Unhydrolyzable Analogues of Adenosine 3':5'--Monophosphate Demonstrating Growth Inhibition and Differentiation in Human Cancer Cells

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ABSTRACT

A set of adenosine 3':5'-monophosphate (cAMP) analogues that combine exocyclic sulfur substitutions in the equatorial (Rp) or the axial (Sp) position of the cyclophosphosphate ring with modifications in the adenine base of cAMP were tested for their effect on the growth of HL-60 human promyelocytic leukemia cells and LS-174T human colon carcinoma cells. Both diastereomers of the phosphorothioate derivatives were growth inhibitory, exhibiting a concentration inhibiting 50% of cell proliferation of 3-100 μM. Among the analogues tested, Rp-8-Cl-cAMPS and Sp-8-Br-cAMPS were the two most potent. Rp-8-Cl-cAMPS was 5- to 10-fold less potent than 8-Cl-cAMP while Sp-8-Br-cAMPS was approximately 6-fold more potent than 8-Br-cAMP. The growth inhibition was not due to a block in a specific phase of the cell cycle or due to cytotoxicity. Rp-8-Cl-cAMPS enhanced its growth-inhibitory effect when added together with 8-Cl-cAMP and increased differentiation in combination with N'-benzyl-cAMP. The binding kinetics data showed that these Sp and Rp modifications brought about a greater decrease in affinity for Site B than for Site A of RI (the regulatory subunit of type I cAMP-dependent protein kinase) and a substantial decrease of affinity for Site A of RII (the regulatory subunit of type II protein kinase) but only a small decrease in affinity for Site B of RII, indicating the importance of the Site B binding of RII in the growth-inhibitory effect. These results show that the phosphorothioate analogues of cAMP are useful tools to investigate the mechanism of action of cAMP in growth control and differentiation and may have practical implication in the suppression of malignancy.

INTRODUCTION

cAMP in mammalian cells acts through its receptor protein, cAMP-dependent protein kinase (1, 2). There are two general types of cAMP-dependent protein kinase, designated as type I and type II, that contain distinct regulatory subunits, RI and RII, respectively, but share a common catalytic subunit (3).

It has been shown (4–6) that differential regulation of type I versus type II protein kinase is critically related to the growth-inhibitory effect of site-selective cAMP analogues. Namely, the potency of site-selective cAMP analogues in growth inhibition correlates with a marked down-regulation of type I protein kinase along with an up-regulation of type II protein kinase (7, 8). Unlike parent cAMP, site-selective cAMP analogues demonstrate selective binding toward either one of the two known cAMP binding sites, [Site A (Site 2) and Site B (Site 1)] (9, 10), of protein kinase, resulting in preferential binding and activation of either protein kinase isozymes (11, 12). The striking growth-inhibitory effect of 8-Cl-cAMP (13) is in fact related to its selective binding and activation of protein kinase isozymes. It binds to type II protein kinase with a high affinity for Site B but with a low affinity for Site A, keeping this isoform in holoenzyme form while binding with moderately high affinity for both Site A and Site B to type I protein kinase, facilitating the RI subunit dissociation and down-regulation of this isoform (6, 8).

Rp-cAMPS, which has a single sulfur substitution for the exocyclic equatorial oxygen, acts as an agonist of cAMP-induced activation of cAMP-dependent protein kinase, while Sp-cAMPS, which has a single sulfur substitution in the exocyclic axial oxygen position, acts as a cAMP agonist (14–16). Previous studies have shown (16) that the antagonist binds to both type I and type II protein kinase holoenzyme and locks the R subunits in a configuration that has a high affinity for the catalytic subunit, thus preventing activation of the holoenzyme. The selectivity of phosphoroethioates for the two different cAMP binding sites has also been addressed (17). Dostmann et al. (18) showed that for type I protein kinase, Sp and Rp forms of cAMP analogues show preference for Site A and Site B, respectively, while for type II protein kinase, both Sp- and Rp-cAMP analogues exhibit preference for Site B.

To elucidate the importance of an intact cyclic phosphate structure for the mechanism of inhibition of tumor cell proliferation and the induction of differentiation and to clarify how important site selectivity for an analogue to Site B of protein kinase type II is, we selected a set of new cAMP analogues according to the test kit concept (19, 20). Systematic modifications were chosen to (a) prevent hydrolysis of an analogue and thus interference with metabolite formation and (b) find out whether the biological data obtained in cell culture are yielding the same sequence of biological activity as exhibited by the kinetic binding data obtained for Sites A and B of protein kinases, type I and II. The first aim was reached by substitution of the exocyclic oxygen atom of cAMP and its analogues by sulfur, yielding the diastereomers Sp- (axial substitution) and Rp- (equatorial substitution). In addition these modifications lead to a more pronounced site selectivity ratio in favor of protein kinase type II Site B as compared with parent compounds cAMP (18).

We examined the effect of Sp and Rp forms of cAMP analogues on the growth of HL-60 human promyelocytic leukemia and LS-174T human colon carcinoma cells in comparison to that of the corresponding cyclophosphate analogue, and the results were analyzed in relation to the binding affinity of the analogue for and activation potency of protein kinase isozymes.

MATERIALS AND METHODS

Chemicals. 8-Cl-cAMP was obtained from the National Cancer Institute, Division of Cancer Treatment, Bethesda, MD; 8-Br-cAMP was obtained from Sigma Chemical Company (St. Louis, MO). Rp- and Sp-cAMPS and Sp-cAMPS analogues were synthesized as described previously (17, 21, 22).

Cell Culture. HL-60 leukemic cells, obtained from the American
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Type Culture Collection (Rockville, MD), were grown in suspension culture in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (500 μg/ml), penicillin (50 units/ml), and glutamine (1 mM) (Gibco, Grand Island, NY). For cell growth experiments, cells were seeded at the density of 2 x 10⁵/ml and treated with cAMP analogues 3 h after seeding. Cell counts were performed at desired times on a Coulter Counter. Cell membrane integrity was measured by trypan blue exclusion test, and cellular integrity was measured by forward and side scatter with FACSscan (Becton Dickinson, San Jose, CA). Cell cycle distribution was determined by DNA content analysis by propidium iodide staining as described previously (23). Briefly, cells were fixed in 70% ethanol and stored at 4°C before analysis. Nuclear DNA was stained with propidium iodide, 50 μg/ml, for 30 min at room temperature. RNase was added to avoid double-helix RNA staining. The DNA formed by a Cell-FIT program using the method of Dean (24) (Becton Dickinson Immunocytochemistry Systems). Pulse area versus pulse width gating was performed to exclude doublets from the G₁-M region. For each sample, 20,000 events were stored in list mode. Cell surface antigen analysis was performed by flow cytometry with FACSscan using a panel of monoclonal antibodies reactive with either monocytic or myeloid cells.

The LS-174T human colon carcinoma cell line (kindly provided by J. Greiner, National Cancer Institute) was grown in Eagle’s minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, Eagle’s minimum essential medium nonessential amino acids, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM glutamine, and penicillin-streptomycin (Gibco, Grand Island, NY); the medium was changed every 48 h. For cell growth experiments, 2 x 10⁴ cells/60-mm dish were seeded; 24 h later the medium was removed, and fresh medium and cAMP analogues using 100× concentrated stock solutions were added. At desired times, cell counts were performed in duplicate on a Coulter Counter after harvesting cells with gentle trypsinization.

Protein Kinase Assays. Protein kinase assays were performed by measuring the transfer of ³²P from [γ-³²P]ATP to Kemp tide essentially as described elsewhere (25).

RESULTS

Binding Affinities of Rp- and Sp-Phosphorothioate Analogues of cAMP. The affinities of the cAMP analogues for Site A and Site B of RI and RII were determined and standardized against the relative affinity of cAMP for the same sites

\[ K_i \text{(analogue)} = \frac{K_i \text{cAMP}}{K_i \text{analogue}} \]

Dostman et al. (18) have shown that the substitution of sulfur for the exocyclic axial oxygen (Sp) in general shows a slight reduction in affinity for Site A of RI and a substantially higher decrease in affinity for Site B of RI. The mean reduction in affinity for RII, resulting from the Sp modification, is much larger for Site A compared with Site B, which in general shows a small decrease in affinity compared with the corresponding cyclophosphate. As shown in Table 1, both Sp-8-Cl-cAMPS and Sp-8-Br-cAMPS exhibited a greater decrease in affinity for Site B (54- and 19-fold decrease, respectively) than Site A (15- and 7-fold decrease, respectively) of RII, while showing preferential decrease in affinity for Site A (22- and 37-fold decrease, respectively) over Site B (1.9- and 3.1-fold decrease, respectively) of RII as compared with the corresponding cyclophosphate.

It has been shown (18) that the introduction of sulfur for the exocyclic axial oxygen leads to a substantial decrease in affinity for all four binding sites in both RI and RII. As shown in Table 1, Rp-8-Cl-cAMPS exhibited a 340- and 480-fold decrease in affinity for Site A and B, respectively, of RI and a 180-fold decrease in affinity for Site A of RII as compared with its cyclophosphate, 8-Cl-cAMP. However, Site B of RII showed the smallest reduction (a 10-fold decrease) (Table 1). Thus, a greater decrease in affinity for Site B than for Site A of RI and a substantial decrease in affinity for Site A of RII with a small decrease in the Site B affinity of RII are the common changes resulting from the phosphorothioate modifications of 8-Cl-cAMP.

Table 2 shows the protein kinase activation constants \( K_a \) of these Sp- and Rp-phosphorothioate cAMP analogues. Sp-8-Cl-cAMPS had an 11- and a 2.5-fold increase in the \( K_a \) for protein kinase type I and type II, respectively, as compared with 8-Cl-cAMP. Rp-8-Cl-cAMPS was incapable of activating type I protein kinase but was a weak agonist for type II protein kinase (23-fold increase in the \( K_a \) as compared with 8-Cl-cAMP).

Inhibition of Growth of HL-60 Cells by Phosphorothioate Derivatives of cAMP Analogues. The phosphorothioate derivatives of cAMP analogues were tested for their growth-inhibitory effect in HL-60 leukemia cells in comparison with their homologous cyclophosphate derivatives. Fig. 1 displays the dose-response curves for 3 days of treatment. 8-Cl-cAMP is a potent growth inhibitor of HL-60 cells (5) (Fig. 1). Rp-8-Cl-cAMPS, the exocyclic sulfur substitution in the equatorial position of the cyclophosphate ring, was less inhibitory than 8-Cl-cAMP but was more potent than Sp-8-Cl-cAMPS, which has a sulfur substitution in the exocyclic axial oxygen position. The growth-inhibitory effect of 8-Br-cAMP was low (<5% growth inhibition at 20 μM). However, a marked enhancement of growth inhibition was observed with Sp-8-Br-cAMPS, which has a sulfur substitution in the exocyclic axial oxygen position (47% growth inhibition at 20 μM concentration). The growth-

![Table 1](image)
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The inhibitory effect of Rp- and Sp-cAMPS was negligible (<5% at 20 μM).

Table 3 shows IC₅₀ values of cAMP analogues. The IC₅₀ values of all the analogues at day 6 were 10- to 12-fold lower than those at day 3, indicating that the sensitivity of cells to analogues increased with the length of time of treatment. The IC₅₀ values of day 6 indicated that the Rp- and Sp-phosphorothioates of 8-Cl-cAMP were 10- and 20-fold less potent, respectively, than 8-Cl-cAMP. In contrast, Sp-8-Br-cAMPS exhibiting the IC₅₀ value identical to that of Rp-8-Cl-cAMPS was 6-fold more potent than 8-Br-cAMP.

Using a propidium iodide staining method (28, 29), we examined whether the reduced cell proliferation observed in HL-60 cells was due to a specific block in one phase of the cell cycle. As shown in Table 4, the fractions of cells in G₁, S, and G₂-M phases were not appreciably different between control cells (untreated) and those treated with Rp- or Sp-8-Cl-cAMPS for 96 h.

Inhibition of Growth of LS-174T Human Colon Carcinoma Cells by Phosphorothioates of cAMP Analogues. The growth-inhibitory effect of phosphorothioates of cAMP and cAMP analogues on LS-174T cells is shown in Table 5. The analogues are listed in order from the most to the least potent for growth inhibition. Rp-8-Cl-cAMPS and Sp-8-Br-cAMPS exhibited the greatest potency, Sp-6-SETcPMPS, Sp-8-Cl-cAMPS, and Rp-cAMPS showed moderate potency, and Sp-cAMPS and Sp-2-Cl-cAMPS exhibited the least potency.

Rp-phosphorothioate of cAMP acts as an antagonist of cAMP-dependent protein kinase activation (14). We examined whether Rp-phosphorothioate would interfere with the growth-inhibitory effect of homologous or heterologous cycl phosphorothioate derivative. Neither Rp-8-Cl-cAMPS nor Rp-cAMPS interfered with the growth-inhibitory effect of 8-Cl-cAMP, and both exhibited an additive effect (Table 6).

Differentiation of HL-60 Leukemia Cells by Rp-8-Cl-cAMPS. We examined the effect of Rp- and Sp-8-Cl-cAMPS 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. Cells treated for 3 days with 50 μM Rp-8-Cl-cAMPS showing a 90% cell viability exhibited an

Table 3 Growth inhibition of HL-60 human leukemia cells by phosphorothioate derivatives of cAMP analogues

The IC₅₀ values were determined from the dose-response curve experiments like those shown in Fig. 1 and represent an average value obtained for each analogue from two or more separate experiments carried out for 3 days or 6 days (see “Materials and Methods”). The values of all the analogues at day 6 were 10- to 12-fold lower than those at day 3 of culture time after treatment of cells with each analogue at given concentrations at day zero (3 h after seeding) (see “Materials and Methods”).

Table 4 Effect of Rp- and Sp-8-Cl-cAMPS on cell cycle kinetics of HL-60 cells

DNA content analysis by propidium iodide staining was performed; see “Materials and Methods.”

Table 5 Effect of phosphorothioate derivatives of cAMP analogues on the growth of LS-174T human colon carcinoma cells

The percentage growth inhibition values shown at 10 and 50 μM analogue concentrations were determined from the dose-response curve experiments like those shown in Fig. 1 and represent an average value obtained for each analogue from two or more separate experiments (see “Materials and Methods”).

Table 6 Additive growth inhibitory effect between phosphorothioate analogue and 8-Cl-cAMP on LS-174T human colon carcinoma cells

The percentage growth inhibition values represent an average value obtained for each analogue from two separate experiments carried out for 4 days. The analogues were added at 24 h after seeding; see “Materials and Methods.”

**Fig. 1.** Effect of cAMP analogue concentration on growth inhibition of HL-60 leukemia cells. The growth inhibition was determined by counting cell number at day 3 of culture time after treatment of cells with each analogue at given concentrations at day zero (3 h after seeding) (see “Materials and Methods”). The percentage of growth inhibition was obtained by comparing the growth of treated cells to that of untreated control cells.
increased the expression of monocyte-specific surface antigens (3- and 2-fold increase of Leu-15 and HLA-DR, respectively, over the control cell levels) as did 5 µM 8-Cl-cAMP (Fig. 2), whereas treatment of cells with 100 µM Sp-8-Cl-cAMPS had no appreciable effect on the surface antigen expression (Fig. 2). We have shown previously that 8-Cl-cAMP in combination with N⁴-benzyl-cAMP demonstrates a synergism on cell differentiation (30). Rp-8-Cl-cAMPS in combination with N⁴-benzyl-cAMP demonstrated the synergistic effect on the surface antigen expression (10- and 6-fold increase of Leu-15 and HLA-DR, respectively, over the control cell levels) (Fig. 2). These results show that Rp-8-Cl-cAMPS can mimic the effect of 8-Cl-cAMP on the differentiation of HL-60 leukemia cells.

DISCUSSION

We have shown here that diastereomeric phosphorothioates of cAMP analogues are potent inhibitors of the growth of human cancer cell lines. Rp-8-Cl-cAMPS and Sp-8-Br-cAMPS, both exhibiting IC₅₀ 3 µM in HL-60 leukemia cells, were the two most potent growth inhibitors among the phosphorothioates tested.

The growth-inhibitory effect of Rp-8-Cl-cAMPS was about 10-fold lower than that of 8-Cl-cAMP but 3-fold greater than that of Sp-8-Cl-cAMPS. The mechanism of action of Rp-8-Cl-cAMPS in growth inhibition appears to be similar to that of 8-Cl-cAMP. The growth inhibition by Rp-8-Cl-cAMPS did not bring about any change in cell cycle phase as was shown with 8-Cl-cAMP (5), and Rp-8-Cl-cAMPS enhanced its growth-inhibitory effect when added together with 8-Cl-cAMP and increased differentiation in combination with N⁴-benzyl-cAMP as was shown with 8-Cl-cAMP plus N⁴-benzyl-cAMP (30).

The striking potency of 8-Cl-cAMP in growth inhibition has been related to its selective bifunctional effect on protein kinase isozyme type I versus type II. It drastically down-regulates type I protein kinase by dissociating it into its subunits while up-regulating type II protein kinase in its holoenzyme form (6, 8). The mechanism of action of 8-Cl-cAMP involves nuclear translocation of RII (6) and an increase in binding of nuclear factor(s) to a synthetic cyclic AMP response element (31). It has been proposed that 8-Cl-cAMP induces the nuclear translocation of type II protein kinase holoenzyme through binding to Site B of RII (8). The selective nuclear translocation of type II protein kinase but not type I protein kinase (6) has been interpreted (13) to be due to the primary structure of RII subunit which contains four positively charged amino acids 262KKKR265K (32), the nuclear location signal (33), near the Site B cAMP binding site. It may be that the selective Site B binding by 8-Cl-cAMP provides a controlled exposure of this sequence, which might be the signal for regulating the transport of this protein into the nucleus (34). Thus, preferential Site B binding to RII could be the critical step in the effect of cAMP analogues in growth regulation.

Previous work with diastereomeric phosphorothioate analogues of cAMP has demonstrated that the cyclic phosphate position of the cAMP molecule and, in particular, the integrity of the exocyclic oxygens, are important for determining the binding affinity of the analogues and for initiating the molecular events leading to dissociation of the holoenzyme (14-16). The sulfur substitution of either exocyclic oxygen has been shown to result in a significant loss in cAMP-binding affinity (18). However, it was also found (18) that two cAMP binding sites (Site A and B) contribute differently to this reduction. The average reduction for the Sp modification is about 6-fold higher for BII (Site B of RII) compared with AI (Site A of RI) and approximately 5-fold higher for AII (Site A of RII) compared with BII (Site B of RII). For the Rp modifications, the reduction is 2-fold higher for AI than for BII and 4-fold higher for AII than for BII. Thus, both Sp and Rp modifications bring about only a small decrease in BII binding but a significant loss in binding for other sites.

The present study demonstrated that the Sp and Rp modifications of 8-Cl-cAMP affected BII affinity minimally while exerting a great decrease in affinity for other sites (Table 1). It appears, however, that rather than the absolute affinity of BII, the ratio of affinity for BII/AII is important in the growth-inhibitory effect of these analogues, since Rp-8-Cl-cAMPS, which showed a greater potency in growth inhibition than Sp-8-Cl-cAMPS, had the absolute affinity for BII much lower than Sp-8-Cl-cAMPS (Table 1). Our results also showed that Rp-8-Cl-cAMPS was incapable of activating type I protein kinase, whereas Sp-8-Cl-cAMPS, despite its significant loss in affinity for AI and BII, was capable of activating type I protein kinase (Table 2). It appears from these results that activation of type I protein kinase is not essential but facilitates growth inhibition since Rp-8-Cl-cAMPS was more potent in growth inhibition than Sp-8-Cl-cAMPS but was less potent than 8-Cl-cAMP.

Interestingly, Sp-8-Br-cAMPS was much more potent in growth inhibition than 8-Br-cAMP, despite the fact that the absolute binding affinities of this phosphorothioate to RI and RII are much lower than the cyclophosphate (Table 1) (18). The Sp modification of 8-Br-cAMP brought about enhancement of relative preference for Site A over Site B of RI and Site B over Site A of RII (Table 1) (18). Thus, rather than the absolute binding affinity, the changes in relative preference for the specific site, especially Site B of RII, appear to be involved in the growth-inhibitory potency of the analogue.

Our data showed that the Rp derivatives of cAMP or 8-Cl-cAMP exhibited not an antagonistic but an agonistic effect on the growth inhibition. If only phosphorylation controlled by cAMP would be responsible for the effects observed, at least Rp-cAMPS, which is undoubtedly an antagonist of holoenzyme dissociation (16), should not show inhibition of proliferation at

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Fig. 2. Effect of phosphorothioate derivatives of cAMP analogue on the surface marker expression of HL-60 cells. Control (untreated) cells and cells treated with cAMP analogues for 96 h were analyzed for the expression of monocyte (Leu-M3, HLA-DR, and Leu-15) and myeloid (Leu-M1) specific antigens. The data represent an average of three separate experiments. For each sample, 2 x 10⁴ cells were analyzed, and cell gating was performed using forward and side scatter. □, Leu-M3; ■, HLA-DR; □, Leu-15; ■, Leu-M1. Rp-8-Cl-S, Rp-8-Cl-cAMPS; N⁴-benzyl-cAMP; Sp-8-Cl-S, Sp-8-Cl-cAMPS; 8-Cl, 8-Cl-cAMP.
all. The observation of growth inhibition by the Rp derivatives, especially by those which have little or no potency in protein kinase activation (i.e., dissociation of R subunit from C subunit), suggests a role for protein kinase holoenzyme or R subunit independent of protein phosphorylation in the regulation of cell proliferation.

In general, Sp and Rp modifications render cAMP analogues more lipophilic and resistant to hydrolysis by phosphodiesterase (35). Thus, phosphorothioate analogues may be superior candidates over the cyclophosphates in practical applications especially when the analogues demonstrate a greater potency than homologous cyclophosphates as was the case for Sp-8-Br-cAMPs. Further studies on the structure-function analysis of phosphorothioate derivatives of cAMP would provide insights into the mechanism of action of cAMP in growth regulation and differentiation.

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