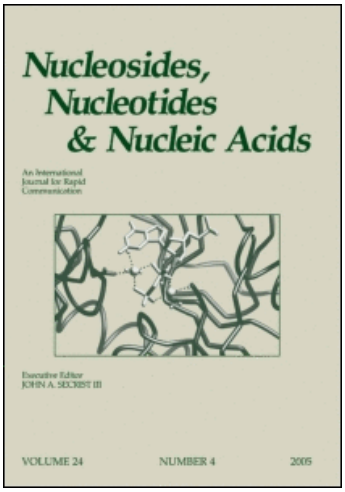

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis and Biological Action of a New Cyclic AMP Analogue

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To cite this Article Fujihashi, Toshiaki , Ogata, Takahiro , Ohkuma, Tetsuo , Endo, Tohru and Kaji, Akira(1992) 'Synthesis and Biological Action of a New Cyclic AMP Analogue', Nucleosides, Nucleotides and Nucleic Acids, 11: 2, 295 — 310
To link to this Article: DOI: 10.1080/07328319208021704
URL: http://dx.doi.org/10.1080/07328319208021704

NUCLEOSIDES & NUCLEOTIDES, 11(2-4), 295-310 (1992)

SYNTHESIS AND BIOLOGICAL ACTION OF A NEW CYCLIC AMP ANALOGUE

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Abstract: A new cyclic AMP analogue, adenosine- 3', 5'-cyclic methyl phosphonate (cAMP-Me) was chemically synthesized. This compound was not a substrate for phosphodiesterase, and it did not activate cAMP-dependent protein kinases (type I or type II). However, it inhibited cAMP phosphodiesterase and protein kinase at milimolar concentration levels. It also inhibited malignant cell proliferation in vitro.

The role of cyclic AMP (cAMP) in the regulation of metabolic processes has been well established in various types of cells. It may also play an important role in differentiation and growth regulation of normal and tumor cells (1). The physiological effects of various agents which raise the intracellular cAMP level on tumor cell growth are not consistent, depending on experimental conditions (2). However, it has been suggested that in normal cells, the level of cAMP is raised upon contact inhibition of growth in cell culture, while in tumor cells, this mechanism is altered in such a way that the cAMP level does not raise upon contact and no contact inhibition of growth is observed (3). Furthermore, pharmacological manipulation for raising the intracellular cAMP level, such as the addition of prostaglandin E_1 to the medium, has been reported to cause growth cessation in some tumor cells (4). cAMP in

This paper is dedicated to the memory of the late Professor Tohru Ueda.

mammalian cells functions by binding to its receptor protein, a regulatory subunit of cAMP-dependent kinase (5). Two distinct isozymes, type I and type II protein kinases, have been identified (6), and their quantitative ratio is different between normal and malignant tissues (7). It has also been suggested that type I kinase is involved in cell growth while type II is involved in cell differentiation (8, 9). The compound which exerts inhibitory action selectively against type I protein kinase or stimulatory action selectively on type II kinase, would be expected as an antitumor agent.

The early observation that exogenously administered cAMP causes a dramatic effect on the growth of tumor cells (10) was the beginning of numerous studies done on experimental cancer chemotherapy with cAMP. However, the application of exogenous cAMP in vivo did not produce the desired response; perhaps due to both the rapid degradation by either extracellular or intracellular cyclic nucleotide phosphodiesterase and the poor penetration of cAMP into cells (11). In an attempt to overcome these problems, N^6,2'-*O*-dibutyryl cAMP (DBcAMP) was synthesized and shown to have various pharmacological actions in vivo, including the inhibition of tumor growth (12), stimulation of tracheal relaxation (13), and the activation of glycolysis in liver (14). However, the antitumor activity of DBcAMP has not been proven unequivocally.

In this communication, we describe the synthesis and some of the biological properties of a new cAMP analogue, 3',5'-cyclic methylphosphonate of adenosine. It had no stimulatory action on both type I and type II protein kinase activities, but had some inhibitory action on these enzyme activities in the presence of cAMP. This cAMP analogue was not hydrolyzed by cAMP phosphodiesterase, and it inhibited phosphodiesterase activity. It also inhibited the growth rate of human malignant cells in vitro.

RESULTS

Synthesis and Characterization of cAMP-Me. The synthetic scheme of cAMP-Me (**5**) is shown in FIG. 1. The starting material, adenosine, was first treated with 1,1,3,3-tetra isopropyl disiloxane dichloride ($TIPDSiCl_2$) to yield the 3',5'-*O*-silyl derivative (**1**). **1** was treated with 2,3-dihydropyran to yield **2**. Deprotection of silyl group of **2** with tetra-*n*-butylammonium fluoride (TBAF) led to the corresponding dialcohol (mixture of isomers of 2'-position, **3**). The

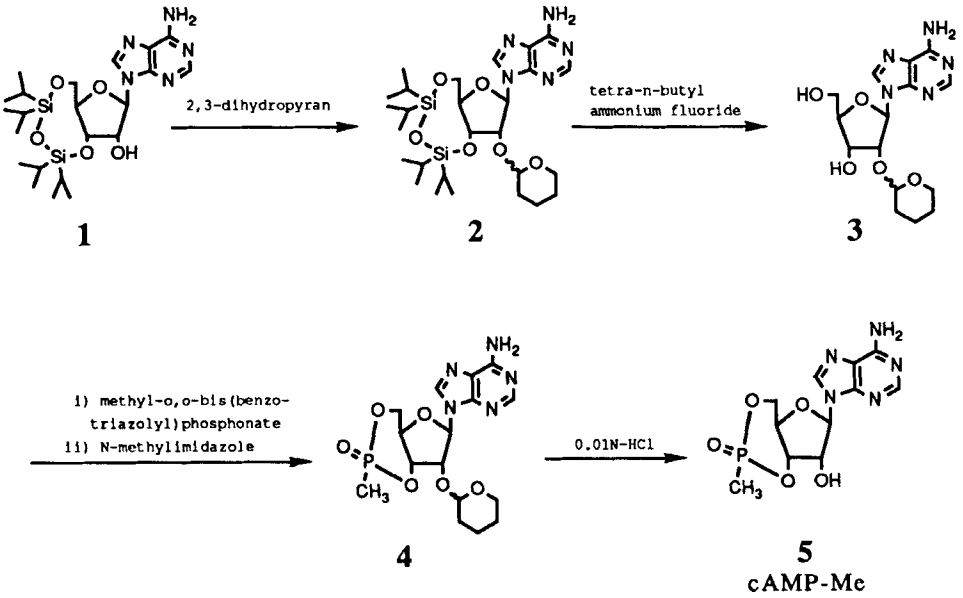

FIG. 1. Synthetic scheme for preparation of cAMP-Me (5).

final product (cAMP-Me, 5) was obtained by the phosphorylation of 3 with methyl-*O,O*-bis (benzotriazolyl) phosphonate followed by the deprotection of the tetrahydropyranyl group with HCl in an overall yield of 33%. The ^{1}H-NMR spectrum of cAMP-Me (in CD_3OD) showed a doublet at δ 1.74 (J=18Hz), which was assigned to the methyl proton linked to the phosphorous atom. The chemical shift in ^{31}P-NMR spectrum at δ 37.68 (singlet) was due to the methylphosphonate group. As shown in FIG. 2, the reverse phase high performance liquid chromatography (RP-HPLC) of purified material showed a major peak indicating over 99% purity. Two minor peaks were proven to be adenosine 5'-methylphosphonate (5'-AMP-Me) and adenosine 3'-methylphosphonate (3'-AMP-Me) in a separate experiment.

Effect of cAMP-Me on cAMP-dependent protein kinase. To examine the possibility that cAMP-Me may substitute cAMP in the activation of cAMP-dependent protein kinase, the experiment indicated in FIG. 3 was performed. Since the protein kinase reaction with histone as a substrate proceeded almost linearly for up to 20 min, the effect of cAMP-Me or 8-azide cAMP (8-N_3-cAMP) on protein kinase was determined by measuring the phosphorylation of

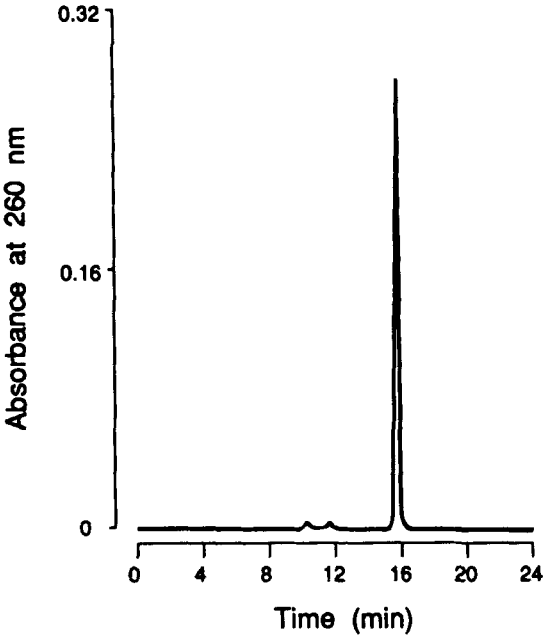

FIG. 2. RP-HPLC analysis of purified cAMP-Me. The HPLC conditions are described in Experimental Procedures.

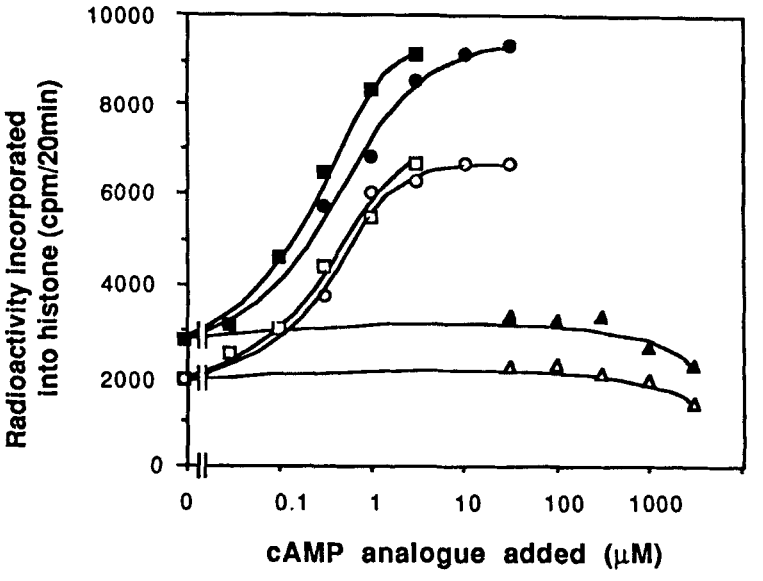

FIG. 3. Agonistic effect of various concentrations of cAMP analogues on the two types of cAMP-dependent protein kinases. The kinase activity in the presence of various concentrations of each cAMP analogue was assayed for 20 min. Incorporation of ^{32}P-phosphate from [γ-^{32}P]-ATP was plotted against the concentration of cAMP analoues added. Open and closed symbols indicate the activity of type I and type II protein kinase, respectively. Circles (○,●), cAMP; triangles(△,▲), cAMP-Me; squares (□,■), 8-N_3-cAMP.

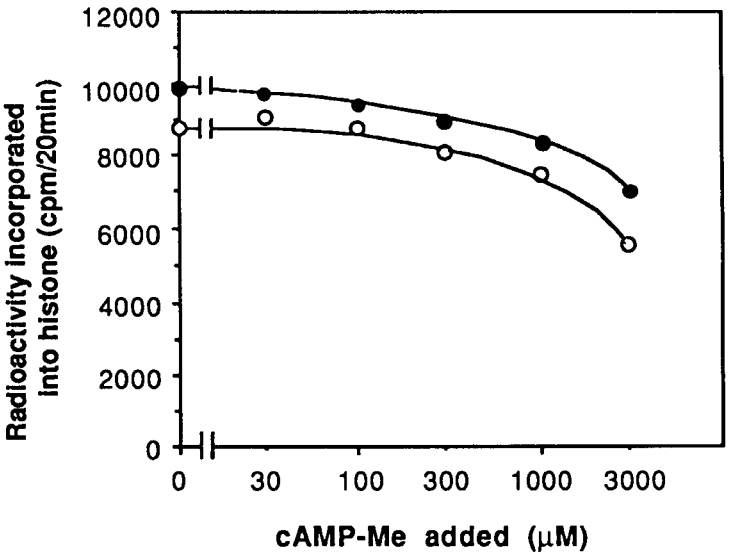

FIG. 4. Antagonistic effect of cAMP-Me on the two types of cAMP-dependent protein kinases. The protein kinase with 10 μM cAMP was incubated for 20 min in the presence of various concentrations of cAMP-Me. Open and closed symbols indicate the activity of type I and type II protein kinase, respectively.

histone at 20 min after the reaction was started (FIG. 3). It is clear from this data that cAMP-Me does not play the role of cAMP in the activation of either type I or type II enzyme, while 8-N_3-cAMP activates these protein kinases in a similar fashion to cAMP. Although cAMP-Me does not substitute for cAMP, high concentration of this compound appears to exert modest inhibitory effect on these enzyme activities in the presence of cAMP as indicated in FIG. 4. As shown in this figure, the concentration required for cAMP-Me to obtain 30 % (type I) or 20 % (type II) inhibition was more than 100 fold that of cAMP present in the reaction mixture.

Interaction of cAMP-Me with cAMP phosphodiesterase. To examine that cAMP-Me may affect the activity of phosphodiesterase, the experiment indicated in FIG. 5 was performed. Twenty min after the onset of the reaction, the enzymatic reaction was proceeding in a linear fashion, and the effects of theophylline and cAMP-Me were studied at this time period. Various concentrations of inhibitors were added, and the 5'-AMP formed from cAMP was plotted against the concentrations of inhibitors added. This figure proved

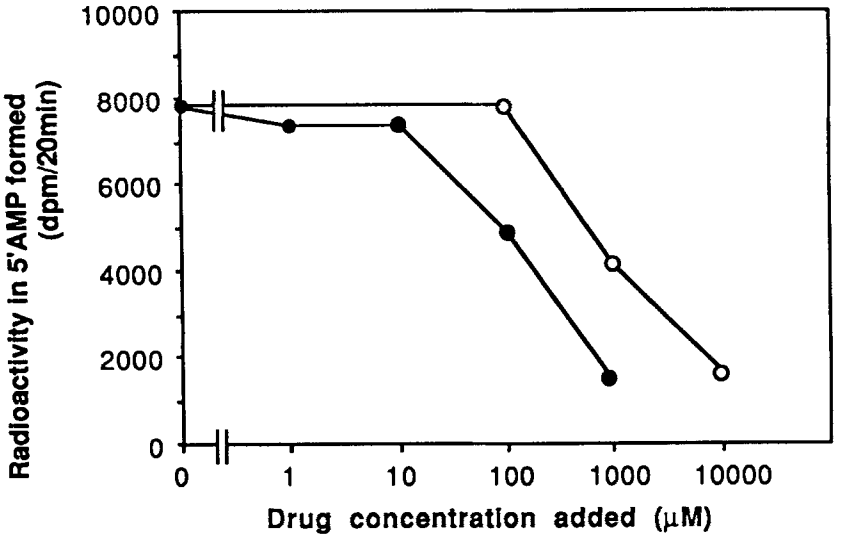

FIG. 5. Effect of cAMP-Me and theophylline on the activity of cAMP phosphodiesterase. The phophodiesterase reaction with 1 μM cAMP and [^{3}H]- cAMP was carried out for 20 min in the presence of various concentrations of cAMP-Me (O) or theophylline (●), and [^{3}H]-5'AMP formed is plotted against time of incubation. Details are described in the Experimental Procedures.

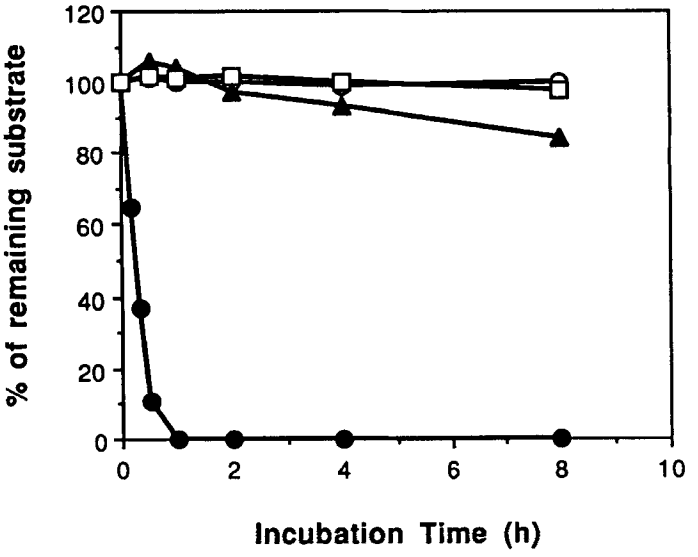

FIG. 6. Stability of cAMP analogues against cAMP phosphodiesterase. Each of 0.4 mM cAMP analogues was incubated with phosphodiesterase for various periods, and remaining amount of cAMP analogue was determined as described in the Experimental Procedures. □, cAMP-Me; ●, cAMP; O, DBcAMP; ▲, 8-N_3-cAMP

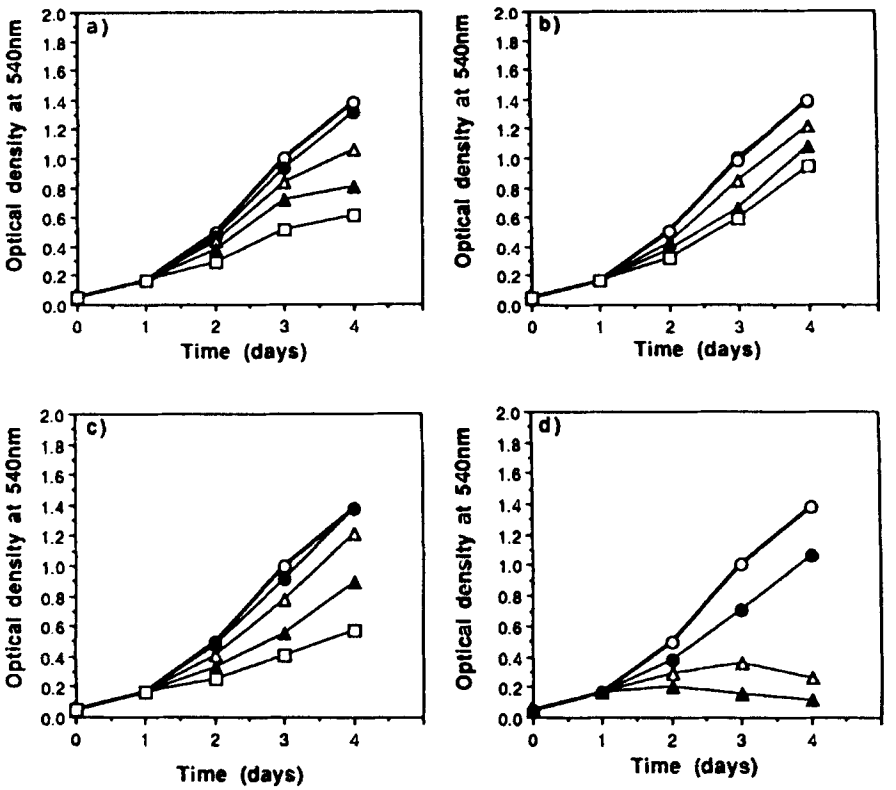

FIG. 7. Effect of cAMP analogues on the growth of DT cells. The cells were seeded at 3 x 10^4 cells/mL in growth medium and incubated with cAMP analogues (a, cAMP-Me; b, cAMP; c, DBcAMP; d, 8-N_3-cAMP; O, no cAMP analogue added; ● , 0.1 mM; Δ , 0.3 mM; ▲ , 1 mM; □ , 3 mM). At the various intervals, the cell growth was measured by the MTT dye method. Values represent the mean of triplicate measurements for each experimental point.

that 50 % inhibition was obtained with 250 μM theophylline and 2 mM cAMP-Me. This indicates that cAMP-Me is approximately 8 fold less active as an inhibitor than theophylline, a well-known inhibitor of cAMP phosphodiesterase. On the other hand, 5'-AMP-Me did not affect the activity of cAMP phosphodiesterase with the concentration of 1 mM (data not shown). In the experiment shown in FIG. 6, cAMP-Me was incubated with phosphodiesterase to examine if this analogue can be a substrate for this enzyme. It is clear from this figure that enzymatic hydrolysis does not occur with cAMP-Me or DBcAMP. 8-N_3-cAMP was only slightly degraded by this enzyme. Under the identical conditions, cAMP was rapidly degraded, and disappeared completely within 60 min.

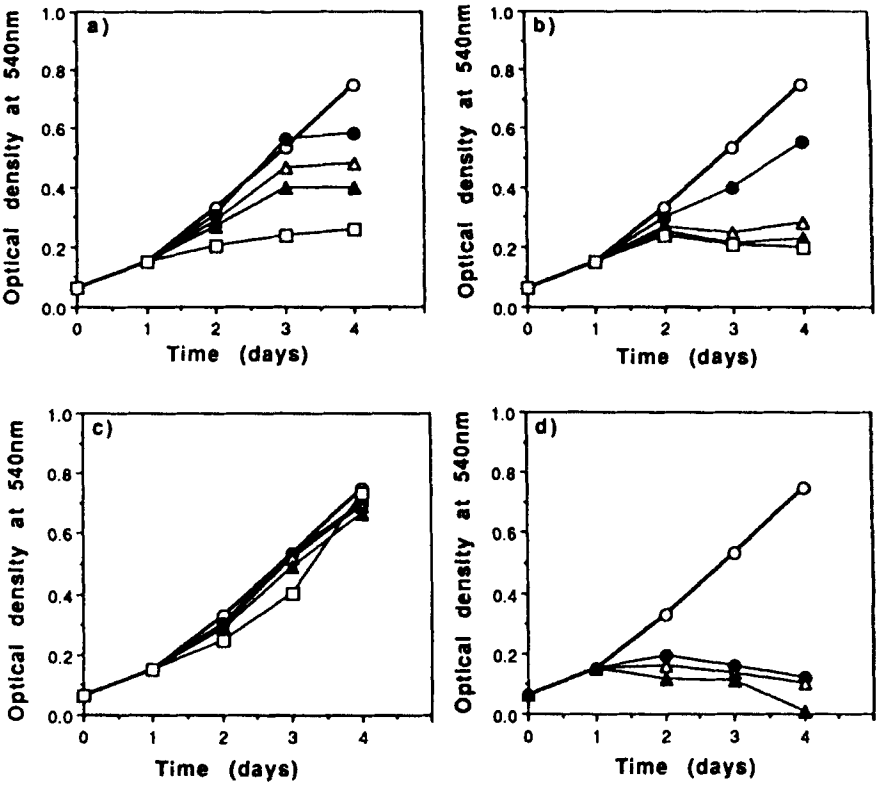

FIG. 8. Effect of cAMP analogues on the growth of HeLa cells. Details are the same as legend in FIG. 7.

Antiproliferative effect of cAMP-Me on tumor cells. The representative data on the effect of cAMP and its analogues on the proliferation of human or murine tumor cells are shown in FIG. 7 and FIG. 8. All four cAMP analogues tested here inhibited the growth of DT cells at 0.3 mM or higher concentration levels. The action of 8-N_3-cAMP was the most potent, followed by cAMP-Me, DBcAMP, and cAMP. As shown in FIG. 8, 8-N_3-cAMP inhibited also the growth of HeLa cells the most effectively. Both cAMP-Me and cAMP inhibited the growth of HeLa cells more effectively than DBcAMP, but less than 8-N_3-cAMP. The 50 % inhibitory concentrations (IC_{50}) of cAMP analogues on several cell lines are listed in TABLE 1. cAMP-Me and cAMP inhibited growth of HeLa, T24 and A375 human tumor cell lines more effectively than DBcAMP. MCF7 cell line appears to be the most resistant to cAMP and its analogues. Thus, it gave highest IC_{50} values for all analogues. Even 8-N_3-cAMP, the most potent

TABLE 1. Effect of cAMP analogues on the growth of tumor cell lines.

	50 % inhibitory concentration (mM) [a]			
Cell line	cAMP-Me	cAMP	DBcAMP	8-N_3-cAMP
DT	1.86±0.94	>3 (80± 1)[b]	2.37±1.07	0.30±0.12
3T3	2.61±0.68	>3 (94±20)	2.71±0.51	0.18±0.09
T24	0.86±0.75	0.93±0.42	>3 (113±30)	0.02±0.01
HeLa	1.15±0.74	0.98±0.80	>3 (96± 4)	0.21±0.28
MCF7	2.79±0.30	>3 (96±11)	>3 (61±37)	>1 (53±18)
A375	0.67±0.18	1.31±0.72	>3 (85±11)	0.03±0.00

The cells were seeded at 3 x 10^4 cells/mL in growth medium and incubated in the presence of cAMP analogues for 48 h. Fifty % inhibitory concentrations were determined by relative cell growth measured by the MTT dye method.

[a] The data represent mean IC_{50} value and standard deviation calculated from separate 3 experiments. [b] Fifty % inhibition was not achieved at the highest concentration tested. Figures in parentheses represent relative cell growth refers to the percentage of the increase in MTT value at the highest concentration tested compared with that of the untreated control group.

TABLE 2. Effect of 5'-AMP-Me and 3'-AMP-Me on the growth of tumor cell lines. Details are the same as legend in TABLE 1.

	50 % inhibitory concentration (mM)	
Cell line	5'-AMP-Me	3'-AMP-Me
DT	>3	>3
3T3	>3	>3
T24	0.14±0.01	>3
HeLa	>3	>3
MCF7	>3	>3
A375	0.61±0.00	>3

analogue, did not exert significant inhibition except at higher concentration in our experiments. Because of the presence of minor components in our cAMP-Me preparation (FIG. 2), the activities of these contaminants against the proliferation of tumor cells were also examined. As shown in TABLE 2, in general, the antiproliferative action of purified 5'-AMP-Me and 3'-AMP-Me was not so potent as cAMP-Me, though in some tumor cell lines almost equally (A375) or more potent as cAMP-Me (T24). IC_{50} of purified 3'-AMP-Me were more than 3 mM against all cell lines tested. Therefore, the biological action of cAMP-Me described in Fig. 7 and 8 must be due to cAMP-Me itself, and not to minor components which existed in our cAMP-Me preparation in quantity less than 1%. In general, 8-N_3-cAMP showed the most potent inhibitory activity in all cell lines tested and cAMP-Me was occasionally more potent than cAMP or DBcAMP, especially with human malignant cells.

DISCUSSION

In this study, we intended to increase the penetration of cAMP into cells, and the stability against phosphodiesterases by adding a methyl group to the phosphorous atom of cAMP. Such alkylphosphonate analogues of nucleotides have been shown to have better transport across the cell membrane (15). Although methylphosphonate analogues of 3',5'-cyclic pyrimidine nucleoside such as thymidine (16) and uridine (17) were synthesized, their biological properties have not been reported. Similar analogue of purine nucleoside has not been synthesized to the best of our knowledge. We synthesized cAMP-Me in satisfactory yields, using the bifunctional phosphorylating reagent (17). The product we obtained (cAMP-Me) is thought to be only one diastereoisomer, because ^{31}P-NMR showed only one singlet signal (δ 37.68), and the RP-HPLC also showed only one major peak (FIG. 2). Marugg et al. (17) also reported that uridine 3',5'-cyclic methylphosphonate was synthesized and obtained one stable diastereoisomer. The reason why only one diastereoisomer in these reactions was formed remains obscure at the present moment.

This newly synthesized cAMP analogue, cAMP-Me, was investigated for biological action against cAMP related enzymes. The data shown in FIG. 3 indicated that cAMP-Me has no stimulatory action on both type I and type II protein kinases partially purified from rabbit skeletal muscle. In sharp contrast, 8-N_3-cAMP stimulated these protein kinase activities even at 0.1 μM. The regulatory subunit of these protein kinases would not recognize cAMP-Me

as an activator, probably because of loss of negative charge on the phosphate moiety of cAMP by the addition of the methyl group. In a similar fashion, Rode et al. (18) reported that 5-fluoro-2'-deoxyuridine 5'-methylphosphonate has an extremely reduced affinity for the thymidylate synthetase compared with 5-fluoro-2'-deoxyuridine 5'-monophosphate. Our data indicated that cAMP-Me is a rather potent inhibitor against cAMP phosphodiesterase. Furthermore, it was shown to be resistant to hydrolysis by cAMP phosphodiesterase, to a similar degree as DBcAMP which is a poor substrate for this enzyme (14).

We expected that cAMP-Me might inhibit the tumor cell growth because of its inhibitory action on cAMP phosphodiesterase, which would raise cAMP level in tumor cells (4). Stability against phosphodiesterases and increase in permeability into tumor cells (15) are preferable characters for exerting pharmacological action. In support of this notion, the inhibitory action of cAMP-Me on the growth of tumor cells was more potent than cAMP and DBcAMP. DBcAMP has been reported to inhibit tumor cell growth in vitro (19, 20). However, DBcAMP is required to be converted to proximal metabolite (21) to act as a cAMP agonist. The tumor cell lines tested here may not have such esterases enough to convert DBcAMP to active metabolites. cAMP-Me is more potent than cAMP, because cAMP would be degraded by cAMP phosphodiesterases before exhibiting its action and less permeable to the tumor cells. Recently one of cAMP analogues, 8-chloro cAMP, was reported to be a potent growth inhibitor of human and rodent cancer cells (22, 23). We compared the effect of available 8-substituted analogue, 8-N_3-cAMP, on tumor cells with that of cAMP-Me. 8-N_3-cAMP was the most potent inhibitor among cAMP analogues tested, and more than five times as potent as cAMP-Me. 8-N_3-cAMP was not only potent agonist to cAMP-dependent protein kinase, but also rather resistant to cAMP phosphodiesterase.

The mechanisms of inhibitory action of cAMP-Me on human tumor cell proliferation is not clear at the present moment. It has been reported that exogenous application of cAMP agonists such as DBcAMP and 8-N_3-cAMP suppress ras oncogene expression in malignant cells (24, 25, 26). It is well established that the ras oncogene is one of the transforming genes in various types of human cancer (27, 28). The ras transformed fibroblasts have been reported to have altered cAMP regulatory mechanisms (29). DBcAMP is also reported to induce the normal morphological phenotype as well as to inhibit the proliferation of some malignant cells (19, 20). On the basis of these

considerations, we investigated the effect of cAMP-Me on the ras specific tumorigenic cell system of DT cells. In our hands, the effect of DBcAMP was marginal, but 8-N_3-cAMP markedly slowed the growth rate of DT cells and induced the flattened morphology at the concentration level of 1 mM (data not shown). The treatment with cAMP-Me had obvious effect on the growth of DT cells, but no morphological change was observed. Therefore, it is unlikely that cAMP-Me inhibits cell proliferation via the elevation in intracellular cAMP or as an agonist of cAMP in this experimental system. We have no knowledge concerning the action of cAMP-Me on any type of cAMP protein kinases differently distributed in various tissues. It is possible that cAMP-Me can exert antitumor activity in vivo, because it is a stable agent which has presumed high permeability.

EXPERIMENTAL PROCEDURES

Materials and Methods. cAMP phosphodiesterase from a bovine heart was purchased from Boeringer Mannheim. cAMP-dependent protein kinases (type I and type II) from rabbit muscle, snake venom nucleotidase, calf thymus histone, cAMP, 8-N_3-cAMP and DBcAMP were obtained from Sigma. [2,8-^{3}H] - cAMP (1.15 TBq/mmol) was obtained from New England Nuclear, and [γ-^{32}P] - ATP (185 TBq/mmol) was from Amersham. $TIPDSiCl_2$ and methyl phosphonic dichloride were purchased from Tokyo Kasei Kogyo and Aldrich, respectively. Other chemical reagents were commercially available. ^{1}H-NMR and ^{31}P-NMR spectra were recorded on a JEOL-FX 90 spectrometer and a Bruker AM 500 spectrometer, respectively.

Synthesis of adenosine 3',5'-cyclic methylphosphonate. The synthetic scheme is shown in FIG. 1. Silylated adenosine (1 of FIG. 1) was synthesized by the reaction of adenosine with $TIPDSiCl_2$ in 94% yield according to the published procedure (30). 2'-Tetrahydropyranyl adenosine (3 of FIG. 1) was prepared as follows; Dry dioxane (10 mL) containing 1 g of 1 was mixed with 406 mg of *p*-toluenesulfonic acid monohydrate and 2 g of 2,3-dihydropyran. The mixture was stirred vigorously for 1h, treated with 0.25 mL of 28% ammonia and the resulting precipitate was removed by filtration. The filtrate was evaporated under reduced pressure. The residue was purified by silica gel chromatography, and 1.1 g of 3',5'-tetraisopropyldisiloxyl 2'-

tetrahydropyranyl adenosine (2 of FIG. 1) was obtained with 93% yield. The purified 2 (900 mg, 1.52 mmol) was dissolved in 15 mL of dioxane, and 3 mmol of TBAF was added. The mixture was stirred at room temperature for 20 min and was concentrated under reduced pressure. The oily residue thus obtained was further purified by silica gel column chromatography, and 503 mg of 3 was obtained with 95% yield.

Compound 3 was converted to adenosine 3',5'-cyclic methylphosphonate (5 of FIG. 1: cAMP-Me) according to the method described by Marugg et al. (17). In brief, compound 3 (367 mg, 1 mmol) was added to 10 mL dioxane solution containing 1.1 mmol of methyl-*O*, *O*-bis(benzotriazolyl) phosphonate. The reaction mixture was stirred for 30 min at 20 °C. Five mmol of dry *N*-methylimidazole (0.4 mL) was added, and allowed to stand for 2 h at 20 °C. A few drops of 1 M triethylammonium bicarbonate (TEAB) was added to this solution. The reaction mixture was diluted with $CHCl_3$-dioxane (9:1 v/v, 20 mL) and washed with 20 mL of 1 M TEAB, subsequently with water. The organic layer was dried on $MgSO_4$ and evaporated to dryness. The oily residue was subjected to a silica gel chromatography, eluted with $CHCl_3$-CH_3OH and 164 mg of 2'-*O*-tetrahydropyranyl adenosine 3',5'-cyclic methylphosphonate (4 of FIG. 1) was obtained with 40% yield. One hundred mg of 4 was treated with 5 mL of 0.01 N HCl in dioxane-H_2O solution (9:1 v/v) at 20 °C overnight. After neutrizing with 28 % ammonia, the reaction mixture was concentrated, and the resulting residue was chromatographed on silica gel. Seventy-seven mg of adenosine 3',5'-cyclic methylphosphonate (5 of FIG. 1) was obtained with 99% yield. The product was subjected to NMR and FAB MS, and the following data were obtained. ^1H NMR (90 MHz, CD_3OD) δ 1.74 (s, 3H, J=18Hz), 6.15 (d, 1H), 8.44 (s, 1H), 8.49 (s, 1H); ^{31}P NMR (D_2O) δ 37.68 (s); FAB MS (m/z) 328 (M+H).

Assay of cAMP dependent protein kinase. Protein kinase activity was assayed by a modification of the reported method (31). The standard reaction mixture (100 μL) contained 100 mM potassium phosphate (pH 7.5), 20 mM $Mg(OAc)_2$, 1 mM theophylline, 0.6 mg/mL of calf thymus histone, 0.5 mM [γ-^{32}P] -ATP ($5x10^5$ cpm) and 2 mg/mL of rabbit muscle protein kinase type I or type II, in the presence or absence of 10 μM cAMP. A test compound was added to the reaction mixture and preincubated for 3 min. The reaction was initiated by the addition of [γ-^{32}P] - ATP, and this reaction mixture was incubated for 20 min at 30 °C. Aliquots of the mixture were transfered onto Whatman 3MM paper disks and immersed into ice-cold 5% trichloroacetic acid

(TCA). The radioactivity of the mixture, which is insoluble in cold 5% TCA, was counted by Cherenchov luminescence (Beckman LS5801).

Assay of cAMP phosphodiesterase. cAMP phosphodiesterase activity was assayed according to the reported method (32). The standard reaction mixture (100 μL) contained 40 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 50 mM $CaCl_2$, 1 μM [^{3}H] -cAMP ($2x10^5$ dpm) and 50 μunits of beef heart cAMP phosphodiesterase, with or without the possible inhibitors. The reaction was initiated by the addition of [^{3}H] -cAMP, incubated for 20 min at 30 °C, and stopped by immersing the test tube into boiling water for 3 min. Snake venom nucleotidase (20 μg) was added to the cooled reaction mixture and the whole mixture was reincubated for 10 min at 30 °C. This was followed by the addition of anion-exchange resin (Bio-Rad AG 1x2). After vigorous shaking, the resin was removed by centrifugation for 5 min at 5000 rpm, and the radioactivities of aliquots of supernatant were measured with a liquid scintillation counter (Beckman LS5801).

Determination of the degradation of cAMP analogues by cAMP phosphodiesterase. The reaction mixture (500 μL) contained 50 mM Tris-HCl buffer (pH 6.8) and 0.01 unit of cAMP phosphodiesterase, was incubated with one of possible substrates, cAMP, DBcAMP, 8-N_3-cAMP or cAMP-Me (0.4 mM each) at 30 °C. During the course of the reaction, 10μL aliquots of the mixture were removed and the reaction was stopped by immerersing into boiling water for 1 min. The mixture was then analyzed for remaining amount of cAMP analogue with RP-HPLC. RP-HPLC was carried out with a Wakosil-II $_5C_{18}$ column (6 x 150 mm, purchased from Wako Pure Chemical Industries, LTD.) at a flow rate of 1 mL/min. The substrates were eluted with the gradient system and monitored at 260 nm. Linear gradient of solution A (50 mM triethylamine acetate, pH 6.8) and solution B (acetonitrile) were used. Gradient was 5% B at 0 min and 25% B at 20 min.

Cell lines Mouse fibroblast NIH/3T3 cells (33), Kirsten murine sarcoma virus-transformed NIH/3T3 cells (DT cells: 34), human bladder carcinoma T24 cells (35), human cervix carcinoma HeLa cells (36) , human melanoma A375 cells (37), and human mammary carcinoma MCF7 cells (38) were grown in Eagle's minimum essential medium or RPMI1640 medium, containing 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin and 100 μg/mL of streptomycin. Cells were cultured at 37 °C in 5% CO_2.

Measurement of Cell growth. When cells were confluent, they were harvested by trypsinization, resuspended in growth medium, and seeded into 96-well culture plate (Costar3596) at the cell density of $3x10^3$ cells/100 μL/well. After overnight cultivation, one of cAMP analogues was added to the adherent cell cultures and incubated at 37 °C in 5% CO_2 atmosphere for 1 to 3 days. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye method (39, 40) with some modifications. A solution (20μL) containing 5 mg/mL of MTT was added to each test well and incubated for 4 h. Formazan crystal produced from MTT was dissolved by the addition of 100 μL of 10% sodium dodecyl sulfate in 0.01 N HCl. After subsequent overnight incubation at 37 °C, the optical density was measured with an enzyme-linked immunosorbent assay reader (Titertek Multiskan) at 540 nm.

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Received 8/5/91
Accepted 11/7/91