Synthesis and Antitumor and Antiviral Activities of a Series of 1-β-D-Ribofuranosyl-5-halocytosine (5-Halocytidine) Cyclic 3′,5′-Monophosphates

Jozsef Beres,[†,‡] Wesley G. Bentrude,*[,§] Gabor Kruppa,[‡] Patricia A. McKernan,[‖] and Roland K. Robins[‖]

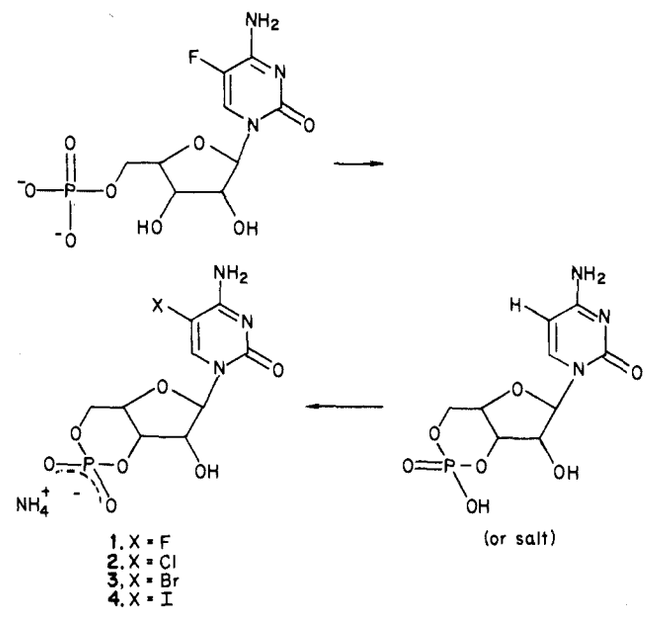
Department of Chemistry, University of Utah, Salt Lake City, Utah 84112, Central Research Institute for Chemistry of the Hungarian Academy of Sciences, H-1525 Budapest, Hungary, and the Cancer Research Center, Department of Chemistry, Brigham Young University, Provo, Utah 84602. *Received June 8, 1984*

A series of 1-β-ribofuranosyl-5-halocytosine cyclic 3′,5′-monophosphates (1–4) has been prepared. Direct halogenation of cytidine 3′,5′-monophosphate (cCMP) yielded the Cl, Br, and I compounds while 5-F-cCMP (1) was obtained on cyclization of the 5′-monophosphate. On in vitro testing of 1–4 against L1210 and P388 leukemias, only 1 showed significant low-level activity (ID_{50} = 3.1 × 10^{-4} mmol/L). Derivatives 2–4 were inactive at 10^{-1} mmol/L and also proved to have low viral ratings against a series of RNA and DNA virus strains in vitro. By contrast the 5-F-cCMP showed moderate activity against VV, HSV-1, and HSV-2 strains (VR ≈ 0.6–0.9). Both 5-fluorocytidine and 5-fluorocytidine 5′-monophosphate had marked antiviral activity (VR = 1.0–2.1) with the above viruses as well as with parainfluenza virus type 3. The nucleoside and nucleotide also were more active than 5-F-cCMP against L1210 and P388 cells. However, comparison of the cytotoxicities and antiviral ED_{50} values of 5-F-cCMP, 5-fluorocytidine 5′-monophosphate, and 5-fluorocytidine suggests a potential therapeutic advantage for 5-F-cCMP. Possible rationales for these activities are discussed in terms of 5-F-cCMP and the corresponding 5′-monophosphate as potential prodrugs and as sources, following enzymatic deamination, of cytotoxic 5-fluorouridine or its 5′-monophosphate.

The frequent association of reduced levels of intracellular adenosine cyclic 3′,5′-monophosphate (cAMP) with the malignant state and the normalization of cell morphology and growth rate observed in a number of cases by the introduction of cAMP and its analogues into the growth media have strongly implicated cyclic nucleotide metabolism as having an important role in the transformation process.[1] Of further significance is a recent report[2] suggesting that cyclic 3′,5′-monophosphates as metabolites of certain nucleosides could be responsible for their antiproliferative and antiviral activities. Moreover, cyclic 3′,5′-monophosphates of nucleosides such as ara-A[3], ara-C[4-6], and the 6-mercapto- and 6-(methylthio)-9-β-D-ribofuranosylpurines[7] have been shown to have good in vitro antitumor properties. Of special interest is the reported in vivo activity of ara-cCMP against L1210 leukemia in mice[6] and wth ara-C resistant strains.[5] 6-Mercapto- and 6-(methylthio)-9-β-D-ribofuranosylpurine cyclic 3′,5′-monophosphate were reported to be cytotoxic to rat hepatoma cells but probably only after PDE-catalyzed hydrolysis to the 5′-monophosphates.[8] The cytotoxicity of the 6-methylthiopurine cyclic nucleotide to cells resistant to the nucleoside by virtue of lack of adenosine kinase activity also is notable.[7] Such results suggest a drug development rationale in which the cyclic nucleotide passes the cell membrane and is then hydrolyzed intracellularly to the active 5′-monophosphate, thereby obviating the need for phosphorylation of the nucleoside in cells which are drug resistant by virtue of loss of kinase. The above nucleosides[3-7] and in addition ribavirin[9] all proved to have good antiviral activities when derivatized as cyclic 3′,5′-monophosphates.

In this paper we report syntheses along with in vitro antitumor and antiviral biological activities of a series of 5-halocytidine cyclic 3′,5′-monosphosphates (1–4). For the relatively active 5-fluoro case (1), comparison data also are presented for the corresponding base, nucleoside, and 5′-monophosphate. The 5-halocytidine derivatives 1–4 are especially of interest since the same deamination by deaminase which renders ara-C inactive would convert them into potential cytotoxins, the corresponding 5-halouridine derivatives. Indeed 5-fluorouridine is an active antitumor agent,[10] and its potential precursor, 5-fluorocytidine, is active against ascites Sarcoma 180 in mice[11] and certain

Scheme I



1. X = F
2. X = Cl
3. X = Br
4. X = I

† Postdoctoral Fellow, University of Utah, 1982–1984.
‡ Hungarian Academy of Sciences.
§ University of Utah.
‖ Brigham Young University.

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Table I. Synthetic Data for 1–4

compd	formula	anal.	isolated yields,[a] %	R_f[c] 1[d]	R_f[c] 2[d]
1	$C_9H_{14}FN_4O_7P$	C, H, F, N, P	27[b]	0.37	0.75
2	$C_9H_{14}ClN_4O_7P$	C, H, Cl, N, P	34	0.41	0.75
3	$C_9H_{14}BrN_4O_7P$	C, H, Br, N, P	24	0.42	0.75
4	$C_9H_{14}IN_4O_7P$	C, H, I, N, P	27	0.45	0.75

[a] Direct halogenations. [b] Redox ring closure. [c] On silica gel TLC plates. [d] For solvent system, see Experimental Section.

Table II. ^{13}C NMR Parameters for 1–4 in D_2O

	$\delta(^{13}C)$[a]									coupling constants, Hz				
no.	C2	C4	C5	C6	C1'	C2'	C3'	C4'	C5'	$^2J_{FC6}$	$^3J_{PC2'}$	$^2J_{PC3'}$	$^3J_{PC4'}$	$^2J_{PC5'}$
1	154.9	158.6	b	126.2	94.9	72.6	77.3	72.1	67.5	32.9	7.8	4.3	4.1	6.8
2	155.9	163.0	b	139.8	95.3	72.8	77.4	72.4	67.7		7.9	4.3	4.2	6.8
3	156.0	163.4	b	142.6	95.5	72.8	77.5	72.4	67.8		7.8	4.2	4.2	6.8
4	156.3	165.1	95.5	148.2	95.5	72.8	77.5	72.4	67.8		7.9	4.4	4.3	7.2

[a] In ppm downfield from Me_4Si (dioxane internal standard). [b] Not observed, presumably because of long relaxation times resulting from the absence of hydrogen substituent.

other transplanted tumors.[12] 5-Fluorocytidine has been shown[13] to be more rapidly deaminated by crude cytidine deaminase from mouse kidney than is cytidine itself. The facile deamination of 5-fluorocytidine to 5-fluorouridine has been demonstrated in human lymphoblastic cells.[14] Even with L5178Y cells deficient in cytidine deaminase activity, 5-fluorocytidine was strongly antiproliferative by inhibition of thymidylate synthetase, likely after conversion to 5-fluoro-2'-deoxyuridine 5'-monophosphate.[15] 5-Fluorocytidine also has been reported to be incorporated into tRNA.[16]

Results and Discussion

Chemistry. A series of 1-β-D-ribofuranosyl-5-halocytosine cyclic 3',5'-monophosphates, **1–4**, has been prepared (Scheme I and Table I). The 5-Cl-cCMP, **2**, resulted on direct halogenation of the *N,N'*-dicyclohexyl-4-morpholinecarboxamidine salt of cCMP itself by use of 2 equiv of *N*-chlorosuccinimide (NCS) in dry *N,N*-dimethylformamide at 115–120 °C (1 h). A 1-equiv excess of NCS was required to drive the reaction to completion. Thus, nearly half of the cCMP remained unreacted after reaction with 1.3 equiv of NCS. At room temperature, only a trace of 5-Cl-cCMP could be detected by TLC after 3 days.

Similarly, *N*-bromosuccinimide (NBS) underwent reaction at room temperature with the above cCMP salt in glacial acetic acid to yield the 5-bromo derivative **3**, previously prepared by cyclization of 5-Br-CMP.[17] The cCMP ammonium salt, probably because of its limited solubility in glacial acetic acid, underwent no more than 50% reaction.

Direct I_2/HIO_3 iodination of cCMP (ammonium salt) in an acetic acid/CCl_4/H_2O medium at 45–50 °C, followed by further reaction overnight at room temperature, yielded the corresponding 5-iodo derivative, **4**. Prolonged reaction time decreased the yield of **4**, perhaps as a result of its hydrolysis.[18] NCS,[19] NBS,[20] and I_2/HIO_3[21] were previously used in the halogenations of heterocyclic bases, nucleosides, and nucleotides but not for the preparation of halo derivatives of cyclic 3',5'-monophosphates, except that the 5-bromouridine.[22] 5-F-cCMP (**1**) was prepared by a redox cyclization[23] of the *N,N'*-dicyclohexyl-4-morpholinecarboxamidine salt of the 5'-monophosphate by use of 2,2'-dipyridyl disulfide/Ph_3P in refluxing pyridine.

The required 5-fluorocytidine 5'-monophosphate (5-F-5'-CMP) precursor to 5-F-cCMP was obtained by the Yoshikawa method.[24] Limited room-temperature solubility of the 5-fluorocytidine hydrochloride required that it be dissolved at 140–150 °C. Above 150 °C the nucleoside decomposed slowly, a problem also encountered if dissolution of the nucleoside was not carried out quickly. ^1H and ^{13}C NMR data confirmed the structure of 5-F-5'-CMP and gave no evidence for the formation of the 3'-monophosphate, a potential side product.[25] This was confirmed by HPLC.[26]

Conventional cyclization of the 5-F-5'-CMP with dicyclohexylcarbodiimide in refluxing pyridine/DMF (5/1, v/v) according to the procedure recently described for the preparation of cCMP[27] led to isolation (yield 34%) of a two-component mixture containing 60–67% of the major component (^1H NMR, HPLC). HPLC showed this to be the cyclic 3',5'-monophosphate contaminated (33–40%) by the isomeric 2',3'-cyclic monophosphate which were identified as described elsewhere.[26] Presumably, the 2',3'-cyclic monophosphate results from cyclization of the 3'-monophosphate which may be produced from the cyclic 3',5'-monophosphate by an unknown process peculiar to the 5-fluoro compound, as this complication is not observed

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Table III. In Vitro Antitumor Properties of 1–4 and Comparison Compounds

compd	ID_{50},[a] mmol/L	
	L1210	P388
5-fluorocytosine	[b]	[b]
5-fluorocytidine hydrochloride	2.6×10^{-6}	3.0×10^{-6}
5-F-5′-CMP	3.4×10^{-6}	3.1×10^{-6}
1	3.1×10^{-4}	3.1×10^{-4}
2	[b]	[b]
3	[b]	[b]
4	[b]	[b]
8-Cl-cAMP	42%[c]	33%[c]
8-*p*-chlorophenylthio-cAMP	20%	18%
8-*p*-bromophenylthio-cAMP	30%	22%
aracytidine	2.5×10^{-6}	1.2×10^{-6}
cCMP	26%[c]	34%[c]

[a] Concentration (millimoles/liter) corresponding to 50% inhibition of cell growth except as otherwise indicated. Single determination. [b] Inactive at 10^{-1} mmol/L. [c] Indicate percent growth inhibition at 10^{-1} mmol/L concentration.

with 5′-CMP itself even in refluxing pyridine/DMF. As noted earlier, the 5-F-5′-CMP failed to yield the cyclic 2′,3′-monophosphate on cyclization with 2,2′-dipyridyl disulfide/Ph_3P in refluxing pyridine in the absence of DMF. Whether this is a result of the lower reflux temperature or absence of some special solvent property of DMF is not known. The absence of 3′-monophosphate contaminant in the 5-fluorocytidine 5′-monophosphate (vide supra) rules out the former as a direct source of the 2′,3′-monophosphate.

The 5-halo-cCMPs (**1–4**) were routinely isolated by DEAE-Sephadex A-25 anion-exchange column chromatography using a linear aqueous salt gradient. As the starting cCMP has identical chromatographic properties under these conditions, it is imperative that the halogenations be run to completion.

The structures of **1–4** were confirmed by ^1H and ^{13}C NMR spectroscopy (Table II) as well as mass spectrometry and quantitative elemental analysis (Table I). In the ^1H NMR spectra, the presence of sharp singlet signals in the range δ 5.58–6.00 confirmed the β nature of the configuration at anomeric Cl′.[28] Diagnostic of the presence of the 5-fluoro substituent in **1** was the 5.8-Hz $^3J_{HF}$ value noted for H6.[29] For **2–4** a *singlet* at about δ 7.9 was observed, which is indicative of halogen substitution at C5. The ^{13}C chemical shifts and J_{PC} values for **1–4** (Table II) are typical of those found for other nucleoside cyclic 3′,5′-monophosphates.[30] The chemical shift assignments to C3′ and C4′ follow the revisions recently made for similar diesters on the basis of single-frequency decoupling experiments.[31] The 32.9-Hz $^2J_{CF}$ value found for C6 of **1** and the upfield shifted position of the C5 resonance for **4** are helpful in confirming those structures as well.[32] Trimethylsilylation of **1–3** prior to electron-impact mass spectrometry led to di- and trisilylation as evidenced by the observation of the corresponding molecular ion and M − 15 peaks (Experimental Section). Evidently the molecular ion of **4** loses iodine too rapidly for the analogous ions to be observed in that case.

Antitumor Properties. Of the four 5-halo-cCMPs, only **1** shows significant activity (Table III). This activity occurs at very low concentration levels although **1** has an ID_{50} about 100X greater than those of the parent nucleoside, the 5′-monophosphate, and the reference compound aracytidine, also tested. Nonetheless, **1** is highly cytotoxic and merits in vivo study. The parent unsubstituted compound, cCMP, was noncytotoxic. The mode of action of **1** of course is not defined. It may indeed pass the cell membrane and possess cytotoxicity of its own. Alternatively, in line with the rationale developed in the introduction, intracellular **1** may be hydrolyzed to its 5′-monophosphate and in that form act as a thymidylate synthetase inhibitor.[15] The reduced activity of **1** relative to the nucleoside and 5′-monophosphate could result from an inefficiency in its hydrolysis since pyrimidine cyclic 3′,5′-monophosphates are generally relatively less reactive than purine analogues toward cAMP and cGMP phosphodiesterases.[18,33] Diester **1** is a potential target for the recently discovered pyrimidine phosphodiesterases.[34] The presence of a cCMP-specific phosphodiesterase in leukemia L1210 cell extracts has been claimed.[35] However, nothing about its presence and activity in P388 cells is known. Of course **1** and 5-F-5′-CMP may simply be prodrug forms of 5-fluorocytidine and release the latter outside the cell.

The poor activity of the cAMP analogues used for comparison suggests that **1** does not exert its cytotoxicity merely by mimicking cAMP activity. Thus, 8-*p*-chlorophenylthio-cAMP is a more potent inducer of protein kinase II activity in Reuber H35 rat hepatoma cells than is N^6,$O^{2'}$-dibutyryl-cAMP.[36] Yet 8-*p*-chlorophenylthio-cAMP has low cytotoxicity toward L1210 and P388 cells (Table III).

Antiviral Tests. Again **1** has antiviral activity (virus rating) much greater than **2–4**; and against the DNA viruses VV, HSV-1, and HSV-2 it displays moderate activity (Table IV). However, toward the RNA viruses, Para 3, VSV, Cox B1, and Reo 3, the activity of **1** is negligible. 5-F-5′-CMP and the 5-fluorocytidine hydrochloride show marked activity toward the three DNA viruses and moderate to marked activity against Para 3. Since 5-fluorocytidine is known to be incorporated into the tRNA of certain cells,[16] it perhaps is surprising that **1** is relatively ineffective against the RNA viruses. The contrastingly high activity of 5-F-5′-CMP, however, is worthy of note. Again, the cAMP analogues are relatively inactive. Reference compound aracytidine is highly active as expected, while cCMP itself is relatively inactive.

On the basis of VRs alone, the nucleoside and 5′-monophosphate appear to have an advantage over **1**. However, comparison of the ED_{50} values (Table V) with the concentrations at which slight toxicity is first noted for the above three 5-fluoro compounds (cytotoxicities of Table IV) suggests a potentially greater therapeutic index for **1** over the other two. Thus **1** shows trace toxicity at 400-fold higher concentration than do 5-F-5′-CMP and 5-fluorocytidine hydrochloride. Yet the ED_{50}s against VV and HSV-1 are only 10–100 times greater for **1** than for the other two compounds. This apparent advantage of the cyclic diester is greater with respect to the 5′-monophosphate than it is by comparison to the nucleoside. It appears from these results that in vivo antiviral tests are in order, especially with HSV-2, for which moderate to

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Table IV. In Vitro Antiviral Properties of 1–4 and Comparison Compounds in Vero Cells

compd	cytotoxicity,[d] mmol/L	VR[a]						
		VV[c]	HSV-1	HSV-2	Para 3	VSV	Cox B1	Reo 3
5-fluorocytosine	5 (trace)	0	0	0.5	0.4	*b*	*b*	*b*
5-fluorocytidine hydrochloride	5×10^{-4} (trace) 0.5 (extensive)	1.3	1.0	2.1	0.8	*b*	*b*	*b*
5-F-5'-CMP	5×10^{-4} (trace) 0.5 (extensive)	1.4	1.2	2.0	1.0	*b*	*b*	*b*
1	0.2 (trace) 0.5 (extensive)	0.6	0.6	0.9	0.1	0.2	0	0
2	5 (extensive)	0	0	0.1	0	0	0	0
3	2 (trace) 5 (extensive)	0.1	0.1	0.2	0	0	0	0
4	(2 trace) 5 (extensive)	0	0	0	0	0.2	0	0
8-Cl-cAMP	0.05 (extensive)	0.2	0	0.2	0.2	0	0	0
8-*p*-chlorophenylthio-cAMP	0.02 (extensive)	0	0	0.4	0.2	0	0	0
8-*p*-bromophenylthio-cAMP	0.02 (trace) 0.05 (extensive)	0	0	0.3	0	0.1	0	0
aracytidine	5×10^{-4} (trace)	2.8	2.7	2.7	0	*b*	*b*	*b*
cCMP	none	0	0	0.2	0	*b*	*b*	*b*

[a] Virus rating. Average of duplicate determinations. [b] Not determined. [c] Virus designations given in the Experimental Section. [d] Single determinations. Trace: rounding up or alteration of morphology affecting usually <10% of cells. Extensive: same effects on 25% or more of cells.

Table V. ED_{50} of 1–4 and Comparison Compounds against Selected Viruses

compd	ED_{50},[a] mmol/L						
	VV[c]	HSV-1	HSV-2	Para 3	VSV	Cox B1	Reo 3
5-fluorocytosine	>5	>5	>5	>5	*b*	*b*	*b*
5-fluorocytidine hydrochloride	5×10^{-3}	2×10^{-2}	$<5 \times 10^{-4}$	1.3×10^{-1}	*b*	*b*	*b*
5-F-5'-CMP	9×10^{-3}	2×10^{-2}	$<5 \times 10^{-4}$	2×10^{-2}	*b*	*b*	*b*
1	2.9×10^{-1}	2.2×10^{-1}	9.2×10^{-2}	>5	5	>5	>5
2	>5	>5	>5	>5	>5	>5	>5
3	5	>5	5	>5	>5	>5	>5
4	>5	>5	>5	>5	>5	>5	>5
8-Cl-cAMP	3.2×10^{-2}	>5	>5	>5	>5	>5	>5
8-*p*-chlorophenylthio-cAMP	>5	>5	>5	>5	>5	>5	>5
8-*p*-bromophenylthio-cAMP	>5	>5	>5	>5	>5	>5	>5
aracytidine	3.4×10^{-4}	3.4×10^{-4}	1.1×10^{-3}	$>5 \times 10^{-1}$	*b*	*b*	*b*
cCMP	>5	>5	>5	>5	*b*	*b*	*b*

[a] Determined as described in the Experimental Section. [b] Not determined. [c] Virus descriptions given in the Experimental Section.

marked activity was found for all four 5-fluoro compounds. 5-Fluorocytosine shows moderate antiviral activity, and the ED_{50}/cytotoxicity comparison suggests potential selectivity in its action as well.

Protein Kinase Activity. Preliminary results showed 1–4 to interact with protein kinase type I (rabbit skeletal muscle) with K_a (concentration for half-maximal activation) values ranging 0.22–3.7 μM. (K_a for cAMP, 30 nM.) Apparently, 1–4, having pyrimidine rather than purine bases, are not strong activators of the kinase. Details of these experiments will be reported elsewhere.

Experimental Section

Chromatography. Precoated TLC plates (Kieselgel 60 F_{254}, 0.2 mm × 20 cm × 20 cm, Merck, Darmstadt, FRG) were used to follow the reactions and check the purity of the products. Solvent systems (v/v) for silical gel TLC were as follows: (1) isobutyric acid:25% ammonium hydroxide:water = 66:1:33 and (2) 2-propanol:25% ammonium hydroxide:water = 7:1:2 with added H_3BO_3. DEAE-Sephadex A-25 used for anion-exchange column chromatography was purchased from Pharmacia Fine Chemicals, Sweden.

Spectroscopy and Analyses. Proton spectra of 1–4 were recorded in D_2O solvent with a Varian XL-100 FT NMR system operating at 100.1 MHz with dioxane (δ 3.70) for internal reference. A proton spectrum of 3 was also taken in Me_2SO-d_6 at 300.3 MHz (Varian SC-300). Carbon-13 spectra were acquired in D_2O solvent with a Varian XL-100/15 disk-augmented FT NMR system operating at 25.2 MHz. Dioxane served as internal reference (67.71 ppm relative to Me_4Si). Chemical shifts (δ) are in ppm and coupling constants (J) in hertz. Mass spectral measurements were carried out on an AEI MS-902 double-focusing instrument (70 eV) at an ion source temperature of 200 °C with direct probe sample introduction. The compounds 1–4 were silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in dry pyridine for 15 min at 150 °C before MS analysis. UV spectra were recorded at three different pH levels with a Varian Cary 17D UV–vis spectrophotometer system (pH 2, 10^{-2} M HCl; pH 7, 0.02 M KH_2PO_4/0.02 M K_2HPO_4; pH 11, 10^{-3} M NaOH). Quantitative elemental analyses were carried out by Galbraith Laboratories, Knoxville, TN, and at the Hungarian Academy of Sciences.

Cytidine cyclic 3',5'-monophosphate ammonium salt was synthesized from 5'-CMP·2Na (Sigma Chemical Co.) according to published procedures.[27] 5-Fluorocytidine hydrochloride was prepared according to a new synthetic route, which will be published elsewhere.

5-Fluorocytidine 5'-Monophosphate Diammonium Salt. 5-Fluorocytidine hydrochloride (0.527 g, 1.77 mmol) was dissolved in 4.4 mL of dry triethyl phosphate at 140–150 °C as quickly as possible. (The solution became pale red.) The solution was cooled to 0 °C with ice–water, and distilled phosphorus oxychloride (0.32 mL, 3.54 mmol) was added. After 5 h at 0 °C, the solution was poured into 20 mL of ice–water, and then a 2 M sodium hydroxide solution (ca. 8 mL) was added to adjust the pH to 7. After standing overnight at ~0 °C, this solution was applied to a 4.7 × 90 cm DEAE-Sephadex A-25 (HCO_3^-) column, washed with water (2 L), and eluted with a linear gradient of water (3 L) and 1.5 M ammonium hydrogen carbonate (3 L). The flow rate was 20 mL/5 min per fraction. The main product appeared in fractions 130–136. After repeating this purification procedure, 5-fluorocytidine 5'-monophosphate diammonium salt (0.324 g, 0.85 mmol, 48%) was isolated.

Relevant ^1H NMR data (D_2O): δ 8.15 (d, J_{FH} = 5.8 Hz, 1 H, H6), 6.03 (s, 1 H, H1'). ^{13}C NMR (D_2O): δ 159.0 (d, J_{CF} = 14.6 Hz, C4), 156.4 (C2), 126.4 (d, J_{CF} = 33.0 Hz, C6), 90.5 (C1'), 83.6

(d, J_{CP} = 8.5 Hz, C4'), 75.1 (C3'), 70.1 (C2'), 64.6 (d, J_{CP} = 4.9 Hz, C5'). Anal. ($C_9H_{19}FN_5O_8P$) N, P, F. R_f (system 1) 0.28, (system 2) 0.16.

5-Fluorocytidine Cyclic 3',5'-Monophosphate Ammonium Salt (1). 5-Fluorocytidine 5'-monophosphate diammonium salt (0.30 g, 0.80 mmol) and *N,N'*-dicyclohexyl-4-morpholinecarboxamidine (0.234 g, 0.80 mmol) were dissolved in 120 mL of dry refluxing pyridine. (A small insoluble part was filtered off.) To this hot solution were added quickly triphenylphosphine (1.05 g, 4.0 mmol) and 2,2'-dipyridyl disulfide (0.881 g, 4.0 mmol) in 40 mL of dry pyridine. After 3 h at reflux temperature, the solution was evaporated to dryness, and 100 mL of dichloromethane and 100 mL of water were added. After separation the aqueous solutions was concentrated and then applied to a 2.7 × 60 cm DEAE-Sephadex A-25 (HCO_3^-) column. Elution was carried out with a linear gradient of water (1.5 L) and 0.75 M ammonium bicarbonate solution (1.5 L) at a flow rate of 20 mL/10 min per fraction. The desired product (0.074 g, 0.22 mmol, 27%) was isolated by evaporation of fractions 61–66. UV: λ_{max} 286, 211, λ_{min} 249 (pH 2); λ_{max} 279, 237, λ_{min} 259 (pH 7); λ_{max} 279, 237, λ_{min} 258 nm (pH 11). EI-MS: *m/e* (relative intensity) 467, M^+ + 2 Me_3Si (2); 452, M^+ + 2 Me_3Si −15 (27); 539, M^+ + 3 Me_3Si (8); 524, M^+ + 3 Me_3Si −15 (100). Relevant ^1H NMR data (D_2O): δ 7.86 (d, J_{HF} = 5.8 Hz, 1 H, H6), 5.90 (s, 1 H, H1').

5-Chlorocytidine Cyclic 3',5'-Monophosphate Ammonium Salt (2). Cytidine 3',5'-cyclic monophoshate (0.031 g, 0.10 mmol), as the free acid, dried over P_2O_5 in vacuo, and *N,N'*-dicyclohexyl-4-morpholinecarboxamidine (0.029 g, 0.10 mmol) were dissolved in 1 mL of dry dimethylformamide at 115–120 °C. To this solution was added *N*-chlorosuccinimide (0.026 g, 0.2 mmol). After 1 h at 115–120 °C, 1 mL of ethanol was added, and the crude product (0.038 g) was precipitated with ether. This was dissolved in water, the pH adjusted to 7 with 1 M NaOH, and the resulting solution applied to a 2.7 × 25 cm DEAE-Sephadex A-25 (HCO_3^-) column. The column was washed with water (100 mL) and then eluted with a linear gradient of water (1 L) and 1 M NH_4HCO_3 (1 L) at a flow rate of 18 mL/12 min per fraction. Salt **2** (0.012 g, 0.030 mmol, 34%) was isolated by evaporation of fractions 31–32. EI-MS: *m/e* (relative intensity) 483, M^+ + 2 Me_3Si (1): 468, M^+ + 2 Me_3Si −15 (100): 555, M^+ + 3 Me_3Si (0.4), 540, M^+ + 3 Me_3Si −15 (1.7). Relevant ^1H NMR data (D_2O): δ 7.98 (s, 1 H, H6), 6.00 (s, 1 H, H1').

5-Bromocytidine Cyclic 3',5'-Monophosphate Ammonium Salt (3). A mixture of cytidine 3',5'-cyclic monophosphate ammonium salt (0.122 g, 0.37 mmol), *N,N'*-dicyclohexyl-4-morpholinecarboxamidine (0.111 g, 0.37 mmol), and *N*-bromosuccinimide (0.132 g, 0.71 mmol) in 3.0 mL of glacial acetic acid was stirred at room temperature for 20 min. (After ca. 10 min the mixture became a yellow solution). The reaction mixture was evaporated to dryness, and the solid residue was washed with acetone and then with diethyl ether. This crude product was purified on a 2.7 × 65 cm DEAE-Sephadex A-25 (HCO_3^-) column. The column was washed with water (100 mL) and then eluted with a linear gradient of water (2 L) and 1.0 M ammonium bicarbonate solution (2 L) at a flow rate of 20 mL/10 min per fraction. In all, 0.037 g (0.09 mmol, 24%) of 5-bromocytidine 3',5'-cyclic monophosphate ammonium salt (**3**) was isolated on evaporation of fractions 73–76. EI-MS: *m/e* (relative intensity) 527, M^+ + 2 Me_3Si (0.8); 512, M^+ + 2 Me_3Si −15 (32); 599, M^+ + 3 Me_3Si (2); 584, M^+ + 3 Me_3Si −15 (100). ^1H NMR data (Me_2SO-d_6): δ 7.79 (s, 1 H, H6), 5.58 (s, 1 H, H1'), 4.19–4.11 (m, 2 H, H3', H2'), 4.06 (m, 1 H, H5'), 3.87 (m, 1 H, H5''), 3.38 (m, 1 H, H4').

5-Iodocytidine Cyclic 3',5'-Monophosphate Ammonium Salt (4). A mixture of cytidine 3',5'-cyclic monophosphate ammonium salt (0.322 g, 1.0 mmol), iodic acid (0.200 g, 1.14 mmol), and iodine (0.166 g, 0.65 mmol) in 4.4 mL of acetic acid, 1.1 mL of carbon tetrachloride, and 3.4 mL of water was stirred at 45–50 °C for 4 h and then overnight (18 h) at room temperature. The solution was evaporated to dryness, and the residue was washed with acetone and then with diethyl ether. This solid crude product was then dissolved in water, and the solution was applied to a DEAE-Sephadex A-25 (HCO_3^-) column (2.7 × 60 cm) eluted with a linear gradient of water (2 L) and 0.75 M ammonium bicarbonate solution (2 L) at a flow rate of 20 mL/10 min per fraction. The major product appeared in fractions 78–82, which on evaporation yielded 0.122 g (0.27 mmol, 27%) of 5-iodocytidine 3',5'-cyclic monophosphate ammonium salt (**4**). Relevant ^1H NMR data (D_2O): δ 7.91 (s, 1 H, H6), 5.73 (s, 1 H, H1').

Antitumor Evaluation. Compounds were evaluated for their ability to inhibit growth of murine leukemia L1210 and lymphoid neoplasm P388 (American Type Culture Collection, Rockville, MD) maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY) and 20 mM Hepes buffer. For growth experiments, cells were adjusted to 1 × 10^5 cells/mL and distributed into 13 × 100 mm culture tubes (1 mL/tube). Test compounds were dissolved in growth medium, sterilized by passage through a 0.22-μm membrane filter, and added to tubes of cells (1 mL/tube). Compounds were tested in duplicate at log concentrations ranging from 1 × 10^{-9} M to 1 × 10^{-4} M. Following 48 h of incubation at 37 °C, cell counts were determined with a Coulter Model ZF cell counter. Cell growth in the presence of test compounds was expressed as a percentage of growth in untreated control wells, and the concentration of compound producing 50% inhibition of cell growth was determined (ID_{50}).

Antiviral Evaluation. Test compounds were evaluated for their ability to inhibit virus-induced cytopathic effect (CPE) produced by herpes simplex virus type 1 strain KOS (HSV-1), herpes simplex virus type 2 strain MS (HSV-2), vaccinia virus strain Elstree (VV), parainfluenza virus type 3 strain C 243 (Para 3), vesicular stomatitis virus strain Indiana (VSV), coxsackie B1 strain HA 201468 (Cox B1), and reovirus type 3 strain Abney (Reo 3) in African green monkey kidney (Vero) cells (American Type Culture Collection, Rockville, MD). Vero cells were maintained in antibiotic free Eagle minimum essential medium (EMEM) with Earle's salts supplemented with 10% heat-inactivated newborn bovine serum (Grand Island Biological Co., Grand Island, NY). For antiviral experiments, cells were inoculated into 96-well tissue culture plates (Corning Glassworks, Corning, NY) at a concentration of 4 × 10^4 cells/0.2 mL per well and cultured for 24 h at 37 °C in 5% CO_2 to confluency.

Monolayers were inoculated with a predetermined number of $TCID_{50}$ (50% tissue culture infective dose) units of virus that will produce complete destruction of the cell monolayer in 72 h. The number of $TCID_{50}$ units in 0.1 mL/well were as follows: HSV-1, 60; HSV-2, 100; VV, 200; Para 3, 60; VSV, 2; Cox B1, 200; and Reo 3, 470. After 30 min of adsorption at 37 °C, test compounds were added (0.1 mL/well) in seven 0.5 log concentrations ranging from 1 × 10^{-5} to 1 × 10^{-2} M resulting in final well concentrations of 5 × 10^{-6} to 5 × 10^{-3} M. At each concentration, duplicate wells were used for evaluation of antiviral activity and single uninfected wells for cytotoxicity evaluation.

The degree of inhibition of viral-induced CPE and compound cytotoxicity were observed microscopically after 72 h of incubation at 37 °C in 5% CO_2. CPE was scored numerically from 0 (normal control cells) to 4 (100% cell destruction as in virus controls) to calculate a virus rating (VR) as previously described.[37] Significance of VR values have been assigned as follows: <0.5, inactive or slight activity; 0.5–0.9, moderate activity; >1.0, marked activity. The dose of test compounds that inhibits viral CPE by 50% (ED_{50}) were scored microscopically during VR readings and double-checked from the crystal-violet-stained 96-well plates.

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Registry No. 1, 94427-51-7; 1 (free acid), 89469-87-4; **2**, 94427-52-8; **2** (free acid), 94427-53-9; **3**, 94427-54-0; **3** (free acid), 72645-67-1; **4**, 94427-55-1; **4** (free acid), 94427-56-2; cytidine cyclic 3',5'-monophosphate ammonium salt, 55727-00-9; 5-fluorocytidine 5'-monophosphate diammonium salt, 94427-57-3; 5-fluorocytidine hydrochloride, 4590-33-4; cytidine 3',5'-cyclic monophosphate, 3616-08-8.

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