123–124 °C (from ethyl acetate); ^{1}H NMR (Me_2SO-d_6) δ 6.72 (s, 1, H-4), 3.85 [s, 2, $CH_2N(CH_2CH_2Cl)_2$], 3.67 (t, 4, CH_2Cl), 2.90 [t, 4, $N(CH_2CH_2Cl)_2$]. Anal. ($C_9H_{14}N_4OCl_2$) C, H, N.

Ethyl 1-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-3-[[bis(2-chloroethyl)amino]methyl]pyrazole-5-carboxylate (12a). A solution of **5a** (0.56 g, 1 mmol) in dry acetone (20 mL) was treated with bis(2-chloroethyl)amine (0.28 g, 2 mmol) recently liberated from the hydrochloride, and the whole was allowed to stand at room temperature for 24 h. The solvent was removed and the residue extracted with ether. The ether was evaporated to give a syrup, which was purified by preparative TLC using ethyl acetate–petroleum ether (1:1). Elution of the major band gave 0.44 g (70%) of **12a** as a homogeneous syrup: $[\alpha]^{25}_D$ −6° (*c* 0.52, chloroform); ^{1}H NMR ($CDCl_3$) δ 6.96 (s, 1, H-4), 6.55 (d, 1, $J_{1',2'}$ = 9 Hz, H-1'), 3.85 [s, 2, $CH_2N(CH_2CH_2Cl)_2$], 3.60 (t, 4, CH_2Cl), 2.98 [t, 4, $N(CH_2CH_2Cl)_2$]. Anal. ($C_{25}H_{35}N_3O_{11}Cl_2$) C, H, N.

Ethyl 1-(2,3,4-Tri-*O*-acetyl-β-D-ribopyranosyl)-3-[[bis(2-chloroethyl)amino]methyl]pyrazole-5-carboxylate (12c). By a method identical with that described for **12a**, **5c** (0.30 g, 0.75 mmol) and bis(2-chloroethyl)amine (0.21 g, 1.50 mmol) led to **12c** (0.28 g, 82%) as a homogeneous syrup: $[\alpha]^{25}_D$ −4° (*c* 0.5, chloroform); ^{1}H NMR ($CDCl_3$) δ 6.96 (s, 1, H-4), 6.75 (d, 1, $J_{1',2'}$ = 9 Hz, H-1'), 3.85 [s, 2, $CH_2N(CH_2CH_2Cl)_2$], 3.60 (t, 4, CH_2Cl), 3.03 [t, 4, $N(CH_2CH_2Cl)_2$]. Anal. ($C_{22}H_{31}N_3O_9Cl_2$) C, H, N.

Ethyl 1-(2,3,5-Tri-*O*-acetyl-β-D-ribofuranosyl)-3-[[bis(2-chloroethyl)amino]methyl]pyrazole-5-carboxylate (12d). By a procedure identical with that described for **12a** and **12c**, **5d** (0.49 g, 1 mmol) and bis(2-chloroethyl)amine (0.28 g, 2 mmol) gave **12d** (0.44 g, 80%) as a homogeneous syrup: $[\alpha]^{25}_D$ −32° (*c* 0.65, chloroform); ^{1}H NMR ($CDCl_3$) δ 6.96 (s, 1, H-4), 7.04 (d, 2, $J_{1',2'}$ = 2 Hz), 3.90 [s, 2, $CH_2N(CH_2CH_2Cl)_2$], 3.62 (t, 4, CH_2Cl), 3.10 [t, 4, $N(CH_2CH_2Cl)_2$]. Anal. ($C_{22}H_{31}N_3O_9Cl_2$) C, H, N.

1-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-3-[[bis(2-chloroethyl)amino]methyl]pyrazole-5-carboxamide (13a). A solution of **7a** (0.51 g, 1 mmol) in dry acetone (20 mL) was treated with bis(2-chloroethyl)amine (0.28 g, 2 mmol) freshly liberated from its hydrochloride and allowed to stand at room temperature for 24 h. The solvent was removed, the residue was treated with ethyl acetate and filtered, and the filtrate was evaporated to give a syrup, which was purified by TLC using ethyl acetate–petroleum ether (9:1). Elution of the major band afforded **13a** (0.39 g, 65%) as a homogeneous syrup: $[\alpha]^{25}_D$ −17.5° (*c* 0.5, chloroform); ^{1}H NMR ($CDCl_3$) δ 6.83 (s, 1, H-4), 6.68 (d, 1, $J_{1',2'}$ = 9 Hz, H-1'), 3.88 [s, 2, $CH_2N(CH_2CH_2Cl)_2$], 3.55 (t, 4, CH_2Cl), 3.10 [t, 4, $N(CH_2CH_2Cl)_2$]. Anal. ($C_{23}H_{32}N_4O_{10}Cl_2$) C, H, N.

1-(2,3,4-Tri-*O*-acetyl-β-D-ribopyranosyl)-3-[[bis(2-chloroethyl)amino]methyl]pyrazole-5-carboxamide (13c). By a procedure identical with that described for **13a**, **7c** (0.17 g, 0.37 mmol) and bis(2-chloroethyl)amine (0.11 g, 0.75 mmol) led to **13c** (0.10 g, 50%) as a homogeneous syrup: $[\alpha]^{25}_D$ −12.5° (*c* 0.5, chloroform); ^{1}H NMR ($CDCl_3$) δ 6.86 (s, 1, H-4), 6.64 (d, 1, $J_{1',2'}$ = 9 Hz, H-1'), 3.98 [s, 2, $CH_2N(CH_2CH_2Cl)_2$], 3.68 (t, 4, CH_2Cl), 3.05 [t, 4, $N(CH_2CH_2Cl)_2$]. Anal. ($C_{20}H_{28}N_4O_8Cl_2$) C, H, N.

1-(2,3,5-Tri-*O*-acetyl-β-D-ribofuranosyl)-3-[[bis(2-chloroethyl)amino]methyl]pyrazole-5-carboxamide (13d). By a procedure identical with that described for **13a** and **13c**, **7d** (0.17 g, 0.37 mmol) and bis(2-chloroethyl)amine (0.11 g, 0.75 mmol) led to **13d** (0.12 g, 60%) as a homogeneous syrup: $[\alpha]^{25}_D$ −10° (*c* 0.6, chloroform); ^{1}H NMR ($CDCl_3$) δ 6.85 (s, 1, H-4), 7.02 (d, 1, $J_{1',2'}$ = 2 Hz, H-1'), 3.98 [s, 2, $CH_2N(CH_2CH_2Cl)_2$], 3.70 (t, 4, CH_2Cl), 3.09 [t, 4, $N(CH_2CH_2Cl)_2$]. Anal. ($C_{20}H_{28}N_4O_8Cl_2$·1AcOEt) C, H, N.

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References and Notes

(1) F. G. de las Heras, R. Alonso, and G. Alonso, *J. Med. Chem.*, **22**, 496 (1979).
(2) A. Contreras, R. M. Sanchez-Perez, and G. Alonso, *Cancer Chemother. Pharmacol.*, **1**, 243 (1978).
(3) E. Mugnaini and P. Grunnanger, *Atti. Accad. Naz. Lincei, Cl. Sci. Fis. Mat. Nat., Rend.*, **14**, 958 (1953); *Chem. Abstr.*, **48**, 2684 (1954).
(4) G. R. Revankar and L. B. Townsend, *J. Heterocycl. Chem.*, **7**, 1329 (1970).
(5) L. B. Townsend, *Synth. Proced. Nucleic Acid Chem.*, **2**, 297 (1973).
(6) L. B. Townsend, *Synth. Proced. Nucleic Acid Chem.*, **2**, 333 (1973).
(7) S. R. Naik, J. T. Witkowski, and R. K. Robins, *J. Heterocycl. Chem.*, 11, 57 (1974).
(8) J. A. Montgomery in "Antineoplastic and Immunosuppressive Agents", A. C. Sartorelli and D. G. Johns, Eds., Part I, Springer-Verlag, Berlin, Heidelberg, and New York, 1974, p 98.
(9) W. C. J. Ross in ref 8, p 33.
(10) H. C. Koppel, R. H. Springer, R. K. Robins, F. H. Schneider, and C. C. Cheng, *J. Org. Chem.*, **27**, 2173 (1962).
(11) J. Farkas and F. Sorm, *Collect. Czech. Chem. Commun.*, **26**, 893 (1961).
(12) J. Farkas and F. Sorm, *Collect. Czech. Chem. Commun.*, **34**, 1696 (1969).
(13) T. I. Geran, N. H. Greenberg, M. M. Mc Donald, A. M. Schumacher, and B. J. Abbot, *Cancer Chemother. Rep., Part 3*, **3**, 432 (1959).
(14) H. Eagle, *Science*, **130**, 432 (1959).
(15) V. I. Oyama and H. Eagle, *Proc. Soc. Exp. Biol. Med.*, **91**, 305 (1956).

2′-*O*-Acyl-6-thioinosine Cyclic 3′,5′-Phosphates as Prodrugs of Thioinosinic Acid[1]

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A series of 2′-*O*-acyl derivatives of 6-thioinosine cyclic 3′,5′-phosphate (6-HS-cRMP) were prepared and examined for their cytotoxic effects on S49 mouse lymphoma cells which were deficient in hypoxanthine–guanine phosphoribosyltransferase (HGPRTase). Cytotoxicity increased with the lipophilicity of the acyl group to a lowest EC_{50} of 65 μM for the 2′-*O*-palmityl derivative. Addition of a mutation in the gene for cAMP-dependent protein kinase to the HGPRTase-deficient cell line confers resistance to 2′-*O*-butyryl-cAMP but not to 2′-*O*-butyryl-6-HS-cRMP, indicating that the latter does not exert its toxic effect via activation of protein kinase. The time course of cell kill by 2′-*O*-palmityl-6-HS-cRMP resembled that of 6-mercaptopurine and not that of cyclic AMP in these cells. The data suggest that the intact cyclic nucleotides are penetrating the cells and being converted, by phosphodiesterase action and deacylation, to the first toxic metabolite of 6-mercaptopurine, thioinosinic acid.

All analogues of naturally occurring purine and pyrimidine bases and nucleosides currently in clinical use for cancer chemotherapy must be converted to their respective nucleotides to exert their cytostatic or cytotoxic effects.

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Furthermore, almost all other such analogues which have shown antineoplastic activity must be similarly transformed. The "fraudulent" nucleotides which the cell synthesizes from the purines, pyrimidines, or nucleosides act at various sites: enzymes of de novo purine and pyrimidine nucleotide synthesis may be inhibited by nucleoside 5′-phosphate analogues; other nucleotide analogues may act by metabolism to the 5′-triphosphates, which may be incorporated into DNA or RNA, or may inhibit nucleic acid polymerases.

The antileukemia drug 6-mercaptopurine (6-MP) is among the most interesting of all these analogues due to the multiplicity of its metabolic transformations and sites of action. First, and most importantly, it must be converted by hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8, HGPRTase) to 6-thioinosinic acid (6-HS-RMP) in order to exert any cytotoxic effects. This nucleotide inhibits IMP dehydrogenase and adenylosuccinate synthetase, and, more importantly, 6-HS-RMP (and its *S*-methyl derivative) is a potent allosteric feedback inhibitor of the first committed step in purine nucleotide synthesis, phosphoribosyl pyrophosphate:glutamine amidotransferase.[2] Furthermore, the delayed cytotoxicity of 6-MP is a result of its incorporation into DNA as a thioguanine nucleotide, a process mediated by several enzymes.[3,4]

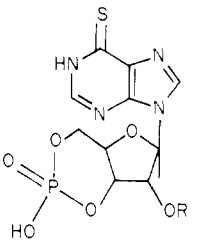
The lack of HGPRTase or a markedly reduced affinity of that enzyme for 6-MP renders leukemia cells resistant to 6-MP.[5-7] Direct administration of 6-HS-RMP could overcome this resistance, but 5′-nucleotides do not penetrate cell membranes. The development of new anticancer drugs based on inhibitors of nucleotide synthesis is hindered by this same problem. Even if a nucleotide were found that could inhibit nucleotide biosynthetic enzymes, it must currently be administered as the base (which must be intracellularly phosphoribosylated) or the nucleoside (which must be phosphorylated) to be able to penetrate cell membranes. Thus, the design of such compounds is restricted to close structural analogues of natural compounds.

We are interested in the design and evaluation of prodrugs of preformed nucleotides for two reasons: (a) such compounds might bypass resistance mechanisms to known anticancer drugs, such as 6-MP, and (b) they may provide directions for useful modifications to new *nucleotide* analogues (whose base or nucleoside would not be expected to be intracellularly activated) which would be clinically efficatious.

In this paper, we describe two nucleotide modifications: (a) removal of one of the phosphate charges by cyclization of the 5′-phosphate to the 3′-OH and (b) addition of a removable lipophilic group to the compounds by acylation of the 2′-OH with carboxylic acids of various chain lengths. The critical event in intracellular "activation" of these compounds is cleavage of the cyclic phosphate by cyclic nucleotide phosphodiesterase to regenerate the active 5′-phosphate; the 2′-*O*-acyl group, if not enzymatically removed before cyclic phosphate cleavage, would certainly be chemically labile after liberation of the 3′-OH.[8]

The nucleotide selected for modifications in this study was 6-thioinosine cyclic 3′,5′-phosphate (6-HS-cRMP) because it is known to be a phosphodiesterase substrate[9] and because 6-MP-resistant strains of the S49 lymphoma cells used in these studies are readily available. These are very deficient in HGPRTase. Additionally, these cells have a well-documented response to adenosine cyclic 3′,5′-phosphate (cAMP), and this allowed an evaluation of whether the cytotoxic action of the cyclic nucleotides was due to their acting as cAMP analogues or thioinosinic acid prodrugs.

Table I. Cytotoxicity of 2′-*O*-Acyl-6-thioinosine Cyclic 3′,5′-Phosphates



R	EC_{50}, μM[a] wild type	EC_{50}, μM[a] HGPRTase mutants[b]
H	200[c]	>1000 (2)
$COCH_3$	210, 220	655 ± 41 (3)
$CO(CH_2)_2CH_3$	137	426 ± 86 (4)
$COCH(CH_3)_2$	23	479 ± 174 (4)
$COC(CH_3)_3$	33	237 ± 100 (3)
$CO(CH_2)_4CH_3$	10, 20	210 ± 62 (4)
$CO(CH_2)_{10}CH_3$	10, 10	109 ± 71 (3)
$CO(CH_2)_{14}CH_3$	6	65 ± 12 (6)
$CO(CH_2)_{20}CH_3$		120, 190
controls		
isobutyric acid		NE at 1000[d]
hexanoic acid		>1000
docecanoic acid		NE at 100[d]
palmitic acid		500
6-MP	8	>1000
6-thioinosine	0.25	400

[a] The EC_{50} is the concentration giving 50% inhibition of growth as described under the Experimental Section. Values are expressed as the mean of all determinations plus or minus standard error (number of determinations). Individual results are given where only one or two determinations were made. [b] Selected for resistance to 1 μM 6-thioinosine (see Experimental Section). [c] Forty percent inhibition at this value. [d] No effect at this value.

Results and Discussion

Preparation of 2′-*O*-Acyl-6-thioinosine Cyclic 3′,5′-Phosphates. The synthesis of 6-HS-cRMP on a large scale was accomplished by direct sulfhydrolysis of adenosine cyclic 3′,5′-phosphate as previously described.[10,11] The 2′-OH was acylated with the carboxylic anhydrides in pyridine with 4-(dimethylamino)pyridine added to facilitate the reaction.[12] Some deacylation was observed when Dowex 50 (H^+) was used to regenerate the free acids of the acylated nucleotides; deacylation was also observed on prolonged storage of the compounds. Before the compounds were tested in cell culture, their purity was examined on reverse-phase LC using the eluants described under the Experimental Section. In all cases, 6-HS-cRMP was the *sole* impurity, normally comprising <5% of the product. Since the effect of this compound was minimal on the cells (see Table I) and since it is produced in any case by acyl cleavage in the media (see below), it was not deemed necessary to further purify the products. The ^1H NMR of all compounds showed an apparent singlet at δ 6.0 for the anomeric proton, indicating the cyclic 3′,5′-phosphate was intact.[9,13] A 1-ppm downfield shift (to about δ 5.0) was observed for the 2′ proton (which appeared as a doublet) in all acylated derivatives as compared to 6-HS-cRMP, indicating the position of acylation. All new derivatives gave a λ_{max} in alcohol of 321 nm, indicating that the mercaptopurine base was unchanged. Small samples of each compound were purified by preparative LC for elemental analysis. Despite several repeat determinations,

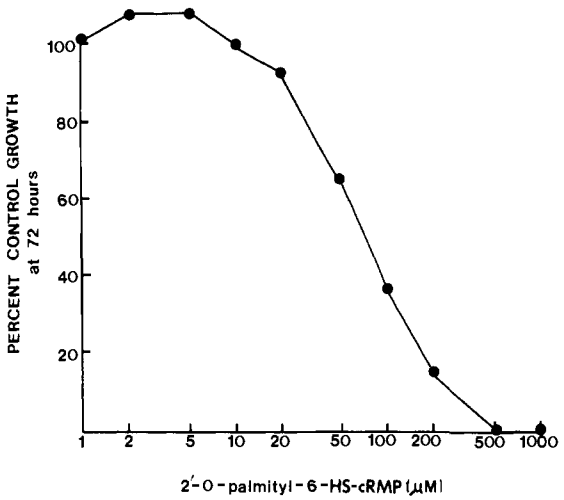


Figure 1. Cytotoxicity of 2′-*O*-palmityl-6-HS-cRMP on S49 cells deficient in hypoxanthine–guanine phosphoribosyltransferase activity.

values obtained for some samples were unpredictably variable.

Cytotoxicity of the Acylated 6-HS-cRMP Derivatives. The nucleotides were examined for their ability to inhibit the growth of S49 murine lymphoma cells in culture. In order to preclude the possibility of extracellular metabolites being the actual cytotoxic agents, the cells used for most of the assays were deficient in HGPRTase. The results are given in Table I. Cytotoxicity is expressed as EC_{50}, the concentration at which 50% inhibition of growth after 72 h of the test cultures compared to the control is observed. The values were obtained by plotting percent growth inhibition against the concentration of drug. A typical growth-inhibition curve, for 2′-*O*-palmityl-6-HS-cRMP, is shown in Figure 1.

Appropriate controls were examined to determine whether extracellular breakdown was responsible for the production of cytotoxic metabolites. The EC_{50} values of 6-mercaptopurine and thioinosine were over 400 μM in the HGPRTase-deficient mutants. Solubility limited the testing of some of the carboxylic acid controls, but all possessed EC_{50} values well above the 6-HS-cRMP derivative acylated with that acid. The EC_{50} values of mercaptopurine and thioinosine were below 10 μM with the wild-type cells.

As shown in Table I, simple removal of one negative charge from thioinosinic acid by formation of the cyclic phosphate has a minimal effect on apparent cell penetration, as judged by cytotoxicity. Montgomery originally prepared 6-HS-cRMP and found little activity in HGPRTase-deficient cells, presumably due to extracellular breakdown.[14] Nevertheless, other workers have found that this modification yields compounds active in tumor strains resistant to the parent nucleoside. These include 6-MeS-cRMP,[15-17] 6-HS-cRMP,[16-18] and 9-(β-D-arabinofuranosyl)adenine cyclic 3′,5′-phosphate.[18] Concentrations used in these experiments were relatively high, however.

Addition of lipophilicity to the prodrugs gives a marked enhancement in their cytotoxicity. A progressively increasing effect is observed as the number of carbons in the acid esterified to the 2′-hydroxyl is increased from 2 to 16, with the maximum effect being observed with 2′-*O*-palmityl-6-HS-cRMP (EC_{50} = 65 μM, or about 15% of the concentration of the parent unacylated compound). The weaker activity of the analogue with the C_{22} side chain may be due to its poor solubility or other physical factors.

Degradation of Drugs under Incubation Conditions. To test the effects of the media and horse serum on the modified nucleotides used in this study, 2′-*O*-palmityl-6-HS-cRMP was incubated with media and serum under conditions identical with the cytotoxicity studies, except that cells were excluded. After 3 days, 20% of the original acylated cyclic nucleotide remained unchanged. Approximately 40% of the original drug was found as 6-thioinosine, the product of deacylation, phosphodiesterase action, and dephosphorylation. The remainder of the drug was found as 6-HS-cRMP (30–40%) and 6-HS-RMP (10%). The 6-HS-cRMP peak was obscured by a component of the incubation mixture and, therefore, difficult to accurately determine. No 6-MP was found. It is unlikely, therefore, that the extracellular metabolites play an important role in the cytotoxicity of these compounds.

Effect of the Cyclic Nucleotides on cAMP-Resistant Cells. The S49 mouse lymphoma cells used in this study are well characterized with respect to their response to cAMP: they undergo growth arrest[19] and ultimately cytolysis[20] in the presence of exogenous N^6,2′-*O*-dibutyryl-cAMP and 8-Br-cAMP. This effect is thought to be mediated by a cAMP-dependent protein kinase.[21] A cell line deficient in protein kinase will not be killed by cAMP or an analogue acting as cAMP. Such a mutant which *also* lacked HGPRTase was examined for its susceptibility to 2′-*O*-butyryl-cAMP and to 2′-*O*-butyryl-6-HS-cRMP. The former compound showed little or no effect at 1 mM, whereas the latter possessed an EC_{50} of 250 μM. Conversely, the mutant lacking HGPRTase but containing cAMP-dependent protein kinase was completely killed by 1 mM 2′-*O*-butyryl-cAMP.

Substantial interest has arisen in the possibility of exploitation of the cell-growth modulating effect of cAMP itself in anticancer chemotherapy. Since 6-HS-cRMP can stimulate cAMP-dependent protein kinase,[9] it was necessary to show that these compounds were not acting as cAMP analogues via protein kinase. The inhibition of growth of several tumor cell lines by cAMP analogues has been demonstrated.[22,23] In our mutants, loss of protein kinase confers resistance to cAMP but not to the acylated 6-HS-cRMP derivatives. It therefore appears that the prodrugs are acting by conversion to thioinosinic acid.

Time Course of 6-HS-cRMP Toxicity vs. cAMP Toxicity. The cytotoxic effect of cAMP on S49 cells discussed above is not manifested until after the first 24 h following exposure to cAMP analogues.[20] Exposure of wild-type cells to 6-mercaptopurine, however, causes rapid inhibition of cell growth. The HGPRTase-deficient S49 mutants were given either N^6,2′-*O*-dibutyryl-cAMP (500 μM) plus 200 μM theophylline or 2′-*O*-palmityl-6-HS-cRMP (500 μM), and the number of cells surviving was counted after 20 and 49 h. As can be seen in Figure 2, the time courses of cell kill by these two agents are dramatically different. These data are also consistent with the interpretation that the mercaptopurine nucleotides are not acting as cAMP analogues via protein kinase but rather via conversion to thioinosinic acid.

Role of Phosphodiesterase. Since 6-HS-cRMP is a phosphodiesterase substrate,[9] it was of interest to determine whether phosphodiesterase action was rate limiting in the expression of the cytotoxic effects. When HGPRTase S49 cells were incubated with varying concentrations of 2′-*O*-palmityl-6-HS-cRMP in the presence of either 100 μM theophylline or 50 μM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolone, however, no protection of cell viability was observed. The effect of 2′-*O*-palmityl-6-HS-cRMP on a strain of cells which lacked HGPRTase,

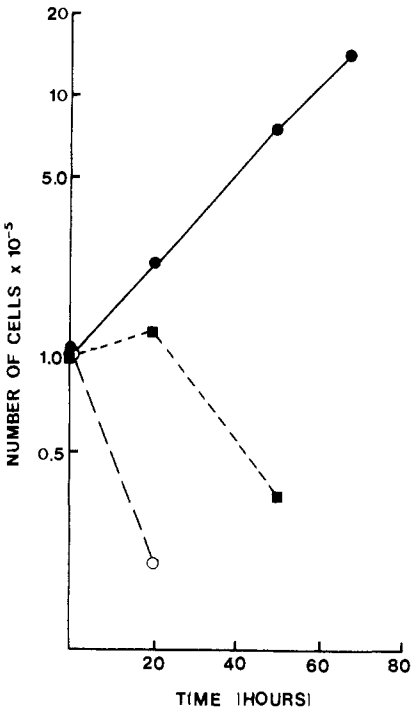


Figure 2. Comparison of time dependence of HGPRTase-deficient S49 cell kill by 0.5 mM 2′-*O*-palmityl-6-HS-cRMP (○) and 0.5 mM N^6,2′-*O*-dibutyryl-cAMP plus 0.2 mM theophylline (■) with growth in the absence of drug (●).

lacked protein kinase, and was very deficient in phosphodiesterase was examined as another probe of the role of phosphodiesterase. No difference, however, was seen in the growth inhibition of this strain by 2′-*O*-palmityl-6-HS-cRMP and the growth inhibition of a similar strain with normal phosphodiesterase levels. These data amplify an expected result in the use of nucleotides as chemotherapeutic agents: the rate-limiting step is most likely the penetration of the cell membrane by the nucleotide and not subsequent enzymatic processes.

It seems reasonable to conclude that (a) the acylated cyclic nucleotides are entering the cells intact, (b) they are not exerting their action by acting as cAMP analogues, and (c) that additional lipophilicity in the form of the 2′-*O*-acyl group facilitates the cell penetration. These results may be of use in the treatment of neoplasms which have become refractory to mercaptopurine due to modification of HGPRTase and in the utilization of nucleotides of various types directly as anticancer agents.

Experimental Section

6-HS-cRMP was prepared either by direct sulfhydrolysis of cAMP or sulfhydrolysis of N^6-methoxyadenosine cyclic 3′,5′-phosphate as previously described.[10,11] Evaporations were performed with a rotary evaporator under high vacuum with a bath temperature of ≤50 °C. Dodecanoic, palmitic, and docosanoic anhydride were prepared by the method of Bleyberg and Ullrich.[24] Hexanoic anhydride was purchased from ICN Life Sciences, Cleveland, Ohio, and other anhydrides were purchased from Aldrich Chemical Co., Milwaukee, Wis. Thin-layer chromatography was performed on EM silica gel 60 F-254 glass-backed plates, and the spots were visualized either by ultraviolet light or by iodine vapor. Liquid chromatography (LC) was conducted with an Altex Model 100 pump, operating at 2 mL/min, fitted with an Altex Model 153 detector monitoring absorbance at 320 nm, unless otherwise specified. An Altex 4.6 × 250 mm reverse-phase (Lichrosorb C_{18}) column was used for all assays. The retention time of 6-HS-cRMP was 5.4 min using 100:1 H_2O–AcOH as eluant.

Synthesis of Acylated 6-HS-cRMP Analogues with C_2–C_6 Acyl Groups. To a solution of 1.2 g (3.3 mmol) of 6-HS-cRMP·H_2O in 50 mL of *N*,*N*-dimethylformamide was added 25 mL of dry toluene. The bulk of the toluene was removed in vacuo, and to this solution was added 10 mmol of acid anhydride, 2 mL of pyridine, and 20 mg of 4-(dimethylamino)pyridine. The mixture was warmed on a steam bath until TLC or LC indicated that the reaction was complete, at which time 1 mL of water was added. After the mixture was left standing for 1 h at room temperature, the bulk of the solvent was removed in vacuo and 25 mL of water was added and removed in vacuo. The residue was dissolved in a minimum of water–methanol (1:1) and was eluted through a 40-mL column of Dowex-50-X8 (hydrogen form) until the eluant did not absorb at 320 nm. The bulk of the methanol in the eluant was removed in vacuo, and the remaining solution was lyophilized to yield the 2′-*O*-acylated derivatives.

Synthesis of Acylated 6-HS-cRMP Analogues with C_{12}–C_{22} Acyl Groups. To a solution of 1.2 g (3.3 mmol) of 6-HS-cRMP in 50 mL of *N*,*N*-dimethylformamide was added 25 mL of dry toluene. The bulk of the toluene was removed in vacuo, and to this solution was added 10 mmol of acid anhydride, 2 mL of pyridine, and 20 mg of 4-(dimethylamino)pyridine. The mixture was warmed on a steam bath until TLC or LC indicated that the reaction was complete (4–18 h), at which time 1 mL of water was added. After the mixture was left standing for 1 h at room temperature, the bulk of the solvent was removed in vacuo and 25 mL of water was twice added and then removed in vacuo. The residue was dissolved in a minimum of 1:1 methanol–chloroform, and this solution was eluted with the same solvent mixture through a 40-mL column of Dowex 50W-X8 (hydrogen form) until the eluant did not absorb at 320 nm. The collected eluant was evaporated in vacuo, and the residue was sonicated in 75 mL of hot hexane and filtered through Celite, which was washed with an additional 50 mL of hot hexane. The Celite and the gummy solid collected upon it were stirred in 75 mL of hot methanol–chloroform (1:1) and filtered, and the Celite was washed with 50 mL of additional hot methanol–chloroform. The filtrate was evaporated in vacuo, and the residue was triturated with 2-propanol–ethyl ether–hexane (1:10:10) to yield the 2′-*O*-acylated derivatives of 6-HS-cRMP as light yellow solids.

All products contained minor amounts (<5%) of 6-HS-cRMP (deacylation by Dowex 50) as the sole contaminate as judged by LC, except for the 2′-*O*-acetate, which contained about 20% starting material. Since the starting material did not interfere with the test results (see Table I), they were used as such in the cell culture experiments. For analysis, small samples were purified on an Altex Lichrosorb C_{18} 10 × 250 mm preparative LC column, using the solvent given below as eluants. For all new compounds, the following spectral data were obtained: NMR (Me_2SO-d_6) δ 8.2 (s, 1, purine), 8.5 (s, 1, purine), 6.0–6.1 (s, 1, H-1′), 5.6 (d, 1, H-2′); UV λ_{max} (MeOH) 321 nm.

The following compounds were obtained by these methods [2′-*O*-substituent, yield, LC eluant (H_2O–MeOH–AcOH, v/v), retention time in minutes]: $CO(CH_2)_2CH_3$, 83%, 80:20:1, 9.2 [Anal. ($C_{14}H_{17}N_4O_7PS\cdot H_2O$) C, H, N]; $COCH(CH_3)_2$, 90%, 80:20:1, 4.6 [Anal. ($C_{14}H_{17}N_4O_7PS\cdot H_2O$) C, H; N: calcd, 12.90; found, 11.32]; $COC(CH_3)_3$, 67%, 80:20:1, 9.9 [Anal. ($C_{15}H_{19}N_4O_7PS\cdot H_2O$) H, N; C: calcd, 40.18; found, 37.34]; $CO(CH_2)_4CH_3$, 91%, 80:20:1, 28.0 [Anal. ($C_{16}H_{21}N_4O_7PS\cdot H_2O$) C, N; H: calcd, 5.24; found, 4.63]; $CO(CH_2)_{10}CH_3$, 93%, 40:40:1, 5.4 [Anal. ($C_{22}H_{33}N_4O_7PS\cdot 1.5H_2O$) C, H; N: calcd, 10.08; found, 8.87]; $CO(CH_2)_{14}CH_3$, 95%, 30:70:1, 9.0 [Anal. ($C_{26}H_{41}N_4O_7PS\cdot H_2O$) C, H, N]; $CO(CH_2)_{20}CH_3$, 87%, 10:90:1, 3.0 [Anal. ($C_{32}H_{53}N_4O_7PS\cdot 0.75H_2O$) C, H, N].

Cultured Cells. The growth characteristics, methods for maintenance, and lymphocytic properties of the wild-type mouse T cell lymphoma cell line, S49, have been described previously.[25,26]

Growth-Rate Experiments. The experiments to determine the relative growth inhibitory and toxic effects of the 2′-*O*-acylated 6-HS-cRMP derivatives were conducted in Falcon multiwell (24 wells) tissue-culture plates. Selected agents in small volumes (5–50 μL) of 30 mM $NaHCO_3$ were pipetted under sterile conditions into each well, followed by the addition of 10^5 cells in 1.00 mL of complete Dulbecco's modified Eagles medium. To assure asynchronous and exponential growth, cells were previously grown for 24 h in a 75-cm^2 flask. Prior to use, cells were centrifuged and resuspended in old medium at a concentration of 10^5 cell/mL.

After 72 h growth at 37 °C, cells in each well were thoroughly mixed with a Pasteur pipet and counted in a Coulter counter

Model ZB1. After subtraction of the initial cell density from the final cell density, the number of cells in the wells containing drug was plotted as a fraction of the number of cells in wells lacking the drug. After 72 h, the number of cells in control wells typically increased 16- to 25-fold (4 to 4.5 doublings). Less than 10% relative growth was equivalent to complete cytolysis as periodically monitored by Trypan blue exclusion on a hemocytometer.

Cell Line Mutant Selection. S49 cells in complete medium at a density of 10^6/mL were exposed to 1.5 mg/mL *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 3 h. After centrifugation to remove the MNNG, the cells were resuspended in complete medium and grown for 10 days to allow for phenotypic expression. Technics for S49 clonal selections using mouse embryo fibroblast feeder layers and semisolid agarose have been described previously.[27-29] For selections, 15 mL of selective medium without cells was allowed to harden over the mouse embryo fibroblasts before 10 mL of cells in selective medium at a cell density of 10^6/mL was layered on top. Appropriate control plates in nonselective complete medium containing 500–1000 cells were used to monitor cloning efficiency.

HGPRTase cells were selected for resistance to 1 μM 6-thioinosine. Cells lacking cyclic AMP-dependent protein kinase and very deficient in cyclic nucleotide phosphodiesterase were generous gifts from Drs. H. R. Bourne and P. Coffino. The isolation and characteristics of these clones has been reported by Bourne et al.[30] Cells deficient in both HGPRTase and protein kinase were thioguanine-resistant clones derived from strain Alr as described by Bourne et al.[30] HGPRTase-deficient clones were also obtained by the method described above from the strain which lacked protein kinase and was very deficient in phosphodiesterase (strain Blr[30]).

Degradation of 2'-*O*-Palmityl-6-HS-cRMP. A solution of 0.5 mM 2'-*O*-palmityl-6-HS-cRMP in Dulbecco's modified Eagles medium containing 10% heat-inactivated horse serum was incubated for 3 days at 37 °C. Aliquots were removed and diluted 5:1 with methanol and then filtered through a 0.45-μm membrane filter. Portions (20 μL) of the filtrate were examined on reverse-phase LC. The mercaptopurine-containing compounds were monitored at 320 nm. The flow rate was 4 mL/min and the detector sensitivity was 0.04 absorbance unit full scale. Unchanged starting material was determined using 30:70:1 H_2O–methanol–acetic acid (v/v) as eluant. Deacylated metabolites were determined in separate runs using 100:1 H_2O–acetic acid as eluant.

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References and Notes

(1) Portions of this work were presented at the Academy of Pharmaceutical Sciences Meeting, Phoenix, AZ, Nov., 1977.
(2) A. R. P. Paterson and D. M. Tidd, in "Antineoplastic and Immunosuppressive Agents, Part II", A. C. Sartorelli and D. G. Johns, Eds., Springer-Verlag, Berlin, p 695 (1975).
(3) J. A. Nelson, J. W. Carpenter, L. M. Rose, and D. J. Adamson, *Cancer Res.*, **35**, 2872 (1975).
(4) D. M. Tidd and A. R. P. Paterson, *Cancer Res.*, **34**, 738 (1974).
(5) R. W. Brockman, *Adv. Cancer Res.*, **7**, 129 (1963).
(6) M. Rosman, M. H. Lee, W. A. Creasey, and A. C. Sartorelli, *Cancer Res.*, **34**, 1952 (1974).
(7) M. Rosman and H. E. Williams, *Cancer Res.*, **33**, 1202 (1973).
(8) D. Hentzen, P. Mandel, and J.-P. Garel, *Biochim. Biophys. Acta*, **281**, 228 (1972).
(9) R. B. Meyer, Jr., D. A. Shuman, R. K. Robins, R. J. Bauer, M. K. Dimmitt, and L. N. Simon, *Biochemistry*, **11**, 2704 (1972).
(10) R. B. Meyer, Jr., D. A. Shuman, R. K. Robins, J. P. Miller, and L. N. Simon, *J. Med. Chem.*, **16**, 1319 (1973).
(11) R. B. Meyer, Jr., T. E. Stone, and F. P. Heinzel, *J. Heterocycl. Chem.*, **15**, 1511 (1978).
(12) G. Höfle and W. Steglich, *Synthesis*, 619 (1972).
(13) C. D. Jardetsky, *J. Am. Chem. Soc.*, **84**, 62 (1962).
(14) H. J. Thomas and J. A. Montgomery, *J. Med. Chem.*, **11**, 44 (1968).
(15) D. Epps, I.-M. Chang, E. Sherwood, and A. P. Kimball, *Proc. Soc. Exp. Biol. Med.*, **150**, 578 (1975).
(16) J. W. Koontz and W. D. Wicks, *Cancer Res.*, **37**, 651 (1977).
(17) G. A. LePage and E. M. Hersh, *Biochem. Biophys. Res. Commun.*, **46**, 1918 (1972).
(18) T. A. Khwaja, L. Kigwana, and R. K. Robins, *Proc. Am. Assoc. Cancer Res.*, **15**, 94 (1974).
(19) P. Coffino, J. W. Gray, and G. M. Tomkins, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 878 (1975).
(20) V. Daniel, G. Litwack, and G. M. Tomkins, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 76 (1973).
(21) P. A. Insel, H. R. Bourne, P. Coffino, and G. M. Tomkins, *Science*, **190**, 896 (1975).
(22) R. Van Wijk, W. D. Wicks, and K. Clay, *Cancer Res.*, **32**, 1905 (1972).
(23) Y. S. Cho-Chung, *Cancer Res.*, **34**, 3492 (1974).
(24) W. Bleyberg and H. Ulrich, *Chem. Ber.*, **64**, 2504 (1931).
(25) K. Horibata and A. W. Harris, *Exp. Cell Res.*, **60**, 61 (1970).
(26) B. Ullman, A. Cohen, and D. W. Martin, Jr., *Cell*, **9**, 205 (1976).
(27) P. Coffino, R. Baumal, R. Laskov, and M. D. Scharff, *J. Cell. Physiol.*, **79**, 429 (1972).
(28) L. J. Gudas, A. Cohen, B. Ullman and D. W. Martin Jr., *Somat. Cell Genet.*, 4, 201 (1978).
(29) B. Ullman, L. J. Gudas, A. Cohen and D. W. Martin, Jr., *Cell*, 14, 365 (1978).
(30) H. R. Bourne, P. Coffino, and G. M. Tomkins, *J. Cell. Physiol.*, **85**, 611 (1975).