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COMMENTARY

NUCLEOSIDE 3′,5′-CYCLIC MONOPHOSPHATE METABOLITES OF PURINE ANALOGS

POSSIBLE ROLE AS PHYSIOLOGICAL MEDIATORS

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It is generally accepted that most purine and pyrimidine antimetabolites are not active *per se* but require metabolism to some type of nucleotide in order to exert their antiproliferative, immunosuppressive or antiviral effects [1–13]. In view of this circumstance, our ability to discern the mechanism of action of this type of drug is often limited by the incompleteness of our knowledge concerning the metabolic interconversions which take place *in vivo* with these agents. It has been shown previously that many such purine and pyrimidine analogs are metabolized to one or more of the following types of nucleotide derivative [1–13]: nucleoside 5′-mono, -di- and triphosphates; 2′-deoxyribonucleoside 5′-di- and triphosphates; nucleotide sugars [14–16]; nucleotide alcohols [17, 18]; analogs of NAD [19–21]; and polynucleotides (RNA and DNA). Moreover, in addition to phosphoribosylation or phosphorylation, some antimetabolites—such as 6-mercaptopurine [22], 8-azainosine [23] and 5-fluorouracil [24]—may also require subsequent modification of the purine or pyrimidine moiety for complete metabolic "activation". Despite our ever-increasing body of knowledge concerning this class of drugs, in relatively few, if any, of these cases has a given species of analog nucleotide been clearly identified as being "*the*" metabolite most critical to the expression of the parent drug's biological activity. Indeed, when multiple nucleotide metabolites are formed within cells, it is likely that more than one of these metabolites will contribute to the overall biological consequences of the parent drug. More recently, yet another type of nucleotide metabolite—nucleoside 3′,5′-cyclic monophosphates—has been demonstrated for several purine nucleoside analogs [25–27].

The purpose of this commentary is 2-fold: (1) to indicate that 3′,5′-cyclic nucleotides may be formed metabolically from a surprisingly large number of purine antimetabolites; and (2) to describe evidence which suggests that these newly recognized cyclic nucleotide metabolites can contribute substantially to the physiological effects associated with the parent drugs. Although this commentary is concerned primarily with purine antimetabolites, the conclusions drawn are likely to be applicable to pyrimidine antimetabolites as well.

Metabolic considerations. Nucleoside 3′,5′-monophosphates (cNMPs) are formed metabolically from their corresponding nucleoside 5′-triphosphates (NTPs) via the action of enzymes known trivially as "cyclases" [nucleoside 5′-triphosphate pyrophosphate-lyases (cyclizing), EC 4.6.1]:

$$\text{NTP} \longrightarrow \text{cNMP} + \text{PP}_i$$

Cells have been shown to contain cyclases specific for the formation of adenosine 3′,5′-monophosphate (cAMP) [28], guanosine 3′,5′-monophosphate (cGMP) [29] and cytidine 3′,5′-monophosphate (cCMP) [30, 31]. Evidence has also been reported for the existence of uridine 3′,5′-monophosphate in both bacterial [32] and mammalian [33] cells, although no enzyme has yet been described for its formation. Hence, in order for a purine analog to be converted intracellularly to its corresponding nucleoside 3′,5′-monophosphate, it is necessary that such a drug first be metabolized to its nucleoside 5′-triphosphate.

The literature abounds with examples of purine analogs which have been shown to undergo metabolism to nucleoside 5′-triphosphates. A partial list of such compounds is presented in Table 1. In order to be metabolized to a nucleoside 5′-triphosphate, it is necessary for an antimetabolite to "fool" at least three separate enzymes—either a phosphoribosyltransferase or a nucleoside kinase, a nucleoside monophosphate kinase and a nucleoside diphosphate kinase—into accepting it as a substrate. Should this be the case with a particular analog, it would not be surprising to find that one more enzyme, a cyclase, could also utilize this analog nucleoside triphosphate as a fraudulent substrate. Indeed, in at least some cases, the cellular accumulation of ATP analogs is accompanied by a substantial decrease in ATP itself [26, 27, 41], thus enhancing the probability for the utilization of the ATP analogs as alternate substrates by adenylate cyclase.

Evidence for metabolic formation of analog purine nucleoside 3′,5′-monophosphates. Experimental evidence for the metabolism of purine analogs to nucleoside 3′,5′-monophosphates has come from both enzymatic and cellular studies. In both types of studies, the limiting factor for progress has been the sensitivity of

Table 1. Selective list of purine derivatives which have been shown to undergo metabolism to nucleoside 5′-triphosphates

Compound	Ref.
2-Azaadenine	34
2-Aminoadenine	34
5-Aminoimidazole-4-carboxamide	35
Hypoxanthine	36
Adenosine N^1-oxide	37
2-Fluoroadenosine	38
N^6-hydroxyladenosine	*
N^6-furfuryladenosine	39
N^6-(Δ^2-isopentenyl)adenosine	39, 40
N^6-benzyladenosine	39
6-Methylmercaptopurine ribonucleoside	41
8-Azaadenosine	23
Tubercidin	42
Sangivamycin	43
Toyocamycin	44
Formycin A	45, 46
2′-Deoxyadenosine	47
Adenine arabinoside	48
3′-Amino-3′-deoxyadenosine	49
6-Thioguanine	50–52
2′-Deoxyguanosine	53

* R. D. Deeprose and T. P. Zimmerman, unpublished observations.

existing analytical procedures. Since cAMP and cGMP are present in mammalian tissues at concentrations of approximately 1 μM or less, it could be anticipated that cyclic nucleotide analogs would be formed metabolically in correspondingly low amounts. In general, radioactive purine analogs of high specific activity, especially the nucleoside 5′-triphosphate analogs necessary for enzymatic studies, have not been readily available to expedite this area of research.

Relatively few structural analogs of ATP or GTP, modified either in the purine or in the ribose moiety, have been examined for substrate activity with isolated preparations of the respective cyclases. From these limited studies, however, it is evident that nucleoside 5′-triphosphates with modifications at the 2′-position of the ribose moiety (e.g. dATP [54–59], adenine arabinoside 5′-triphosphate [60] and dGTP [61]) are accepted as substrates by preparations of cyclases from different sources. Indeed, guanylate cyclase from rat lung exhibited a preference for dGTP compared with GTP [61]. Inosine 5′-triphosphate has also been shown to be an alternate substrate for guanylate cyclase [61].

Studies with intact cells have provided compelling evidence for the metabolism of some purine nucleoside analogs to their 3′,5′-monophosphates. Radiolabeled 2′-deoxyadenosine has been shown to be metabolized to both dATP and 2′-deoxyadenosine 3′,5′-monophosphate in brain slices; in support of the analytical data, the tissue level of 2′-deoxyadenosine 3′,5′-monophosphate could be increased by veratridine, an agent which stimulates cyclic nucleotide formation [25]. By means of a highly sensitive radioimmunoassay with specificity directed toward cAMP, it has been possible to demonstrate the metabolism of non-radioactive 2-fluoroadenosine [26], tubercidin [27] and formycin A [27] to their respective 3′,5′-monophosphates in lymphoid cells of various origins. The identification of these latter analog cyclic nucleotide metabolites was based upon their co-chromatography with authentic standards (and complete separation from cAMP) through sequential columns of aluminium oxide and appropriate ion-exchange resins, the loss of their cross-reactivity in the cAMP radioimmunoassay after treatment with cAMP phosphodiesterase, and the abilities of various pharmacological agents (e.g. adenosine, cholera toxin, prostaglandin E_1 and inhibitors of cAMP phosphodiesterase) to increase their levels in intact cells. In all cases, the cells which formed these cyclic nucleotide analogs were shown by high-performance anion-exchange chromatography to metabolize 2-fluoroadenosine, tubercidin and formycin A extensively to their 5′-triphosphates, the expected biosynthetic precursors of the 3′,5′-monophosphates. In the case of formycin A, an inhibitor of adenosine deaminase was needed to allow optimum metabolism of this purine nucleoside analog to both its 5′-triphosphate and its 3′,5′-monophosphate. Lack of detectable metabolism of radiolabeled 2-chloroadenosine to its corresponding 3′,5′-monophosphate in cultured human fibroblasts [62] and brain slices [25] can be attributed to the poor efficiency with which cells metabolize this agent to its 5′-triphosphate.*

Biological activity of cyclic nucleotide analogs. A vast body of literature exists attesting to the central roles of cAMP and cGMP in the regulation of a wide variety of physiological processes. Within the framework of present knowledge, various hormonal or environmental agents are believed to modulate cellular levels of these cyclic nucleotides, either through stimulation of their production by adenylate or guanylate cyclase or through enhancement of their degradation by 3′,5′-cyclic nucleotide phosphodiesterases (EC 3.1.4.17). In eukaryotic cells, it is generally accepted that the regulation of cellular physiology by intracellular cAMP and cGMP is accomplished via the activation of various cyclic nucleotide-dependent protein kinases (ATP: protein phosphotransferases, EC 2.7.1.37) which are specific for either cAMP [63] or cGMP [64].

Once formed intracellularly, 3′,5′-cyclic nucleotide analogs can conceivably perturb cyclic nucleotide metabolism and function in any of several different ways. Perhaps the most important and best-documented biochemical activity of cyclic nucleotide analogs concerns their interaction with protein kinases. A large number of purine nucleoside 3′,5′-monophosphate derivatives have been prepared chemically and have been shown to exhibit a wide range of activity, relative to cAMP and cGMP, in stimulating preparations of cyclic nucleotide-dependent protein kinases. A partial summary of this literature, restricted to a consideration of those purine analogs known to be metabolized to nucleoside 5′-triphosphates (Table 1), is presented in Table 2. The 3′,5′-cyclic monophosphates of 2-fluoroadenosine, adenosine N^1-oxide, N^6-hydroxyladenosine, N^6-furfuryladenosine, N^6-(Δ^2-isopentenyl)adenosine, N^6-benzyladenosine, 6-methylmercaptopurine ribonucleoside, 8-azaadenosine, tubercidin and toyocamycin have been found to approach or excel cAMP as activators of cAMP-dependent protein kinases. Although the chemical synthesis of formycin A 3′,5′-monophosphate has been described [80, 81], no information has been reported concerning the ability of

* T. P. Zimmerman, G. Wolberg and G. S. Duncan, unpublished observations.

Table 2. Activation of cAMP- and cGMP-dependent protein kinases (PK) by purine nucleoside 3′,5′-monophosphate analogs*

Analog 3′,5′-monophosphate	K_a for cAMP (cGMP)/K_a for analog		Ref.
	cAMP-dependent PK	cGMP-dependent PK	
2-Azaadenosine	0.06–0.10		65
2-Fluoroadenosine	1.8+		26
2-Aminoadenosine	0.12	0.05	66
5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamide	0.03–0.05	0.02	67, 68
Inosine	0.15–0.59	0.01–0.26	68–70
Adenosine N^1-oxide	0.40–0.45	0.023	71, 72
N^6-hydroxyladenosine	1.0		67
N^6-furfuryladenosine	0.95+		73
N^6-(Δ^2-isopentenyl)adenosine	1.1+		73
N^6-benzyladenosine	0.75–0.95+	0.02	68, 73
6-Methylmercaptopurine ribonucleoside	1.2–1.5	0.42	69
8-Azaadenosine	0.53–0.85		65
Tubercidin	0.49–1.9	0.09	65, 74–76
Sangivamycin	0.19–0.23		76
Toyocamycin	0.44–0.52		76
2′-Deoxyadenosine	0.004–0.005		77
Adenine arabinoside	0.001–0.003		77
3′-Amino-3′-deoxyadenosine	< 0.001–0.028+		78
6-Thioguanosine	0.002	0.18	66
2′-Deoxyguanosine	0.002	0.01	79

* The K_a values (activation constants) are the concentrations of each cyclic nucleotide that cause a half-maximal stimulation of the protein kinase [75]. Multiple values in this table represent data obtained with different preparations of protein kinase.

+ These values are estimates derived from available data.

this cyclic nucleotide analog to stimulate protein kinase. The cyclic nucleotide derivatives of 6-methylmercaptopurine ribonucleoside and 6-thioguanosine were found to be fair-to-good activators of cGMP-dependent protein kinases.* Thus, it is clear that a considerable number of cyclic nucleotide analogs which are likely to be formed metabolically from appropriate precursors (cf. Table 1) are capable of stimulating cyclic nucleotide-dependent protein kinases. Should a relatively high cellular concentration of such a cyclic nucleotide analog be realized or should a protein kinase exhibit an extraordinary affinity for a particular cyclic nucleotide analog, a state of hyperstimulation of cellular protein kinase would be expected to result. Such a circumstance would presumably represent a deleterious loss of regulation of cellular physiology.

In view of current interest in the mechanism of action of 2′-deoxyadenosine, adenine arabinoside and 2′-deoxyguanosine in various biological systems, it is pertinent to note in Table 2 that the 3′,5′-cyclic nucleotides of all three of these purine nucleosides are essentially inactive in the stimulation of the appropriate protein kinases. Hence, even if formed metabolically, these three cyclic nucleotide derivatives would not appear capable of affecting cellular physiology via direct effects on protein kinases. Nevertheless, the possibility remains that some cyclic nucleotide analogs may inhibit the binding of their natural counterparts (cAMP or cGMP) to protein kinases, without themselves causing significant stimulation of this enzyme [82], and may thereby prevent normal modulation of protein kinase activity within cells.

3′,5′-Cyclic nucleotide phosphodiesterases represent another potential intracellular site of action for cyclic nucleotide analogs. Several of the analogs considered in Table 2 have been found to inhibit competitively different preparations of low K_m phosphodiesterases specific for cAMP or cGMP and to have apparent K_i values in the micromolar range (Table 3). As is apparent in this table, phosphodiesterases from different tissues can have widely different K_i values for any given cyclic nucleotide analog. Thus, it is possible that metabolically formed cyclic nucleotide analogs might cause a selective elevation in the cellular levels of cAMP and/or cGMP due to tissue-specific inhibition of phosphodiesterases.

Many cAMP analogs, when compared with cAMP itself, have been found to be relatively resistant to hydrolysis by phosphodiesterases (Table 3). This circumstance is favorable to the metabolic buildup of such cyclic nucleotide analogs within cells.

In addition to protein kinases and cyclic nucleotide phosphodiesterases, other cellular enzymes may be found to be sensitive to cyclic nucleotide analogs. For example, 5-phospho-α-D-ribose-1-pyrophosphate synthetase, a key enzyme in the *de novo* pathway of purine and pyrimidine biosynthesis, is stimulated directly (i.e.

* It is important to note that these cyclic nucleotides were tested against a cGMP-dependent protein kinase from lobster muscle. No information has been published concerning the activity of these analogs with a cGMP-dependent protein kinase from a mammalian source.

Table 3. Substrate and inhibitory properties of purine nucleoside 3′,5′-monophosphate analogs with cAMP-specific phosphodiesterase

Analog 3′,5′-monophosphate	K_i* (μM)	Relative rate of hydrolysis+	Ref.
2-Azaadenosine	5.8–30	0.5–2.0	65, 83
2-Aminoadenosine		0.93	66
5-Amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide	57–65	0.22–0.38	67, 84
Inosine‡	78	0.46–1.4	69, 85, 86
Adenosine N^1-oxide	17–23	0.15–1.0	71, 72, 87
N^6-hydroxyladenosine	8.5–50	0.44	67
N^6-furfuryladenosine		0.02	73
N^6-(Δ^2-isopentenyl)adenosine		0.12	73
N^6-benzyladenosine		0.20	73
6-Methylmercaptopurine ribonucleoside		0.17–0.60	69, 70, 88
8-Azaadenosine	33–170		65
Tubercidin	6.7–214	0.34–3.0	65, 74, 76, 86, 89–91
Sangivamycin	1.7–4.4	0.16–0.29	76
Toyocamycin	0.8–1.6	0.39–0.53	76
2′-Deoxyadenosine	7.2–81	0.38–0.92	77, 84, 92
Adenine arabinoside	8.0–460	0.15–0.25	77
3′-Amino-3′-deoxyadenosine		0.28	78
2′-Deoxyguanosine§		0.21–0.44	79, 92

* These apparent K_i values were determined with the low K_m cAMP-specific phosphodiesterase isolated from several mammalian tissues.

+ The relative rate of hydrolysis of each cyclic nucleotide analog versus that of cAMP was determined with the high K_m cAMP-specific phosphodiesterase isolated from several mammalian tissues.

‡ An apparent K_i value of 1.1 μM for inosine 3′,5′-monophosphate has been determined with the low K_m cGMP-specific phosphodiesterase purified from guinea pig lung [84].

§ An apparent K_i value of 1.7 μM for 2′-deoxyguanosine 3′,5′-monophosphate has been determined with the low K_m cGMP-specific phosphodiesterase purified from guinea pig lung [84].

independently of the action of protein kinases) by physiological concentrations of cGMP, and this effect is antagonized by cAMP [93, 94].

Studies with intact cells have provided further evidence for the biological activity of some cyclic nucleotide analogs. 2-Fluoroadenosine is an irreversible inhibitor of lymphocyte-mediated cytolysis and is metabolized extensively and irreversibly (i.e. during the 1- to 2-hr experimental period) to both 2-fluoroadenosine 5′-triphosphate and 2-fluoroadenosine 3′,5′-monophosphate (F-cAMP) within the cytolytic lymphocytes [26]. Agents which prevent the uptake and phosphorylation of 2-fluoroadenosine by these lymphocytes also prevent the irreversible inhibition of cytolysis otherwise observed with this adenosine analog [95]. Cytolytic lymphocytes pretreated with different concentrations of 2-fluoroadenosine and then transferred to drug-free medium retain proportionate levels of analog nucleotides (including F-cAMP) and exhibit inversely proportionate degrees of cytolytic activity. Subsequent treatment of these 2-fluoroadenosine-pretreated lymphocytes with an inhibitor of cAMP phosphodiesterase resulted in increased inhibition of cytolytic function and an increased cellular content of F-cAMP without an increase in cAMP above control levels [95]. These results suggest that F-cAMP is the metabolite of 2-fluoroadenosine which is responsible for the irreversible inhibition of this lymphocyte function.

6-Methylmercaptopurine ribonucleoside 3′,5′-monophosphate (cMMPRMP), like N^6,$O^{2'}$-dibutyryl cAMP, has been shown to cause induction of tyrosine aminotransferase *in vivo* in rat liver [70]. With cultured rat hepatoma cells, cMMPRMP was found to cause an increase in the activities of both tyrosine aminotransferase and phosphoenolpyruvate carboxykinase [96]. Since 6-methylmercaptopurine ribonucleoside, which is metabolized rapidly to its 5′-monophosphate and much more slowly to its 5′-di- and triphosphates [41], was unable to mimic this activity of cMMPRMP [97], the effect observed appears to have been due to the cyclic nucleotide analog *per se* and not to a 5′-nucleotide derived from it. In further support of this conclusion, cMMPRMP has been shown to stimulate protein kinase activity in intact hepatoma cells under conditions where this cyclic nucleotide analog also induced tyrosine aminotransferase [98]. It is pertinent to note that the above findings represent short-term (i.e. 5 hr or less) effects of cMMPRMP on non-growing cells. By contrast, the antiproliferative [96, 97, 99] and antiviral [100] activities of cMMPRMP are long-term (i.e. 2 to 3 days) effects which are likely to result in part from the intracellular conversion of cMMPRMP to its 5′-monophosphate and to the subsequent biochemical effects of this metabolite [88, 97, 100].

N^6-benzyladenosine 3′,5′-monophosphate was found to be more active than cAMP in activating phosphorylase *b* kinase in extracts of liver and muscle [101]. With intact hepatoma cells, this cyclic nucleotide analog was shown both to cause induction of tyrosine aminotransferase and to stimulate protein kinase activity [96, 98]. N^6-benzyladenosine 3′,5′-monophosphate inhibited the growth of several lymphoid cell lines [73].

Tubercidin 3′,5′-monophosphate has been shown to exhibit glycogenolytic activity in perfused rat liver [89] and lipolytic activity in rat adipocytes [91]; in this latter study tubercidin 5′-monophosphate was devoid of lipolytic activity. The growth inhibitory activity of tubercidin 3′,5′-monophosphate toward KB cells was approximately one-sixth that of tubercidin [102].

Other cyclic nucleotide analogs, including N^6-(Δ^2-isopentenyl)adenosine 3′,5′-monophosphate [73], N^6-furfuryladenosine 3′,5′-monophosphate [73], formycin A 3′,5′-monophosphate [81] and adenine arabinoside 3′,5′-monophosphate [99, 103, 104], have been shown to be cytotoxic. In addition, N^6-hydroxladenosine 3′,5′-monophosphate [100] and adenine arabinoside 3′,5′-monophosphate [103] have been shown to inhibit viral replication.

Concluding remarks. It is now apparent that some purine antimetabolites are converted intracellularly to nucleoside 3′,5′-monophosphates and that the cyclic nucleotide derivatives of many purine analogs possess a high degree of biological activity. Among the cellular processes purportedly regulated or influenced by cAMP and/or cGMP are cellular proliferation* [107–113], immune function [107, 114–116] and viral replication [110, 117–120]. Since these three phenomena are among the more common targets for chemotherapy with purine analogs, the cyclic nucleotide metabolites of these drugs, when formed, may contribute in an important way to a successful outcome of therapy. To the extent that these cyclic nucleotide metabolites are important in particular therapeutic applications of purine analogs, it should prove possible to enhance therapeutic efficacy by subsequent administration of a selective inhibitor of cyclic nucleotide phosphodiesterase. This latter expectation appears to have been realized in recent studies concerning the inhibition of lymphocyte-mediated cytolysis by 2-fluoroadenosine [95]. On the other hand, in some therapeutic applications of purine analogs these cyclic nucleotide metabolites may be responsible for undesired side-effects of the drugs. In such cases it may be possible to counteract these unwanted drug effects with pharmacological agents which selectively alter cyclic nucleotide metabolism.

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* cCMP also appears to play a role in the regulation of cellular proliferation [105, 106].

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