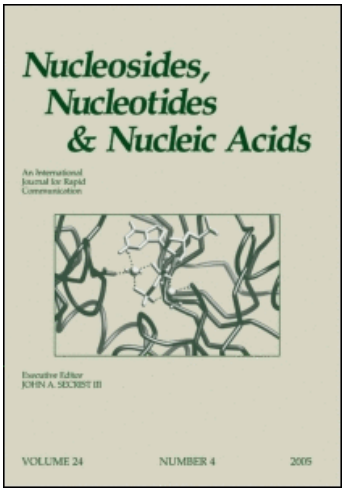

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Purine Nucleoside 3',5'-Cyclic Monophosphates as Hormonal Modulators of Cellular Proliferation, Metastases and Lymphocyte Response

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PURINE NUCLEOSIDE 3',5'-CYCLIC MONOPHOSPHATES AS HORMONAL MODULATORS OF CELLULAR PROLIFERATION, METASTASES AND LYMPHOCYTE RESPONSE

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A review of the potential role of cAMP and cGMP as hormonal regulators of tumor cell proliferation, metastases and lymphocyte activation reveals that several synthetic purine nucleoside 3',5'-cyclic monophosphates are more potent and more selective in modulating certain specific responses than the parent natural cyclic nucleotides. cAMP derivatives have been prepared which will temporarily restore transformed cells to the normal phenotype. cAMP analogs may well be found which will selectively inhibit tumor metastases. Certain cGMP analogs could selectively stimulate the lymphocyte response toward the destruction of tumor cells. The synthesis of new cyclic nucleotides should provide unique nontoxic agents that could combat neoplasia on a hormonal basis.

Recently attention has been focused on the search for new antitumor agents which are not generally cytotoxic. Although a great deal of effort has been expended in the past on the synthesis and study of nucleosides lethal to cancer cells and replicating normal cells alike, surprisingly little effort has been devoted to the concept of employing nucleosides and nucleotides to change a cancerous cell (transformed cell) from the malignant state back to a typically normal cell. If the transformed tumor cell could be controlled so that it behaved normally, this would indeed provide a new approach to the treatment of neoplasia.

Such an approach is not just wishful thinking. In 1971, Ira Pastan and co-workers[1] reported the restoration of several morphological characteristics of normal fibroblasts in sarcoma cells by the treatment of these cells with adenosine-3',5'-cyclic monophosphate (cAMP) administer-

ed as the N^6,2'-O-dibutyryl derivative. Simultaneously, Hsie and Puck[2,3] observed the morphological conversion in vitro of transformed Chinese hamster ovary cells to a fibroblastic form by the treatment of a combination of dibutyryl-cAMP and testosterone. The changes in the following characteristics were consistent with the conversion from a malignant to a normal fibroblastic state.[3]

1. Acquisition of contact inhibition;
2. Change from a random growth pattern to a parallel pattern;
3. Decrease in the ability to be agglutinated;
4. Disappearance of knob-like structures around the cell periphery;
5. Return of anchorage dependence.

These properties of the cAMP treated malignant cell have been confirmed and extended by use of the electron microscope.[4] Pastan and co-workers[1] suggested that cAMP might be an important factor in the morphology of normal fibroblasts and this function may be altered or lost during the transformation process. As illustrated by these experiments, cAMP has been shown to play an important role in cell growth and differentiation,[5] which represents one of the most exciting areas of cancer research today. It has recently been postulated[6] that the microtubular-microfilamentous structure of the cell conveys growth regulatory information from the cell membrane via cAMP to the nucleus and its disorganization leads to malignant growth.

The malignant cell is characterized by a cell cycle which is apparently identical to that of normal cells; the major differences between the two cell types is the loss of growth regulatory mechanisms which allow transformed cells to enter or remain in the G_0 phase or quiescent state. There is considerable evidence which indicates that cGMP levels fall markedly in cultured fibroblasts as they enter quiescence. Furthermore, a dramatic transient rise in cGMP is observed after release from quiescence by serum readdition.[7,8] If cGMP is the mediator of the G_1 phase, then addition of cGMP or its analogs to quiescent cultures should cause cells to enter DNA synthesis and cell division. Seifert and Rudland[9] have reported such experiments and note definite success using cGMP with a 15% stimulation of DNA synthesis. Thus, it now appears that cell proliferation may be stimulated by either or both of the following:[10,11]

1. A fall in cAMP levels
2. A rise in cGMP levels

A reverse of (1) and (2) inhibits cell proliferation.[10,11] The simple concept that cAMP acts as a negative and cGMP acts as a positive signal for cellular growth is receiving continual support from various laboratories.[12-15]

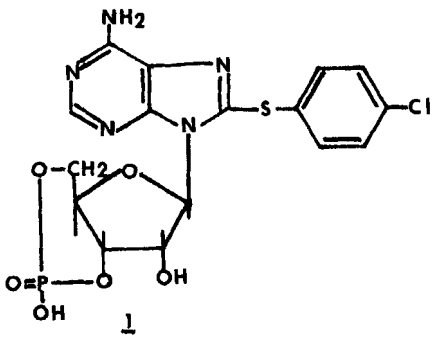
The cAMP content of fibroblasts transformed by viruses is decreased to about 50% of normal. The lower level of cAMP is thought to be responsible for most of the properties of transformed cells,[15] including their altered morphology. Willingham and Pastan[16] have reported electron microscopic evidence that dibutyryl-cAMP promotes formation and altered distribution of cytoplasmic microtubules and microfilaments which are responsible for changes in morphology, mobility and adhesiveness.

One of the most striking studies of the effect of cAMP on transformed cells is that of Pollack and co-workers.[17] The dependence on anchorage for growth is lost in transformed cells. Pollack et al.[17,18] showed that there was a correlation between revertant lines that had regained their normal high cAMP levels and those that regained anchorage dependence. Thus, loss in anchorage dependence correlated well with in vivo tumorigenicity.[18]

Recently, Zeilig and Goldberg[19] reported that in a fast growing rat hepatoma cell line, cGMP levels vary independently and reciprocally with cAMP levels during the cell cycle and cGMP levels increase up to ten fold at the onset of mitosis. These results are consistent with possible regulatory roles for both cAMP and cGMP in cell proliferation.[20] Johnson and Hadden[21] have recently shown that cGMP increased the activity of RNA polymerase I and III apparently by specifically binding to these enzymes. Chawla et al.[22] have recently shown that plasma and urine levels of cGMP were considerably increased in patients with disseminated cancer over those of control patients with other diseases.

In a recent study, Puck and Robins[23] found that the restoration of fibroblast morphology of CHO-K1 cells was restored most readily by 8-p-chlorophenylthio-cAMP, 1, which gave approximately 50% conversion of the "knobbed" cells to the normal "stretched" type at 10^{-4} molar. N^6,O-2'-Dibutyryl-cAMP required a concentration of 3×10^{-3} molar concentration

to achieve the same effect. The synthesis of 8-p-chlorophenylthio-cAMP, 1, was reported from our laboratory[24] in 1973. It is of interest to note that 1 was 18 times more active in the stimulation of cAMP dependent protein kinase isolated from bovine brain than cAMP itself.[24] Recently Imada and co-workers[25] have shown that the CHO-K1 cells are stim-



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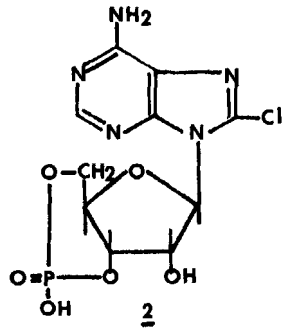
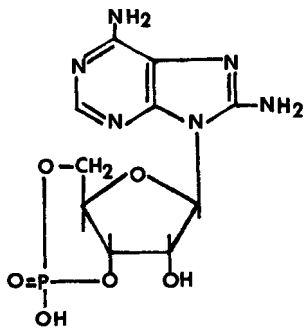


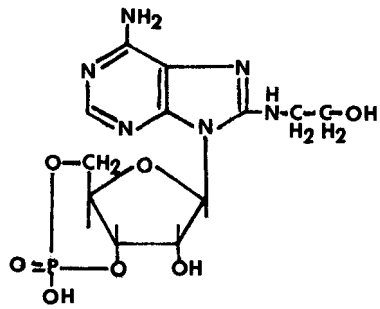
2

lated by 8-p-chlorophenylthio-cAMP which induces a membrane glycoprotein of 135,000 mol. wt. This membrane glycoprotein, which contains D-glucosamine, is strongly associated with the cell membrane and could be responsible for the major morphological changes which are seen in the reversion to normal cell phenotype. Hsie[25a] has recently summarized the reverse transformation of CHO cells by cAMP and cAMP derivatives. 8-Bromo-cAMP, 6, has recently been shown to have a potent inhibitory effect on the morphological transformation of Syrian hamster cells exposed to N-methyl-N'-nitro-N'-nitrosoguanidine, whereas 8-bromo-cGMP enhanced such transformation.[25b]

As early as 1969, Gericke and Chandra[26] showed that cAMP definitely inhibited the growth of transplanted NKL-lymphosarcoma in mice. Cho-Chung[27] showed that dibutyryl-cAMP, 8-methylthio-cAMP and 8-bromo-cAMP significantly inhibited MTW9 and Walker 256 mammary carcinomas and 5123 hepatoma in experimental animals. Growth inhibition was dose dependent. Niles and co-workers[28] have shown that 8-methylthio-cAMP inhibited human prostatic ephthelial cell replication. More recently, Niles has noted that 8-chloro-cAMP, 2, supplied from our laboratory,[29] significantly inhibited human carcinoma in cell culture.[30] Koontz and Wicks[31] have recently shown that 8-(β-hydroxyethylamino)cAMP,[32] 4, and 8-amino-cAMP,[32] 3, were lethal to growing H35 cultured rat hepatoma cells.



8-amino-cAMP
3



8-(β-hydroxyethylamino)-cAMP
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Nucleoside 3',5'-Cyclic Monophosphates as Potential Inhibitors of Tumor Metastases

Most cancer deaths in humans are not caused by the primary tumor, but rather by secondary tumor growth elsewhere in the body.[33] Therefore the main problem in treating cancer is not the excision of the primary tumor mass, but the elimination of metastases.[34] Metastasis is the formation of a cancerous lesion that no longer has continuity with the primary tumor. The ability to metastasize is uniquely the property of a malignant tumor. Malignancy is not a characteristic shared by all tumor cells.[35] Definitive evidence that malignant primary tumors contain subpopulations with differing metastatic capabilities has been obtained by Fidler and Kripke[36] utilizing various cloning techniques. The cloned sublines differed markedly in their metastatic potential. Thus, highly metastatic tumor cell variants pre-exist in the parent tumor.[36] Such marked heterogeneity in metastatic potential has now been found in a substantial number of different tumor lines.[35] Schabel et al.[37] have recently pointed out that most of the methods presently used to define the drug action on experimental animal tumors could be misleading since they are based on the assumption that transplantable tumors are homogeneous.

Indeed, it would appear that attention should be focused on the attenuation of certain of the cells with high metastatic capability as a new approach to cancer chemotherapy. Using the technique of somatic cell hybridization between A9 and TLX5 cells, Tisdale and Phillips[38] were able to correlate intracellular cAMP levels to the malignancy of cell lines. Cells of high malignancy had low levels of cAMP while cells

of low malignancy had a high level of cAMP. Cyclic AMP has been shown to suppress the tumorigenicity of CELO virus-transformed hamster skin cells in vivo. This was brought about by pretreating the transformed cells with dibutyryl cAMP before inoculation into the animal.[39] Treatment of the human lymphocytic cell line RPMI 1788 with dibutyryl-cAMP caused attachment of the cells to the culture vessels and their conversion to the normal fibroblast-like forms.[40] Shields[41] has shown that dibutyryl-cAMP greatly increased the adhesion of cells to a culture dish. This suggests that cAMP and cAMP analogs could be useful to inhibit proliferation of metastasizing tumor cells.[42] Tisdale[42] has recently pointed out that a great potential of cAMP in cancer therapy may lie in its ability to cause redifferentiation of certain types of tumors. Dibutyryl-cAMP induces irreversible morphological differentiation of mouse neuroblastoma cells in culture to a form characteristic of mature neurons.[43]

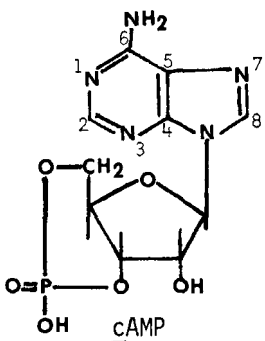
Pardee et al.[44] have postulated that uncontrolled growth leading to malignancy is brought about by permanent transformation of the cell surface (caused by carcinogenic chemicals or tumor viruses). The changes in the membrane are such as to inhibit the membrane bound adenylate cyclase which in turn lowers the cAMP concentration inside the cell. There is a resultant increase in cGMP and rapid cellular proliferation.[44] Pastan and co-workers[45,46] have shown that chick embryo fibroblasts transformed by Rous sarcoma virus have decreased adenylate cyclase activity. This decreased enzymatic activity which leads to a lowering of cAMP would appear to be responsible for most of the abnormal properties of transformed cells, including an increased rate of cellular proliferation.[46] In lymphocytes of patients with chronic lymphatic leukemia, adenylate cyclase activity is decreased as compared to normal human lymphocytes.[47] The altered adenylate cyclase activity has more recently been correlated with in vitro viral transformation of various cell lines.[48]

Lowe and Henderson[49] noted that caffeine considerably enhanced the growth inhibitory effects of various antimetabolites against lymphoma L-5178Y cells in culture. Caffeine has shown antineoplastic activity in a number of systems.[50,51] Since caffeine is known to be a good inhibitor of cAMP phosphodiesterase (PDE) it is quite likely that these antitumor effects are due to an increased level of cAMP. Recently Bertram

and co-workers[52] have studied 1-methyl-3-isobutylxanthine, (a potent PDE inhibitor) using a cloned line of Lewis lung carcinoma adapted to cell culture. 1-Methyl-3-isobutylxanthine greatly reduced tumor colony formation. In mice 1-methyl-3-isobutylxanthine resulted in a 10-fold decrease in the formation of lung nodules resulting from metastases. Only four of 24 treated animals had lung metastases as compared to 24 out of 25 control animals exhibiting lung metastases.[52] Treatment beginning two days after removal of the primary tumor was equally effective in reducing lung metastases. This dramatic *in vivo* study strongly suggests *c*AMP involvement since this inhibition by methylisobutylxanthine did correlate with its ability to elevate *c*AMP in the cells.[52] Similarly, Bertram and co-workers[52a] have recently shown that the PDE inhibitor RO20-1724 doubled the life span of such mice inoculated with Lewis Lung carcinoma cells resulting in lung metastases. Janik and Bertram[53] conclude that the observed effect of methylisobutylxanthine are not due to immune mechanisms since it is also active in nude mice injected with Lewis lung carcinoma cells. It appears highly likely that intercellular inhibitory growth control signals have been stimulated *via* *c*AMP.

Recently Niles and co-workers[54] have shown that 8-chloro-*c*AMP,[20] **2**, was a most effective growth inhibitor in a human carcinoma cell line. Combination of 3-isobutyl-1-methylisobutylxanthine with 8-chloro-*c*AMP gave much better inhibition than with either compound alone. The ability of 8-halogen substituted *c*-AMP to inhibit the growth of the human carcinoma *in vitro* correlated well the ability to activate a protein kinase in the same cell line[54] 8-chloro-*c*AMP, **2**, which was the most effective growth inhibitor significantly stimulated protein kinase at 10^{-8} molar. Substitution of an alkylureido group at the N^6 position of *c*-AMP produced agents with a greater antiproliferative activity towards cells of malignant origin than cells from a normal individual.[55] Tumor cells may undergo morphological changes which represent spontaneous maturational sequences.[56] The final product of such changes is a highly differentiated, fully mature cell which has often lost many of the neoplastic traits.[57] There is considerable evidence that supports the view that cyclic nucleotides are involved in the regulation of tumor cell differentiation and maturation.[42] Oat cell carcinoma, considered to be of epidermal bronchial origin, has been shown to differ-

entiate to a form similar to cultured neuroblastoma cells in the presence of dibutyryl cAMP.[59] cAMP accelerates the production of mature, highly differentiated melanoma cells with reduced proliferative ability from murine melanoma B-16 cells.[59] The cells were less malignant when injected into syngenic hosts. Human melanoma cells behaved similarly



cAMP

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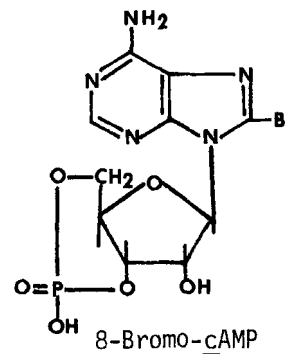


8-Bromo-cAMP

6

to murine melanoma cells after exposure to cAMP.[60] It has recently been reported that 8-bromo-cAMP,[32] 6, effects biochemical and morphological changes in murine neuroblastoma cloned cells without concomitant inhibition of cell division.[61] The response of malignant muscle cells appears to be toward differentiation after cAMP treatment.[62]

Karpatkin and Pearlstein[63] have recently summarized the evidence that platelets play a role in the development of tumor metastases. Certain tumor cells aggregate platelets *in vitro*. There would appear to be a correlation between the ability of certain tumor cells to aggregate platelets in vitro and their metastatic potency *in vivo*.[64] Greenberg and co-workers[65] postulate that platelets might contribute to metastases by promoting vascular attachment of the circulating tumor cells.

Gasic and co-workers[66] correlated the degree of *in vivo* metastases with the ability of tumor cells to aggregate platelets *in vitro*. The murine tumors with capacity to aggregate platelets *in vitro* produced more lung metastases than tumors lacking this ability. The ability of tumor cells to interact with and attach to host platelets increases their potential to adhere to the vascular endothelium. Abnormalities in blood coagulation are common in patients with advanced malignant dis-

ease[66a] and tumor cells have been shown to have procoagulant activity.[66b] Honn and co-workers[66c] have concluded that the ability of tumor cells to metastasize may be related to their ability to promote aggregation of host platelets. Honn and co-workers[67] have recently studied prostacyclin (PGI_2), the most potent antithrombogenic agent known, and its effect on the intravenous injection of B-16 amelanotic melanoma tumor cells into syngeneic mice, which produces metastases in the lung, liver and spleen. Intravenous injection of 100 μg of PGI_2 injected before tumor cell administration reduced metastatic foci in the lungs by 70%[67] and totally prevented metastases to the other organs. Since PGI_2 is known to act as a platelet aggregation inhibitor by activation of adenyl cyclase and increasing the level of cAMP[68] Honn[67] reasoned that a cAMP phosphodiesterase inhibitor should potentiate the antimetastatic effect of PGI_2 by prolonging the half-life of cAMP. This indeed proved to be the case and when 100 μg of theobefore PGI_2 and B-16 cells the metastases three percent.[67]

The potency of PGI_2 as an inhibitor of human platelet aggregation has recently been shown to correlate directly with the elevation of cAMP.[69] Thus these data lend support to the concept that cAMP mediates the effect of PGI_2 on platelet function by activation of adenyl cyclase.

Inhibition of platelet aggregation by cAMP was first reported by Marcus and Zucker[70] in 1965. Salzman and Levine[71] showed that N^6-2'-O-dibutyryl-cAMP inhibited platelet aggregation induced by ADP, epinephrine, collagen or thrombin which confirmed the earlier work of Marquis and co-workers[72] who also showed that PGE_1 inhibits platelet aggregation and stimulates cAMP synthesis by human platelet membrane fractions by stimulation of adenyl cyclase. Caffeine was shown by these workers[72] to inhibit platelet phosphodiesterase resulting in increased cAMP levels. Exogenous cAMP itself inhibits platelet aggregation.[72a] Robison and co-workers[72b] have reviewed the supporting evidence that cAMP plays a major role as an inhibitor of platelet aggregation.

A major surface property of tumor cells which is relevant to their metastatic potential is adhesiveness. Available evidence is such that the carbohydrate moiety is believed to be involved in cell adhesion.[73] Oppenheimer[74] has shown that cellular aggregation requires the synthesis of oligosaccharides as part of the cellular surface. A study of the role of adhesion in determining the metastatic spread of malignant

cells, suggests that tumor cells are less adhesive than their normal cellular counterparts.[75] Thus these cells are more readily released from the primary tumor.

In 1972, Robert W. Holley[76] proposed the changes of the cell surface in the malignant cell, and the resulting increased cellular permeability, as the main factors responsible for increased cellular proliferation. Pardee,[77] as early as 1964, suggested that the cell membrane exerted a selective control upon the transport of regulatory molecules which could exert either a positive or negative effect on growth control. Pardee and Foster[78] have shown that polyoma transformed 3T3 cells transported certain amino acids approximately three times faster than did untransformed 3T3 cells. Several viral transformed cell lines have been found to produce a protease.[79] Pardee and co-workers propose that the activation of such a protease creates cell surface changes which transform the cell and result in rapid cellular proliferation.[80] Pardee et al.[80] propose that membrane bound hormones such as adenyl cyclase may be altered as a result of membrane surface changes.

The cAMP content of virally transformed fibroblasts is decreased to about 50% of controls.[81] The lowered level of cAMP is believed to be responsible for lowered adhesiveness.[82] This dramatic effect on cellular membrane is thought to be due to changes in microtubles and microfilaments.[81] Pastan[83] has proposed that the src gene product modifies membrane bound adenyl cyclase leading to a decreased level of cAMP. It has recently been demonstrated that cAMP enhances sulfate incorporation into membrane proteoglycans suppressing cell division.[84] Thus the lack of cAMP in transformed cells could alter normal cellular membrane glycoprotein by inhibiting sulfate incorporation. Since adenyl cyclase is an integral part of the plasma membrane, alteration of the membrane by oncogenic virus, chemicals or radiation could alter the properties and activity of this enzyme. Pastan and co-workers[85,86] have shown that chick embryo fibroblasts transformed by Rous sarcoma virus have decreased adenylcyclase activity. This decrease in enzyme activity which leads to the lowering of cAMP would appear to be responsible for most of the abnormal properties of transformed cells.[48]

It has recently been postulated by Bertram et al.[52a] that the transformed tumor cell lacks a growth inhibitory factor characteristic of the normal cell line. Confluent monolayers of mouse fibroblast cells

have the ability to cause reversible growth inhibition of cocultured transformed cells.[52a] Information between cells is usually transferred via gap junctions[86a] which consist of hydrophilic channels between cels which allow molecules of up to molecular weight of 1,000 to pass between adjacent cells. Lowenstein and co-workers[86b] have recently shown that when cAMP was added to mammalian cells in culture, junction permeability increased significantly, which was correlated with an increase in the number of gap junctions. The passage via gap junctions of small molecular weight growth inhibitory substances between contacting cells could account for the growth inhibitory effect of cAMP. It is of interest that addition of dibutyryl cAMP and theophylline to a transformed Syrian hamster fibroblast cell line caused a partial restoration of a gap-junction defect in these cells.[86c] This concept is further supported by recent data[86d] with a Cl-1D mouse tumor cell line which fails to make permeable junctions in confluent culture. Upon administration of cAMP or dibutyryl cAMP the cells acquired permeable junctions characteristic of normal cell-to-cell channels.

Recently, Cho-Chung[87] has shown that dibutyryl-cAMP and L-arginine caused inhibition of cell replication of human breast cancer cells. This inhibition was accomplished by a change in cell morphology to normal phenotype. Several recent papers[88-90] have suggested that hormonal regulation of cellular proliferation by modulation of cAMP could offer a more fruitful approach to cancer chemotherapy than the direct inhibition of DNA synthesis which results in direct cell kill and considerable host toxicity.

New cAMP Analogs as Potential Inhibitors of Metastases

A review of structure activity relationship of cAMP derivatives indicate[91] that a substituent such as methylthio, bromo or alkyl at position 8 in the purine ring prevents enzymatic opening of the 3',5'-cyclic phosphate ring. It would appear that the presence of an 8-substituent gives a predominant syn conformation which is apparently not attacked by phosphodiesterase.[91-94] This is particularly important since it has recently been shown that it is the syn form of cAMP which binds to the cAMP receptor protein[95] purified from E. Coli as noted by binding studies using a 270 MHZ nuclear magnetic resonance spectrometer.

Zimmerman[96] has recently pointed out that nucleoside 3',5'-cyclic monophosphates may be formed metabolically from a surprisingly large number of purine antimetabolites. These 3',5'-cyclic nucleotides formed *in vivo* may indeed be responsible for many of the biological effects previously ascribed to the simple purine antimetabolites.[96-99] A number of workers have attempted to define the features of the cAMP molecule which are generally important in the binding of cAMP to various protein kinases. Jastorff et al.[97,97a] have studied the cAMP receptor site in the regulatory subunit of cAMP-dependent protein kinase type I using various cAMP analogs and have proposed that the cyclic phosphate-ribose moiety of cAMP is bound to the kinase *via* its 3' and 5'-oxygens, the 2'-hydroxyl and the negative charge of the phosphate anion (see 5). These workers propose that the adenine moiety is bound in a hydrophobic cleft *without* significant hydrogen bonding interactions. These conclusions are in general agreement with those previously reached by Miller and Robins[100] for cAMP protein kinase type II with the exception that N_3 of adenine appears to be a significant binding site[101] in the type II kinase. The Jastorff model[97] suggests that specificity of activating these protein kinases might be achieved by substitution in the purine moiety. Such a proposal is supported by good experimental data.[100] Severin and co-workers[99] suggest that a hydrogen bond from the protein kinase enzyme to the 6-substituent (NH_2) is also vital for cAMP activation of protein kinase from pig brain.[99] Of course, different cAMP-dependent protein kinases from various sources may indeed possess different allosteric binding requirements and may allow bulk tolerance of different types. Such concepts are indeed the basis of the hoped for specificity[101-104] for antitumor action. It has recently been shown[105] that in proliferating cells the ratio of type I to type II cAMP-dependent protein kinase was 0.37. 8-Bromo-cAMP added to these cells resulted in growth arrest and a type I to type II ratio of 3.76. It has recently been shown[106] that protein kinase I has stricter steric requirements than protein kinase II for the binding locale adjacent to position 2 of cAMP. LeCam et al.[107] have shown that the decrease in growth rate and change in morphology of CHO cells seen with cAMP can be correlated with the phosphorylation by cAMP-dependent protein kinase type II of a 52,000-dalton protein. In selecting the cAMP derivatives for synthesis we would favor those base analogs which have the structural requirement

of cAMP-dependent protein kinase type II.[101] It should be noted that the presence of the amino group at position 6 does not appear to be necessary[102,103] for activation of protein kinase II isolated from bovine cardiac muscle or bovine brain.

It should be pointed out that N^6-O-2'-dibutyryl-cAMP and various lipid-soluble analogs of cAMP penetrate cells more readily than cAMP itself. Endogenous cAMP does indeed cross cellular membranes. Tritium labelled cAMP administered intravenously to rats was recovered in 20% yield in the urine.[108] In E. coli the uptake of cAMP is by facilitated diffusion, whereas efflux is via an energy-dependent active transport process.[109] The cells regulate the number of cAMP transport carriers.[109] In human erythrocytes cAMP can enter erythrocytes in sufficient amounts to alter the activity of cAMP-dependent protein kinases.[110]

In considering certain cAMP analogs which would be superior to cAMP as potential inhibitors of metastasis the following criteria would seem to be important. The 3',5'-cyclic nucleotide analogs selected for studies should be:

1. Good activators of platelet protein kinase type II.
2. Good inhibitors of platelet cAMP PDE.
3. Poor substrates for PDE.
4. Selective for activators of protein kinase type II.
5. Rather nonpolar to ensure good transport into cells.
6. Nucleotides which are not readily incorporated into cellular RNA and DNA which would result in cellular toxicity.

cAMP and the Lymphocyte Immune Response

According to the concept of immune surveillance,[111,112] the development of a tumor represents a failure on the part of the immune system to attack and kill emerging tumor cells. There is now evidence to suggest that immune responsiveness is regulated in part by cyclic adenosine 3',5'-monophosphate (cAMP).[113,114] The bulk of this evidence suggests that cAMP inhibits immune responsiveness. It thus seemed possible that some cases of tumor development might result from or be associated with, abnormal cyclic nucleotide metabolism in immunocytes. Most and perhaps all of the physiologically important effects of cAMP are

thought to be mediated by the activity of a cAMP-dependent protein kinase.[115] Preliminary studies suggested that in mice the melanoma tumor-bearing state was associated with a loss of cAMP-dependent protein kinase activity from human peripheral blood lymphocytes.[116] Ortez and co-workers[116] have recently shown that cAMP-dependent protein kinase activity was depressed in whole thymus and spleen as well as isolated splenic lymphocytes from B-16 melanoma bearing C57 Bl/6J mice as compared to control animals. A similar loss of enzyme activity was observed in human peripheral blood lymphocytes from melanoma bearing patients as compared to normal subjects.[116] An unaltered level of activity in the heart of tumor bearing mice suggested some specificity for the lymphoid system. This depressed enzyme activity was the result of a diminished V_{max} for cAMP stimulated calf histone phosphorylation.[116] The tumor bearing state in the mouse was also accompanied by a depletion of small lymphocytes from both thymus and spleen, and it was hypothesized that the losses of lymphocytes and cAMP-dependent protein kinase activity are related.[116] It has been suggested[116] that the tumor results in selective neutralization of a lymphocyte subpopulation rich in cAMP protein kinase activity. This neutralization may be the result of cellular destruction *via* tumor elaborated lymphotoxins or it may result from selective removal of lymphocytes from the circulation *via* chemotactic factors. Since the effect is most pronounced in the thymus, it is suggested that the lymphocyte type removed is a T cell. This hypothesis receives support from the observations of Hamaoka *et al.*[117] that transferred T cells are poorly recovered from Ehrlich tumor-bearing mice and the report of Levy *et al.*[118] that T cell depressants can be isolated from the sera of rhabdomyosarcoma tumor-bearing mice. Additional support is the recent demonstration by Niaudet *et al.*[119] that T cells contain more cAMP and adenylate cyclase than B cells. It seems quite possible that T cells may also contain more cAMP protein kinase than B cells and that selective removal of T cells is therefore responsible for the drop in cAMP protein kinase activity associated with mixed lymphocyte preparations from tumor-bearing patients and mice.

Tisdale and Thompson[120] have recently shown that patients with chronic lymphocytic leukemia had a different ratio of cAMP-dependent protein kinase I to II than the normal population. Human peripheral blood lymphocytes *in vitro* using chicken erythrocytes as target cells

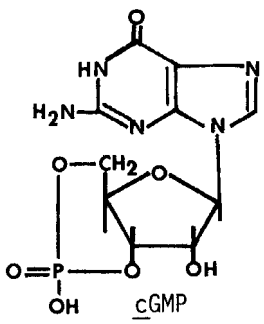
were recently studied in the presence of agents which increased cAMP concentrations.[121] Such agents strongly inhibited the cytotoxic effects of lymphocytes.[121] Such studies support the early observation[122] that cAMP inhibits *in vitro* antibody synthesis by spleen cells. Parker[123] has summarized studies of this type involving cAMP and lymphocytes and concludes that the predominant action of cAMP is that of inhibition of lymphocyte function. It would appear that this negative effect on the proliferation of lymphocytes and their function may be correlated with an activation of cAMP-dependent protein kinase II within the cell.[124]

cGMP and the Lymphocyte Immune Response

The lymphocyte, the fundamental cell of immune function in man, has been intensively studied over the past ten years; however, although the phenomenon of lymphocyte transformation, triggered by surface interaction with antigens is readily observed in cell culture and correlates well with cellular immunity *in vivo*, the detailed molecular control mechanism is still under study. Goldberg and co-workers[125] were the first to suggest that guanosine-3',5'-cyclic monophosphate (cGMP) is a positive intracellular mediator in lymphocytes. These workers showed that the mitogens, phytohemagglutinin and concanavalin A produced up to 50-fold increases of lymphocytic cGMP within 20 minutes.

cGMP has been shown[126] to reverse the inhibitory effect of cAMP on induction of humoral antibody response to heterologous erythrocyte antigens in spleen cultures. cGMP has been found to stimulate nucleic synthesis in lymphocytes as the result of its role as an intracellular mediator,[127] through mitogen-recepter interaction at the surface of the lymphocyte cell. The involvement of cGMP in the regulation of lymphocyte activation has recently been summarized by Hadden et al.[127a] The mitogenic action of cGMP and 8-Br-cGMP, 5, (first prepared[128] in our laboratory in 1973) has been reported,[129,130] in cultured lymphocytes. These effects are opposite to those of exogenous cAMP and 8-Br-cAMP, 3. The effects of cGMP and cAMP on lymphocyte mitogenesis are likely due to their opposing action on the phosphorylation of certain lymphocyte nuclear proteins.[131] cGMP, 7, and 8-Br-cGMP, 8, enhance phosphorylation rapidly while cAMP is inhibitory.[131] These and similar data from other

laboratories[127,132,133] are consistent with the concept that cGMP and cAMP are antagonistic toward each other in their influence on lymphocyte mitogenesis. It has been reported that homogenates of human peripheral blood lymphocytes contain a single phosphodiesterase which hydrolyzes only cAMP.[134] No cyclic GMP phosphodiesterase activity was



cGMP

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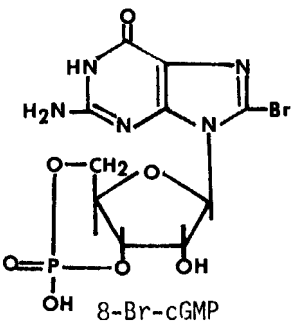

8-Br-cGMP

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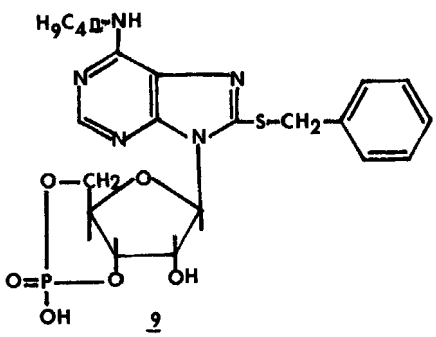
detected in homogenates of these cells. The cyclic AMP phosphodiesterase from the normal donor lymphocytes was 85% inhibited by a 10-fold excess of cyclic GMP, while the enzyme from the leukemic patients' lymphocytes, or from the lymphoblastic lines, was inhibited only 15%. The phosphodiesterase activities from the lymphoblastoid cell lines and from the leukemic lymphocytes were considerably higher than those of the normal lymphocytes.[135] Thus we see that lymphocytes are especially sensitive to activation by cGMP and cGMP analogs since cGMP induces a change in cyclic AMP phosphodiesterase to a form which is more active and less susceptible to inhibition by cGMP. The resulting increase in cAMP hydrolysis contributes to the reciprocal changes in these two cyclic nucleotides which are seen during mitogenic transformation.

The activity of PRPP synthetase which is induced 9-fold in splenic cells 48 hours after mitogenic stimulation has been shown to be stimulated by the presence of cGMP.[136] The release of lysosomal enzymes from isolated hepatic lyposomes is potentiated by cGMP and inhibited by cAMP.[137] Exogenous cGMP has also been shown to reverse the inhibitory effect of cAMP on mitogen-induced proliferation.[60,138] Whitfield and co-workers[139-141] have demonstrated enhanced proliferation of active mouse lymphoblasts with concentrations of cGMP as low as 10^{-11} molar. Thus activation at such a low concentration is truly amazing and sug-

gests that certain specific cGMP analogs could be prepared to selectively activate lymphocytes against tumor cells.

Interferon increases the specific cytotoxicity of sensitized murine spleen cells[142] and the generation of specific natural killer cells in lymphocyte culture.[143] Interferon preparations also enhance the spontaneous cytotoxicity of human lymphocytes *in vitro* against tumor cells.[144-146] The modulation of human blood lymphocytes has been noted to be markedly enhanced by lymphoblastoid interferon.[147] The capacity of natural killer (NK) cells to spontaneously lyse a number of tumor cells *in vitro* is increased in the presence of interferon.[145,148] The recent work of Tovey *et al.*[149] has shown mouse interferon resulted in a rapid (10 min.) four-fold increase in the intracellular concentration of cGMP in mouse leukemia L-1210 cells. Such a rapid increase in the level of cGMP may be responsible for the triggering of the NK cells. The increased awareness of the role of NK cells as a novel cell type having the spontaneous ability to destroy tumor cells is summarized in a recent issue of *Science*.[150]

It is interesting to note that the immunopotentiation effect of levamisole has been postulated to be due to the fact that levamisole inhibits cGMP phosphodiesterase which results in the increase of cGMP within the lymphocyte and results in increased lymphocyte proliferation.[151] Recently, fresh human lymphocytes isolated from hospitalized patients were cultured with various cyclic nucleotides in addition to mitogen. cGMP enhanced the immuno and proliferative response where cAMP had a definite suppressive effect.[152] In view of these data it would appear possible to selectively modulate the antitumor activity of normal lymphocytes in a manner which would result in activation of NK cells toward tumor destruction. It should be pointed out that as discussed earlier, cGMP can also contribute to cellular proliferation. Indeed, dibutyryl cGMP has recently been shown to promote the growth[153] of tumor HXK4. In concept, however, it should be possible to prepare analogs of cGMP which would be specific activators of lymphocytes to natural killer cells without stimulating tumor cell proliferation. The success of preparing cyclic nucleotides which will provide a specific physiological response has recently been reviewed.[154] A specific example is the potent inotropic activity of N^6-n-butyl-8-benzylthio-cAMP, 9,



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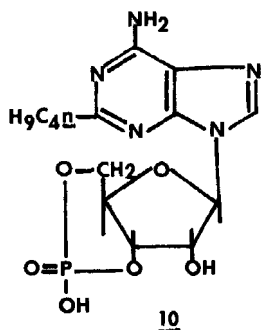

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exhibited in dogs.[155] Another example is 2-n-butyl-cAMP,[156] 10, which is orally effective and superior to aldomet in the hypertensive rat.[154] In dogs, 10 results in a 50% drop in blood pressure with a 10-hour duration with no tachycardia or significant increase in heart rate.[154] This type of physiological response is ample evidence that these cyclic nucleotides, 9 and 10, are indeed crossing the cellular membrane. Recently, a study of the cellular uptake of cAMP was made in intact human erythrocytes.[157] Not only was cAMP shown to be transported across membranes into human erythrocytes, but it appears that there is a shared transport channel for cAMP and anion transport.[157] Thus this data suggests that a carrier-mediated transport system for cAMP exists at least in certain types of cells.[157] Thus one may be able to utilize a cyclic nucleotide form of a nucleoside which would successfully cross cell membranes and then be opened to the desired 5'-phosphate within the cell by certain cellular cAMP phosphodiesterases.

It is clear that the nucleoside 3',5'-cyclic monophosphates offer a unique opportunity for drug design which could provide such nucleotides with a highly specific hormonal regulatory response. Such hormonal modulators could well regulate cell proliferation, revert cell transformation, prevent metastases and/or stimulate lymphocyte response. The challenge is thus to prepare nucleoside 3',5'-cyclic monophosphates which will be highly specific in each of these areas and then utilize such agents in a desired combination to affect the required control over the neoplastic cell. Such projected research offers a unique challenge to the nucleoside and nucleotide chemist.

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