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Site-selective cyclic AMP analogs provide a new approach in the control of cancer cell growth

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Site-selective cyclic AMP analogs bind to site 1 or site 2 of the known cAMP-binding sites depending on the position of substituents on the purine ring, either at C-2 and C-8 (site 1) or at C-6 (site 2). The growth inhibitory effect of such site-selective cAMP analogs used in this investigation with 15 human cancer cell lines surpassed that of analogs previously tested. The most potent analogs were 8-chloro, N^6-benzyl and N^6-phenyl-8-*p*-chlorophenylthio-cAMP. The combination of a C-8 with an N^6 analog had synergistic effects. The 24 site-selective analogs tested produced growth inhibition ranging from 30 to 80% at micromolar concentrations with no sign of toxic effects. Growth inhibition was not due to a block in a specific phase of the cell cycle but paralleled a change in cell morphology, an increase of the R^{II} cAMP receptor protein and a decrease of p21 *ras* protein. Since the adenosine counterpart of the 8-chloro analog produced G_1 synchronization without affecting the R^{II} and p21 *ras* protein levels, it is unlikely that an adenosine metabolite is involved in the analog effect. Site-selective cAMP analogs thus provide a new biological tool for control of cancer growth.

Cyclic AMP; Protein kinase; Proto-oncogene; (Human cancer line)

1. INTRODUCTION

Cyclic adenosine 3′,5′-monophosphate (cAMP) has been implicated as a regulatory agent in cell growth and differentiation [1–4], but its precise role has not yet been uncovered. cAMP in mammalian cells functions by binding to its receptor protein, cAMP-dependent protein kinase [5,6]. Two distinct isozymes, type I and type II protein kinases, have been identified [7,8], and differential expression of these isozymes has been linked to the regulation of cell growth and differentiation [9–12]. Since a mixture of type I and type II kinase isozymes is present in most tissues [7,8], selective modulation of these isozymes in intact cells may be a crucial function of cAMP.

Both isozymes contain dimeric regulatory (R) subunits and two catalytic (C) subunits [7,8]. The R subunits (R^I, R^{II}) of type I and type II isozymes differ, but the C subunits of isozymes are identical [7,8]. Both R^I and R^{II} contain two types of binding sites for cAMP, site 1 and site 2 [13,14], and cAMP analogs that selectively bind to either one of the two sites are known as site 1 selective (C-2, C-8 analogs) and site 2 selective (C-6 analogs), respectively [13,14].

Site-selective analogs in appropriate combinations demonstrate synergism of binding and specificity toward either type I or type II kinases [15,16]. Application of these in vitro findings on

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the site-selective analogs to cAMP effects in vivo in intact cells or tissues has been scarce [17–19]. Therefore, we investigated the effect of site-selective cAMP analogs on the growth of human cancer lines of various cell types.

2. MATERIALS AND METHODS

cAMP, N^6,$O^{2'}$-dibutyryl-cAMP (DBcAMP) and 8-Br-cAMP were from Boehringer Mannheim (Indianapolis, IN). All other cAMP analogs were synthesized [20] at the Nucleic Acid Research Institute (Costa Mesa, CA).

The human cancer cell lines used included MCF-7, MCF-7*ras*, MDA-MB-231, T-47D, ZR-75-1, BT-20 and HBL-100 breast cancers; WiDr, LS-174T and HT-29 colon carcinomas; A549 lung carcinoma; HT-1080 fibrosarcoma; A4573 Ewing's sarcoma; FOG and U251 gliomas. All breast cancer cell lines were grown in improved minimal essential medium supplemented with 10% fetal bovine serum (FBS), 20 mM Hepes, penicillin-streptomycin and extra glutamine; colon carcinoma cell lines and A549 and HT-1080 cell lines were grown in Eagle's minimal essential medium supplemented with 10% FBS, Eagle's minimal essential medium non-essential amino acids, 20 mM Hepes, extra glutamine and penicillin-streptomycin. A4573 cell line was grown in RPMI supplemented with glutamine, penicillin-streptomycin, 20 mM Hepes and 10% FBS. FOG and U251 cell lines were grown in Dulbecco's modified minimal essential medium supplemented with penicillin-streptomycin, glutamine, 20 mM Hepes and 10% FBS. All cells were grown at 37°C in humidified incubators in an atmosphere of 5% CO_2.

For cell growth experiments, 2–3 × 10^5 cells/60 mm dish were seeded, and 24 h later (day 0), the medium was removed, and fresh medium and the additives were added then and every 48 h thereafter. The cAMP analogs were added using 100× concentrated stock solutions. As desired times, cell counts in duplicate were performed on a Coulter counter after harvesting cells with gentle trypsinization.

Cell extracts were prepared at 0–4°C. The cell pellets (2 × 10^7 cells), after two washes with phosphate-buffered saline, were suspended in 0.5 ml buffer Ten (0.1 M NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% Na deoxycholate, 2 KIU/ml bovine aprotinin and 20 mM Tris-HCl, pH 7.4), vortex-mixed, passed through a 22-gauge needle 10 times, allowed to stand for 30 min at 4°C, and centrifuged at 750 × *g* for 20 min; the resulting supernatants were used as cell lysates.

3. RESULTS AND DISCUSSION

Various site-selective cAMP analogs, C-2, C-6 and C-8 monosubstituted and C-2 and C-8 or C-6 and C-8 disubstituted, were tested for their effect on the growth of cancer lines. Table 1 shows values for the growth inhibitory effect of 24 cAMP analogs. The values for percentage growth inhibition were determined from dose-response curve experiments. The analogs are listed in order from the most to the least potent for growth inhibition for a given modification on the adenine ring. In breast (MCF-7 and MCF-7*ras*) and colon (LS-174T) cancer cell lines, analogs modified with a halogen or thio moiety at the C-8 position were more potent than those modified with an amino moiety at the C-8 position. Thus, at 50 μM, 8-Cl-, 8-thiomethyl- and 8-Br-cAMP exhibited 40–80% growth inhibition, while 8-amino-β-hydroxyethyl-, 8-aminomethyl- and 8-*N*,*N*-dimethylamino-cAMP exhibited growth inhibition of ≤20%. Halogen or thio derivatives of C-8 analogs have generally greater specificity for type II protein kinase activation, while amino derivatives of C-8 analogs demonstrate greater specificity for type I protein kinase activation [15,16]. Our data showing the greater growth inhibition by C-8 halogen or thio derivatives than C-8 amino derivatives suggest the involvement of the cellular type II protein kinase in growth inhibition. C-6 analogs were generally less potent in growth inhibition than the C-8 analogs. At 50 μM, N^6-benzyl- and N^6-carbonylethoxy-cAMP demonstrated 30–70% growth inhibition. Dibutyryl-cAMP, at 50 μM, exhibited no growth inhibition of either breast or colon cancer cells.

The C-6,-8 analog, N^6-phenyl-8-thio-*p*-chlorophenyl-cAMP, having structural similarity within both N^6-benzyl-cAMP (the most potent C-6 analog) and 8-Cl-cAMP (the most potent C-8 analog), exhibited the greatest potency among the disubstituted analogs tested, whereas the analogs with a structural similarity in only one of the two positions, such as N^6-*n*-butyl-8-thio-*p*-chloro-

Table 1

Effect of site-selective cAMP analogs on the growth of breast (MCF-7, MCF-7*ras*) and colon (LS-174T) cancer cell lines

	Cyclic nucleotide analog: abbreviated name	% growth inhibition at 50 μM MCF-7	MCF-7*ras*	LS-174T
C-8	8-chloro	67	80	75
	8-thiomethyl	46	48	42
	8-bromo	41	47	40
	8-iodo	26	28	25
	8-thio-*p*-chlorophenyl	23	22	24
	8-amino-β-hydroxyethyl	15	15	20
	8-aminomethyl	14	18	15
	8-*N*,*N*-dimethylamino	0	0	0
C-6	N^6-benzyl	56	65	70
	N^6-carbonylethoxy	32	42	40
	N^6-benzoyl	30	33	28
	N^6-carbamoylphenyl	10	20	0
	N^6-butyryl	0	0	0
	N^6,$O^{2'}$-dibutyryl	0	0	0
C-2	2-*n*-hexyl	36	25	42
	2-thiobenzyl	28	30	22
	2-phenyl	10	15	15
C-6,-8	N^6-phenyl-8-thio-*p*-chlorophenyl	60	55	47
	N^6,N^6-diethyl-8-thio-*p*-chlorophenyl	43	43	45
	6-piperidino-8-thio-*p*-chlorophenyl	33	30	25
	N^6-benzyl-8-thiobenzyl	25	30	20
	N^6-*n*-butyl-8-thio-*p*-chlorophenyl	15	20	25
C-2,-8	2-*n*-butyl-8-bromo	46	37	36
	2-methyl-8-thio-*p*-chlorophenyl	30	36	22

The values of percentage growth inhibition were determined from dose-response curve experiments. Each value represents an average value obtained from three or more separate experiments. Cell counting was performed on days 3 and 4

phenyl-cAMP or N^6-benzyl-8-thiobenzyl-cAMP, exhibited less potency. C-2 analogs and C-2,-8 analogs tested here were in general less potent.

8-Cl-, N^6-benzyl- and N^6-phenyl-8-thio-*p*-chlorophenyl-cAMP were the three most potent growth inhibitors for all 15 cancer cell lines. These analogs at 20 μM demonstrated 40–76% growth inhibition (table 2). Thus, the site-selective cAMP analogs exerted a potent growth inhibition in a wide spectrum of human cancer cell lines: hormone-dependent (MCF-7, T-47D, ZR-75-1 [21]) and hormone-independent (MDA-MB-231, MCF-7*ras*, BT-20 [22,23]) breast cancer lines, colon and lung cancer lines, fibrosarcoma and glioma cell lines. The majority of these cancer cell lines have shown a resistance to dibutyryl-cAMP treatment, even at 1 mM, and to cAMP phosphodiesterase inhibitors.

Previous studies have demonstrated that binding of a cAMP analog selective for either intrachain site on the regulatory subunit of protein kinase stimulates binding of a cAMP analog selective for the other site [24,25], and two such site-selective analogs in combination demonstrate synergism in protein kinase activation [15,16].

To test the effect of cAMP analog combinations, C-8 analog (site 1 selective) and C-6 analog (site 2 selective) were combined such that alone they exhibit growth inhibition of 10–20% each, and the effects were quantified by synergism quotient [16,17]. Synergism quotient was defined as the net growth inhibitory effect of the analog combination divided by the sum of the net individual analog effects on growth inhibition. The results showed that, in both breast and colon cancer lines, 8-Cl-cAMP (1 μM) in combination with either N^6-benzyl- (0.5 μM) or N^6-benzoyl-cAMP (0.5

Table 2

Growth inhibition of human cancer cell lines by site-selective cAMP analogs

Cell line		% growth inhibition at 20 μM		
		8-Cl	N^6-Benzyl	N^6-Phenyl-8-thio-*p*-chlorophenyl
Breast cancer	MCF-7	65 (60–70)	50 (42–60)	57 (55–60)
	MCF-7*ras*	75 (70–80)	60 (55–70)	50 (45–55)
	MDA-MB-231	60 (54–63)	52 (44–56)	42 (36–52)
	T-47D	52 (44–60)	50 (45–55)	55 (45–60)
	ZR-75-1	51 (42–57)	53 (46–60)	55 (45–60)
	BT-20	54 (45–65)	42 (37–52)	53 (45–60)
	HBL-100	47 (40–52)	53 (48–59)	41 (36–55)
Colon cancer	LS-174T	70 (64–75)	60 (55–65)	45 (42–52)
	WiDr	68 (65–78)	62 (52–70)	46 (35–50)
	HT-29	70 (62–80)	45 (43–48)	48 (38–56)
Lung cancer	A549	71 (65–79)	60 (50–70)	51 (41–61)
Fibrosarcoma	HT-1080	56 (45–60)	55 (45–65)	40 (35–50)
Ewing's sarcoma	A4573	60 (55–70)	62 (50–72)	52 (47–55)
Glioma	FOG	60 (55–65)	76 (66–81)	62 (53–70)
	U251	45 (40–50)	60 (55–65)	60 (55–65)

The values for percentage growth inhibition were determined from dose-response curve experiments and represent an average value and range (in parentheses) obtained for each analog from three or more separate experiments. Cell counting was performed on days 3 and 4

μM) produced the greatest degree of growth inhibition compared with that expected from the sum of the individual analogs alone (synergism quotient = 1.66–1.77). The combination, therefore, produced growth inhibition equivalent to that shown by 10–15 μM of either analog alone. The N^6 analogs also demonstrated synergism of growth inhibition with other C-8 analogs, such as 8-thiomethyl-cAMP (synergism quotient = 1.50–1.87) and 8-Br-cAMP (synergism quotient = 1.5). Only a limited degree of synergism was expressed, however, when the N^6 analogs were combined with 8-amino derivatives (synergism quotient = 1.08–1.25). Bovine heart type II and rat heart type I protein kinases exhibit a different C-8 analog specificity for stimulation of binding and synergism of activation when combined with a C-6 analog [15,16]. Specifically, when used in combination with a C-6 analog, those analogs with sulfur or halogen attached to C-8 act more synergistically for type II protein kinase, and type I kinase exhibits greater synergism using analogs with nitrogen attached at C-8 [15,16]. Our results, showing that the synergism of growth inhibition by N^6 analogs when combined with 8-thio and 8-Cl analogs far exceeds that by N^6 analogs in combination with 8-amino derivatives, suggest a response of type II protein kinase present in the cancer cells.

The hormone-dependent and -independent breast, colon and lung cancer cells, and fibrosarcoma and glioma cells all demonstrated a characteristic change in their cell morphology after treatment with 8-Cl- or N^6-benzyl-cAMP at 10–20 μM for 4–5 days. The cells exhibited enlargement of the cytoplasm with a stretched fibroblast-like appearance (not shown). Thus, growth inhibition produced by the site-selective analogs brought about a change in cell morphology.

Using a propidium iodide staining method [26], we examined whether the reduced cell proliferation observed in the cancer cell lines after treatment with the analogs was due to a specific block in one phase of the cell cycle. The results showed that the fractions of cells in G_1, S and G_2/M phases were not appreciably different between control cells (untreated) and those treated with 8-Cl-cAMP. Thus, the inhibition of cell growth induced by the cAMP analogs was not associated with a specific block in one phase of the cell cycle. However, 8-Cl-adenosine treatment induced an appreciable in-

crease in G_1 with a marked reduction in S phase of the cell population. These data indicated that the growth inhibition produced by 8-Cl-cAMP treatment was not due to its adenosine metabolite.

It has been considered that the type I isozyme of cAMP-dependent protein kinase is involved in cell proliferation and transformation, while type II isozyme is involved in cell differentiation and inhibition of cell growth [9–12]. Since the type I and type II protein kinases differ only in their regulatory subunits (the cAMP-binding receptor protein) [7,8], we measured, using the photoaffinity ligand 8-N_3-[^{32}P]cAMP [27], the cAMP receptor protein during the analog treatment of these cancer cells. As shown in fig. 1A, the untreated breast

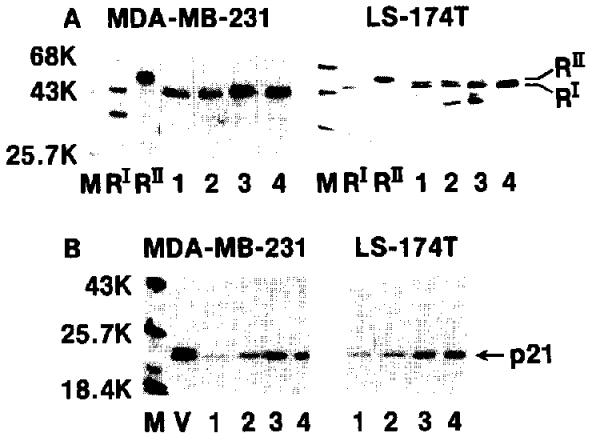

Fig. 1. The effect of site-selective cAMP analog treatment on the levels of cAMP receptor proteins and p21 *ras* protein. (A) Photoactivated incorporation of 8-N_3-[^{32}P]cAMP; (B), Western blotting of p21 protein. R^{I}, the 48 kDa R^{I} cAMP receptor protein; R^{II}, the 56 kDa R^{II} cAMP receptor protein; V, cell lysate from Ha-MuSV-transformed NIH 3T3 clone 13-3B-4 [18]. Lanes 1–3, cells, each treated for 3 days with 8-Cl-cAMP (10 μM), N^6-benzyl-cAMP (20 μM) and N^6,$O^{2'}$-dibutyryl-cAMP (1 mM), respectively; lane 4, untreated control cells; M, marker proteins of known molecular mass (Bethesda Research Laboratories). Each lane contained 100 μg protein for SDS-PAGE. The photoactivated incorporation of 8-N_3-[^{32}P]cAMP was performed as in [27] at cAMP exchange condition of 23°C for 60 min, and the samples were then subjected to SDS-PAGE. Western blotting of p21 protein was performed on the cell lysate as described [31,32] using p21 monoclonal antiserum Y13-259 [33]. To provide a reference p21, a cell lysate from Ha-MuSV-transformed NIH 3T3 clone 13-3B-4 [18] was used.

(MDA-MB-231) and colon (LS-174T) cancer cells contained a major cAMP receptor protein of 48 kDa (lane 4), the R^{I} cAMP receptor protein (the regulatory subunit of type I protein kinase) [5]. When the cells were treated for 3 days with 8-Cl-cAMP (lane 1) or N^6-benzyl-cAMP (lane 2), the 52 kDa R^{II} (the regulatory subunit of type II protein kinase) [28,29] increased appreciably with concomitant decrease of the R^{I} receptor protein. When the cells were treated with dibutyryl-cAMP, which did not appreciably inhibit cell growth, the R^{I} and R^{II} receptor levels remained unchanged (cf. lanes 3,4). Quantification by densitometric tracings of the autoradiograms showed that the site-selective analog treatments brought about a 70–80% decrease in the R^{I} level and a 10–12-fold increase in the R^{II} receptor level, resulting in a 4–9-fold increase in the ratio of R^{II} to R^{I}. These results were further confirmed with DEAE-cellulose chromatography of the cell extracts. Treatment with 8-Cl-cAMP induced an increase of both R^{II} and type II protein kinase holoenzyme with concomitant decrease of R^{I} and type I protein kinase holoenzyme (not shown).

The changing levels of the R^{I} and R^{II} cAMP receptor proteins paralleled a reduction of the cellular transforming gene product, p21 *ras* protein [30], as measured by Western blotting analysis. As shown in fig.1B, treatment with 8-Cl-cAMP (lane 1) or N^6-benzyl-cAMP (lane 2) caused a decrease in p21 *ras* protein level, whereas dibutyryl-cAMP (lane 3), which did not affect cell growth, caused no reduction in the p21 level. Thus, the same C-6 and C-8 analogs caused a decrease in the R^{I} cAMP receptor and p21 protein levels and an increase in the R^{II} cAMP receptor level.

The changes in R^{I}, R^{II}, and p21 levels were not found in the growth-arrested cells treated with 8-Cl-adenosine. Thus, 8-Cl-adenosine failed to produce the biochemical change induced by 8-Cl-cAMP.

To our knowledge, our data represent the first unequivocal demonstration that site-selective cAMP analogs are capable of exerting a major effect on the growth of a spectrum of human cancer cell lines. We have shown that the site-selective analogs are effective at micromolar concentrations. All previously reported studies of cAMP regulation of cell growth, using dibutyryl-cAMP derivatives, reported effective concentrations in

the unphysiologic millimolar range [1–3]. The analogs appear to work directly through the cAMP receptor protein, cAMP-dependent protein kinase present in the cells, by substituting endogenous cellular cAMP as recently demonstrated in rat hepatocytes [34] and rat heart and fat cells [35]. Among the site-selective analogs tested, 8-Cl-cAMP, which has a high site 1 selectivety for type II protein kinase (90-fold over that of cAMP; Ogreid, D., personal communication), exhibited the most potency in all cancer lines tested. In fact, an increase in the R^{II} cAMP receptor protein with a decrease in the R^{I} receptor protein correlated with the growth inhibitory potency of the analogs. This unique behavior of the site-selective analogs, demonstrating selective modulation of the R^{I} and R^{II} cAMP receptor proteins in cancer cells, is not mimicked by the early known analogs, such as dibutyryl-cAMP, cAMP itself or agents that increase cellular cAMP level. cAMP at high levels, having no site selectivity, activates both type I and type II protein kinase isozymes maximally and equally without discrimination [5–8].

The synergistic effect of the C-6 and C-8 analog combinations demonstrated on growth inhibition provides further support for analog efficacy being dependent on its ability to activate selectively type II protein kinase. However, the analogs could vary considerably in their cell penetration rate, phosphodiesterase sensitivity, and in other properties that could also contribute to their potencies.

The grown inhibitory effect of the site-selective cAMP analogs was not due to the cytotoxic effect of its adenosine metabolites as experimentally documented using 8-Cl-adenosine. Thus, the growth inhibitory effect of site-selective cAMP analogs as described here is different from that in previous reports, which have shown strong cytotoxicity by some of the amino-substituted C-8 analogs [36] and cyclic nucleotides of purine analogs [37].

Growth inhibition produced by the analogs brought about biochemical and morphological changes but did not produce G_1 arrest in the cancer cells tested here. Therefore, the analogs may produce growth inhibition by slowing down the cell cycle progression and perhaps promoting cell differentiation. In fact, the role of site-selective cAMP analogs in the promotion of differentiation has been shown in leukemic cell lines [38].

The site-selective analogs were able to arrest the growth of cancer cells that are resistant to dibutyryl-cAMP. Therefore, the site-selective cAMP analogs tested here may be of use as growth inhibitors for a wide spectrum of cancer cells, including those previously found to be resistant to other cAMP analogs or to agents that increase intracellular cAMP.

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