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CONCISE REPORT

Site-Selective cAMP Analogs at Micromolar Concentrations Induce Growth Arrest and Differentiation of Acute Promyelocytic, Chronic Myelocytic, and Acute Lymphocytic Human Leukemia Cell Lines

By Giampaolo Tortora, Pierosandro Tagliaferri, Timothy Clair, Oscar Colamonici, Leonard M. Neckers, Roland K. Robins, and Yoon Sang Cho-Chung

Cyclic AMP (cAMP)-dependent protein kinase may play a role in the functional and morphological differentiation of leukemic cells. In this study, we showed that the cAMP analogs, potent activators of protein kinase recently shown to be selective for either site 1 or site 2 cAMP binding sites of protein kinase, demonstrate potent growth inhibition of acute promyelocytic, chronic myelocytic, and acute lymphocytic leukemic cell lines with no sign of toxicity. The growth inhibition accompanied monocytic differentiation in HL-60 cells and a loss of nuclear terminal deoxynucleotidyl transferase activity in Molt-4 leukemic cells. The growth inhibition also paralleled a decrease in c-*myc* protein and R^I cAMP receptor protein. Thus, cAMP analogs selective for either site 1 or site 2 of the protein kinase appear to restore a coupling of proliferation and maturation in leukemic cells.

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THE CURRENT new approach to treating leukemia is to promote cell differentiation rather than cell killing. In a recent report,[1] we demonstrated that site-selective cAMP analogs, which are manyfold more active in protein kinase activation than the previously studied cAMP analogs, exert a major growth regulatory effect on a spectrum of human cancer cell lines. By using experimental models of human leukemic cell lines, we investigated whether the growth regulatory effect of site-selective cAMP analogs accompanies cell differentiation.

cAMP in mammalian cells functions through binding to its receptor protein, cAMP-dependent protein kinase.[2,3] Two distinct isozymes, type I and type II protein kinases, have been identified,[4,5] and differential expression of these isozymes has been linked to regulation of cell growth and differentiation.[6-9] The regulatory subunits (R^I, R^{II}) of type I and type II isozymes contain two types of binding sites for cAMP, site 1 and site 2,[10,11] and cAMP analogs that selectively bind to either one of the two sites are known as site 1 selective and site 2 selective, respectively.[11] Generally, analogs modified at the C-8 position are site 1 selective and those modified at the C-6 position are site 2 selective.

Furthermore, these site-selective analogs in appropriate combinations demonstrate synergism of binding and specificity toward either type I or type II kinases.[12,13] This unique binding specificity of cAMP analogs that demonstrate site selectivity is not mimicked by cAMP itself or by previously studied analogs.

In this study, we correlated the effect of site-selective cAMP analogs on the growth and differentiation of leukemic cells to the response of type I and type II protein kinases present in the leukemic cells.

MATERIALS AND METHODS

cAMP, N^6,$O^{2'}$-dibutyryl cAMP (DBcAMP) and 8-Br-cAMP were from Boehringer Mannheim Biochemicals (Indianapolis). All other cAMP analogs were synthesized[14] at the Nucleic Acid Research Institute (Costa Mesa, CA). The leukemic cell lines used include HL-60 (acute promyelocytic), K-562 (chronic myelocytic), *myc*-K562 (chronic myelocytic), and Molt-4 (acute T lymphocytic). All leukemic cell lines except *myc*-K562 were obtained from American Type Culture Collection (Rockville, MD), and the *myc*-K562 cell line was produced by infecting K-562 with a retroviral vector supplied by L. Wolff (National Cancer Institute, NIH, Bethesda, MD).

Cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/mL), streptomycin (500 μg/mL), 10 mmol/L HEPES buffer, and extra glutamine. For cell growth experiments, cells were treated with cAMP analogs one time at 3 hours after seeding, and cell counts in duplicate were performed on a Coulter counter 48 and 72 hours later. Surface antigen analysis of HL-60 cells was performed by flow cytometry using a panel of monoclonal antibodies reactive with either myeloid cells or monocytic cells. Terminal deoxynucleotidyl transferase (TdT)[15] was assayed by an immunoperoxidase method using the Bethesda Research Laboratory's (Gaithersburg, MD) TdT fluorescence kit. Western blotting of c-*myc* protein was performed by the method previously described[16,17] using c-*myc* antibody 15206D11 (Scripps Clinic and Research Foundation, La Jolla, CA). Photoaffinity labeling of cAMP receptor proteins with 8-N_3-[^{32}P]cAMP (ICN Pharmaceuticals, Irvine, CA) was performed as previously described.[18]

RESULTS

A variety of cAMP analogs, modified at either the C-6 or C-8 positions of the adenine moiety at various concentrations, were tested for their growth inhibitory effect on leukemic cell lines (Table 1). Among the C-8 analogs (site 1 selective) tested, 8-Cl-cAMP exhibited the most potency, demonstrating 50% growth inhibition at 5-20 μmol/L concentrations (IC_{50}) in all four leukemic cell lines. 8-Br-,

From the Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, and the Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD; and the Nucleic Acid Research Institute, Costa Mesa, CA.

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Address reprint requests to Dr Yoon Sang Cho-Chung, Laboratory of Tumor Immunology and Biology, NCI, NIH, Building 10, Room 5B38, Bethesda, MD 20892.

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Table 1. Effect of Site-Selective cAMP Analogs on Growth of Leukemic Cell Lines

Cyclic Nucleotide Analog	cAMP Analog	Inhibition of Growth IC_{50} (μmol/L) HL-60	Molt-4	K-562	*myc*-K562
C-8	8-Chloro	10	5	20	20
	8-Bromo	50	100	100	100
	8-Thiomethyl	100	100	100	100
	8-Aminomethyl	100	100	100	100
C-6	N^6-Benzyl	20	10	27	30
	N^6-Benzoyl	50	40	45	50
	N^6, $O^{2'}$-Dibutyryl	500	—*	1,000	1,000

*No growth inhibition.

Table 2. Modulation of Differentiation Markers in HL-60 Cells by 8-Cl-cAMP

Markers	Control (%) Positive	8-Cl-cAMP* (20 μmol/L) Positive
My7	81	11
My9	75	54
Leu M1	72	0
Leu M5	0	0
MO_2	0	75
OKM_5	0	51

*Seventy percent growth inhibition with 90% cell viability.

8-thiomethyl-, and 8-aminomethyl-cAMP were 5 to 20 times less potent than 8-Cl-cAMP. N^6-benzyl-cAMP was the most potent of the C-6 analogs (site 2 selective) tested with IC_{50} values of 10 to 30 μmol/L. N^6-benzoyl-cAMP, which has structural similarity with N^6-benzyl-cAMP, exhibited IC_{50} values of 40 to 50 μmol/L (Table 1). DBcAMP, the analog most commonly used in the past studies,[19-21] exhibited the least potency, with IC_{50} values of 500 to 1,000 μmol/L, and in Molt-4, the 50% growth inhibition (IC_{50}) could not be obtained (Table 1). Growth inhibition by the site-selective cAMP analogs was not due to cell killing; the cells were 80% to 90% viable as determined by exclusion of trypan blue dye.

Phosphodiesterase inhibitors, such as theophylline (0.1 mmol/L) or 1-methyl-3-isobutylxanthine (0.5 mmol/L), each alone had little or no growth inhibitory effect, and the inhibitors could not enhance the analog effect when added in combination with the analog (data not shown). These results suggest that the analogs produced growth inhibition at concentrations below which the degradation by phosphodiesterase could take place and also that the growth inhibition was not due to raising cellular cAMP. Our results are compatible with recent reports that in intact rat hepatocytes[22] and rat heart and rat fat cells[23] site-selective cAMP analogs caused a decrease rather than an increase in cellular cAMP.

We examined the effect of site-selective cAMP analogs on the expression of differentiation markers in HL-60 cells to determine if the growth-arrested HL-60 cells are more differentiated than the untreated cells. Treatment for 3 days with 8-Cl-cAMP exhibiting 90% cell viability induced a marked increase in the expression of monocyte-specific surface antigens (MO_2, OKM_5) and a decrease in markers related to the immature progenitor cells (My7, My9) (Table 2).[24,25] The 8-Cl-cAMP–treated cells became strongly positive for α-naphthyl butyrate esterase, a cytochemical marker for monocytes, and underwent a monocytic morphological transformation characterized by a decreased nuclear-to-cytoplasm ratio, abundant ruffled and vacuolated cytoplasm, and loss of nucleoli (data not shown).

Disappearance of cellular TdT has been considered as a differentiation marker for human T lymphocytic leukemia.[15] Treatment of Molt-4 (acute T lymphocytic) leukemia cells with 8-Cl-cAMP (10 μmol/L) caused a time-dependent decrease in TdT activity; at 2 days after the treatment, TdT activity decreased to 50% of that in untreated control cells, and by day 4, the activity decreased to 10% of the untreated control levels. Moreover, treatment for 4 days with 8-Cl-cAMP in combination with N^6-benzyl cAMP (20 μmol/L) caused almost complete loss (>95%) of TdT activity (data not shown). These cells exhibiting the loss of TdT demonstrated >90% viability.

By using a propidium iodide staining method, we examined whether the reduced cell proliferation observed in the leukemic cell lines after treatment with the analogs was due to a specific block in one phase of the cell cycle.[26,27] The results showed that the fractions of cells in each phase of cell cycle were not appreciably different between the control cells and the cells treated with the analogs (data not shown).

The type I isozyme of cAMP-dependent protein kinase has been considered to be involved in cell proliferation and transformation, whereas the type II isozyme is involved in cell differentiation and inhibition of cell growth.[6-9] Because type I and type II protein kinase differ only in their ***regulatory*** subunits (the cAMP binding receptor protein),[4,5] we measured, using the photoaffinity ligand 8-N_3-[^{32}P]cAMP,[18] the cAMP receptor protein during the analog treatment of these leukemic cells. As shown in Fig 1A, the untreated Molt-4 leukemic cells contained a major cAMP receptor protein with a mol wt of 48,000 (lane 1), the R^I cAMP receptor protein (the regulatory subunit of type I protein kinase).[2] When the cells were treated for 3 days with 8-Cl-cAMP (lane 2), the R^I receptor protein markedly decreased, whereas the R^I protein remained without appreciable change when the cells were treated with DBcAMP (lane 3). That the decrease of the R^I receptor photoaffinity labeling found after 8-Cl-cAMP treatment could be due to the presence of bound 8-Cl-cAMP to the R^I receptor is unlikely; 8-Cl-cAMP, like 8-piperidino-cAMP,[13] selectively binds to site 1 of R^{II} but binds to site 2 of R^I receptor (D. Øgreid, confidential personal communication); thus, 8-Cl-cAMP bound to site 2 of R^I would synergistically enhance, instead of interfering with, the site 1-selective binding of 8-N_3-[^{32}P]cAMP.

8-Cl-cAMP also caused a marked reduction of c-*myc* protein level (Fig 1B, lane 2), whereas DBcAMP (lane 3) did not affect the c-*myc* protein level . 8-Cl-adenosine (5 μmol/L), despite its strong growth inhibitory effect (85%), affected neither the R^I level nor the c-*myc* protein level (Table of Fig 1), indicating that a decrease in R^I and c-*myc* protein levels caused by 8-Cl-cAMP treatment does not merely reflect

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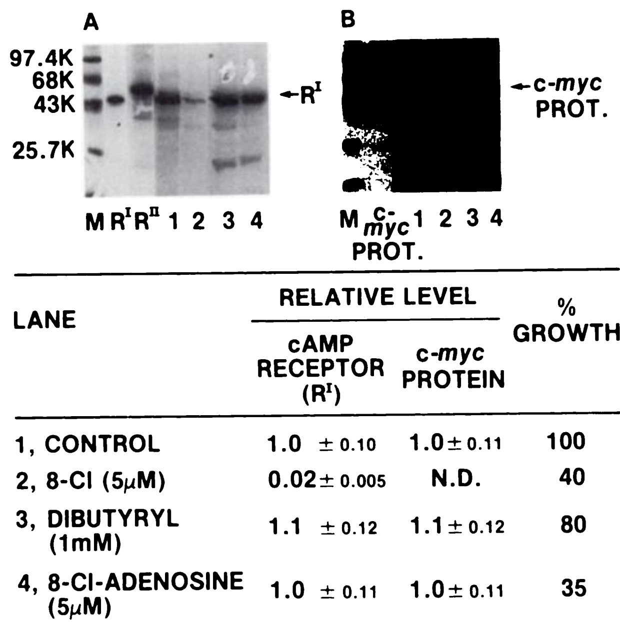

LANE	RELATIVE LEVEL		% GROWTH
	cAMP RECEPTOR (R^I)	c-*myc* PROTEIN	
1, CONTROL	1.0 ± 0.10	1.0 ± 0.11	100
2, 8-Cl (5μM)	0.02 ± 0.005	N.D.	40
3, DIBUTYRYL (1mM)	1.1 ± 0.12	1.1 ± 0.12	80
4, 8-Cl-ADENOSINE (5μM)	1.0 ± 0.11	1.0 ± 0.11	35

Fig 1. Effect of site-selective cAMP analog treatment on the levels of cAMP receptor protein and c-*myc* protein. (A) Photoactivated incorporation of 8-N_3-[^{32}P]cAMP; (B) Western blotting of c-*myc* protein. R^I, the 48,000 mol wt R^I cAMP receptor protein[2]; R^{II}, the 56,000 mol wt R^{II} cAMP receptor protein[28]; c-*myc* protein, a purified preparation of c-*myc* protein.[29] Lane 1, untreated control cells; lanes 2 through 4, the cells treated for 3 days with 8-Cl-cAMP (5 μmol/L), DBcAMP (1 mmol/L), and 8-Cl-adenosine (5 μmol/L), respectively. M, marker proteins of known mol wt (Bethesda Research Laboratories). Each lane contained 100 μg protein for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The cell pellets, after two washes with phosphate-buffered saline, were suspended in buffer ten (0.1 mol/L NaCl, 5 mmol/L $MgCl_2$, 1% Nonidet P-40, 0.5% Na deoxycholate, 2 KIU/mL bovine aprotinin, 20 mmol/L Tris-HCl, pH 7.4) (2 × 10^7 cells/mL), vortexed, passed through a 22-gauge needle ten times, allowed to stand for 30 minutes at 4°C, and centrifuged at 750 g for 20 minutes at 4°C; the resulting supernatants were used as cell lysates. Numbers in panel represent the average value ± SE of seven separate experiments. ND, nondetectable.

growth inhibition or cell death in general. A similar decrease in R^I and c-*myc* protein levels also occurred in other leukemic lines, K-562, *myc*-K562, and HL-60, after 8-Cl-cAMP treatment. The R^{II} cAMP receptor protein was not detected in Molt-4 but was measurable in other leukemic cell lines. The analog treatment did not affect the R^{II} levels in these leukemic cells.

DISCUSSION

To our knowledge, the present data represent the first unequivocal demonstration that site-selective cAMP analogs are capable of exerting a major effect on the growth of promyelocytic, chronic myelocytic, and acute T lymphocytic human leukemic cell lines at micromolar concentrations. All previously reported studies of cAMP regulation of cell growth, using DBcAMP, reported effective concentrations in an unphysiologic millimolar range.[19-21] The analog effect was not due to raising of cellular cAMP level as was previously believed, because phosphodiesterase inhibitors in combination with the analog did not enhance the analog effect. The analogs worked directly through cAMP receptor protein, the regulatory subunit of cAMP-dependent protein kinase.[4,5] Among the site-selective analogs tested, 8-Cl-cAMP, which has a strong site 1 selectivity for type II protein kinase (90-fold more than that of cAMP, D. Øgreid, confidential personal communication), exhibited the most potency. The analog effect correlated with a selective modulation of two types of cAMP receptor proteins—a marked reduction in the R^I receptor, which was previously related to cell growth and transformation,[7,8] with no change in the R^{II} receptor, which was related to growth arrest and differentiation.[6,9]

This selective modulation of the R^I and R^{II} cAMP receptor protein was not achieved by the early-known analog, DBcAMP. The growth inhibition also caused a marked reduction in c-*myc* protein level. The decrease in the R^I cAMP receptor and c-*myc* protein was not observed when cells were growth arrested by 8-Cl-adenosine, indicating that the analog effect was not due to its adenosine metabolite.

The growth arrest by the analogs accompanied differentiation of the leukemic cells, as shown by the expression of several surface antigens specific for monocytic differentiation in HL-60 cells and a loss of the activity of TdT, a marker enzyme for cell immaturity in Molt-4 cells. Despite the appearance of markers of mature phenotype and definitive growth arrest shown in the analog-treated cells, the cell cycle phase distribution between the treated and untreated cells was similar; namely, the treated cells exhibited no G_0/G_1 arrest.

In normal myeloid cell precursors, the growth inducers induce cell viability and cell multiplication and also production of differentiation inducers.[30-33] In leukemic cells, therefore, continuous production of growth inducers may be essential for continuous production of differentiation inducers to achieve their terminal differentiation. The site-selective cAMP analogs, which produce growth arrest while allowing the cells to progress through their normal cell cycle but at a slower rate, may be ideal agents for terminal differentiation of leukemic cells because they would allow continuous production of differentiation inducers. Thus, site-selective cAMP analogs appear to restore the balance between proliferation and maturation of leukemic cells.

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