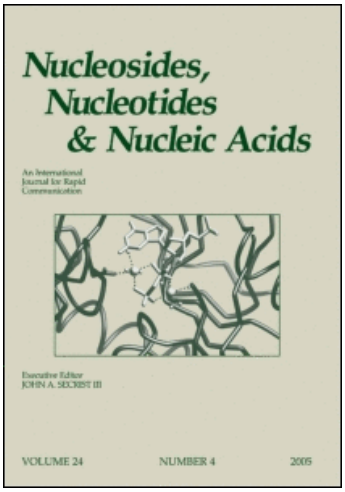

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BIOLOGICAL ACTIVITY AND PHOSPHORYLATION OF 2'-AZIDO-2'-DEOXYURIDINE AND 2'-AZIDO-2'-DEOXYCYTIDINE

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Abstract: 2'-Azido-2'-deoxyuridine and 2'-azido-2'-deoxycytidine were evaluated for their inhibitory activity against ribonucleotide reductase and for subsequent cell growth inhibition. Their mono- and di-phosphates were synthesized and their inhibitory activities against the reductase were also determined in a permeabilized cell system, along with the two nucleosides. The results of the present study identify the first phosphorylation step involved in the conversion of the two azidonucleosides to the corresponding diphosphates to be rate-limiting in the overall activation.

INTRODUCTION

2'-Azido-2'-deoxyuridine 5'-diphosphate (N_3dUDP) and 2'-azido-2'-deoxycytidine 5'-diphosphate (N_3dCDP) are potent inhibitors of ribonucleotide reductase (EC 1.17.4.1), an important target for therapeutic intervention in cancer.[1,2] Recently, inhibition of the reductase has been also promoted as a new strategy of treating AIDS.[3,4] As studied in a cell free system, N_3dUDP was reported to inactivate the enzyme from *E. coli* at a stoichiometric ratio.[5] These diphosphates are, however, of little therapeutic utility due to membrane-impermeability caused by the negative charges on the 5'-diphosphate. One of these diphosphate precursors, 2'-azido-2'-deoxycytidine (N_3dCyd), inhibits DNA synthesis in several cell lines via reductase inhibition, after phosphorylated to N_3dCDP by cellular kinases.[6,7] Interestingly, however, 2'-azido-2'-deoxyuridine (N_3dUrd) has received little attention, although its diphosphate appears to be as potent as N_3dCDP in the reductase inhibition in a cell-free assay system.[5,8] The weak activity of N_3dUrd most

likely reflects the apparent difficulty of intracellular phosphorylation, which usually constitutes the rate-limiting step in its activation to the corresponding nucleotides.

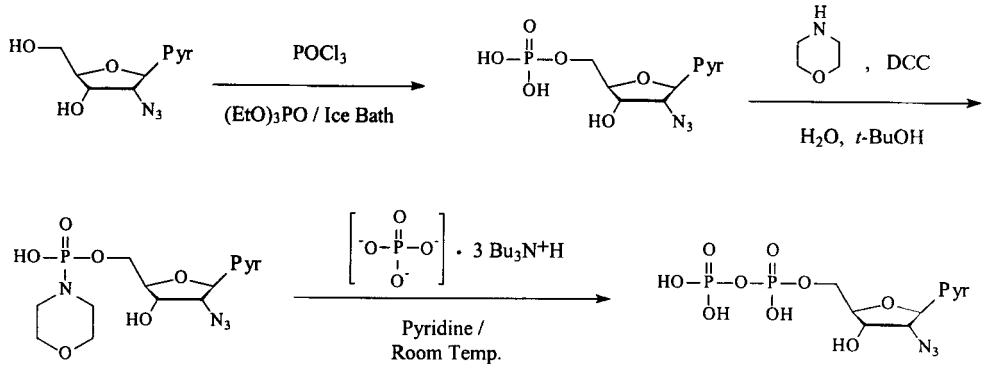
In the present study, we examined the ease with which N_3dUrd and N_3dCyd are phosphorylated by measuring the biological activity in two different cell lines, 3T6 and CHO. These cells have different levels of deoxycytidine kinase. We further report inhibitory activity of the azidonucleoside monophosphates (N_3dCMP and N_3dUMP) and diphosphates (N_3dCDP and N_3dUDP) towards the reductase in a permeabilized cell system. The results support that the first phosphorylation step is rate-limiting in the activation of both azidonucleosides, thus justifying a prodrug approach of azidonucleoside monophosphate.

SYNTHESIS

Monophosphates of azidonculeosides were obtained in anhydrous triethyl phosphate without protecting the hydroxyl group at 3′ position with $POCl_3$ as described elsewhere.[9] 5′-Diphosphates were then prepared via the 5′-phosphomorpholidate.[10] All of the azidonucleotides used in the present study were purified on a preparative HPLC and then converted to sodium salts.

BIOLOGICAL ACTIVITY

The azidonucleosides must be phosphorylated twice to become an inhibitor of the nucleotide reductase. The first phosphorylation step was examined by correlating the deoxycytidine kinase level with the cell growth inhibition. Two cell lines, 3T6 and CHO cells, were incubated with the azidonucleosides, N_3dUrd and N_3dCyd, for 48 hr. As shown in **Fig. 1**, N_3dCyd significantly inhibited the growth of 3T6 cells with IC_{50} approximately at 50 μM. However, against CHO cells, its activity was relatively weak with $IC_{50} > 0.3$ M. N_3dUrd was active against neither 3T6 nor CHO cells. The level of deoxycytidine kinase was measured as described in the literature.[11] The enzyme activity was approximately 3-fold higher in 3T6 than in CHO cells: 0.27 vs. 0.086 mmol/mg protein/min. These findings are consistent with the hypothesis that the higher level of



Scheme 1. Preparation of 2'-azido-2'-deoxynucleoside 5'-diphosphate from the azidonucleoside via its 5'-monophosphate, where Pyr is cytosine or uracil.

the kinase renders cells more sensitive to the azidonucleosides. Thus, N_3dUrd appears to be a poor substrate for deoxycytidine kinase or other cellular kinases.

In investigating the conversion of the monophosphate to diphosphate, a permeabilized cell system was adopted. It allows not only the nucleoside but also the charged nucleotides to access cellular kinases as well as ribonucleotide reductase without affecting normal cellular function. It was reported that CHO cells become permeable to nucleotides during incubation in 1% polyoxyethylene sorbitan monooleate (Tween 80) containing 0.25 M sucrose.[12] Such an assay system has been successfully applied to determination of ribonucleotide reductase.[13] The procedure provided an opportunity to determine the efficiency at which the azidonucleosides and their 5'-monophosphates are phosphorylated to corresponding diphosphates to result in reductase inhibition.

The permeabilized cells, either CHO or 3T6 cells, were incubated for 30 min at 37 °C with varying concentrations of N_3dCyd and the N_3dUrd derivatives. The reductase activity was then determined.[13] As summarized in **Table 1**, both N_3dCMP and N_3dCDP inhibited the reductase activity in CHO cells in a concentration-dependent manner, while N_3dCyd did not. The value of IC_{50} observed with N_3dCMP (5.8 μM) is slightly higher than that of N_3dCDP (2.3 μM). Similarly, both N_3dUMP and N_3dUDP suppressed the reductase activity. As in the case with intact cells, however, N_3dUrd again failed to show any significant inhibitory activity up to 350 μM. Similar trend was observed with the

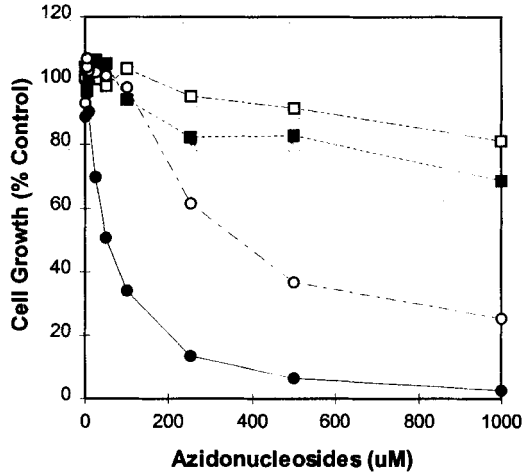


Figure 1. Inhibition of CHO (open symbols) and 3T6 (closed symbols) cell growth by the azidonucleosides, N_3dCyd (circles) and N_3dUrd (squares) after 48-hr incubation. Each point is the average of triplicate determinations. The ranges were less than 5% in all cases.

Table 1. Effect of N_3dCyd and N_3dUrd on ribonucleotide reductase activity in the permeabilized cells. Each value is the average of duplicate determinations. The ranges were less than 10 % in all cases.

Agents Tested	IC_{50} (μM)	
	in CHO cell	in 3T6 Cell
N_3dCyd	>350	>350
N_3dCMP	5.8	34
N_3dCDP	2.3	26
N_3dUrd	>350	>350
N_3dUMP	38	60
N_3dUDP	21	65

permeabilized 3T6 cell system. Taken together with the results described in the previous section, these observations present a convincing argument that the key step in the activation of the azidonucleosides is the first phosphorylation.

The second phosphorylation step was further examined by blocking it with an ATP analog. In the catalytic action of ribonucleotide reductase, a nucleoside 5′-triphosphate serves as an allosteric effector rather than as a phosphate donor or energy source.[1] As a

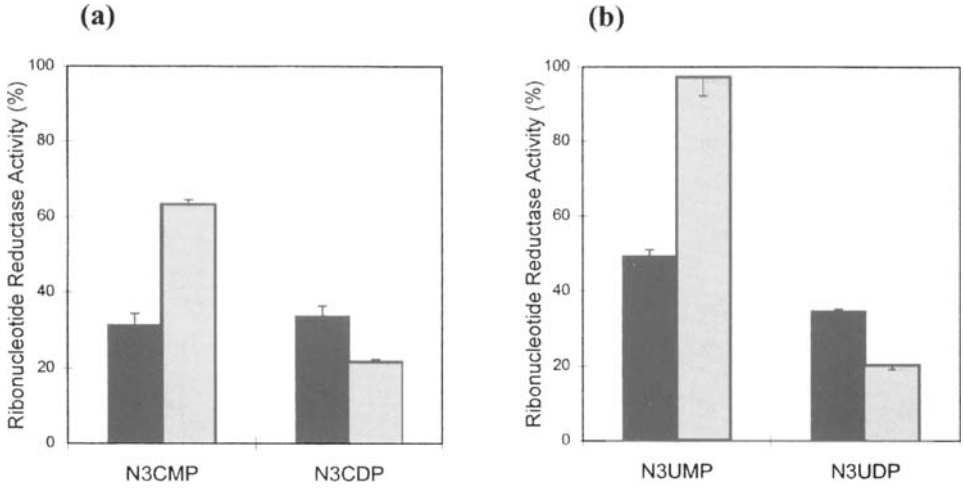


Figure 2. Ribonucleotide reductase activity determined in permeabilized CHO cells in the presence of 10μM N_3dCMP or N_3dCDP (**a**) and 50μM N_3dUMP or N_3dUDP (**b**) with ATP (dark shade) or 5'-adenylylimidodiphosphate (AMP-PNP; light shade). The enzyme activity in the absence of any test compound with ATP was taken 100%. The bar represents standard deviation (n=3).

positive allosteric effector of cytidine diphosphate reduction, ATP can be effectively replaced with its analog 5'-adenylylimidodiphosphate (AMP-PNP).[14] The terminal phosphate group in AMP-PNP is connected to adenosine diphosphate via a non-hydrolyzable bond and thus unavailable in the phosphate transfer catalyzed by a kinase. As a result, if ATP is replaced with AMP-PNP, the reaction catalyzed by the reductase remains intact, but phosphorylation by the cellular kinases should decrease significantly.

When ATP was replaced with the same concentration (2 mM) of AMP-PNP, the inhibition of the reductase by the azidonucleoside 5'-monophosphates was greatly reduced, but the azidonucleoside diphosphates still maintained their inhibitory activity. As shown in **Fig. 2a**, 10 μM N_3dCMP inhibited the enzyme as much as 67% with ATP but only 37% with AMP-PNP. On the other hand, the enzyme activity was not significantly affected by 10 μM N_3dCDP: 66% vs. 78% inhibition with ATP and with AMP-PNP, respectively. These results indicated that N_3dCDP is the true inhibitor of ribonucleotide reductase and that N_3dCMP is merely a precursor whose activity depends

on its phosphorylation. That the inhibition of the reductase by N_3dCMP was not completely abolished in the presence of AMP-PNP is likely due to phosphorylation with the residual endogenous ATP.

In the assay with 2 mM ATP, N_3dCDP can be further phosphorylated to its triphosphate N_3dCTP. The latter can engage itself in the regulation of the reductase as well as other cellular biochemistry in some capacity. For instance, it is possible that N_3dCTP can serve as an allosteric effector. If N_3dCTP does not significantly influence the reductase activity, the decrease in the effective concentration of N_3dCDP by phosphorylation to the triphosphate should result in a decreased inhibition of the reductase. If, on the other hand, N_3dCTP interferes with the reductase as a negative effector to a greater extent than N_3dCDP, the inhibition observed with ATP will be greater than with AMP-PNP. The observation that the enzyme inhibition by N_3dCDP was almost equal in both cases supports that N_3dCTP does not affect the reductase activity.

Similar results were also observed with uridine series (**Fig. 2b**). N_3dUMP showed a decreased inhibitory activity in the assay with AMP-PNP. At 50 μM N_3dUMP, 50% of the reductase activity was inhibited with ATP but little inhibition, less than 3%, was observed when ATP was replaced with AMP-PNP. The greater inhibition of the reductase by N_3dUDP with AMP-PNP than with ATP is attributed to the higher effective concentration of the diphosphates resulted by its impaired phosphorylation to the triphosphate.

The results obtained with AMP-PNP thus further support the conclusion that the reductase inhibition observed with the azidonucleoside monophosphates originates from their corresponding diphosphates that are in turn generated by phosphorylation of the monophosphates.

The processes described thus far are summarized in **Fig. 3**. The phosphorylation of the azidonucleosides to their monophosphates is slow especially for N_3dUrd. The azidonucleoside monophosphates are, however, readily phosphorylated to the corresponding diphosphates which inhibit ribonucleotide reductase.

As a result, both azidonucleoside monophosphates and diphosphates showed the inhibitory activity against the reductase at an almost identical potency (**Table 1**).

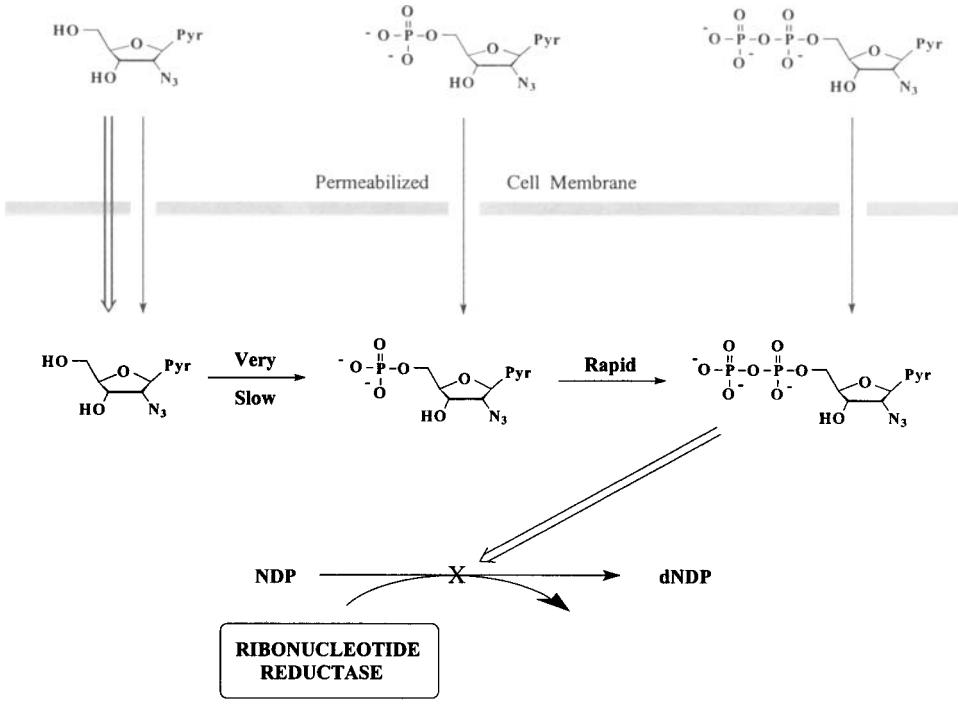


Figure 3. Phosphorylation of the azidonucleosides (N_3dCyd and N_3dUrd) and their monophosphates (N_3dCMP and N_3dUMP) to the diphosphates (N_3dCDP and N_3dUDP) in the permeabilized cell system. Pyr is cytosine or uracil. The reduction of nucleoside diphosphate (NDP) to deoxynucleoside diphosphate (dNDP) by ribonucleotide reductase is inhibited by azidonucleoside diphosphate that can enter the cell through pores transiently formed by 1% Tween 80 or be generated from N_3dUMP by phosphorylation.

Cellular nucleoside kinases responsible for phosphorylation of nucleoside analogs generally show a rather stringent substrate specificity,[15] while nucleoside monophosphate kinases do not.[16] Our results with the azidonucleosides and the azidonucleotides also support this consensus.

In summary, the present study presents evidence that the phosphorylation of N_3dUrd and N_3dCyd in the cell is the critical step in conversion to their corresponding diphosphates, potent inhibitors of nucleotide reductase. It thus provides a rationale for developing a prodrug strategy with their monophosphates in circumvent the rate-limiting step.[17]

EXPERIMENTAL

Nuclear magnetic resonance spectra, ^1H NMR and ^{31}P NMR, were recorded on a Varian VXR-300S NMR spectrometer. ^1H and ^{31}P chemical shifts are reported in ppm relative to TMS (δ) and to 85 % H_3PO_4 (δ), respectively. Mass spectra were obtained on a Finnigan TSQ-700 triple quadrupole mass spectrometer, using atmospheric pressure chemical ionization (APCI). Analytical high-pressure liquid chromatography (HPLC) was performed on a Hewlett Packard 1090 Chromatograph on a reversed phase column (BDS-Hypersil®-C8, 250 x 4.6 mm; Keystone Scientific Inc.). Preparative HPLC was performed on a Rainin HPXL Chromatograph using a reversed phase column (Zorbax®-C18, 250 x 21.2 mm, DuPont). UV spectra were recorded on a Hewlett Packard 300 Diode Array Spectrophotometer. 2′-Azido-2′-deoxyuridine and 2′-azido-2′-deoxycytidine were purchased from Sigma Chemical Co. (St. Louis, MO). All the other chemicals and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chinese hamster ovary (CHO) and 3T6 cells were obtained from American Type Culture Collection (Rockville, MD).

2′-Azido-2′-deoxyuridine 5′-Monophosphates (N_3dUMP): A mixture of 2′-azido-2′-deoxyuridine (200 mg, 0.74 mmol) and $POCl_3$ (300 μL, 3.2 mmol) in anhydrous $(EtO)_3PO$ (2 mL) was stirred overnight at 4 °C. Triethylammonium bicarbonate (1 M, pH 8.5, 6.5 mL) was added and the resulting mixture was extracted with Et_2O (3 × 10 mL). The product N_3dUMP in the aqueous layer was collected and purified on a 2.5 x 30 cm column of Sephadex A-25 with 0-0.5 M linear gradient (2 L) of $Et_3NH^+HCO_3^-$ (pH 7.5). The appropriate fractions were identified by UV absorbance, pooled, and evaporated in vacuo. To remove the inorganic phosphate still remaining, the residue was dissolved in H_2O (10 mL) and $Ba(OH)_2$ (185 mM) was then added until the pH of the solution was 12. Carbon dioxide was then bubbled in to adjust the pH to 7. The mixture was centrifuged to remove precipitated $Ba_3(PO_4)_2$ and the barium salt of N_3dUMP (^1H-decoupled ^{31}P NMR (D_2O): δ 4.22, singlet) was obtained from the supernatant. The barium salt of N_3dUMP in H_2O was passed through a column of Dowex-50 x 8 (H^+ form) ion exchange resin. Elution with H_2O followed by lyophilization to give 170 mg of N_3dUMP (free acid, 0.49 mmol) in 65 % yield. UV: λ_{max} (H_2O) 262 nm (ε 9.6 × 10^3); ^1H NMR (D_2O): δ 7.77

(d, 1H, J = 8.1 Hz, H-6), 5.84 (d, 1H, J = 4.9 Hz, H-1'), 5.78 (d, 1H, J = 8.1 Hz, H-5), 4.40 (t, 1H, H-3'), 4.19 (t, 1H, H-2'), 3.97-4.11 (m, 3H, H-5' and H-4'); ^{1}H-decoupled ^{31}P NMR (D_2O): δ -0.06 (s); MS: *m/z* 348 ([M - H]$^-$).

2'-Azido-2'-deoxycytidine 5'-Monophosphates (N$_3$dCMP): N_3dCMP was prepared by the same synthetic method as N_3dUMP. The overall yield was 57 %. UV: λ_{max}(H_2O) 272 nm (ε 7.7 × 10^3). ^{1}H NMR(D_2O): δ 7.98 (d, 1 H, J = 7.6 Hz, H-6), 5.99 (d, 1 H, J = 7.6 Hz, H-5), 5.92 (d, 1 H, J = 4.6 Hz, H-1'), 4.40 (t, 1 H, H-3'), 4.21 (t, 1 H, H-2'), 4.05-4.08 (m, 1 H, H-4'), 3.77-3.93 (m, 2 H, H-5'). ^{31}P NMR(D_2O): δ 3.82 (s). MS: *m/z* 347 ([M-H]$^-$).

2'-Azido-2'-deoxyuridine 5'-Diphosphates (N$_3$dUDP): Freshly distilled dicyclohexyl carbodiimide (670 mg, 3.3 mmol) in *t*-butanol (15 mL) was added dropwise to a mixture of morpholinium salt of N_3dUMP (0.7 mmol), water (10 mL), morpholine (0.28 mL) and *t*-butanol (10 mL) over a period of 5 hr while the reaction mixture was under reflux. The mixture was further refluxed overnight, by which time the reaction was completed as judged by ^{31}P NMR. After cooled to room temperature, the mixture was diluted with water and then filtered. The filtrate was evaporated in vacuo to near dryness. These processes were repeated until no more solid appeared upon addition of water. The solid thus obtained was once again dissolved in water (15 mL), washed with diethyl ether (3 × 10 mL), and then evaporated in vacuo to dryness. The crude phosphomorpholidate of N_3dUrd [^{31}P NMR(D_2O): δ 7.36, singlet] was further dried three times by evaporation with 10 mL of anhydrous pyridine. Separately, after tributylammonium phosphate is prepared by mixing tributyl amine (500 μL, 2.1 mmol) and 85 % *o*-phosphoric acid (144 μL, 2.1 mmol) in 10 mL pyridine, it was also dried in the same way with pyridine. The two pyridine solutions were combined and evaporated to dryness, with successive addition and evaporation of two 10-mL portions of pyridine. Finally, the residue was dissolved in pyridine and the solution was stirred at room temperature under an argon atmosphere for 4 days or until the reaction was complete as judged by ^{31}P NMR. After removal of pyridine by evaporation in vacuo, crude N_3dCDP was purified by HPLC on Zorbax C18 column. The overall yield was 60%. UV: λ_{max}(H_2O) 262 nm. ^{1}H NMR(D_2O): δ 7.75 (d, 1 H, J = 8.1 Hz, H-6), 5.82 (d, 1 H, J = 4.9 Hz, H-1'), 5.76 (d, 1 H, J = 8.1 Hz, H-5), 4.38 (t, 1 H, H-3'), 4.17 (t, 1 H, H-2'), 3.96-4.10 (m, 3 H, H-5' and

H-4'). ^{31}P NMR (D_2O): δ 8.05 (d, J = 21.3 Hz), 11.16 (d, J = 21.3 Hz). MS: *m/z* 428 ([M-H]$^-$).

2'-Azido-2'-deoxycytidine 5'-Diphosphates (N_3dCDP): N_3dCDP was prepared by the same synthetic method as N_3dUDP. Phosphomorpholidate of N_3dCyd was again confirmed prior to phosphorylation [^{31}P NMR(D_2O): δ 7.89, singlet]. The overall yield was 55 %. UV: λ_{max}(H_2O) 272 nm. ^{1}H NMR(D_2O): δ 7.86 (d, 1 H, J = 7.6 Hz, H-6), 5.98 (d, 1 H, J = 7.6 Hz, H-5), 5.86 (d, 1 H, J = 3.9 Hz, H-1'), 4.46 (t, 1 H, H-3'), 4.16-4.19 (m, 1 H, H-2'), 4.04-4.10 (m, 3 H, H-4' and H-5'). ^{31}P NMR(D_2O): δ 7.89 (d, J = 21.9 Hz), 11.09 (d, J = 21.9 Hz). MS: *m/z* 427 ([M-H]$^-$).

Cell Growth Test The growth of 3T6 and CHO cells with or without N_3dUrd or N_3dCyd was determined using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay as described elsewhere.[18] Briefly, rapidly growing cells were harvested, counted, and seeded into 96-well microtitre plates. For both cell lines, 3,000 cells in 150 μL were seeded to each well of the plate. After 24 hr of incubation, 20 μL of the azidonucleoside solution was added to each culture well in triplicate (time-zero), and further incubated for 48 hr at 37 °C. To each well, 20 μL of a freshly prepared 5 mg MTT/ml PBS was added and continuously incubated for another 4 hr. After the medium was carefully suctioned off, the plate was placed inverted to ensure that all of the residual solution was removed. Finally, 200 μL of 0.05 N-HCl/ DMSO was added to dissolve the MTT formazan produced by viable cells. Absorbance at 490 nm was measured with a Bio-Rad Model 3350 microplate reader.

Determination of Deoxycytidine Kinase Activity The dCyd kinase activity was determined following a literature procedure.[11] In essence, the radioactivity associated with the deoxycytidine 5'-phosphates produced by phosphorylation of [^{14}C]dCyd is measured after [^{14}C]dCyd is incubated at 37°C for a given period of time with cell extracts. The separation of the radio-labeled nucleotide product from remaining [^{14}C]-dCyd can be easily achieved by using an anionic exchange paper disc. The assay system consisted of [^{14}C]dCyd (50 μM; 0.073 μCi), ATP (5 mM), DTT (3 mM), magnesium chloride (5 mM), and the cell extract (100 μg protein) in a 50 mM HEPES buffer at pH 7.2. The final volume of the reaction mixture was 30 μL. The assay was run in triplicate

at several time points during the incubation; 5, 10, 15, 20, and 35 min. The reaction was quenched by placing the reaction mixture for 3 min in boiling water. Heated controls served as background counts. After centrifugation, 15 μL of reaction mixture was spotted on DE81 ion exchange paper disc (Whatman, Fairfield, NJ). The disc was air-dried and then washed three times, each with 10 ml of distilled water. The disc was finally placed in a scintillation vial and 1 ml of 0.4 N KCl/0.1 N HCl was added to the vial to elute out the nucleotide product from the disc. After mixing with scintillation cocktail, the radioactivity was counted on a Packard Model 4430 liquid scintillation counter.

Ribonucleotide Reductase Assay in Permeabilized Cell System Tween 80 was used to make cell membarne permeable to nucleotides. Exponentially growing monolayer cells were harvested with 0.05% trypsin buffer (GIBCO) and suspended in a fresh culture medium at a concentration of 10^7 cells/mL in the permeabilizing buffer. The latter consisted of 1% Tween 80 (Sigma), 0.25 M sucrose, and 2 mM dithiothreitol (DTT) in 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer at pH 7.2. The cells were incubated at room temperature for 30 min with occasional agitation and then centrifuged. The cell pellet was resuspended in a fresh permeabilizing buffer at the concentration of 2.5×10^7 cells/mL. An aliquot of 200 μL, which contained 5×10^6 cells, was then added to 100 μL of an assay buffer containing [^{14}C]CDP (the substrate) and an inhibitor. The reaction mixture, at a final volume of 300 μL, contained 50 mM HEPES buffer at pH 7.2, 2 mM ATP, 6 mM DTT, 8 mM $MgCl_2$, 10 μM [^{14}C]CDP (1.0 μCi /assay), 0.67% Tween 80, 0.17 mM sucrose and a given concentration of an inhibitor. The mixture was agitated on a shaker (Precision Scientific) for 30 min at 37°C, and then the reaction was terminated by boiling. After centrifugation, 200 μL of the supernatant was added to 1.0 mL of 21 mM Tris buffer at pH 9.0, which contained 1.5 mg of *Crotalus adamanteus* venom, 5.3 mM $MgCl_2$, and 4.4 mM deoxycytidine (dCyd). This dephosphorylation was performed for 30 min at 37 °C and then terminated by boiling. The nucleosides converted from the corresponding nucleotides by treatment of the snake venom were separated on a 10 × 80 mm Dowex-1-borate column. The radioactive deoxycytidine ([^{14}C]dCyd) originated from [^{14}C]CDP via [^{14}C]dCDP was eluted from the column with 4 mL of distilled water. [^{14}C]dCDP was expected from the action of the reductase. To the eluent was added 16 mL of Scintiverse (Fisher Scientific, Fair Lawn,

NJ) and then radioactivity was determined with a Packard model 4430 liquid scintillation counter. The radioactivity recovered in the eluent reflects the reductase activity.

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