.0/26.2	252–253	260
6	55.5/55.3	3.8/3.8	27.0/26.6	277–278	260
7	47.4/47.6	3.3/3.5	23.0/22.5	222–223	304
8	47.4/47.1	3.3/3.4	23.0/23.1	256–257	304
9	47.4/47.8	3.3/3.4	23.0/22.3	292–293	304
10	41.0/41.4	2.8/2.8	20.0/19.5	248–249	352
11	65.2/65.0	5.5/5.8	29.3/28.8	267–268	240
12	65.2/64.8	5.5/6.0	29.3/29.1	232–235	240
13	65.2/65.8	5.5/5.4	29.3/27.5	288–290	240
16	61.2/60.7	5.1/5.3	27.4/26.7	263–265	256
17	53.3/53.1	3.7/3.6	31.1/30.8	356–357	271
18	53.3/53.1	3.7/3.5	31.1/30.9	335–337	271
19	49.0/49.4	3.1/3.1	23.8/23.1	309–311	295
20	49.0/49.1	3.1/3.2	23.8/23.0	332–333	295
21	58.9/59.3	5.3/5.5	24.5/23.5	229–230	286
22	58.9/59.3	5.3/4.9	24.5/23.5	189–191	286
23	58.9/59.7	5.3/5.6	24.5/23.8	262–263	286
24	58.9/59.7	5.3/5.3	24.5/23.4	311–313	286
25	57.1/57.3	5.4/5.3	22.2/21.9	208–210	316
26	57.1/57.2	5.4/5.2	22.2/21.9	227–229	316
27	55.2/55.2	3.5/3.4	26.8/26.2	255–256	262
28	55.2/54.4	3.5/3.2	26.8/26.4	261–262	262
29	51.6/52.2	2.9/2.7	25.1/24.5	280–281	280
30	51.6/51.2	2.9/2.7	25.1/24.5	280–281	280
31	51.6/50.6	2.9/2.8	25.1/24.5	274–275	280
32	51.9/51.8	3.3/3.2	25.2/24.9	172–173	279
33	51.9/51.8	3.3/3.3	25.2/24.7	262–263	279
34	57.6/57.8	4.8/4.8	25.8/25.5	253–254	272
35	57.6/57.1	4.8/4.0	25.8/24.9	253–254	272
36	56.0/55.4	4.3/4.8	27.2/26.6	256–257	258
37	56.0/56.4	4.3/4.0	27.2/27.5	273–274	258
38	55.8/56.1	5.0/5.0	23.2/22.9	247–248	302

CH_3O and Cl, were achieved by condensing 6-(methylmercapto)purine with 2–3 molecular equiv of corresponding amines.[20] However, this method usually gives lower yields, compared with our results, which were achieved using 6-chloropurine. The melting points, reported for the compounds prepared using an earlier method, are also somewhat lower. The preparation of some BAP derivatives, substituted by single halogen atom on the phenyl ring, using 6-(methylmercapto)purine has been previously reported by other authors.[28,29] However, no physico-chemical data to validate the identity and purity of the prepared compounds are present in these reports. Moreover, because different assays were used to evaluate their biological activity, it is difficult to compare the results.

2.2. Cytokinin activity in bioassays

The effect of introduced substituents on BAP has been investigated in three cytokinin bioassays, based on the stimulation of different physiological processes in plants (Table 3). As a comparison, BAP itself, a highly active and widely practically used cytokinin, was employed as standard compound.

Derivatives **1** and **2** containing a fluorine atom in the *ortho* and/or *meta* position of the phenyl ring topped BAP activity in the *Amaranthus* bioassay by 16% and 40%, respectively. Among the monosubstituted BAP derivatives, any substituent at the *para*-position (compounds **3**, **6**, **9**, **13**, **16** and **18**) reduced the activity drastically in this bioassay. In contrast, all tested di- and trifluoro derivatives **27**–**31** were found to be very active (in all three biotests), even those containing the substituent in the *para* position (**27**, **29** and **31**). However, di- and trisubstituted hydroxy/methoxy derivatives (**21**–**26** and **33**–**37**) have been found to be inactive in this assay. A similar pattern of relative activity was also obtained in the callus bioassay, except for the high activity of **3**.

In contrast, the senescence bioassay revealed responses different from those of the other two biotests. As we previously reported for endogenously occurring 2- and 3-methoxy derivatives of BAP,[19] their activity was either comparable or slightly lower in the *Amaranthus* and tobacco callus bioassays, but double that of BAP in the senescence assay. This indicates that these compounds may preferentially affect physiological processes associated with the impact of different stress factors on

Table 3. Relative cytokinin bioassay activity of prepared 6-benzylaminopurine derivatives at optimal concentration compared with the activity of 6-benzylaminopurine (BAP) (100% means 10^{-5} M BAP for the *Amaranthus* betacyanin bioassay, 10^{-4} M BAP in the case of the senescence bioassay and 10^{-6} M BAP for the tobacco callus bioassay)

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^{-4}	116 (±3)	10^{-4}	169 (±20)	10^{-6}	111 (±21)
2	10^{-4}	140 (±5)	10^{-4}	200 (±25)	10^{-5}	135 (±8)
3	10^{-5}	44 (±4)	10^{-4}	95.5 (±3.5)	10^{-6}	122 (±12)
4	10^{-5}	109 (±8)	10^{-4}	116.5 (±6.5)	10^{-6}	93 (±4)
5	10^{-5}	96 (±5)	10^{-4}	82 (±2)	10^{-5}	94 (±6)
6	10^{-5}	35 (±7)	10^{-4}	64.5 (±13.5)	10^{-6}	64 (±8)
7	10^{-4}	94 (±6)	10^{-4}	52 (±14)	10^{-5}	102 (±5)
8	10^{-4}	71 (±5)	10^{-4}	48 (±6)	10^{-6}	85 (±11)
9	10^{-5}	17 (±7)	10^{-5}	30 (±15)	10^{-6}	15 (±9)
10	10^{-5}	79 (±3)	10^{-4}	83.5 (±23)	10^{-5}	76 (±8)
11	10^{-5}	98 (±22)	10^{-4}	158 (±29)	10^{-6}	118 (±3)
12	10^{-5}	84 (±14)	10^{-4}	111 (±16)	10^{-6}	79 (±5)
13	10^{-5}	26 (±10)	10^{-4}	35 (±23)	10^{-6}	52 (±8)
16	10^{-4}	23 (±2)	10^{-4}	79 (±6)	10^{-6}	39 (±17)
17	10^{-4}	66 (±7)	10^{-4}	60 (±15)	nt	nt
18	10^{-4}	25 (±2)	10^{-4}	83 (±9)	nt	nt
19	10^{-4}	19 (±8)	10^{-5}	0	nt	nt
20	10^{-4}	63 (±10)	10^{-4}	117 (±22)	nt	nt
21	10^{-4}	22 (±3)	10^{-4}	109 (±5)	10^{-6}	7 (±3)
22	10^{-5}	12 (±2)	10^{-7}	26 (±11)	nt	nt
23	10^{-4}	2 (±1)	10^{-4}	16 (±1)	nt	nt
24	10^{-4}	27 (±6)	10^{-4}	43 (±17)	nt	nt
25	10^{-4}	3 (±3)	nt	nt	nt	nt
26	10^{-4}	2 (±2)	10^{-4}	25 (±2)	nt	nt
27	10^{-4}	88 (±7)	10^{-5}	139 (±2)	10^{-6}	95 (±1)
28	10^{-4}	107 (±2)	10^{-5}	156 (±4)	nt	nt
29	10^{-4}	107 (±5)	10^{-5}	131 (±22)	10^{-6}	76 (±4)
31	10^{-4}	108 (±1)	nt	nt	10^{-5}	101 (±1)
33	10^{-4}	91 (±7)	10^{-5}	141 (±20)	10^{-6}	90 (±1)
34	10^{-4}	19 (±3)	10^{-5}	34 (±5)	nt	nt
37	10^{-5}	43 (±9)	10^{-4}	134 (±12)	10^{-6}	39 (±6)

the photosynthetic apparatus. In the present study, we found this tendency to be more or less general among aromatic cytokinins (Table 3). Moreover, 6-(2,3-dimethoxybenzylamino)purine (**21**), an inactive compound in the two other biotests, was still able to exceed the activity of BAP in the senescence bioassay. Strong differences in activities of the same cytokinin compounds (e.g., **3**, **18** and **21**) in the different bioassays indicate that it may be possible to design-specific compounds that can be used to modulate cytokinin-dependent processes in a targeted fashion. This may also indicate that another receptor and/or signalling system is involved in mediating the influence of cytokinins on the senescence process than in mediating their influence on cell growth and division.

Three membrane-located sensor histidine kinases of *A. thaliana* have been identified recently to function as cytokinin receptors. The AHK4 protein (identical to CRE1 and WOL)[6–8] was shown to bind directly cytokinins[8,23] and two of its homologues, AHK2 and AHK3, have also been found to be cytokinin-binding receptors.[23,31] We employed Δ*rcsC Escherichia coli* strains that express AHK4 and AHK3[7,23] to study the relative sensitivity of these receptors to the prepared compounds. This system was previously successfully used to systematically investigate the relative activities of natural cytokinin metabolites.[24] However, in contrast with our bioassay results, only few BAP derivatives tested were able to activate AHK4 significantly (namely **2**, **3** and **15**), although with much lower sensitivity (below 10%), when compared to *trans*-zeatin (Fig. 1). Most of the prepared compounds were recognised by the AHK3 receptor (**1**–**5**, **14**, **17**, **20**, **27** and **29**), but also only with low sensitivity, between 10% and 32% of *trans*-zeatin activity (Fig. 1). The differences of activities in bioassays and the receptor assay may indicate that either AHK2. the cytokinin receptor that was not tested in this study, preferentially recognises BAP derivatives or that another recognition system, also able to interact with BAP derivatives, exists in plants.

2.3. Antitumour and kinase assays

We also tested the prepared compounds for their antitumour 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 (MCF-7) and mouse melanoma (B16). The cytotoxicity of selected compounds for NIH-3T3 (normal mouse fibroblasts)

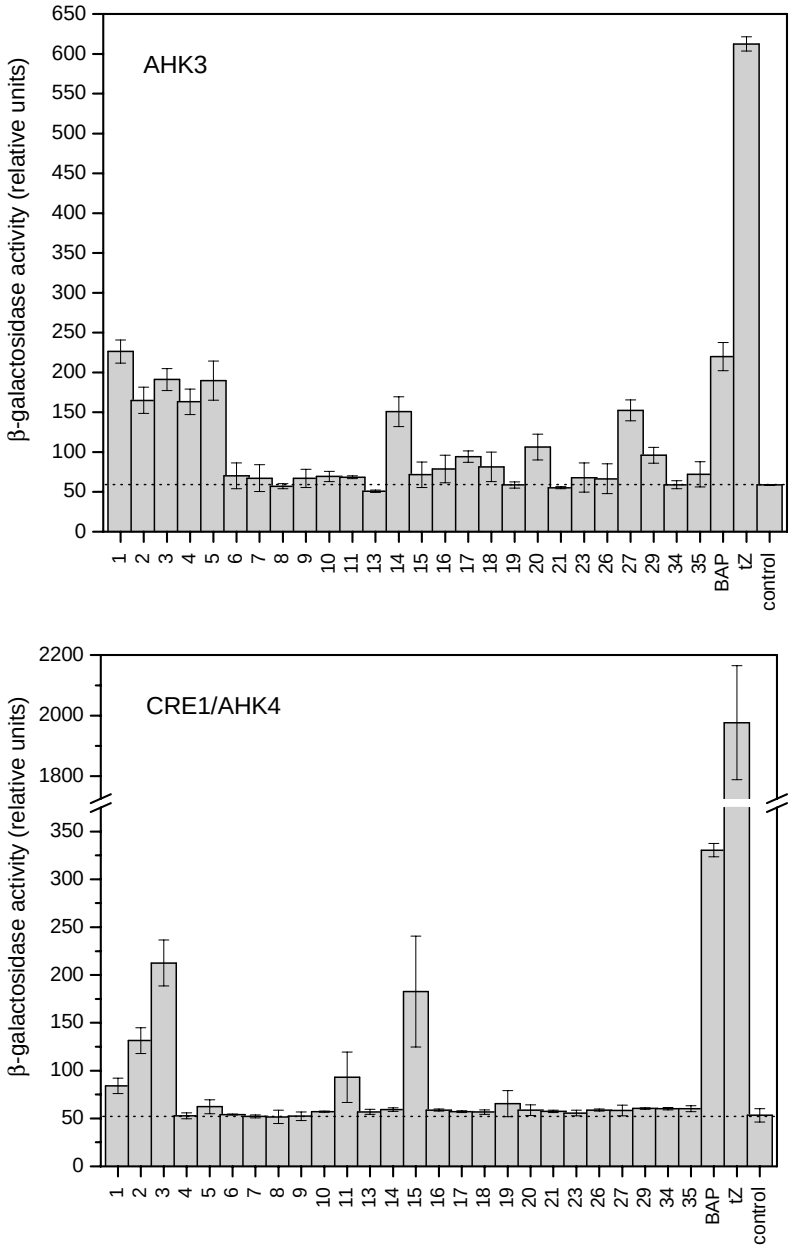


Figure 1. Comparison of the sensitivities of CRE1/AHK4 and AHK3 to different cytokinin derivatives in the *Escherichia coli* assay. Concentration of tested compounds was 1 μM. The β-galactosidase activity in non-induced strains (control) is indicated by the dotted line. Error bars show SD (n = 3). tZ means *trans*-zeatin.

was examined as well. The data obtained from a calcein AM viability/cytotoxicity assay are presented in Table 4. The obtained IC_{50} values show interesting cytotoxic properties of compound **34** (IC_{50} = 26.6 μmol/l for MCF-7), in contrast to other very similar derivatives (e.g., compounds **35** and **36**), which displayed no activity in this assay. Weak cytotoxicity of several other mono-substituted halogen-, methyl- and methoxy-BAPs (**1**, **3**, **4**, **13**, **15** and **16**) was also confirmed (Table 4). The cytotoxic properties as well as the cytokinin activity of related ribosides will be described elsewhere (Doležal et al., manuscript in preparation).

The growth-inhibiting ability of several well-known cytokinins as well as their ribosides on the HL-60 cells has been previously reported.[32–36] Ribosides have been shown to be potent inducers of apoptosis. In contrast, cytokinin free bases did effectively induce HL-60 cell differentiation and transformation into mature granulocytes.

It is also known that natural cytokinins are weak and non-specific inhibitors of various protein kinases.[16] However, systematic screening of purine derivatives of cytokinin origin led to the discovery of 2,6,9-trisubstituted purines and revealed a large number of highly active CDK inhibitors such as olomoucine and roscovitine.[16,17] To verify the anticancer properties of BAP analogues, they were screened for the ability to inhibit human recombinant CDK2 (summarised in

Table 4. Cytotoxicity of prepared BAP derivatives in calcein AM assay (IC_{50}, μmol/l)

Compound	Cell line/IC_{50} (μmol/l)							
	HOS	HL 60	K-562	MCF7	NIH-3T3	G-361	B16	CEM
BAP	>166.7	>166.7	138.9	>166.7	nt	nt	nt	>166.7
1	>166.7	147.1	132.6	>166.7	nt	116.0	79.1	111.4
2	>166.7	>166.7	105.2		nt	>166.7	nt	>166.7
3	>166.7	59.2	>166.7	>166.7	nt	>166.7	nt	66.4
4	122.6	109.6	95.7	146.2	>166.7	56.6	16.9	58.4
5	>166.7	>166.7	>166.7	>166.7	nt	148.6	nt	>166.7
6	nt	>166.7	>166.7	>166.7	nt	nt	nt	>166.7
10	>166.7	133.3	94.7	>166.7	nt	>166.7	nt	124.6
11	>166.7	>166.7	156.6	>166.7	nt	>166.7	nt	>166.7
12	>166.7	>166.7	>166.7	102.5	nt	>166.7	164.1	>166.7
13	>166.7	88	72.8	>166.7	nt	160.4	nt	72.1
14	>166.7	>166.7	>166.7	115.8	nt		134.9	107.8
15	>166.7	>166.7	121.7	158.1	>166.7	124.7	41.5	>166.7
16	>166.7	>166.7	142.4	>166.7	>166.7	>166.7	84.7	>166.7
17	nt	>166.7	>166.7	>166.7	nt	nt	nt	>166.7
18	nt	>166.7	>166.7	>166.7	nt	nt	nt	>166.7
24	nt	>166.7	>166.7	>166.7	nt	nt	nt	>166.7
25	nt	153.4	>166.7	>166.7	nt	nt	>166.7	99.5
27	nt	>166.7	>166.7	>166.7	nt	nt	nt	139.7
28	nt	nt	>166.7	>166.7	nt	nt	nt	nt
29	nt	>166.7	>166.7	>166.7	nt	nt	nt	32.6
31	nt	nt	>166.7	>166.7	nt	nt	nt	nt
33	nt	>166.7	>166.7	>166.7	nt	nt	>166.7	124.4
34	nt	64.3	>166.7	26.6	nt	nt	69	46.3
35	nt	nt	>166.7	>166.7	nt	nt	nt	nt
36	>166.7	nt	>166.7	>166.7	nt	>166.7	nt	nt
37	>166.7	nt	>166.7	>166.7	nt	>166.7	nt	nt
38	>166.7	nt	>166.7	>166.7	nt	>166.7	nt	nt

The following cell lines were used: CEM—human T-lymphoblastic leukaemia, HL-60—promyelocytic leukaemia, G-361—human malignant melanoma, K-562—human chronic myelogenous leukaemia, HOS—human osteogenic sarcoma, MCF-7—human breast carcinoma, B 16—mouse melanoma and NIH/T3T—mouse normal fibroblasts.

Table 5). The results showed that the attachment of one halogen (compounds **1**–**10**), methyl (**11** and **13**) or methoxy (**14**–**16**) group at any position of the benzyl ring led to an approximately 2-fold increase in inhibitory activity compared to BAP. Nitro derivatives (**17** and **18**) were however less potent. Certain losses of inhibitory activity were also observed after the introduction of more than one hydroxy and/or methoxy substituents to the benzyl moiety (**21**, almost completely inactive **23** and **26**). On the other hand, difluoro, trifluoro and corresponding chloro derivatives (**27**–**32**) still led to a slight increase in activity. Some recent studies have also suggested that different substituents on the benzyl ring (Cl and OCH_3) may further enhance the ability of purines to block CDK1 activity.[37,38] Although none of the 6-substituted purines tested here was found to be a very potent CDK2/cyclin E inhibitor, we assume that our results may also be useful for the future design and synthesis of the next successful class of related trisubstituted purine-based inhibitors.

In a long-term experiment, the treatment of tobacco callus with cytokinin stimulates the rate of cell division. The optimal cytokinin concentration for cell proliferation is typically 10^{-9} to 10^{-5} M. At higher concentrations (above 10^{-5} M) cytokinins become less effective; above about 10^{-4} M they become inhibitory. This is shown in Figure 2 where the growth curve falls below the dashed line, which represents growth in the absence of exogenous cytokinins. While this is valid for many of the compounds tested, it is not so for all of them (nitro derivatives) (Fig. 2). Using high concentrations of different BAP derivatives, a good correlation between callus growth inhibition and the ability to inhibit human recombinant CDK2/cyclin E kinase has been found. This was further verified by testing selected BAP derivatives in a plant kinase inhibition assay. Figure 3 shows that several of the cytokinins tested (isopentenyladenine (iP), BAP, **4**) also have the ability to inhibit plant CDKs (in concentrations higher than 50 μM). On the other hand, 6-(3-nitrobenzylamino)purine **17**, the compound, which did not show any inhibition of callus growth, was also inactive in the plant CDK assay (see Figs. 2 and 3 for comparison). Because these results again confirmed our previous hypothesis, it might be concluded that the tobacco callus growth inhibition, which is caused by excessive cytokinin concentrations, is at least partially due to the cytokinin inhibition of CDK activity. However, many other enzymes besides CDKs, involved in regulation of cell growth and division, could also be inhibited.[16]

3. Conclusions

In summary, a group of 6-benzylaminopurine derivatives with different phenyl ring substituents has been prepared. Many of them have been found to be very active in different cytokinin bioassays. In contrast, only

Table 5. Inhibition of CDK2/cyclin E by BAP derivatives at 50 μM concentration, with iP and olomoucine, 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine, as controls

Compound	Residual CDK2 activity (%)
1	36.0 (±1.5)
2	37.4 (±1.2)
3	40.8 (±1.8)
4	27.3 (±0.3)
5	36.2 (±1.1)
6	39.6 (±2.4)
7	26.2 (±0.6)
8	27.8 (±3.2)
9	37.4 (±3.1)
10	33.6 (±3.1)
11	34.0 (±1.9)
13	47.6 (±2.8)
14	68.3 (±5.0)
15	48.7 (±3.7)
16	34.4 (±3.9)
17	78.7 (±5.9)
18	67.8 (±7.2)
19	34.9 (±9.4)
20	47.9 (±3.8)
21	56.4 (±8.0)
23	92.6 (±9.8)
26	99.1 (±2.4)
27	44.1 (±1.9)
29	47.5 (±4.3)
34	43.9 (±3.3)
BAP	62.0 (±4.6)
iP	50.6 (±5.0)
Olomoucine	7.1 (±1.8)

All values are averaged from three independent experiments.

a few were able to interact with the cytokinin AHK receptors. One tentative conclusion is that a different sensing mechanism for aromatic cytokinins may exist in plants.

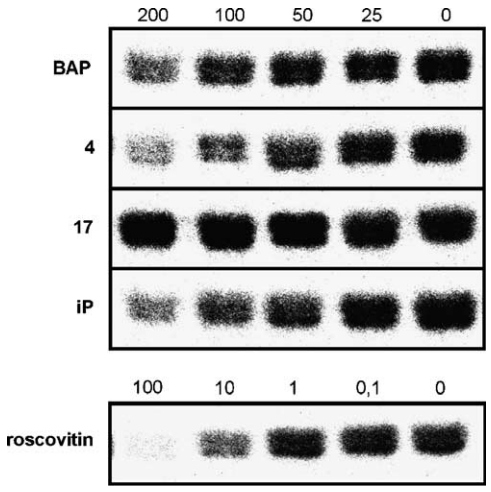


Figure 3. Inhibition of histone kinase activity by purine derivatives. CDK-like protein kinases were purified from *Arabidopsis thaliana* callus by binding to $p13^{suc1}$-Sepharose and assayed in the presence of increasing concentrations of BAP, **4** and **17**. Effects of isopentenyladenine (iP) and roscovitine, 2-{[(1-hydroxymethyl)propyl]amino}-6-benzylamino-9-isopropylpurine, were evaluated as controls in the same conditions.

On the other hand, some of the prepared compounds displayed (in higher concentrations) cytotoxic activity against both animal and plant cells. This effect may be, at least partially, caused by the inhibition of CDK activity in these cells. This would provide a mechanistic explanation for the dose–response curves of the callus growth test to cytokinins. The results from the antitumour assay and human recombinant CDK2 inhibition assay can also help to design novel trisubstituted compounds potent as CDK inhibitors and apoptosis inducers in cancer cells.

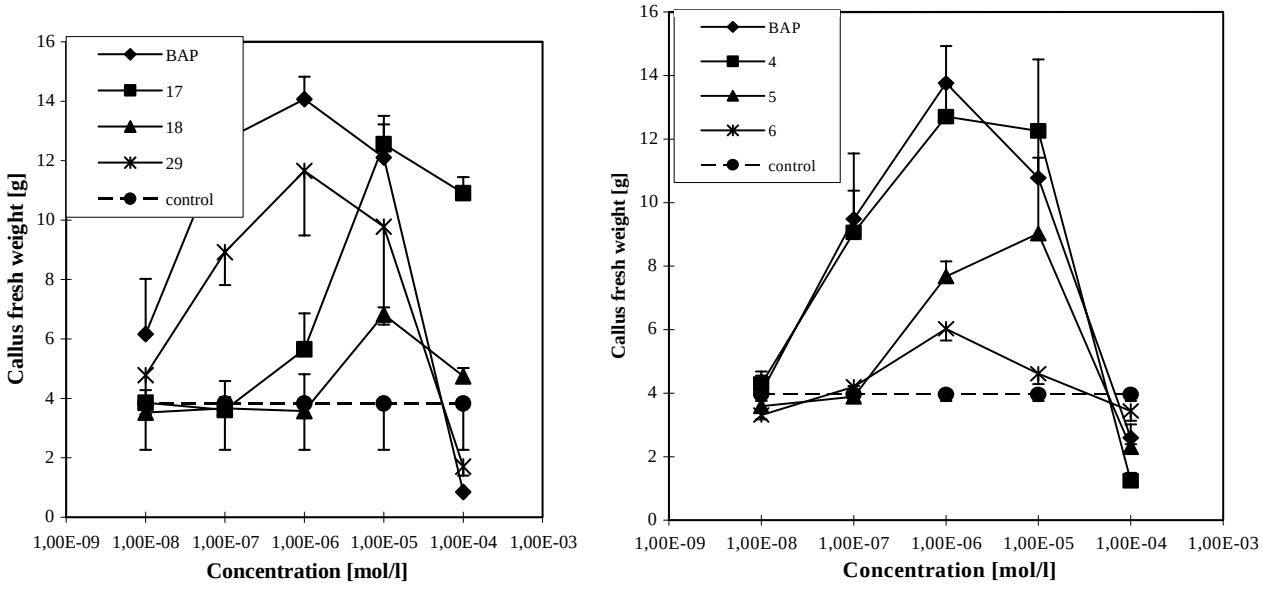


Figure 2. Typical dose–response curves for cytokinin-induced growth of cytokinin-dependent tobacco callus. Error bars show standard deviations of the mean for 10 replicate determinations. Dashed line indicates the value for the control treatment without any cytokinin, 4.0 ± 0.4 g.

4. Materials and methods

4.1. General procedures

The melting points were determined on a Boetius stage apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck). Elemental analyses (C, H and N) were performed using an EA1108 CHN analyser (Fissons Instruments). ES + mass spectra were recorded using direct probe on Waters ZMD 2000 mass spectrometer. The mass monitoring interval was 10–1500 amu. The spectra were collected using 3.0 s cyclical scans and applying sample cone voltage 25 V at source block temperature 150 °C, desolvation temperature 80 °C and 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 and a frequency of 300.13 MHz (^{1}H) and 75.48 MHz (^{13}C), 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, 6-chloropurine, 4-methyl umbelliferyl galactoside, 4-methylumbelliferone, DMEM, trypsin and DMSO were purchased from Sigma. Aldrich supplied 2-fluorobenzylamine, 3-fluorobenzylamine, 4-fluorobenzylamine, 3-chlorobenzylamine, 4-chlorobenzylamine, 2-bromobenzylamine hydrochloride, 3-bromobenzylamine hydrochloride, 4-bromobenzylamine hydrochloride, 2-iodobenzylamine, 2-methylbenzylamine, 3-methylbenzylamine, 4-methylbenzylamine, 3-methoxylbenzylamine, 3-nitrobenzylamine hydrochloride, 4-nitrobenzylamine hydrochloride, 2,4-dichlorobenzylamine, 3,4-dichlorobenzylamine, 2,3-dimethoxylbenzylamine, 3,4-dimethoxylbenzylamine, 3,5-dimethoxylbenzylamine, 2,4-dimethoxylbenzylamine hydrochloride, 3,4,5-trimethoxylbenzylamine and 2,4,6-trimethoxylbenzylamine hydrochloride. Fluka supplied 2-methoxylbenzylamine, 4-methoxylbenzylamine, 2-chlorobenzylamine, 2,4-difluorobenzylamine, 3,5-difluorobenzylamine, 2,4,5-trifluorobenzylamine, 2,3,6-trifluorobenzylamine, 2,4,5-trifluorobenzylamine, 2-chloro-4-fluorobenzylamine and 3-chloro-4-fluorobenzylamine were purchased from Fluorochem. Merck supplied casamino acids, calcein AM was purchased from Molecular Probes. [γ-^{33}P]ATP was provided by MP Biomedicals. NiNTA column was purchased from Qiagen. 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 (Olomouc, Czech Republic). Milli-Q water was used throughout. The other solvents and chemicals used were all of standard pa quality.

4.3. Synthesis of 6-benzylaminopurines

The preparation and cytokinin activity of 6-(2-methoxybenzylamino)purine **14** and 6-(3-methoxybenzylamino)purine **15** have been described elsewhere.[19] The general procedures for the preparation of 6-benzylaminopurines were described earlier.[4,19,20] In brief, 6-chloropurine 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 crystallised from dimethylformamide. The identity and purity of the synthesised compounds were confirmed by elemental and melting point analyses, analytical thin-layer chromatography, high performance liquid chromatography, MS (Table 2) and NMR (Supplementary data).

4.4. Preparation of 3,5-dihydroxybenzylamine hydrobromide

3,5-Dimethoxybenzylamine was converted to its hydroxylated analogue as previously described.[21,22] Acetanhydride (15 ml) was added, by syringe, to 3,5-dimethoxybenzylamine solution in 47% HBr under nitrogen. The stirred reaction mixture was subsequently heated to 107 °C for 5 h. After evaporation of the acid in vacuo, ethanol (15 ml) was added to the residue, and the solvent was again evaporated. The crude product (1.8 g) was recrystallised from EtOH. Elemental analysis (C, H and N) (Found: C, 37.9; H, 4.7; N, 6.6. $C_7H_{10}NO_2Br$ calcd: C, 38.2; H, 4.6; N, 6.4); TLC (chloroform:methanol:ammonia (8:2:0.1, v/v/v) as the mobile phase): single spot; mp 197–198 °C; ES + MS: *m/z* 140 $[MH]^+$; ^{1}H NMR (DMSO-d_6, 300 MHz): 9.45 (NH_2), 8.10 (OH), 6.29d (2H, 6H), 6.23tr (4H), 3.82s (7H); ^{13}C NMR (DMSO-d_6, 75 MHz): 159.03 (3C, 5C), 136.05 (1C), 107.18 (2C, 6C), 102.89 (4C), 42.70 (7C).

4.5. Preparation of 2-hydroxy-3-methoxybenzylamine hydrobromide and 2,3-dihydroxybenzylamine hydrobromide

The compounds were prepared from 2,3-dimethoxybenzylamine (1.5 ml; 10 mmol) using a procedure similar to that for 3,5-dihydroxybenzylamine. 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-3-methoxybenzylamine hydrobromide were filtered off, washed with acetone and diethyl ether, dried and recrystallised from acetone. Elemental analysis (C, H and N) (Found: C, 41.0; H, 5.4; N, 5.9. $C_8H_{12}NO_2Br$ calcd: C, 41.1; H, 5.2; N, 6.0); TLC (chloroform:methanol:ammonia (8:2:0.1, v/v/v) as the mobile phase): single spot; mp 209–211 °C; ES + MS: *m/z* 154 $[MH]^+$; ^{1}H NMR (DMSO-d_6, 300 MHz): 9.42 (NH_2), 8.28 (OH), 7.00kv (5H), 6.92kv (6H), 6.80tr (4H), 3.95s (7H), 3.80s (–OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): 147.92 (3C), 144.17 (2C), 128.45 (1C), 122.29 (5C), 120.81 (6C), 112.77 (4C), 56.44 (–OCH_3), 37.88 (7C).

The remaining filtrate was evaporated in vacuo, ethanol (10 ml) was added to the residue and the solvent was again evaporated. Ether (20 ml) was subsequently added, the solid residue was dried and recrystallised from ethanol to give 0.25 g of 2,3-dihydroxybenzylamine hydrobromide. Elemental analysis (C, H and N) (Found: C, 38.2; H, 4.1; N, 6.1. $C_7H_{10}NO_2Br$ calcd: C, 38.2; H, 4.6;

N, 6.4); TLC (chloroform:methanol:ammonia (8:2:0.1, v/v/v) as the mobile phase): single spot; mp 153–154 °C; ES + MS: *m/z* 140 $[MH]^+$; ^1H NMR (DMSO-d_6, 300 MHz): 9.55 (NH_2), 8.15 (OH), 6.85kv (5H), 6.78kv (6H), 6.65tr (4H), 3.92s (7H); ^{13}C NMR (DMSO-d_6, 75 MHz): 145.59 (3C), 145.05 (2C), 129.46 (1C), 121.15 (5C), 120.83 (6C), 116.29 (4C), 38.09 (7C).

4.6. Preparation of 4-hydroxy-3,5-dimethoxybenzylamine hydroiodide

3,4,5-Trimethoxybenzylamine was dissolved in 55% HI and acetanhydride was added by syringe. The reaction mixture was subsequently magnetically stirred at room temperature for 4 h. The crude product was recrystallised from ethanol. Elemental analysis (C, H and N) (Found: C, 34.9; H, 4.2; N, 4.8. $C_9H_{14}NO_3I$ 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 213–314 °C; ES + MS: *m/z* 196 $[MH]^+$; ^1H NMR (DMSO-d_6, 300 MHz): 8.55 (NH_2), 8.02 (OH), 6.75s (2H, 6H), 3.75s (–OCH_3), 3.92s (7H); ^{13}C NMR (DMSO-d_6, 75 MHz): 148.34 (3C, 5C), 136.13 (4C), 124.01 (1C), 107.13 (2C, 6C), 56.64 (–OCH_3), 43.04 (7C).

4.7. Testing for cytokinin activity

The cytokinin activity of all the prepared derivatives was tested in 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, as previously described.[5,30] Prior to testing, stock solutions of cytokinins in DMSO were prepared and further diluted in the media used for each biotest. The final concentration of DMSO in the media did not exceed 0.5%.

Activity testing was done over the 10^{-4} to 10^{-8} M concentration range. Five replicates were prepared for each cytokinin concentration and the entire test was repeated at least three times. From these data, the concentration with the highest biological response and the relative activity at this concentration for each compound were calculated (Table 3). The activity of BAP at the optimal concentration was set at 100 and the activities of the tested compounds were related to that of BAP. The optimal BAP concentrations, used for calculations, were 10^{-5} M for the *Amaranthus* betacyanin bioassay, 10^{-4} M in the case of the senescence bioassay and 10^{-6} M for the tobacco callus bioassay.

4.8. Bacterial cytokinin assay

The preparation of *Escherichia coli* strain KMI001 harbouring the plasmid pIN-III-AHK4 or pSTV28-AHK3 and the bacterial cytokinin assay were performed as described,[7,23,24] albeit with slight modifications. The *E. coli* strains were grown overnight at 25 °C in M9 media enriched with 0.1% casamino acids[25] 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 further grown at 25 °C. Incubation times of 17 h 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 plate containing 2 μl of 50 mM 4-methyl umbelliferyl galactoside, which was subsequently incubated for 1 h at 37 °C. The reaction was stopped by adding 100 μl 0.2 M of Na_2CO_3. Fluorescence was measured using a Fluoroscan Ascent (Labsystems, Finland) at the excitation and emission wavelengths of 365 and 460 nm, respectively. The OD_{600} of remaining culture was determined and β-galactosidase activity was calculated as nanomole 4-methylumbelliferone $\times$ $OD_{600}^{-1} \times h^{-1}$.

4.9. Antitumour activity testing

Human T-lymphoblastic leukaemia (CEM), 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 for a cytotoxicity determination of the prepared compounds by a calcein AM assay, as we have previously described.[26] 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 (in the 0.5–170 μM final concentration range) were added. Incubation lasted for 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 Fluoroscan Ascent reader (Labsystems, Finland). IC_{50} values, the compound concentrations lethal to 50% of the tumour cells, were estimated. 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%.

4.10. Human kinase assay

Human CDK2/cyclin E kinase assay was performed according to a published method.[37] The Sf9 culture co-infected with the appropriate baculoviruses was harvested 70 h post infection, incubated in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 1% Tween 20, 1 mM DTT, 0.1 mM PMSF, 0.5 μg/ml leupeptin and 1 μg/ml aprotonin) for 30 min on ice and the soluble fraction was recovered by centrifugation at 20.000*g* for 10 min. The enzyme was purified on a NiNTA column (Qiagen), stored at 4 °C and used within a week. To carry out the enzyme inhibition assays under linear conditions, the final point test system for kinase activity measurement was used. Kinase was added to the reaction mixture in such a way as to obtain linear activity both with respect to the concentration of the enzyme and time. The kinase inhibition determination involved the use of 1 mg/ml

histone H1 (type III-S, Sigma) in the presence of 15 μM ATP, 0.05 μCi [γ-^{33}P]ATP and of the tested compound in a final volume of 10 μl, all in reaction buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM EGTA, 10 mM 2-glycerolphosphate, 1 mM NaF and 1 mM DTT, pH 7.4). All the compounds tested were dissolved in DMSO at 5 mM, diluted to 250 μM with 5 mM HCl and assayed immediately at a final concentration of 50 μM. After 10 min incubation, reactions were stopped by the addition of 5 μl of 3% H_3PO_4, aliquots were spotted on P-81 phosphocellulose (Whatman, USA) which was subsequently washed 3 × with 5% H_3PO_4 and finally air-dried. The measurements of kinase inhibition employed digital image analyser BAS-1800 (Fujifilm, Japan). Kinase activity was expressed as percentage of maximum activity (i.e., without an inhibitor).

4.11. Plant kinase assay

The kinase inhibition assays with proteins bound to p13^{Suc1}-Sepharose were performed according to a published method.[27] Briefly, the cultured *A. thaliana* cv. Columbia cells were homogenised, the proteins were bound to p13^{Suc1}-Sepharose and assayed in the presence of the tested compound with histone H1 (Sigma type III-S) and 10 μM ATP + 0.05 μCi of [γ-^{33}P]ATP in a final volume of 20 μl. The reactions were incubated at 30 °C for 15 min and terminated by 3 × SDS sample buffer. After separation on 12% SDS PAGE, the dried gels were analysed by bioimager BAS-1800 (Fujifilm, Japan).

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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.2005.09.004.

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