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LEADING ARTICLE

Antiproliferative effect of plant cytokinin analogues with an inhibitory activity on cyclin-dependent kinases

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In this study, analogues of olomoucine, a previously described plant cytokinin analogue with cyclin-dependent kinase (CDK) inhibitory activity, were investigated for effect on CDK1 and CDK2 and for effect on cell proliferation. Eight new compounds exhibit stronger inhibitory activity on CDK1 and CDK2 and on cell proliferation than olomoucine. Some active compounds showed low inhibition of proliferation of normal myeloid growth. Improvement of inhibitory activity of known compounds with a C6-benzylamino group was brought about by substitution with one hydroxyl. Also, new C2 substituents associated with inhibitory activity on CDK and on cell proliferation are described. There was a significant correlation between effect on CDK and antiproliferative effect on the KG1 and Molt3 cell lines and on primary human lymphocytes, strongly suggesting that at least part of the antiproliferative effect of cytokinin analogues was due to inhibition of CDK activity. Cytokinin analogues induced apoptosis in a time- and concentration-dependent manner and changes in cell cycle distribution. The antiproliferative and pro-apoptotic effects of plant cytokinin analogues suggest that they are a new class of cytostatic agents and that they may find an application in the chemotherapy of cancer.
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Introduction

Cytokinins are one out of five known classes of plant growth regulators, also called phytohormones.[1] They are important at specific phases of the plant cell cycle.[2–4] To date, a number of compounds which stimulate plant cell division have been isolated and designated as cytokinins. All these naturally occurring cytokinins have an intact adenine and some of them, like 6-benzylaminopurine (BAP), contain an aromatic ring substituent at C6.[2]

Due to their important role in the plant cell cycle, natural cytokinins have been tested for interaction with CDK, the known cell cycle regulators of animal cells. The natural cytokinins dimethylaminopurine and N^6-isopentenyladenine were found to inhibit CDK1/cyclin B kinase in a non-specific manner.[5–7] On the other hand, screening of chemically synthesized aromatic cytokinin analogues for inhibition of CDK1/cyclin B kinase led to the discovery of the highly specific inhibitor olomoucine, ie 6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine.[8] Olomoucine has been found to inhibit cell proliferation and to induce apoptosis in tumor cells.[9,10] Olomoucine also potentiated mitoxantrone-induced apoptosis[11] and initiated apoptosis in a case of dog malignant melanoma.[12] Among 35 highly purified kinases, only the cell cycle regulating CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, brain CDK5/p35 and ERK1/MAP kinase were substantially inhibited by olomoucine. Among 81 cytokinin analogues tested, only C2-, C6-, N9-substituted purines (further designated as '2,6,9-trisubstituted purines', see also Table 1) showed strong inhibitory effect on CDK1 (also called cdc2).[8] Roscovitine, another 2,6,9-trisubstituted purine with inhibition of CDK1, binds CDK2 in a manner similar to that of olomoucine.[13–15] This indicates that the introduction of new substituents at these 2,6,9 positions might lead to enhanced binding affinity and selectivity. Recently, a new group of purine-based structures was identified in a screening of trisubstituted purine combinatorial libraries designed for CDK2 inhibition. This screening led to the development of new CDK2/cyclin A inhibitors, eg purvalanol A and B.[16]

While human tumor development is associated with alterations of CDK and their regulators,[17,18] the potential therapeutic value of CDK inhibitors as a cancer treatment was evaluated in different studies (reviewed in Ref. 19). In the present study, we searched for new inhibitors of CDK that also inhibit cell proliferation. Novel 2,6,9-trisubstituted purines were screened for CDK1/cyclin B and CDK2/cyclin E inhibitory activity. Those 2,6,9-trisubstituted purines were tested on malignant cell proliferation (cell lines KG1 and Molt3) and on normal cell proliferation (hematopoietic myeloid progenitor cells and primary blood lymphocytes). The KG1 cell line was obtained from the bone marrow of a patient with acute myelogenous leukemia.[20] This KG1 cell line is $CD34^+$ and in this study, $CD34^+$ and $CD34^+CD38^-$ hematopoietic progenitors were tested as normal counterparts of this cell line. The Molt3 cell line was derived from the peripheral blood of a patient with a T cell acute lymphoblastic leukemia[21] and primary blood lymphocytes were used as the normal counterpart of this malignant cell type.

Materials and methods

Cytokinin analogues

All the cytokinin analogues (Table 1) were synthesized as described previously.[22] They were dissolved as 50–100 mM solutions in dimethylsulphoxide (DMSO) (Sigma, Bornem, Belgium).

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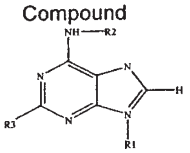
Table 1 Structure of cytokinin analogues used in this study

Compound 	*N9 (R1)*	*C6 (R2)*	*C2 (R3)*
OL (8, 9)	methyl	benzylamino	hydroxyethylamino
P21	methyl	benzylamino	morpholino
P3 (8, 13)	methyl	isopentenylamino	hydroxyethylamino
P7 (13, 15)	isopropyl	benzylamino	1-ethyl-2-hydroxyethylamino
P8 (13, 15)	isopropyl	benzylamino	1-ethyl-2-hydroxyethylamino
P10 (13)	isopropyl	benzylamino	3-hydroxypropylamino
P28 (13)	isopropyl	benzylamino	2-aminoethylamino
P24	isopropyl	benzylamino	5-cyanopentylamino
P22	isopropyl	benzylamino	2-hydroxyethylthio
P12 (13)	isopropyl	benzylamino	3-aminopropylamino
P16	isopropyl	benzylamino	methylthio
P19	isopropyl	p-hydroxybenzylamino	(R/S)-1-ethyl-2-hydroxyethylamino
P18	isopropyl	p-hydroxybenzylamino	3-hydroxypropylamino
P17	isopropyl	p-hydroxybenzylamino	hexylamino
P29	isopropyl	m-hydroxybenzylamino	1-ethyl-2-hydroxyethylamino
P34	isopropyl	m-hydroxybenzylamino	3-hydroxypropylamino
P35	isopropyl	o-hydroxybenzylamino	3-hydroxypropylamino
P23	isopropyl	1-phenyl-2-hydroxyethylamino	hexylamino
P25	isopropyl	benzylthio	3-hydroxypropylamino
P20	isopropyl	3-hydroxypropylamino	3-hydroxypropylamino
P27	isopropyl	adamant-1-ylamino	3-hydroxypropylamino
P26	isopropyl	adamant-1-ylamino	2,3-dihydroxypropylamino

The N9, C6 and C2 substituents (respectively R1, R2 and R3) are indicated. Compounds are grouped, first by N9 substituent (R1) and then by C6 substituent (R2). References of published compounds are mentioned between parentheses after compound number. Other names: OL, olomoucine; P7, roscovitine R; P8, roscovitine S, P10, bohemine.

CDK inhibition assay

CDK1/cyclin B and CDK2/cyclin E complexes were produced in Sf9 insect cells co-infected with appropriate baculoviral constructs. The cells were lysed 70 h post infection and the protein extract was recovered by centrifugation at 14 000 *g* for 10 min. The kinase inhibition determination involved the use of 1 mg/ml histone H1 (type III-S; Sigma) in the presence of 15 μM ATP, 0.2 μCi γ-^{32}P-ATP (Amersham, Uppsala, Sweden) and of the tested compound in a final volume of 10 μl (concentration in the range of 0.001–100 μM), all in reaction buffer (50 mM Hepes pH 7.4, 10 mM $MgCl_2$, 5 mM EGTA, 10 mM 2-glycerol phosphate, 1 mM NaF, 1 mM DTT, pH 7.4). After 10 min incubation, reactions were stopped by adding SDS sample buffer and the proteins were separated using electrophoresis on 12.5% SDS polyacrylamide gels. The measurements of kinase inhibition employed digital image analyzer BAS 1800. The cytokinin analogue concentration at which 50% of the CDK1 and CDK2 kinase activity was inhibited was computed from dose–response curves and designated as IC_{50} (50% inhibitory concentration).

Cell sources and cultures

Human leukemic cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) (KG1: CCL-246; Molt3: CRL-1552) and were cultured in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 200 U/ml penicillin, 200 μg/ml streptomycin and 1 μg/ml amphotericin B (further designated as IMDM/10% FCS), in a 5% CO_2–95% air fully humidified incubator. Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density gradient (LSM; ICN Biomedicals, Costa Mesa, CA, USA) and stimulated for 24–48 h with 5 μg/ml phytohemagglutinin A (PHA) (Sigma). After washing off the PHA, PBMC were further cultured in IMDM/10% FCS with 10 U/ml interleukin (IL)-2 (Genzyme, Leuven, Belgium). Adult bone marrow (ABM) samples were obtained by sternal puncture from hematologically normal donors undergoing cardiac surgery, after obtaining informed consent according to the ethical regulations of the University of Antwerp. Cells were collected in IMDM/10% FCS supplemented with 100 U/ml heparin and separated by density gradient.

Effect of cytokinin analogues on cell proliferation

Cell lines and PBMC were plated at 10 000 cells per well in 200 μl IMDM/10% FCS and incubated at 37°C with different concentrations of cytokinin analogues (in the range of 0–50 μM). After 96 h of culture, absolute cell number was determined with a FACScan flow cytometer (Becton Dickinson) after addition of a known concentration of Fluoresbrite microspheres (Polysciences, Eppelheim, Germany). The absolute number of cells/well was calculated as follows and according to a method described elsewhere:[23,24] {(total number of beads added/well) / (number of beads measured)} × (number of cells measured). The cytokinin analogue concentration at which cell growth was inhibited by 50% (IC_{50}) was calculated from dose–response curves.

Cell sorting

ABM cells (10^7 cells/ml) were labeled with the supernatant of the 43A1 hybridoma as a source of murine monoclonal IgG_3 anti-CD34 antibodies (kindly donated by Dr HJ Bühring, University of Tübingen, Germany),[25] washed and incubated with fluorescein-isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunglobulins (FITC-RAM-Ig) (DAKO, Glostrup, Denmark) (1 μg/10^6 cells). For sorting of $CD34^+CD38^-$ progenitors, cells labeled as described above were further incubated with 10-fold excess of mouse Ig and with phycoerythrin-conjugated murine monoclonal anti-CD38 antibody (anti-CD38-PE) (1 μg/10^6 cells) (Becton Dickinson, Erembodegem, Belgium). After washing twice in IMDM, $CD34^+$ hematopoietic progenitor cells or $CD34^+CD38^-$ immature hematopoietic progenitor cells were sorted on a FACStarPlus cell sorter equipped with a water-cooled argon ion laser (INNOVA Enterprise Ion Laser; Coherent, Santa Clara, CA, USA).

Myeloid colony-forming unit (CFU) assay

Direct myeloid colony formation by $CD34^+$ myeloid progenitor cells was assessed in a CFU assay. These assays were initiated with 500 cells per well and plated in duplicate in methylcellulose (0.9%)[26] and with different concentrations of cytokinin analogues. After 14 days of culture at 37°C in a 7.5% O_2–5% CO_2 fully humidified incubator, cultures were scored microscopically for colony formation. The cytokinin analogue concentration at which colony number was inhibited by 50% (IC_{50}) was calculated from dose–response curves.

Pre-CFU

Pre-CFU assays,[27] which are a functional assay of immature $CD34^+CD38^-$ hematopoietic progenitors,[28] were initiated as a liquid culture with 500 cells/well in 200 μl IMDM/10%FCS[26] and with different concentrations of cytokinin analogues. After 14 days of culture, cells were plated in duplicate at 500/well (in 1000 μl) in secondary methylcellulose CFU cultures as described for CFU assays. Cytokinin analogue IC_{50} values were determined on colony numbers obtained at the end of the secondary CFU culture.

DNA condensation

Cells were incubated with 5 μg/ml of the fluorochromes Hoechst 33342 and ethidium bromide (EB) (Sigma). Intensity of Hoechst 33342 staining and EB staining were evaluated with a Fluovert fluorescence microscope (Leitz, Wetzlar, Germany) and cells were scored as viable (faint Hoechst 33342 fluorescence), apoptotic (intense Hoechst 33342 staining and presence of apoptotic bodies), necrotic (EB red fluorescence) or secondarily necrotic, ie late apoptotic with features of necrosis (EB red fluorescence and apoptotic bodies).[29]

DNA fragmentation and cell cycle analysis

Control and cytokinin analogue-treated cell cultures were washed with phosphate-buffered saline (PBS), fixed in 1% formaldehyde at 4°C and permeabilized in 70% ethanol at −20°C. After rehydration in PBS, cells were incubated with TUNEL (terminal deoxynucleotidyl transferase-mediated FITC-dUTP nick end labelling) reaction mixture according to the manufacturer's (Boehringer Mannheim, Brussels, Belgium) protocol for 40 min at 37°C, washed in PBS and resuspended in 500 μl PBS containing 5 μg/ml EB and 0.1% RNAse. After 30 min of incubation at 4°C, green (FITC-dUTP incorporated in fragmented DNA) and red (EB binding to DNA) fluorescence of nuclei of individual cells was measured on a FACscan flow cytometer.

Statistical analysis

Comparisons were made using Student's *t*-test. Correlation coefficients were calculated using Spearman's rank correlation. IC_{50} values which could not be determined because the dose–response curve did not reach the level of 50% inhibition, were replaced in calculations by the concentration lying just above the highest concentration tested (for instance 51 μM, in the case where the highest concentration tested was 50 μM).

Results

Effects of cytokinin analogues on CDK

Inhibitory activity of cytokinin analogues on CDK1 and CDK2 was seen at concentrations ranging from 0.03 μM to >200 μM (Table 2). Eight of the new products in this series (P17, P18, P19, P22, P23, P29, P34, P35) showed a stronger inhibitory activity on CDK1 and CDK2 than the previously described CDK inhibitor olomoucine, which has an IC_{50} of 7 μM on CDK1 and CDK2. Cytokinin analogues have closely related inhibitory activities in both CDK1 and CDK2 assays ($r = 0.9996$, $P < 0.0001$).

Effects of cytokinin analogues on cell proliferation

Olomoucine showed low inhibition of proliferation of leukemic cell lines while compounds with improved CDK inhibition over olomoucine were more active in inhibiting malignant cell growth (Table 2). IC_{50} values were as low as 8 μM for KG1 (P23 and P35) and 7 μM for Molt3 (P29 and P35). Most compounds also inhibited growth of PBMC (Table 2). For cytokinin analogues with a demonstrable CDK inhibitory activity (ie with an $IC_{50} < 100$ μM), there was a significant correlation between inhibition of CDK and antiproliferative effect (Table 3).

Influence on clonogenic output of $CD34^+$ hematopoietic myeloid progenitors was tested for nine compounds and compared with the effect on the KG1 myeloid cell line, which is also $CD34^+$. Those compounds were chosen as representative examples of: (1)inhibitory activity on CDK and on KG1 cell proliferation (P10, P12, P23, P28); (2) no activity on CDK and activity on KG1 (P27, P16); (3) activity on CDK and no activity on KG1 (olomoucine); (4) and low activity on CDK and none on KG1 (P3). Compounds P23, P27, P12, P16, P10 showed selective growth inhibition of leukemic cells as compared to normal $CD34^+$ myeloid progenitors (Table 4). P23 was also selectively more active on leukemic myeloid cells, as compared to $CD34^+CD38^-$ immature hematopoietic progenitors.

Table 2 Effect of cytokinin analogues on CDK1 and CDK2 enzymatic activity and on proliferation of hematopoietic cell lines KG1 and Molt3 and of primary human lymphocytes (PBMC)

Compound No.	*CDK1 IC_{50} (μM)*	*CDK2 IC_{50} (μM)*	*KG1 IC_{50} (μM)*	*Molt3 IC_{50} (μM)*	*PBMC IC_{50} (μM)*
P35	0.1 ± 0.01	0.08 ± 0.02	8 ± 2	7 ± 1	5 ± 1
P29	0.11 ± 0.01	0.03 ± 0.01	14 ± 2	7 ± 1.8	6 ± 1
P7	0.2 ± 0.02	0.38 ± 0.05	22 ± 1.9	25 ± 4	14 ± 1.9
P8	0.8 ± 0.02	0.8 ± 0.08	14 ± 1	18 ± 0.5	10 ± 2
P19	1 ± 0.15	0.25 ± 0.05	11 ± 1	15 ± 1	5 ± 0.3
P17	1 ± 0.08	2 ± 0.1	29 ± 5	23 ± 6	18 ± 3
P34	1.1 ± 0.05	0.06 ± 0.01	12 ± 0.4	12 ± 1	7 ± 2
P10	1.1 ± 0.06	0.6 ± 0.07	32 ± 7	44 ± 2.6	11 ± 0.2
P28	1.5 ± 0.11	0.42 ± 0.08	14 ± 1	13 ± 1.5	5 ± 1
P18	2.6 ± 0.15	0.1 ± 0.05	19 ± 0.6	21 ± 3	11 ± 2
P23	2.6 ± 02	3.2 ± 0.2	8 ± 0.8	15 ± 1	11 ± 0.2
P22	3.8 ± 0.12	2.2 ± 0.2	13 ± 1.3	16 ± 2	9 ± 1.2
P24	6.3 ± 0.3	5.4 ± 0.4	>50	>50	27 ± 5
P21	6.5 ± 0.2	7 ± 0.5	49 ± 3	>50	41 ± 6
OL	7 ± 0.2	7 ± 0.2	>50	>50	27 ± 7
P12	21 ± 2.5	18 ± 2	16 ± 1.7	19 ± 2	7 ± 2
P20	23 ± 2.8	25 ± 2	>50	>50	35 ± 8
P3	65 ± 6	60 ± 5	>50	>50	>50
P27	>100	>100	9 ± 1.7	13 ± 1	7 ± 1
P26	>100	>100	19 ± 1	22 ± 3.6	19 ± 1
P25	>100	>100	28 ± 8	15 ± 4	17 ± 2
P16	>200	>200	25 ± 1.5	>50	25 ± 1

Results are grouped by decreasing effect on CDK1. CDK data are expressed as mean ± standard deviation. Cell data are expressed as mean ± standard error of the mean (s.e.m.) of at least three experiments.
OL, olomoucine; PBMC, peripheral blood mononuclear cells.

Table 3 Correlation coefficient (*r*) and corresponding *P* value of the comparison between effect of cytokinin analogues with demonstrable anti-CDK activity ($n = 18$) on CDK1 or CDK2 and on cell proliferation

Comparison	*r*	*P*
CDK1 – KG1	0.594	0.014
CDK1 – Molt3	0.664	0.006
CDK1 – Lymphocytes	0.607	0.012
CDK2 – KG1	0.613	0.011
CDK2 – Molt3	0.743	0.002
CDK2 – Lymphocytes	0.737	0.002

Table 4 Effect of cytokinin analogues on colony formation by primary human $CD34^+$ and $CD34^+CD38^-$ hematopoietic progenitor cells

Compound No.	*$CD34^+$ IC_{50} (μM)*	*$CD34^+$ $CD38^-$ IC_{50} (μM)*
P23	>25	>25
P27	>50	13 ± 7
P28	7 ± 2	3 ± 1
P12	32 ± 5	21 ± 2
P16	>50	13 ± 4
P10	>50	>25
OL	>50	13 ± 3
P3	>50	>25

Results are expressed as mean ± s.e.m. of at least three experiments. Compounds are grouped by decreasing effect on the KG1 cell line.

Induction of apoptosis and relationship to the cell cycle

Compounds that were analyzed for effect on apoptosis included: (1) P23 and P28 with activity on CDK and on KG1; (2) P27 without activity on CDK and with activity on KG1; and (3) olomoucine with activity on CDK and without activity on KG1. KG1 cells, treated with olomoucine at different time points and with different concentrations, displayed a low level of typical features of apoptosis, eg DNA condensation (Figure 1a and b) and DNA fragmentation. In contrast to olomoucine, compounds P23, P27 and P28 showed a clearly different dose–response curve (Figure 1a). The time–response curve showed a more rapid induction of apoptosis by P28 than by P23 and P27 (Figure 2b). When analyzing DNA fragmentation with the TUNEL technique, P23 and P28 showed DNA fragmentation after 12 h of incubation, while this was only apparent with P27 after 48 h. Using the TUNEL technique, no significant increase in DNA fragmentation could be detected in olomoucine-treated cells as compared to control KG1 cells, compatible with the low proportion of cells with DNA condensation seen after treatment with that compound.

Flow cytometric detection of DNA fragmentation combined with DNA staining can identify apoptotic cells and simultaneously evaluate their position in the cell cycle.[30,31] In all cultures treated with P23 or with P27 a higher proportion of cells in G_0/G_1 phase was seen within the apoptotic, ie TUNEL-positive cell population (Figure 2), strongly suggesting that P23- and P27-induced apoptosis mainly, but not exclusively, started in that phase of the cell cycle. No significant changes were noticed in any phase of the cell cycle within the non-apoptotic, ie TUNEL-negative cell population in P23-and P27-treated cultures.

P28 treatment resulted in a significantly higher proportion

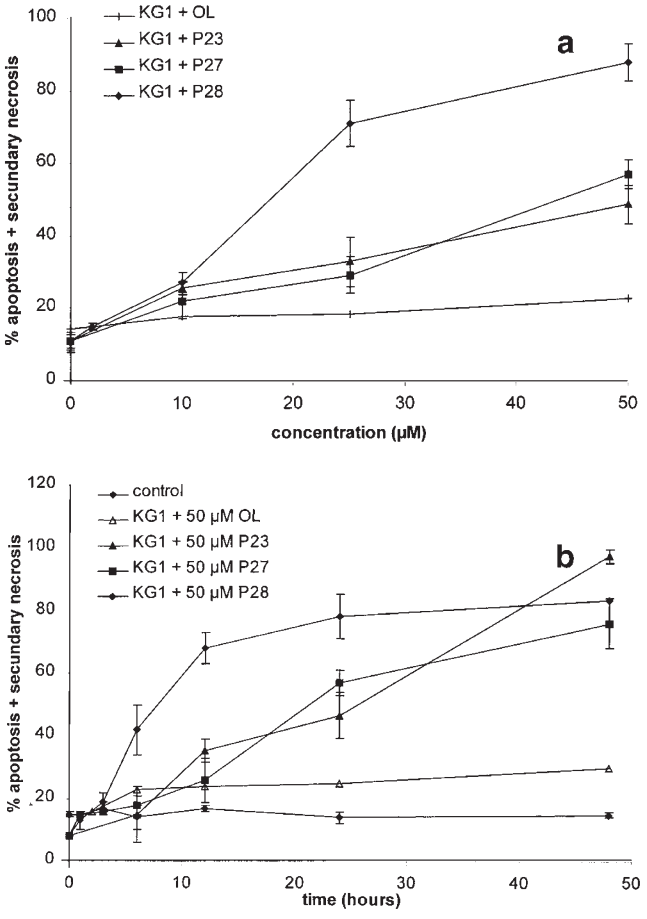


Figure 1 Percentage of apoptotic and secondarily necrotic KG1 cells as determined by Hoechst 33342 DNA condensation combined with ethidium bromide staining. (a) Following a 24 h treatment with different concentrations of P23, P27, P28 and olomoucine; (b) in cultures treated with 50 μM P23, P27, P28 and olomoucine during a 48 h time period.

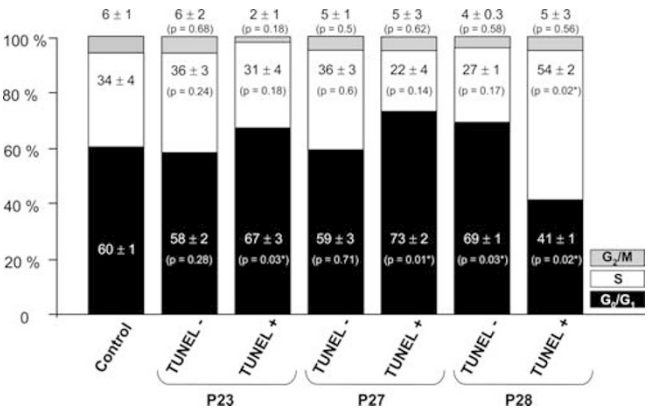


Figure 2 Cell cycle distribution of KG1 cells as detected by ethidium bromide in combination with TUNEL staining. KG1 cells were treated for 12 h with 50 μM P23 and P28 and for 48 h with 50 μM P27. Cell cycle distribution of non-apoptotic population (TUNEL −, TUNEL-negative population) and apoptotic population (TUNEL +, TUNEL-positive population) is shown as compared to cell cycle distribution from an untreated KG1 cell culture, which was uniformly TUNEL negative. Values are percentages of cells in G_0/G_1, S and G_2/M (the latter indicated above the bars) phases of the cell cycle and are expressed as mean ± s.e.m. of at least three experiments. Comparisons were made with control and *P* values are indicated between parentheses; significant *P* values are indicated with an asterisk.

of S-phase cells within the apoptotic population (Figure 2). This coincided with a higher proportion of cells in G_0/G_1 phase in the non-apoptotic population, indicating that cells in the S phase were selectively induced by P28 to undergo apoptosis.

Discussion

In this study cytokinin analogues were designed with structural properties for potential inhibition of cell cycle kinases. They were studied for CDK1/CDK2 inhibition, for inhibition of cell proliferation and for induction of apoptosis.

Structure–activity relationship

Previous studies have suggested that C2, C6 and N9 substituents on adenine are critical for inhibition of CDK.[13,16,22,32,33] In this study new aspects of the structure–activity relationship became apparent, including correlation with effect on cell proliferation.

A benzylamino group at position C6 of the purine ring is thought to be responsible for the specificity of CDK inhibition. In this study, it became apparent that the effects were increased by the addition of one hydroxyl group to the C6-benzylamino substituent of known CDK inhibitors. All the 6-hydroxybenzylamino-substituted cytokinin analogues strongly inhibited cell growth and, in most cases, inhibition of CDK1/CDK2 was also improved. Adding an hydroxyl group to the 6-benzylamino group of roscovitine (P7, P8) led to the development of P29, which has a meta-hydroxybenzylamino at C6, and a more pronounced inhibition of CDK1/CDK2 and of cell proliferation than roscovitine. The roscovitine analogue P19, with p-hydroxybenzylamino at C6, is a stronger inhibitor of cell growth and of CDK2, than roscovitine. Addition of a hydroxyl group at the C6-benzylamino group of bohemine (P10) improved CDK1/CDK2 inhibition in the case of ortho-hydroxyl substitution (P35). In the case of meta- (P34) or para-hydroxyl substitution (P18) only stronger CDK2 inhibition was observed. All three bohemine analogues (P18, P34, P35) had more effect on cell proliferation than bohemine.

Two compounds (P26, P27) without a benzyl substituent at C6 had no activity on CDK1/CDK2 but had antiproliferative effects (Table 2): they have a large substituent at C6, namely NH-adamant-1-yl. Steric hindrance is most likely the reason for low CDK1/CDK2 inhibition, and the known inhibitory effects of adamantyl derivatives on lymphocyte proliferation[34] are in accordance with the anti-proliferative effect of P26 and P27.

The compounds tested here have the highest structural variability in their C2 substituent and show that the C2 position allows for a large variety of substitutions. While the literature reports the need for an hydroxylated C2 substituent,[22] we have shown here that amino- or thiol-containing C2 substituents also inhibit CDK1/CDK2 activity and cell proliferation.

Relationship between inhibition of CDK and antiproliferative effect

Comparison of the behavior in cell-free and cell-based systems can help to determine a possible correlation between

effect on CDK activity and cell proliferation. In our study we found a significant correlation between inhibition of CDK1/CDK2 and inhibition of cell proliferation, strongly suggesting that at least part of the antiproliferative effect was due to CDK inhibition. As far as we know this is the first report discribing such a correlation for a large series of related compounds with an anti-CDK effect. The antiproliferative effect of cytokinin analogues may also be due to additional non-CDK-related effects, as has been reported for another CDK inhibitor, flavopiridol.[35] This may also explain why certain cytokinin analogues without anti-CDK activity (P16, P25, P26, P27) still have an antiproliferative effect. In addition two of these compounds (P26, P27) have an adamantyl substituent, which on its own may account for cytostatic action.

Eight new compounds (P17, P18, P19, P22, P23, P29, P34, P35) have an effect on CDK in a IC_{50} range between 0.08 and 3.8 μM. This is as low as the IC_{50} of flavopiridol (IC_{50} = 0.4 μM)[36] which is the first CDK inhibitor now in clinical trials (reviewed in Refs 35 and 37). All the new cytokinin analogues with CDK inhibition in this study inhibited lymphocytic and leukemic cell growth. The literature reports a variety of cellular effects of chemical CDK inhibitors and supports their evaluation as putative anti-tumoral agents.[35,38,39] Our observation of differential effects between leukemic and normal myeloid cells (especially regarding P23) underscores the potential therapeutic properties of these compounds. That this differential effect seen with myeloid cells was not observed when comparing leukemic with normal T lymphocytes (Molt3 *vs* PBMC), is probably due to cell-type dependent activity, that may reflect differences in cell uptake, differences in metabolization into inactive or active compounds, and/or differences in target proteins important for cell proliferation.[39]

Induction of apoptosis by cytokinin analogues

Cytokinin analogues have been shown in this study to induce apoptosis. The induction of apoptosis is probably the main cellular mechanism for the cytostatic effect of cytokinin analogues. Our data strongly suggest preferential (but not exclusive) induction of apoptosis in specific phases of the cell cycle, rather than a cell cycle phase arrest in the non-apoptotic population leading to a higher apoptotic fraction in that phase. Although CDK1 and CDK2 were inhibited to the same extent by the different compounds, some showed a different cell cycle-related effect. This may be due to the possibility that the correlation of CDK1 and CDK2 inhibition in cell-free systems does not entirely reflect *in vivo* cellular conditions, for instance due to differences in ATP concentration present *in vitro* and *in vivo* and/or due to additional target proteins other than CDK with an impact on cell proliferation and apoptosis.[39] With the apoptosis detection methods used, only the final stages of apoptosis were assessed. We are currently studying early events and mechanisms of cytokinin analogue-induced apoptosis and its relation to the cell cycle, including inhibition of CDK activity.

A new class of cytostatic agents?

In summary, we have shown that new 2,6,9-trisubstituted purines, with an analogy to plant hormone cytokinins, have significant effects both on CDK activity and on cell proliferation and that both activities are at least in part related to each other. Some compounds had more activity on malignant myeloid cells than on their normal counterparts and induced apoptosis in a time- and concentration-dependent manner. The development of now more than 20 compounds with a structure analogy to the cytokinin phytohormones suggests that we are dealing with a new class of chemotherapeutic agents and we propose to designate them as cytokinin analogues.

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