Enhancement of Nucleoside Cytotoxicity through Nucleotide Prodrugs

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Received March 7, 2002

A common reason for the lack of cytotoxicity of certain nucleosides is thought to be their inability to be initially activated to the monophosphate level by a nucleoside kinase or other activating enzyme. In a search for other nucleosides that might be worthwhile anticancer agents, we have begun to examine the utilization of monophosphate prodrugs in order to explore whether any enhanced cytotoxicity might be found for the prodrugs of candidate nucleosides that have little or no cytotoxicity. To that end, 5′-bis(pivaloyloxymethyl) phosphate prodrugs of two weakly cytotoxic compounds, 8-aza-2′-deoxyadenosine (**5**) and 8-bromo-2′-deoxyadenosine (**9**), have been prepared. These prodrugs (**8** and **12**) were examined for their cytotoxicity in CEM cells and were found to possess significantly enhanced cytotoxicity when compared with the corresponding parent nucleosides. Further cell culture experiments were conducted to gain insight into the mechanisms of cytotoxicity of these two prodrugs, and those data are reported.

Introduction

Over the years, we have prepared many nucleosides for evaluation as potential anticancer agents. While many of these compounds proved to have in vitro and/or in vivo activity, a significant number of compounds had little or no cytotoxicity when examined in our in vitro tumor panel. While a number of reasons might be the cause of this lack of activity, a common one is that the nucleoside is not converted to its monophosphate derivative by the initial activating enzyme. In most cases, though not all, the steps from the monophosphate to the triphosphate derivatives are more easily accomplished by the relevant enzymes than is the first step. It has been clear to us for some years that we might have prepared a number of potentially valuable nucleosides if we were able to find a way to get them to the triphosphate level in cells. To that end, the strategy that has been utilized in the antiviral area for the same purpose, that is, to synthesize a monophosphate prodrug, appeared to have promise for our purposes. To evaluate the concept, we selected two weakly cytotoxic compounds, 7-amino-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (8-aza-2′-deoxyadenosine, **5**, Scheme 1) and 8-bromo-2′-deoxyadenosine (**9**, Scheme 2), for conversion to an appropriate monophosphate prodrug. Prior studies in our laboratories had determined that these two nucleoside analogues were poor substrates for mammalian kinases (unpublished observation) and were therefore good candidates to evaluate this strategy. From the variety of possible monophosphate prodrugs that were known when we began this work,[1] we selected the bis(pivaloyloxymethyl) group for the initial test of the concept. We describe herein the synthesis and biological evaluation in cell culture of bis(pivaloyloxymethyl) 8-aza-2′-deoxyadenosine-5′-monophosphate **8** and bis(pivaloyloxymethyl) 8-bromo-2′-deoxyadenosine-5′-monophosphate **12**.

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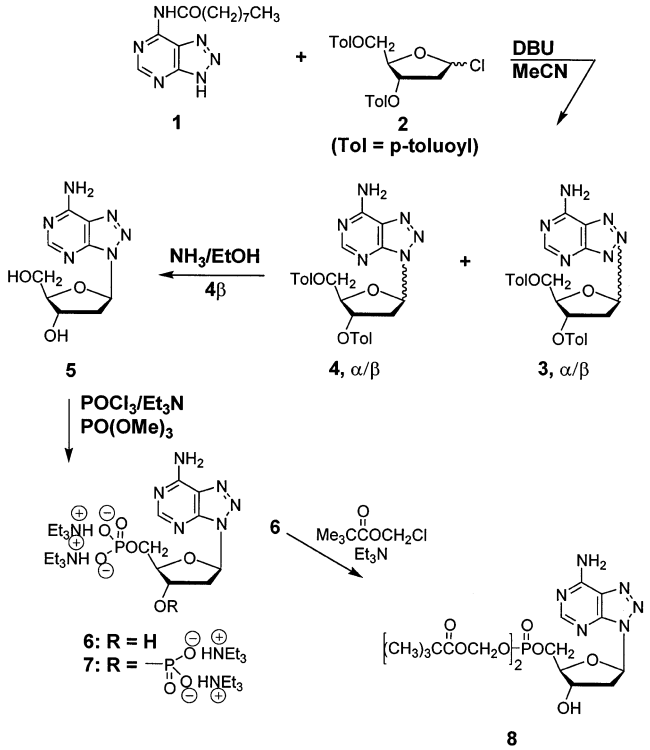
Scheme 1

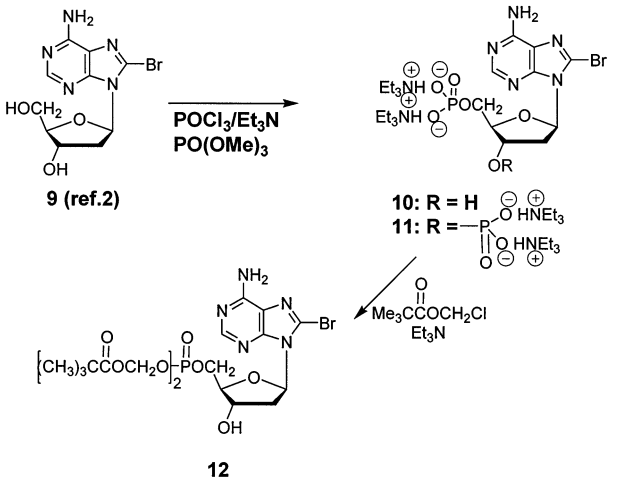


Chemistry

The two nucleoside starting materials are both reported in the literature. Nucleoside **9** is readily prepared by bromination of 2′-deoxyadenosine.[2] The synthesis of **5** has been reported several times,[3−5] including once from our laboratories.[3] None of these routes provides good yields of the desired product, and all of them provide multiple products relative to anomeric mixture and glycosylation site; therefore, we chose to pursue a modified approach. The anion of N^6-nonanoyl-8-azaadenine (**1**),[4] generated by treatment of **1** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU),[6] was coupled with

10.1021/jm020107s CCC: $22.00
Published on Web 08/24/2002

Scheme 2



2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranosyl chloride[7] in acetonitrile. This procedure produced both the α and the *β* anomers of nucleosides attached through the 8- and 9-positions (purine numbering). Separation of the desired 9*β* anomer, which had already lost its nonanoyl group, was followed by conventional deprotection with NH_3/EtOH at 75 °C to afford **5**, with properties in agreement with published data.[3−5]

Conversion of the two nucleosides into the desired prodrugs involved initial phosphorylation followed by introduction of the bis(pivaloyloxymethyl) group. We chose to utilize standard phosphorylation methodology for both nucleosides, involving treatment with 2 equiv of phosphorus oxychloride in trimethyl phosphate at reduced temperature. Phosphorylation of **5** under these conditions at −10 °C produced a mixture of the desired monophosphate **6** and the corresponding 3′,5′-diphosphate **7**, unexpectedly in about equal amounts. Partial separation was possible through chromatographic means, and a sample with about 69% **6** was carried forward.

Various methods have been utilized for the introduction of pivaloyloxymethyl groups into nucleoside phosphate salts,[8,9] and they are generally characterized by low to moderate yields. We chose direct esterification of **6** with commercially available chloromethyl pivalate in Et_3N/1-methyl-2-pyrrolidinone[10] because it satisfied our goal of rapidly obtaining sufficient **8** for biological evaluation without additional synthetic steps. Separation of **8** from the corresponding 3′,5′-diphosphate tetrakis(pivaloyloxymethyl) and tris(pivaloyloxymethyl) esters was accomplished using a silica gel column. Target compound **8** was obtained as a low melting, but tractable, brittle glass.

Scheme 2 outlines the synthesis of target compound **12** from nucleoside **9**. Phosphorylation was accomplished by the method described above except that the temperature was kept in the −14 to −12 °C range. After some chromatographic purification, a mixture comprised of mainly monophosphate **10** (89%) but also containing a small amount of starting nucleoside **9** (9%) and a lesser quantity of diphosphate **11** was carried forward. Reaction of the mixture with chloromethyl pivalate as described above, followed by column purification, gave the desired product **12**. This material appeared to be of high quality by thin-layer chromatography (TLC), but high-pressure liquid chromatography (HPLC) analysis showed 4.9% of a barely resolved second component that was tentatively identified as the tetrakis(pivaloyloxymethyl) ester of the 3′,5′-diphosphate. This sample, containing the small diphosphate impurity, was utilized for the biological experiments.

Biochemical Studies

Monophosphate prodrug **12** was 10-fold more potent as an inhibitor of CEM cell growth than parent nucleoside **9**. The concentration of compound that was required to inhibit CEM cell growth by 50% during a 96 h incubation period was 28 ± 16 vs 275 ± 72 *μ*M (respectively, mean and standard deviation of at least three separate experiments). Nucleoside **5** was known to be toxic to human cells by virtue of its conversion to 8-aza-hypoxanthine through the sequential action of adenosine (Ado) deaminase and purine nucleoside phosphorylase (unpublished observation). Its toxicity can be prevented by addition to the culture medium of deoxycoformycin (dCF), which is an inhibitor of Ado and adenosine 5′-monophosphate (AMP) deaminase. In the presence of dCF, **8** was also more cytotoxic than **5** (IC_{50} of 15 ± 7 vs 190 ± 17 *μ*M, respectively, mean ± standard deviation of at least four separate experiments). The increased potency of **8** and **12** with respect to the parent nucleosides suggested that this strategy may be useful in identifying novel cytotoxic nucleosides that could have antitumor activity.

As expected for these types of compounds,[11,12] they rapidly disappeared from the culture medium with a half-life of approximately 7 h (data not shown). The half-lives were similar in culture medium without serum, culture medium with serum, and culture medium with serum plus cells, which indicated that the degradation of the parent compounds in CEM cell culture was primarily due to a nonenzymatic process. Because the half-life of these two compounds in culture medium was much shorter than the duration of the cytotoxicity experiments, it was possible that the potency of these agents would be enhanced if they were repeatedly administered during the cytotoxicity assay. However, repeated administration of compound (dosing on days 0, 1, 2, and 3) only increased the potency of these compounds by about 2-fold (data not shown). The products of the degradation reaction were not determined in these experiments. If the mono-PIV phosphodiester was the major product, it is possible that it could also penetrate cell membranes and result in cytotoxicity to the cells.

Treatment of CEM cells with either compound resulted in the inhibition of the incorporation of [^{3}H]-uridine (Urd) into RNA and [^{3}H]thymidine into DNA but did not affect the incorporation of [^{3}H]leucine into protein (Table 1). These results, suggesting that both RNA and DNA synthesis were inhibited by these compounds, are quite different from those normally observed with other deoxynucleoside analogues. Inhibition of the incorporation of [^{3}H]Urd into RNA could result from the inhibition of Urd transport, RNA polymerases, or the enzymes involved in Urd metabolism. Although treatment with these two compounds inhibited the utilization of Urd, they did not have any effect on uridine 5′-triphosphate (UTP) levels in CEM cells (data not shown). Therefore, it is not likely that these agents

Table 1. Effect of **8** or **12** on RNA, DNA, and Protein Syntheses[a]

	percent of control		
treatment	DNA synthesis	RNA synthesis	protein synthesis
12	53	14	94
8	11	3	95
8 + dCF	11	3	98

[a] CEM cells were incubated with either 29 μM **12** or 35 μM **8** (plus or minus 10 μM dCF). [^{3}H]thymidine, [^{3}H]Urd, or [^{3}H]leucine were added 30 min after the initiation of the incubation; samples were taken 1, 2, and 4 h after the addition of radioactive labels; and the incorporation of radiolabeled precursors into DNA, RNA, or protein (respectively) was determined as described.[12,13] This experiment has been repeated twice with similar results.

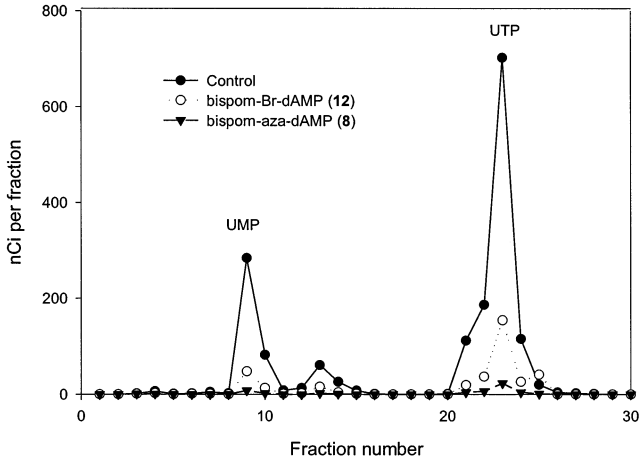


Figure 1. Effect of **12** or **8** on the utilization of [^{3}H]Urd in CEM cells. CEM cells (300 000 cells/mL) were incubated at 37 °C with [^{3}H]Urd (2 μC/mL) plus 29 μM **12**, 35 μM **8**, or no drugs for 2 h. Cells (8 mL) were collected and washed, and an acid soluble extract was prepared and injected onto SAX HPLC as described.[14] One minute fractions were collected as they eluted from the column and were counted for radioactivity. This experiment has been repeated two times with similar results.

inhibited uridylate kinase or nucleotide diphosphate kinase. However, inhibition of Urd transport or Urd kinase activity by these agents could inhibit the utilization of [^{3}H]Urd without affecting pyrimidine nucleotide pools. To test for this possibility, the effect of both compounds on the phosphorylation of [^{3}H]Urd to UTP was determined (Figure 1). Both compounds decreased the metabolism of [^{3}H]Urd, indicating that these agents inhibited either the transport of Urd or the Urd kinase activity and that RNA polymerase was not a target of these compounds. To clarify the effect of these compounds on RNA and DNA synthesis, the effect of both compounds on the incorporation of [^{3}H]adenine into RNA and DNA was determined. Neither compound inhibited the incorporation of [^{3}H]adenine into RNA, which indicated that RNA synthesis was not inhibited by these agents (Figure 2A). However, both compounds inhibited the incorporation of [^{3}H]adenine into DNA (Figure 2B).

Both compounds immediately inhibited the uptake and utilization of the [^{3}H]Urd in CEM cells (data not shown). Unfortunately, because uptake was rate-limiting in the conversion of Urd to UTP in these cells, we could not determine which process was being inhibited, Urd uptake or Urd kinase activity. Neither **12** nor **8** (at

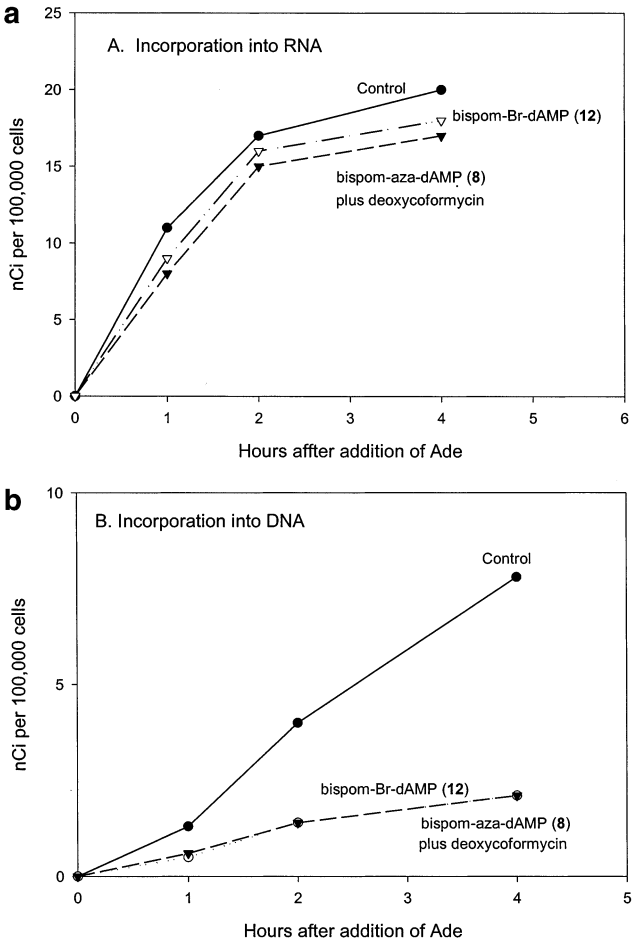


Figure 2. Effect of **12** or **8** on the incorporation of [^{3}H]adenine into RNA and DNA. CEM cells (600 000 cells/mL) were incubated with 1 μM [^{3}H]adenine (1 μCi/mL) plus 29 μM **12**, 35 μM **8** plus 10 μM dCF, or no drugs. The compounds were added 30 min prior to the addition of [^{3}H]adenine. One, two, and four hours after the addition of [^{3}H]adenine, a 4 mL cell sample was taken from each incubation and the amount of radioactivity in RNA (A) and DNA (B) was determined as described.[12,13] This experiment has been repeated with similar results.

20 μg/mL) inhibited Urd kinase activity in cell-free extracts of CEM cells (data not shown), however, which indicated that inhibition of this activity by the parent compounds was not the reason for the decrease in Urd metabolism in these cells. The parent compound was stable for the duration of the experiment. Although inhibition of Urd utilization by these agents is an interesting observation, this effect of these agents would not result in toxicity since CEM cells are not dependent on pyrimidine salvage for generation of UTP; i.e., little or no Urd is provided in the culture medium for the growth of these cells, so inhibition of its utilization would not affect growth of these cells.

Acid soluble extracts were obtained from CEM cells treated with 145 μM **12** and analyzed by strong anion exchange HPLC (SAX) to determine whether 8-Br-dATP was formed in the cells. In our standard SAX HPLC system, 8-Br-dATP (chemically synthesized standard) eluted between adenosine 5′-triphosphate (ATP) and guanosine 5′-triphosphate (GTP) with a retention time of approximately 38 min. Although there was evidence of new peaks in the monophosphate region of the chromatogram, there was no peak that eluted with a

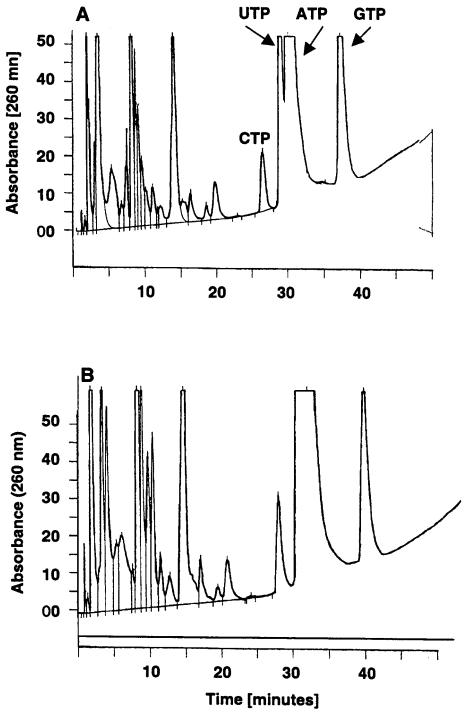


Figure 3. Metabolism of **12** in CEM cells. CEM cells (700 000 cells/mL) were treated with 145 μM **12** (B) or no compounds (A) for 7 h. An acid soluble extract from 50 $\times$ 10^6 cells was obtained and analyzed by SAX HPLC as described.[4] The natural nucleotides and 8-Br-dAMP metabolites were detected by their absorbance at 260 nm as they eluted from the column. The experiment has been repeated with similar results.

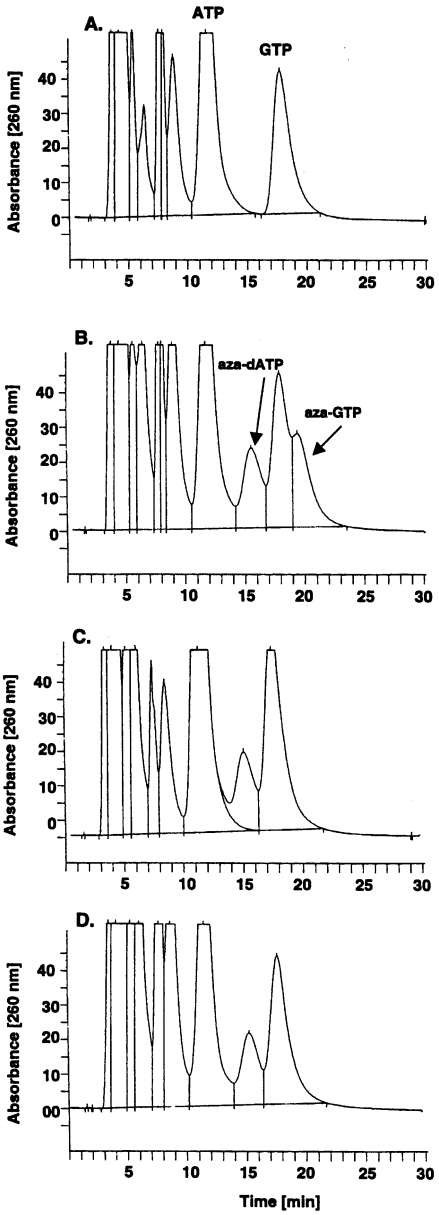


Figure 4. Metabolism of **8** in CEM cells. CEM cells (700 000 cells/mL) were treated with 70 μM **8** (B), 70 μM **8** plus 735 μM hypoxanthine (C), 70 μM **8** plus 10 μM deoxycoformycin (D), or no compounds (A) for 4 h. An acid soluble extract from 21 $\times$ 10^6 cells was obtained and analyzed by SAX HPLC as described in the legend to Figure 3 except that no gradient was used to separate the acid soluble metabolites. The samples were eluted with 500 mM potassium phosphate buffer. The natural nucleotides and 8-aza-dAMP metabolites were detected by their absorbance at 260 nm as they eluted from the column. The experiment has been repeated with similar results.

retention time equal to that of 8-Br-dATP (Figure 3). In contrast to the results with **12**, two new peaks were detected in the triphosphate region of SAX HPLC separation of acid soluble extracts created from cells that were treated with 70 μM **8** (Figure 4). One of the peaks eluted between ATP and GTP with a retention time that was identical to that of an authentic standard of 8-aza-dATP. The other peak eluted just after GTP and had a UV spectrum that was similar to an authentic standard of 8-aza-GMP, which was the standard available in our laboratories. The addition of either hypoxanthine or dCF prevented the synthesis of the peak that eluted just after GTP but had no effect on the generation of 8-aza-dATP from **8**. The fact that hypoxanthine inhibited the synthesis of this peak suggested that the compound must first be cleaved to 8-aza-hypoxanthine prior to conversion to triphosphate. Therefore, the metabolite that eluted just after GTP is likely to be mostly 8-aza-GTP. Hypoxanthine would not be expected to affect the synthesis of this metabolite, if it were 8-aza-dGTP. Once 8-aza-dAMP is formed from the parent compound, however, it is possible that it could be deaminated to 8-aza-dIMP, which could be converted to 8-aza-dGTP. The peak that eluted after GTP but not the one that eluted prior to GTP was also detected in cells treated with **5** (data not shown). These studies are direct evidence that **8** entered the CEM cells and that the bis(pivaloyloxymethyl) groups came off the molecule in the cells liberating 8-aza-dAMP, which was utilized by the cellular kinases to create 8-aza-dATP.

Both 8-Br-dATP and 8-aza-dATP were competitive inhibitors of the incorporation of dATP into DNA by DNA polymerase α (K_i values of 548 $\pm$ 93 and 156 $\pm$ 36 μM, respectively, $N = 3$). The K_m of dATP with DNA polymerase α was 4 $\pm$ 1 μM, which indicated that these compounds did not potently interact with DNA polymerase α. 8-Aza-dATP was able to substitute for dATP in reactions that utilized [^{3}H]dGTP instead of [^{3}H]dATP to label the newly synthesized DNA (Figure 5), which indicated that 8-aza-dATP was not an inhibitor of the DNA polymerase reaction but was instead a good alternative substrate for DNA synthesis. In contrast, 8-Br-dATP was not utilized as efficiently as dATP or 8-aza-dATP for DNA synthesis (Figure 5). However, in this experiment, 8-Br-dATP did not inhibit the incor-

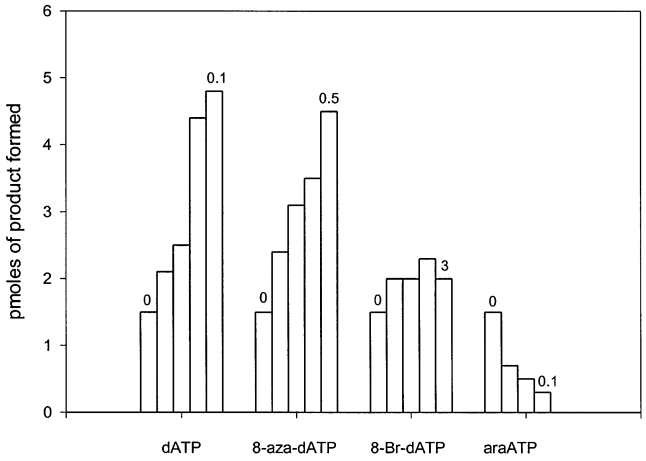


Figure 5. Utilization of 8-Br-dATP and 8-aza-dATP as substrates for DNA synthesis. DNA polymerase α isolated from CEM cells was incubated in 50 μL reactions with dATP (0, 0.5, 1, 10, or 100 μM), aza-dATP (0, 10, 50, 100, or 500 μM), Br-dATP (0, 100, 200, 1000, or 3000 μM), or araATP (1, 10, or 100 μM). The numbers above the bars in the figure are the concentration (expressed in mM) of the deoxyadenosine nucleotides that were used in the experiment. The reactions also included 50 mM Tris (pH 8.0), 1 mg/mL bovine serum albumin, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/mL activated DNA, 100 μM each of dTTP and dCTP, and 10 μM [^{3}H]dGTP (10 000 Ci/mol). After they were incubated at 37 °C for 1 h, the reaction mixtures were spotted on glass fiber filters and washed with 5% trichloroacetic acid and ethanol, and the radioactivity on each filter was determined.[20] This experiment has been repeated three times with similar results.

poration of [^{3}H]dGTP into DNA at concentrations that were above its K_i. The slight increase in DNA synthesis activity suggested that the incorporation of 8-Br-dAMP into the 3′ terminus also did not result in termination of the DNA chain and that the V_{max} of the DNA synthesis reaction with 8-Br-dATP was less than that for dATP and 8-aza-dATP. The effect of arabinofuranosyl ATP (araATP) on the incorporation of [^{3}H]dGTP into DNA is shown in Figure 5 for comparison purposes. AraATP is utilized by DNA polymerase α as a substrate for DNA synthesis, and its incorporation into the 3′ terminus of a DNA chain results in the subsequent inhibition of chain elongation by the polymerase.[13]

Discussion

Although both compounds selectively inhibited DNA synthesis and were cytotoxic to CEM cells, our results suggested that the mechanism of action of these two compounds could be quite different. The major difference between these two compounds was that **8** was readily converted to 8-aza-dATP in CEM cells, whereas no 8-Br-dATP was detected in cells treated with **12**. This difference in the metabolism of these two compounds indicated that one or both of the enzymes involved in the generation of the triphosphate (AMP kinase and nucleoside diphosphate kinase) were able to discriminate between 8-Br- and 8-aza-nucleotides. Given the broad substrate specificity of nucleoside diphosphate kinase, it is likely that 8-Br-dAMP is a poor substrate for AMP kinase.

Currently, it is not clear how **8** inhibits DNA synthesis. Because 8-aza-dATP supported DNA synthesis with DNA polymerase α at a rate that was similar to that for dATP (although at higher concentrations), inhibition of DNA polymerase α cannot account for the inhibition of DNA synthesis. However, numerous DNA polymerases are involved in the synthesis and repair of chromosomal DNA, and it is possible that one or more of these enzymes could be inhibited by 8-aza-dATP. Our results indicated that 8-aza-dAMP would readily be incorporated into chromosomal DNA in place of dAMP. The incorporation of 8-aza-dAMP into DNA could disrupt the function of DNA and result in the inhibition of DNA synthesis. This possibility seems unlikely, however, because DNA synthesis was immediately inhibited by **8**. The 5′-triphosphates of numerous deoxyadenosine analogues are potent inhibitors of ribonucleotide reductase (**6**), and inhibition of this enzyme by 8-aza-dATP would result in the inhibition of DNA synthesis. Finally, it should be noted that although a considerable amount of 8-aza-dATP was generated in CEM cells treated with **8** and triphosphate metabolites of nucleoside analogues are often responsible for the toxicity of these types of agents, it is possible that another metabolite, or the parent prodrug itself, is responsible for the inhibition of DNA synthesis and toxicity of this agent.

Our results indicated that the generation of 8-Br-dATP was not likely to be involved in the mechanism of action of **12**. Although it is possible that small amounts of 8-Br-dATP were formed in CEM cells that were below the levels of detection used in these experiments, it is not likely that generation of small amounts of 8-Br-dATP would have much effect on DNA synthesis or DNA function given the high concentrations needed to inhibit DNA polymerase α.

These biological evaluations indicated that the synthesis of 5′-bis(pivaloyloxymethyl) derivatives of nontoxic nucleoside analogues can lead to toxic molecules that do not need to be activated by nucleoside kinases. It is likely that other nucleotide prodrug systems that allow the by-passing of the kinase activation step will also have utility in this regard.[1] Nucleoside kinases are often the rate-limiting step in the activation of nucleoside analogues used in the treatment of cancer. Thus, the generation of these types of compounds should expand the structural variety of nucleoside analogues that are currently available for evaluation as antitumor agents and could lead to novel classes of antitumor drugs. The concept is a reasonable one to develop novel cytotoxic agents, and we are pursuing its application with other prodrug systems and with other nontoxic nucleosides.

Experimental Section

Chemical Methodology. Melting points were determined by the capillary method on a Mel-Temp apparatus and are uncorrected. All evaporations were carried out in vacuo on a rotary evaporator or under high vacuum by short-path distillation into a glass trap cooled in dry ice/acetone. Samples were dried under high vacuum over P_2O_5 at room temperature (rt) unless an elevated temperature is given. Elemental analyses were performed by the Spectroscopic and Analytical Laboratory of the Southern Research Institute or by Atlantic Microlab, Inc., Atlanta, GA. Where solvents such as EtOH or H_2O are included in a reported analysis, their presence was confirmed by the ^{1}H NMR spectrum. Elemental analyses within ±0.4 of the calculated values are reported only by the symbols of the elements. Analtech precoated (250 μm) silica gel G (F) plates were used for TLC, and spots were detected by irradiation with a 254 nm UV light, by absorption of I_2

vapor, or by charring after a $(NH_4)_2SO_4/H_2SO_4$ spray. Column chromatography in either the flash column or the gravity mode was performed with silica gel 60 (230−400 mesh) using the slurry method of column packing. Mass spectra were recorded on a Varian/MAT 311A double-focusing mass spectrometer in the positive fast-atom bombardment (FAB) mode. A few spectra of nucleoside phosphate salts were also obtained in the negative electrospray mode from Mr. Marion Kirk of the University of Alabama at Birmingham. Addition of a trace of LiCl to some samples confirmed the identification of molecular ions by giving $(M + Li)^+$ cluster peaks that were stronger than the $(M + H)^+$ peaks. ^{1}H NMR spectra were recorded on a Nicolet/GE NT300NB spectrometer operating at 300.635 MHz. Chemical shifts (δ) are reported in parts per million downfield from internal tetramethylsilane. Chemical shifts of multiplets are measured from the approximate center of the multiplet, and coupling constants (J) are reported in Hz. Ultraviolet spectra were determined in 0.1 N HCl (pH 1), pH 7 buffer, and 0.1 N NaOH (pH 13) with a Perkin-Elmer UV−visible near-infrared model Lambda 9 spectrophotometer. Maxima and extinction coefficients are reported in the format: λ_{max} in nanometers ($\epsilon \times 10^{-3}$). HPLC was performed on either Hewlett-Packard or Perkin-Elmer equipment with variable wavelength UV detection by the following methods. Method A (reversed phase): Phenomenex Sphereclone 5 μm, 4.6 mm × 250 mm column; H_2O/MeCN (2:3); 1 mL per minute. Method B (strong anion exchange): Keystone Scientific Partisil, 4.6 mm × 250 mm column; solution 1:5 mM $NH_4H_2PO_4$ (ADHP) adjusted to pH 2.8; solution 2: 750 mM ADHP adjusted to pH 3.7; 50 min linear gradient from 100% 1 to 100% 2 at 2.0 mL/min. Method C (ion-pairing): same column as method A; solution 1: 0.01 M ADHP + 0.005 M tetrabutylammonium phosphate adjusted to pH 5.0 with H_3PO_4; solution 2: MeOH; 20 min linear gradient from 20 to 50% 2 at 1 mL/min. Method D: same column as A; solution 1: 0.01 M ADHP; solution 2: MeOH; 20 min linear gradient from 10 to 90% 2 at 1 mL/min.

8-Aza-2′-deoxy-3′5′-di-O-(p-toluoyl)adenosine (β4). Under a N_2 atmosphere, freshly opened DBU (0.550 g, 3.62 mmol) was added to a stirred suspension of **1**[4] (1.00 g, 3.62 mmol) in dry acetonitrile (30 mL), forming a clear solution in 5 min. After 15 min, **2**[7] (1.62 g, 4.16 mmol, 1.15 eq) was added in one portion, and the mixture became a clear solution in ∼10 min. After 48 h, the solvent was evaporated, and the residual gum was applied directly to a silica gel column. Elution with $CHCl_3$/MeOH (first 99:1 and then 4:1) separated the mixture into three major zones. The first zone (0.7 g) was itself a complex mixture of at least six mobile components. The main components of the second zone (0.99 g, 56%) were an anomeric pair that were shown subsequently to be β**4** (9β isomer) followed by α**4**. The third, least mobile zone (0.54 g, 31%) was likewise an anomeric pair that was mostly α and β**3**. Recolumning of the second zone using cyclohexane/EtOAc (2:3) gave fractions totaling 423 mg (24%) of sufficient purity to allow positive spectral identification as the desired β**4** and trailing fractions totaling 465 mg (26%) that were identified as α**4**.

The pilot reaction above was scaled up using the following quantities of reagents: DBU (8.09 g, 53.2 mmol), **1** (14.69 g, 53.2 mmol), **2** (22.74 g, 58.5 mmol, 1.1 equiv). Evaporation of the reaction mixture after 48 h gave a gum that was triturated with Et_2O (500 mL) and filtered under N_2 pressure to remove most of the insoluble DBU·HCl. Evaporation of the filtrate gave a soft foam (34.8 g) that was chromatographed on a SG column with elution by cyclohexane/EtOAc 2:3 followed by 1:3 to give three major zones as described above. The middle zone (23 g, crude) was recolumned repeatedly (cyclohexane/EtOAc 2:3), selecting the best fractions at each stage for further purification. Finally, samples of the desired β**4** of high isomeric and anomeric purity were obtained totaling 4.58 g (18%). An analytical sample was prepared by recrystallization of a portion from cyclohexane/EtOAc (4:1) with filtration and drying of the white crystalline solid in vacuo over P_2O_5 at 65 °C for 18 h; mp 93−94 °C; on continued heating, it solidifies and melts again 163−164 °C. Spectral properties of β**4**, as well as α**4**, α**3**, and β**3**, have all been published;[5] however, because of the previously unreported melting behavior, our data for β**4** are included for confirmation. MS: m/z 489 $(M + H)^+$, 353 (sugar fragment)$^+$, 137 $(B + 2H)^+$. HPLC (method A): 99.7%; retention time, 8.34 min (α**4**, 7.24 min). TLC (cycl/EtOAc 1:3): R_f 0.47 (α**4**, 0.42). UV(MeOH): 0.1 N HCl: 246 (36.9); pH 7 buffer (slightly cloudy); 249(25.5), 286(17.8); 0.1 N NaOH: 238 (26.1), 277 (12.0). ^{1}H NMR (DMSO-d_6): δ 2.37, 2.41 (2 s, 6H, Tol CH_3), 2.91, 3.55 (2 m, 1 each, H-2′s), 4.46, 4.57 (2 m, 2H, 5′-CH_2), 4.65 (m, 1H, H-4′), 5.93 (m, 1H, H-3′), 6.83 (t, 1H, H-1′, J = 6.4), 7.32, 7.82, 7.96 (m, 8H, Tol CH), 8.21, 8.56 (2 s, 2H, 6-NH_2), 8.31 (s, 1H, H-2′). Anal. Calcd for $C_{25}H_{24}N_6O_5$: C, 61.47; H, 4.95; N, 17.20. Found: C, 61.50; H, 5.15; N, 17.18. For comparison, our α**4** melted 188−189 °C; literature[5] 187 °C; spectral data also agreed with published values.

8-Aza-2′-deoxyadenosine (5). A solution of β**4** (3.70 g, 7.57 mmol) in EtOH saturated with NH_3 (450 mL) was heated for 3 days in a glass-lined stainless steel bomb in a 75 °C oil bath. The cooled solution was evaporated, and the residue was washed twice by trituration and filtration with Et_2O to remove the *p*-toluamide and ethyl *p*-toluate. A solution of the solid filter cake in hot MeOH was evaporated with silica gel (∼50 mL dry volume), and the powder was layered carefully onto a SG column. Elution with $CHCl_3$/MeOH (9:1) gave **5** in fractions totaling 1.63 g (85%). The best of these (1.27 g) was recrystallized from MeOH (200 mL) and dried in vacuo over P_2O_5 for 18 h at 100 °C; recovery, 0.96 g (75%); mp 201−202 °C, resolidifies on continued heating and chars but does not melt below 300 °C (literature[4] 200−201 °C). TLC ($CHCl_3$/MeOH 4:1): R_f 0.53. ^{1}H NMR[5] and UV[4] spectra agreed with the literature. Anal. ($C_9H_{12}N_6O_3$) C, H, N.

Triethylammonium 8-Aza-2′-deoxyadenosine-5′-monophosphate (6) and Triethylammonium 8-Aza-2′-deoxyadenosine-3′,5′-diphosphate (7). Under an atmosphere of dry N_2, a stirred suspension of **5** (137 mg, 0.54 mmol) in a mixture of trimethyl phosphate (2.5 mL) and Et_3N (110 mg, 1.09 mmol, 2 equiv) was cooled to −10 °C in an ice/acetone bath and treated dropwise over 5 min with $POCl_3$ (0.10 mL, 167 mg, 1.09 mmol) added via hypodermic syringe. After 1 h, the mixture was poured into 50 mL of ice/water, and the solution was neutralized (meter) by dropwise addition of cold Et_3N. The pH drifted downward because of the slow hydrolysis of cold $POCl_3$, so it was monitored and adjusted for ∼40 min until it stabilized at pH 7, and then the solution was frozen and lyophilized overnight to give a paste of solid in $PO(OMe)_3$. Trituration with Et_2O (50 mL) and filtration gave a hygroscopic white solid: wt 816 mg (>100% because of extra triethylammonium salts). A mass spectrum in the negative mode showed a strong 331 $(M - 1)^-$ (**6**), a medium 411 $(M - 1)^-$ (**7**), and a weak 491 $(M - 1)^-$ for a triphosphate. An attempt was made to separate the monophosphate and diphosphate components on a column of washed and defined Darco G60 activated charcoal, eluting first with H_2O and then EtOH /H_2O/ concentrated NH_4OH (50:50:1). Some desalting was achieved, but the nucleotides came off in a single broad band; wt 130 mg after lyophilization.

With 925 mg of **5** as starting material, a 6.75-fold scale-up of the phosphorylation reaction was performed as described above except that reaction time was decreased to 45 min. After the crude lyophilized mixture was washed with Et_2O, the solid (5.52 g) was washed by trituration and decantation with $CHCl_3$ (3 × 50 mL) to remove most of the extraneous Et_3N·HCl. HPLC (method B) on the washed solid (2.62 g) showed 46% **6** and 45% **7**. This was combined with 120 mg of similar material from the pilot run. A column (ca. 4 cm × 30 cm) was poured from a slurry of "gravity grade" silica gel (70−230 mesh) in a solvent mixture of MeOH/H_2O/concentrated NH_4OH (6:2:1),[14] and the column was washed with the solvent until the initially turbid effluent ran clear (ca. 250 mL). A solution of the combined products in H_2O (12 mL) was applied, and the column was eluted at its maximum flow rate of ∼1.2 mL/min with continuous UV monitoring at 275 nm. After TLC of selected samples, the fractions containing most of the UV active material were pooled into two samples, which were evaporated, dissolved in H_2O (10 mL), filtered to remove silica,

frozen, and lyophilized overnight. The first of these, from the leading edge of the nucleotide band, weighed 0.93 g and was shown by HPLC (method C) to be 69% **6** and 22% **7** plus trace constituents. The second sample (0.53 g), from the trailing edge, was 9% **6** and 87% **7**. For the 0.93 g sample: MS (positive): *m/z* 333 $(M + H)^+$ of **6**, 413 $(M + H)^+$ of **7**, 137 $(B + 2H)^+$; (negative): 331 $(M - H)^-$, 411 $(M - H)^-$. For the 0.53 g sample: MS (negative): very strong 411 (**7**), weak 331 (**6**). ^{31}P NMR (D_2O, HEPES buffer, EDTA; referenced to external H_3PO_4 as $\delta = 0.0$ ppm): δ 2.58 (d, $J = 7.7$, 5′-PO_4-2), 2.91 (t, $J = 5.4$, 3′-PO_4-2).

Bis(pivaloyloxymethyl) 8-Aza-2′-deoxyadenosine-5′-monophosphate (8).[10] The 0.93 g sample containing 69% **6** was refluxed with benzene (150 mL) using a Dean−Stark trap to collect H_2O. After 20 min, the mixture was cooled and evaporated to give 0.900 g of amorphous powder. A stirred suspension of the powder (69% as **6**·$[Et_3N]_2$ salt $C_{21}H_{43}N_8O_6P$, 621 mg, 1.2 mmol) in anhydrous *N*-methylpyrrolidinone (NMP, 13 mL) and Et_3N (1.74 g, 17.2 mmol) was treated with chloromethyl pivalate (3.33 g, 22.1 mmol) and heated for 5 h in a 60 °C oil bath under a static N_2 atmosphere. After it was cooled, the mixture was diluted with EtOAc (200 mL), and the soft, white precipitate (Et_3N·HCl and unreacted **6**, TLC) was collected under N_2 pressure and washed liberally by trituration on the funnel with $CHCl_3$ (3 × 50 mL) to extract Et_3N·HCl. The weight of recovered **6** was 372 mg (60%). The EtOAc filtrate was washed by shaking with H_2O (3 × 20 mL), and the H_2O wash was back-extracted with EtOAc. The dried (Na_2SO_4) organic layer was evaporated and then evacuated on the oil pump overnight to give a viscous oil (0.69 g) that was shown by MS and TLC to be a crude mixture containing **8** and the corresponding 3′,5′-diphosphate tri- and tetra(pivaloyloxymethyl) esters.

The recovered **6** and various column fractions known to contain **6** were combined and dried (536 mg). The reaction above was repeated with this mixed starting material to give an additional 0.21 g of material similar to the 0.69 g of product. The combined sample was columned twice on SG with $CHCl_3$/MeOH 95:5 as eluent to give **8** as a brittle glass. A concentrated solution in CH_2Cl_2 (2 mL) was evaporated under high vacuum to give a tractable brittle foam, which was dried in vacuo over P_2O_5 at room temperature for 24 h and at 56 °C for 1 h; yield 167 mg (∼24% from 1.2 mmol of **6**); mp softens to a syrup 62−64 °C. MS: *m/z* 561 $(M + H)^+$. TLC ($CHCl_3$/MeOH 95:5): R_f 0.27; diphosphate tetraester, R_f 0.56. UV (EtOH): 0.1 N HCl: 263 (11.8); pH 7 buffer; 279 (10.7); 0.1 N NaOH: 279 (10.7). ^1H NMR ($CDCl_3$): δ 1.23, 1.24 (2 s, 18H, pivaloyl CH_3), 2.69, 3.22 (2 m, 1 each, H-2′), 3.39 (d, 1H, 3′-OH, $J = 3.7$), 4.25 (complex m, 3H, H-4′ and H-5′), 5.09 (br s*, 1H, H-3′), 5.62 (m, 4H, O−CH_2−O), 6.32 (br s, 2H, NH_2), 6.78 (m, 1H, H-1′), 8.46 (s, 1H, H-2). *On expansion, this signal is a poorly resolved multiplet. Anal. ($C_{21}H_{33}N_6O_{10}P$) C, H, N.

Triethylammonium 8-Bromo-2′-deoxyadenosine-5′-monophosphate (10) and Triethylammonium 8-Bromo-2′-deoxyadenosine-3′,5′-diphosphate (11). Under a N_2 atmosphere, a stirred suspension of **9**[2] (3.81 g, 11.4 mmol) in $PO(OMe)_3$ (50 mL) and Et_3N (2.31 g, 22.8 mmol) was chilled below −10 °C in an ice/acetone bath and treated dropwise over 5 min with $POCl_3$ (3.50 g, 22.8 mmol). After 1 h at −14 to −12 °C, the mixture was poured into ice/H_2O (250 mL); the yellow solution was neutralized rapidly by addition of cold Et_3N and monitored (meter) for ∼45 min until it stabilized at pH 7.1. The solution was lyophilized overnight to give a semisolid paste, which was washed by trituration and decantation with Et_2O (2 × 200 mL) followed by $CHCl_3$ (3 × 200 mL). The crude solid residue weighed 7.1 g (102%). A partial solution of this material in H_2O was filtered to remove 202 mg (5%) of unreacted **9**, and the filtrate was applied to a SG column (70−230 mesh, 600 mL dry volume), which was prepared and eluted with MeOH/H_2O/NH_4OH (6:2:1) according to the method given above for **6** to give 2.85 g (40%) of material that was mostly the 5′-monophosphate **10**, 1.49 g (15%) of the 3′,5′-diphosphate **11**, 0.78 g of mixed **10** and **11**, and 60 mg (1.6%) of **10**. The 2.85 g sample of **10** was combined with similar material from a previous pilot run, and the composite (3.7 g) was recolumned as above. The best fractions (TLC) of **10** were combined, concentrated to a small volume, filtered to remove silica, frozen, and lyophilized to give a hygroscopic glass; wt 2.40 g. MS (negative): *m/z* 408 $(M - H)^-$ of **10** with a peak of nearly equal strength at 410 because of the ^{81}Br isotope peak. HPLC (method B): 89.4% **11** (retention time, 5.7 min) and 8.5% **9** (retention time, 2.2 min). TLC (MeCN/1 N NH_4OH 3:2): R_f 0.46. ^1H NMR (D_2O + sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4): δ 2.45, 3.39 (2 m, 1 each, H-2′s), 4.10 (complex m, 3H, 5′-CH_2 and H-4′), 6.52 (t, 1H, H-1′, $J = 7.3$), 8.17 (s, 1H, H-2). $\delta = 0.0$. Likewise, the best column fractions containing **11** were combined, evaporated, and dried as above; wt 0.26 g. MS (negative): *m/z* 488, 490 $(M - H)^-$ of **11**. HPLC (method B): 96% **11** (retention time, 20.0 min). TLC (MeCN/1 N NH_4-OH): R_f 0.07. ^1H NMR (D_2O): 2.63, 3.45 (2 m, 2H, H-2′), 4.08, 4.15 (2 m, 2H, 5′-CH_2), 4.32 (m, 1H, H-4′), 5.01 (m, 1H, H-3′), 6.59 (t, 1H, H-1′, $J = 7.4$), 8.22 (s, 1H, H-2).

Bis(pivaloyloxymethyl) 8-Bromo-2′-deoxyadenosine-5′-monophosphate (12). The remainder after analyses of the 2.4 g sample of **10** was dried by azeotropic distillation with benzene (see **8**) to give 2.12 g of amorphous powder. A stirred suspension of the powder (3.08 mmol calculated as 89% **10**·$[Et_3N]_2$) in a mixture of *N*-methylpyrrolidinone (27 mL, NMP), Et_3N (3.63 g, 35.9 mmol), and chloromethyl pivalate (7.30 g, 48.6 mmol) was heated in a 60 °C oil bath under a N_2 atmosphere for 5 h. The cooled mixture was diluted with EtOAc (400 mL), and the soft precipitate (mostly Et_3N·HCl, TLC) was filtered off. The filtrate was washed by shaking with H_2O (3 × 50 mL), dried (Na_2SO_4), and evaporated to give a mixture of crude **12** and NMP; wt 6.8 g. Column chromatography on SG with $CHCl_3$/MeOH 95:5 as eluent gave 0.82 g (41%) of impure **12**. The best fractions containing **12** were combined (0.45 g) and recolumned. Again, the best fractions (TLC) were pooled, evaporated, and reevaporated with CH_2Cl_2 to give a brittle foam, which was dried in vacuo over P_2O_5 at room temperature for 48 h; yield 379 mg (19%); mp softens to a syrup ∼40 °C. This apparently homogeneous (TLC) sample of **12** was shown by HPLC (method D) to contain 4.9% of another component tentatively believed to be the tetrakis-(pivaloyloxymethyl) ester of the diphosphate. Elemental analyses and calculated extinction coefficients reflect the approximate composition $C_{22}H_{33}BrN_5O_{10}P$·$0.05C_{34}H_{54}BrN_5O_{17}P_2$. MS (positive): *m/z* 638 $(M + H)^+$ and ^{81}Br isotope peak at 640; 214, 216 $(B + 2H)^+$. TLC ($CHCl_3$/MeOH 9:1): R_f 0.37. UV (EtOH): 0.1 N HCl: 263 (18.2); pH 7 buffer; 212 (25.4), 265 (16.7); 0.1 N NaOH: 265 (17.1). ^1H NMR ($CDCl_3$): δ 1.22, 1.23 (2 s, 18.8 H*, pivaloyloxymethyl CH_3), 2.43, 3.55 (2 m, 1 each, H-2′ s), 3.17 (br s, 1H, 3′-OH), 4.19 (m, 1H, H-4′), 4.26, 4.44 (2 m, 1 each, 5′-CH_2), 5.01 (br s, 1H, H-3′), 5.53 (br s, 2H, 6-NH_2), 5.65 (complex m, 4.5H*, O−CH_2−O), 6.44 (t, 1H, H-1′, $J = 6.9$), 8.28 (s, 1H, H-2). Anal. ($C_{22}H_{33}BrN_5O_{10}P$·$0.05C_{34}H_{54}BrN_5O_{17}P_2$) C, H, N, Br. *Both of these signals include the "extra" protons from the minor component.

Biological Methodology. CEM cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1 mg/mL sodium bicarbonate, 10 units/mL penicillin, 10 μg/mL streptomycin, and 50 μg/mL gentamycin. Cell numbers were determined with a Coulter Counter, and the concentration of compound that resulted in 50% inhibition of cell growth over a 96 h incubation period was determined (IC_{50}) and used as a measure of cytotoxicity.

The effect of the compounds on the incorporation of radiolabeled precursors ([8-^{14}C]adenine, [5-^3H]Urd, [methyl-^3H]-thymidine, or [4,5-^3H]leucine; Moravek Biochemicals, Brea, CA) into RNA, DNA, or protein was determined as described.[15,16] The incorporation of Urd or adenine into RNA is determined by subtracting the incorporation of radiolabel into the alkali stable/acid precipitable fraction (DNA) from the total acid precipitable fraction (DNA plus RNA). Urd and adenine are primarily incorporated into RNA but can also be incorporated into DNA as 2′-deoxycytidine or 2′-deoxyadenosine,

respectively. The incorporation of thymidine and leucine into acid precipitable material is a measure of their incorporation into DNA and protein, respectively.

The acid soluble extracts were obtained from CEM cells incubated with the nucleotide analogues and were analyzed using strong anion exchange HPLC as described.[17] 8-Br-dATP and 8-aza-dATP were made by Sierra Bioresearch (Tucson, AZ) from the nucleoside that was provided by us. The structures of both compounds were verified by phosphorus and hydrogen NMR.

Urd kinase activity was measured in cell-free extracts of CEM cells as described.[18] Urd uptake was measured using the oil-stop method described by Paterson et al.[19] DNA polymerase α was purified 52-fold from CEM cells to a specific activity of 120 nmol/mg/hr, and its activity was measured in 50 μL reactions as described.[20] The inhibition constants (K_i) of 8-aza-dATP and 8-Br-dATP were determined from a plot of the slopes of the Lineweaver−Burk lines vs the concentration of inhibitor (three concentrations of each compound were used in the determination of the K_i). Each line of the Lineweaver−Burk plots was determined by linear regression from at least five points. The experiment was done three times.

Acknowledgment. We thank the Analytical and Spectroscopic Laboratory, including Dr. James M. Riordan, Mr. Mark D. Richardson, Ms. Sheila R. Campbell, and Ms. Joan C. Bearden, for analytical data. Support for this research was provided by the National Cancer Institute, National Institutes of Health, through Grant P01 CA34200.

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JM020107S