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Research Article

Cellular Delivery of Nucleoside Diphosphates: A Prodrug Approach

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Purpose. This study is concerned with cellular delivery/generation of 2′-azido-2′-deoxyuridine and -deoxycytidine diphosphate (N_3UDP or N_3CDP), potent inhibitors of ribonucleotide reductase. It characterizes the phosphorylation steps involved in the conversion of 2′-azido-2′-deoxyuridine (N_3Urd) and 2′-azido-2′-deoxycytidine (N_3Cyd) to the corresponding diphosphates and explores a prodrug approach in cellular delivery of the inhibitor which circumvents the requirement of deoxynucleoside kinases.

Methods. Cell growth of CHO and 3T6 cells of known deoxycytidine kinase level was determined in the presence of N_3Urd and N_3Cyd. Activity of ribonucleotide reductase was determined in the presence of the azidonucleosides as well as their mono- or di-phosphates in a Tween 80-containing permeabilizing buffer. A prodrug of 5′-monophosphate of N_3Urd was prepared and its biological activity was evaluated with CHO cells as well as with cells transfected with deoxycytidine kinase.

Results. N_3Urd failed to inhibit the growth of both cell lines, while N_3Cyd was active against 3T6 cells and moderately active against CHO cells. These results correlate with the deoxycytidine kinase levels found in the cells. Importance of the kinase was further established with the finding that the nucleoside analogs were inactive as reductase inhibitors in a permeabilized cell assay system while their mono- and di-phosphates were equally active. The prodrug was active in cell growth inhibition regardless of the deoxycytidine kinase level.

Conclusions. The azidonucleosides become potent inhibitors of the reductase by two sequential phosphorylation steps. The present study indicates that the first step to monophosphate is rate-limiting, justifying a prodrug approach with the monophosphate.

KEY WORDS: 2′-azido-2′-deoxycytidine; 2′-azido-2′-deoxyuridine; 2′-azido-2′-deoxyuridine 5′-diphosphate; ribonucleotide reductase; phosphorylation of nucleoside analogue; prodrug; anticancer agent.

INTRODUCTION

Ribonucleoside diphosphate reductase (EC 1.17.4.1) is involved in *de novo* synthesis of the DNA building blocks, deoxynucleotides (1). The enzyme activity is high in rapidly dividing neoplastic cells while it is almost undetectable in non-dividing cells (2). As such, the reductase is a potential target for therapeutic intervention in cancer (3,4). Similarly, inhibition of the reductase has been recently promoted as a new strategy to treat AIDS (5). Hydroxyurea is widely used in the clinic, however, its reductase inhibition is relatively weak and *in vivo* effectiveness suffers from the difficulty in maintaining a therapeutically meaningful level (6). Although a large number of potentially powerful reductase inhibitors has been investigated for its use in the clinic, none has appeared to be entirely satisfactory (7).

2′-Azido-2′-deoxyuridine 5′-diphosphate (N_3UDP) and 2′-azido-2′-deoxycytidine 5′-diphosphate (N_3CDP) are extremely potent inhibitors of the reductase. N_3UDP, for instance, inactivates the enzyme from *E. coli* at a stoichiometric ratio (8). These diphosphates are, however, of little therapeutic utility, due to membrane-impermeability caused by the negative

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ABBREVIATIONS: AraC, 1-β-D-arabinofuranosylcytosine; CDP, cytidine 5′-diphosphate; CHO, chinese hamster ovary cell; dCyd, deoxycytidine; IC_{50}, inhibitory concentration of 50%; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; N_3CDP, 2′-azido-2′-deoxycytidine 5′-diphosphate; N_3CMP, 2′-azido-2′-deoxycytidine 5′-monophosphate; N_3Cyd, 2′-azido-2′-deoxycytidine; N_3UDP, 2′-azido-2′-deoxyuridine 5′-diphosphate; N_3UMP, 2′-azido-2′-deoxyuridine 5′-monophosphate; N_3Urd, 2′-azido-2′-deoxyuridine; $(POM)_2$-N_3UMP, bis(pivaloyloxymethyl) ester of N_3UMP.

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charges on 5′-diphosphate at a physiological pH. One of these diphosphate precursors, 2′-azido-2′-deoxycytidine (N_3Cyd) inhibits DNA synthesis in several cell lines, via reductase inhibition subsequent to phosphorylation by cellular kinases (9). Interestingly, however, 2′-azido-2′-deoxyuridine (N_3Urd) has received little attention, although its diphosphate appears to be as potent as N_3CDP in the reductase inhibition in a cell-free assay system (8,10).

The present study is concerned with cellular delivery or generation of the diphosphates. Effects of the two azidonucleosides, N_3Urd and N_3Cyd, and related nucleotides on the cytotoxicity and reductase activity were measured in several cell lines under various conditions. In accordance with many other anticancer or antiviral nucleoside analogs in general (11), the study results identify the first phosphorylation step as rate-limiting in the activation and highlight the merit of a prodrug approach with nucleoside monophosphates.

MATERIALS AND METHODS

Chemicals and Instruments

N_3Urd, N_3Cyd, [^{14}C]dCyd, and 1-β-D-arabinofuranosylcytosine (AraC) were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. N_3UMP, N_3CMP, N_3UDP, and N_3CDP were prepared as described in the literature (12). Unless specified otherwise, all other chemicals and cell culture media used in the present study were purchased from either Sigma or Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]cytidine 5′-diphosphate ([^{14}C]CDP) was obtained from Moravek Biochemicals (Brea, CA).

Analytical HPLC was performed on a Hewlett Packard Model 1090 using a reversed-phase column (BDS-Hypersil®-C8, 250 × 4.6 mm; Keystone Scientific Inc.). Preparative HPLC was performed on a Rainin system, Model HPXL, using a reversed-phase column (Zorbax®-C18, 250 × 21.2 mm, DuPont). UV spectra were recorded on a Hewlett Packard Model 300 diode array spectrophotometer.

Cells and Cell Culture

Chinese hamster ovary (CHO) and 3T6 cells were obtained from American Type Culture Collection (Rockville, MD). H1437 cells containing integrated deoxycytidine kinase gene (LNPO-dCK) and β-galactosidase gene (LNPO-LacZ) are described elsewhere (13). The transfected H1437 cell lines were established by selection with Geneticin (Life Technologies, Grand Island, NY) and tested for mRNA and protein expression as described elsewhere (13). CHO cells were maintained in α-minimal essential medium (α-MEM) supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO_2. Similarly 3T6 cells and H1437 cells were cultured in supplemented Dulbecco's Modified Eagle's Medium (DMEM) and in supplemented RPMI 1640, respectively. All experiments were performed with cells in exponentially growing phase. Cell number was determined on microscope with a hemocytometer.

MTT Cell Viability Assay

The growth of CHO, 3T6, and H1437 cells with a test compound was determined using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay as described elsewhere (14). Briefly, rapidly growing cells were harvested, counted, and seeded in 96-well microtitre plates. For CHO and 3T6 cells, 3,000 cells in 150 μl were seeded to each well of the plate and 10,000 cells were seeded for H1437 cells. After 24 hr of incubation, 20 μl of the test compound in solution was added to each well in triplicate (time-zero), and further incubated for 2 (CHO and 3T6) or 4 days (H1437) at 37°C. To each well, 20 μl of a freshly prepared 5 mg MTT/ml phosphate-buffered saline (PBS) was added and continuously incubated for another 4 hr. After the medium was suctioned off, 200 μl of 0.05 N-HCl/dimethylsulfoxide 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 enzyme activity was determined following a literature procedure (15). 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 to time with cell extracts. The separation of the radio-labeled nucleotide product from remaining [^{14}C]-dCyd was achieved with anionic exchange paper discs. The assay was run in triplicate at several time points during the incubation; 5,10,15,20, and 35 min and 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 CHO or 3T6 cells permeable to nucleotides (16). Exponentially growing cells were detached from the culture plates and then resuspended at concentration of 10^7 cells/ml in the permeabilizing buffer consisting of 1% Tween 80 (Sigma), 0.25 M sucrose, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer at pH 7.2 and 2 mM dithiothreitol (DTT). The cells were incubated at room temperature for 30 min and then, after centrifugation, 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 assay buffer containing enzyme substrate ([^{14}C]CDP) and a test compound such as N_3Cyd, N_3CMP, N_3CDP, N_3Urd, N_3UMP, N_3UDP, or hydroxyurea. 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 dCyd. This dephosphorylation was performed for 30 min at 37°C and then terminated by boiling.

The nucleosides converted from the corresponding nucleotides by the snake venom were separated on a 10 × 80 mm Dowex-1-borate column. The radioactive [^{14}C]dCyd derived from [^{14}C]deoxycytidine diphosphate that was formed from [^{14}C]CDP by the action of reductase was eluted from the column

with 4 ml of distilled water. After 16 ml of Scintiverse (Fisher Scientific, Fair Lawn, NJ) was added, radioactivity was determined with a Packard model 4430 liquid scintillation counter. The amount of radioactivity recovered in the eluate reflects the level of the reductase activity.

Synthesis and Stability of Bis[(Pivaloyloxy)Methyl] 2′-Azido-2′-Deoxyuridine 5′-Mono-Phosphate [$(POM)_2$-N_3UMP]

A mixture of N_3UMP (35 mg, 0.10 mmol) and tributylstannyl methoxide (65 mg, 0.20 mmol) in CH_3OH (3 ml) was stirred at 25°C for 30 min. Methanol was removed by evaporation. The last traces of CH_3OH was removed by repeated evaporation of the solution in CH_3CN (3 ml). To the residue in CH_3CN (3 ml), were added $Bu_4N^+Br^-$ (65 mg, 0.20 mmol) and iodomethyl pivalate (324 mg, 1.0 mmol). The latter was prepared by reacting chloromethyl pivalate with NaI in CH_3CN. The mixture was refluxed for 1 hr and then cooled to 25°C. The mixture was concentrated to a small volume (~ 0.3 ml) under reduced pressure and then applied on a silica gel column. The column was eluted with a mixture of CH_2Cl_2 and CH_3OH (95:5, v/v). The appropriate fractions were identified by UV absorption, pooled and evaporated under vacuum. The crude product was further purified by HPLC on a preparative Zorbax C18 column, using 40% CH_3CN/60% H_2O as the mobile phase at a flow rate of 15 ml/min. The desired product appeared at retention time 13 min. The final yield was 43 mg (0.075 mmol; 75% yield). ^1H NMR(CH_3OH-d_4): δ 7.60 (d, 1H, J = 8.1 Hz, H-6), 5.78 (d, 1H, J = 4.4 Hz, H-1′), 5.68 (d, 1H, J = 8.1 Hz, H-5), 5.59–5.64 (m, 4H, OCH_2O), 4.31–4.38 (m, 2H, H-2′ and H-3′), 4.29–4.21 (m, 1H, H-4′), 4.03–4.09 (m, 2H, H-5′), 1.16 (s, 18H, $C(CH_3)_3$); ^1H-decoupled ^{31}P NMR(CH_3OH-d_4): δ −3.71 (s); MS: *m/z* 578 ([M + H]$^+$). *Anal.* Calculated for $C_{21}H_{32}N_5O_{12}P$: C, 43.68; H, 5.59; N, 12.13. Found: C, 43.43; H, 5.54; N, 11.96.

The prodrug [$(POM)_2$−N_3UMP] was incubated at 37°C in the cell culture medium supplemented with 10% heat-inactivated fetal bovine serum, human serum (Sigma), or CHO cell homogenate. Rapidly growing CHO cells were suspended at 2×10^7 cells/ml in phosphate buffered saline (PBS) and homogenized in a glass vessel on ice using a motor-driven Teflon pestle (Thomas Scientific). The initial concentration of the prodrug was 0.1 mM for each experiment and the incubation mixture contained 1.0% EtOH. The stock solution of the prodrug in EtOH and the incubation medium were separately preincubated for 10 min at 37°C. At various time intervals, a 200 μl aliquot of the solution of the prodrug in the cell homogenate was transferred to 500 μl of cold CH_3CN. After vortexing, the mixture was centrifuged for 5 min at 12,000 ×g. The supernatant was directly injected to the HPLC column (BDS-Hypersil®-C8 column, 5 μm, 250 × 4.6 mm). The mobile phase consisted of 55% H_2O and 45% CH_3OH. The column was eluted at a flow rate of 1.0 ml/min. The area under the peak corresponding to the prodrug at capacity factor k′ = 2.0 was used in determining its concentration.

Appearance of N_3UMP from $(POM)_2$-N_3UMP

CHO cell homogenate was prepared at 6.3×10^7 cells/ml in PBS as described above. The initial concentration of $(POM)_2$-N_3UMP was 0.2 mM and the final concentration of EtOH in the incubation mixture was 1.0%. Before mixing, a stock solution of $(POM)_2$-N_3UMP in EtOH and the cell homogenate were separately preincubated for 10 min at 37°C. At various time intervals, an aliquot of 200 μl was transferred to 500 μl of cold CH_3CN. After vortexing, the mixture was centrifuged for 5 min at 12,000 ×g. The supernatant was subject to HPLC analysis. A gradient system described below separated N_3Urd (k′ = 0.4), N_3UMP (k′ = 1.4), monopivaloyloxymethyl ester of N_3UMP [$(POM)_1$-N_3UMP] (k′ = 2.0), and $(POM)_2$-N_3UMP (k′ = 3.0). Solvent **A** was 10 mM $Bu_4N^+H_2PO_4$ in H_2O while Solvent **B** was 100% CH_3CN. The following step gradients were adopted, in which the % of solvent **A** is indicated for a specified period of time. 0–1 min, 70%; 1–3 min, 70–50%; 3–5 min, 50%; 5–8 min, 50–25%; 8–10 min, 25%; 10–11 min, 25–70%; and finally 11–12 min, 70%. The column eluate corresponding to the peak of $(POM)_1$-N_3UMP was collected and identified by mass spectrometry. The other three peaks were identified by comparing their retention times with those of the authentic samples.

RESULTS

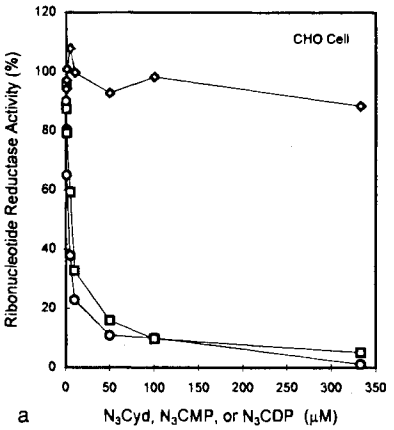
Inhibition of Cell Growth by 2′-Azido-2′-Deoxyribonucleosides and Its Relationship to Deoxycytidine Kinase Activity in 3T6 and CHO Cells

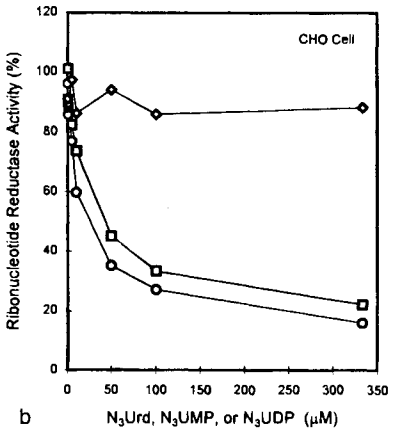
As shown in Table I, N_3Cyd showed a significant cytotoxicity against 3T6 fibroblast cell line with IC_{50} value approximately at 50 μM when the cell was continuously exposed to the compound for 48 hr. However, against another fibroblast cell line CHO cells, its activity was relatively weak with $IC_{50} > 0.3$ M. N_3Cyd has been known to inhibit cell growth by blocking the reductase function at the stage of its diphosphate (9). It was thus reasonable to suspect that the different cytotoxicity observed might be due to difference in rate at which the azidonucleoside is converted to its active form, the diphosphate. When the level of the deoxycytidine kinase was measured for both 3T6 and CHO cells, it was 3-fold higher in 3T6 than in CHO cells (Table I). The different cytotoxicity shown by N_3Cyd towards 3T6 and CHO cells is in agreement with the above hypothesis in that the higher level of the kinase renders cells more sensitive to the azidonucleosides. As shown in Table I, N_3Urd was inactive against either cell line. Towards 3T6 cells, for instance, N_3Cyd appears to be over 20-fold more active than N_3Urd in terms of IC_{50}. It appears that N_3Urd is a poor substrate for deoxycytidine kinase or other cellular kinases.

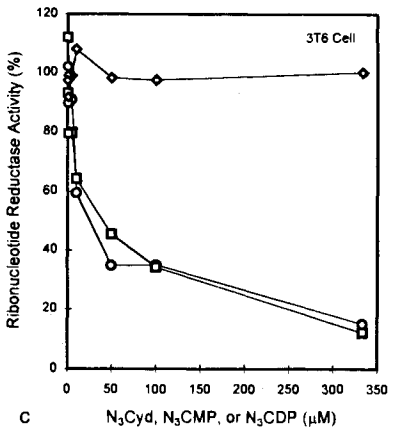
Table I. Effect of Azidonucleosides on the Cell Growth and Its Relationship to Deoxycytidine Kinase Activity in 3T6 and CHO Cell Lines[a]

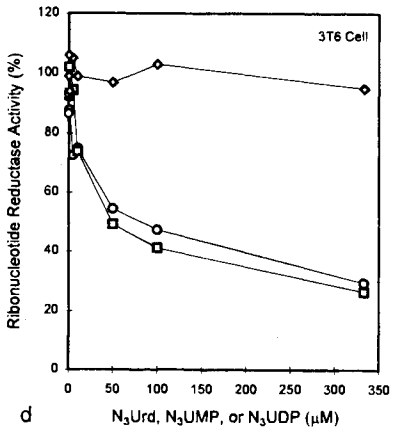
Cell line	IC_{50} (μM)		Enzyme activity (nmol/mg protein/min)
	N_3Cyd	N_3Urd	
3T6	51 ± 3	> 1000	0.265 ± 0.012
CHO	335 ± 15	> 1000	0.086 ± 0.003

[a] Values are the averages of triplicate determinations with standard deviations. IC_{50} is defined as the concentration of a test compound at which the number of cells that survived after 48-hr incubation is one half of the total number of viable cells found in the absence of the test compound.









Inhibition of Ribonucleotide Reductase by the Azidonucleosides and Their Mono- and Di-Phosphates

A permeabilized cell system was used to determine the efficiency at which the azidonucleosides and their 5′-monophosphates are phosphorylated to corresponding diphosphates, resulting in reductase inhibition. In a series of preliminary experiments, the permeabilized cell system was validated for the purpose of the present studies. First, in the presence of 1% Tween 80, the reductase activity was found to be proportional to the CHO cell concentration. Secondly, the permeabilized CHO cell system showed a linear relationship between the double reciprocal of the CDP reduction rate and the substrate (i.e, CDP) concentration. Finally, a well known inhibitor of the reductase, hydroxyurea, resulted in a concentration-dependent reduction of the enzyme activity in this assay system (data not shown).

As shown in Fig. 1a, both N_3CMP and N_3CDP inhibited the reductase activity in a concentration-dependent manner, while N_3Cyd did not. The value of IC_{50} observed with N_3CMP (5.8 μM) is slightly higher than that of N_3CDP (2.3 μM). Similarly, both N_3UMP and N_3UDP suppressed the reductase activity in the permeabilized CHO cell system (Fig. 1b). As with N_3Cyd, however, N_3Urd again failed to show any significant inhibitory activity up to 350 μM.

When the experiment was performed with permeabilized 3T6 cells, the same trend was observed: The azidonucleoside monophosphates were as effective as azidonucleoside diphosphates in inhibiting ribonucleotide reductase activity, while neither N_3Cyd nor N_3Urd was active (Figs. 1c and 1d). As shown in Figs. 1a through 1d, the inhibitory activity of the azidonucleoside monophosphates was similar to that of the diphosphates.

Synthesis and Characterization of Prodrug, $(POM)_2$-N_3UMP

To circumvent the rate-limiting phosphorylation of the azidonucleosides in its activation, a membrane-permeable prodrug of their monophosphates was employed. Since the phosphorylation of N_3Urd appears to be more difficult than N_3Cyd, N_3UMP rather than N_3CMP was chosen to assess the efficiency of the prodrug approach. Bis(pivaloyloxymethyl) ester of N_3UMP [$(POM)_2$-N_3UMP] was synthesized and characterized as described in Materials and Methods. The rate of hydrolysis of $(POM)_2$-N_3UMP (0.2 mM) at 37°C in CHO cell homogenate was proportional to the cell concentration in the homogenate, implying its catalytic function; the apparent first-order rate constant varied from 1.50×10^{-3} min^{-1} ($t_{1/2}$ = 462 min) at 2×10^6 cells/ml to 1.39×10^{-2} min^{-1} ($t_{1/2}$ = 50 min) at 2×10^7 cells/ml. The corresponding value in the cell culture medium

Fig. 1. Inhibition of ribonucleotide reductase in permeabilized CHO cells after 30 min of incubation at 37°C with: N_3Cyd (◇), N_3CMP (□) and N_3CDP (○) (**a**); and N_3Urd (◇), N_3UMP (□) and N_3UDP (○)(**b**). In permeabilized 3T6 cells with: N_3Cyd (◇), N_3CMP (□) and N_3CDP (○) (**c**); and N_3Urd (◇), N_3UMP (□) and N_3UDP (○) (**d**). The enzyme activity in an identical assay system but without any test agent was taken as 100%. The experiment was run twice, duplicate determination in each. At a given concentration of a test agent the variation was less than 7%.

used was 4.8×10^{-3} min^{-1} ($t_{1/2}$ = 143 min). The medium consisted of 90% α-MEM and 10% heat-inactivated fetal bovine serum. Although the latter was heat-inactivated before used, the significant hydrolysis of $(POM)_2$-N_3UMP observed suggested that it contained esterase activity. In comparison, $(POM)_2$-N_3UMP degraded in human serum with an apparent first-order rate constant of 0.13 min^{-1} ($t_{1/2}$ = 5.1 min).

HPLC analysis indicated that the disappearance of $(POM)_2$-N_3UMP was accompanied by initial appearance of the monopivaloyloxymethyl derivative $(POM)_1$-N_3UMP, followed by formation of the complete hydrolysis product N_3UMP with the peak concentration occurring between 100 and 300 min (Figure 2). The nucleoside N_3Urd formed slowly, presumably by enzymatic hydrolysis of N_3UMP. It accumulated to become the most predominant species after 300 min. The appearance and removal of various species during hydrolytic degradation of the prodrug **IV** is depicted in Figure 2. A nonlinear regression analysis (MINSQ; Micromath, Salt Lake City, UT) was carried out based on a kinetic scheme, prodrug $(POM)_2N_3UMP$ (**IV**) → $(POM)_1N_3UMP$ → N_3UMP (**II**) → N_3Urd (**I**). The analysis results indicated that relative rates of the first, second and third steps are 17:1.7:3.2.

As expected, the growth of CHO cells was not affected when they were exposed to the nucleoside analogue N_3Urd or its 5′-monophosphate N_3UMP up to 100 μM. In contrast, $(POM)_2$-N_3UMP inhibited the cell growth with IC_{50} of 3.0 μM (Figure 3).

To further confirm that a prodrug of the monophosphate can overcome the rate-limiting phosphorylation of the nucleoside by cellular kinase, we measured the biological activity of the prodrug in a cell line derived from lung cancer that was transfected with deoxycytidine kinase gene, H1437/dCK. In addition to the wild type, H1437/WT, cells transfected with β-galactosidase gene, H1437/lacZ, were also used as control (13). Theses three cell lines were exposed to N_3Urd, N_3Cyd, the prodrug, and 1-β-D-arabinofuranosylcytosine (AraC). As expected, the four compounds tested showed different activities against each of the cell line, however, each compound showed the same activity against both H1437/WT (Fig. 4a) and H1437/lacZ (Fig. 4b) cells. These results indicate that the transfection with an unrelated gene itself does not affect the sensitivity of the cells to the compounds tested. N_3Urd, which might not be phosphorylated by deoxycytidine kinase, remained inactive up to 1mM against the three cell lines. However, H1437/dCK cells showed an increased sensitivity to N_3Cyd and AraC (Fig. 4c). This is presumably because they are a good substrate of the kinase. Compared with H1437/lacZ, H1437/dCK showed 15- and 70-fold increases in sensitivity towards N_3Cyd and AraC, respectively (Fig 4b and 4c). In contrast, the prodrug of N_3UMP maintained the same potency independent of deoxycytidine kinase level.

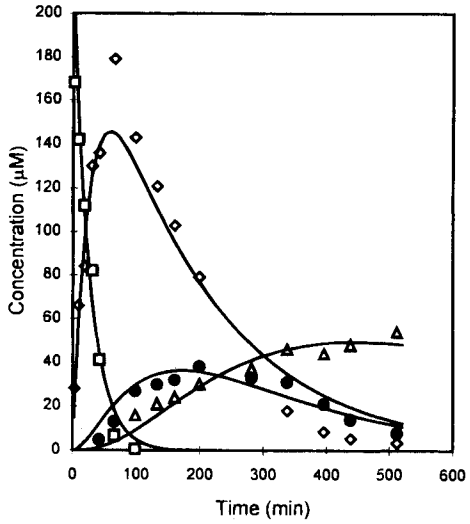


Fig. 2. Degradation of the prodrug $(POM)_2$-N_3UMP (**IV**) in CHO cell homogenate at 37°C. The initial concentration of the prodrug was 0.2 mM; **IV** (□) degraded to $(POM)_1$-N_3UMP (◇), which in turn generated N_3UMP (**II**; ●) and further N_3Urd (**I**; Δ). The solid lines were generated with a nonlinear regression analysis based on a kinetic scheme described in the text.

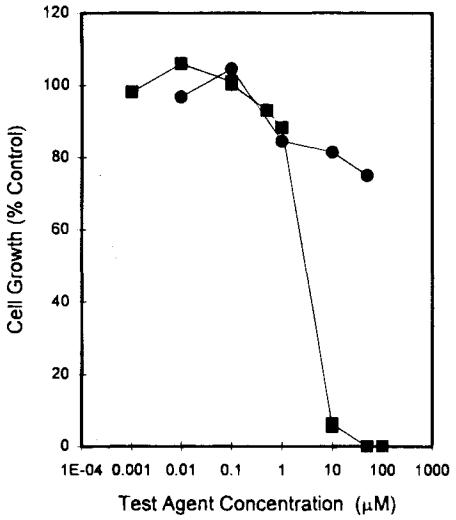


Fig. 3. CHO cell growth after 48-h incubation with varying concentrations of bis(pivaloyloxymethyl) 2′-azido-2′-deoxyuridine 5′-monophosphate (**IV**) (■), N_3UMP (▲), and N_3Urd (●). Each data point represents the average of two experimental results which were within 5% of each other. Cell growth in identical culture medium including 1.0% ethanol constitutes the control at 100%.

DISCUSSION

Most of the nucleoside-based therapeutic agents are precursors of an active nucleotide (17). The nucleotide analog is generated from its precursor by the cellular kinases after the latter enters the cell via a nucleoside transport system and/or a passive diffusion (18). In terms of membrane transport, this is an important advantage of a nucleoside analog over membrane-impermeable nucleotides in that it overcomes the transport barrier against charged molecules imposed by the cell membrane. In this approach, however, phosphorylation of a nucleoside analog can become a new obstacle, since a given synthetic nucleoside analog may not be an ideal substrate of the cellular nucleoside kinases. To further complicate the issue, the kinase activity highly depends on not only cell type but also cell cycle and host species (e.g., human vs. animal) (19). These considerations have well justified a prodrug approach with the monophosphate of nucleoside analogs.

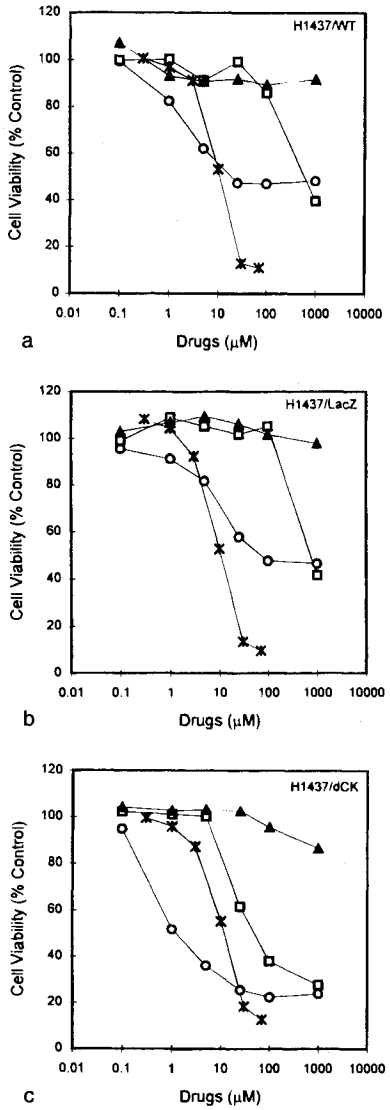


Fig. 4. Sensitivity of H1437/WT (**a**), H1437/lacZ (**b**), and H1437/dCK (**c**) cell lines to N_3Urd (▲), N_3Cyd(□), AraC(○), or the prodrug of N_3UMP (✳). The cells were continuously exposed to each of these test compounds for 4 days. Each data point is the average of triplicate determinations and the standard deviations were invariably less than 5%.

The major goal of the present study is to identify an efficient way to generate the azidonucleoside diphosphate inside the cell. To achieve the cellular delivery of the azidonucleoside diphosphate, the feasibility of delivering its precursors has been investigated. Direct delivery of a triply charged azidonucleoside diphosphate is possible in concept if all negative charges on the 5′-pyrophosphate can be masked with membrane-permeable pro-moieties. However, this particular strategy is not practical for several reasons. First, the synthesis of a desired derivative of a pyrophosphate can not be easily achieved mainly because of its instability (20). Secondly, during the generation of the parent diphosphate, the intermediate pyrophosphate with intact pro-moieties can be converted directly to the monophosphates. Although several pyrophosphate diesters have been reported as nucleoside phospholipid prodrugs (21), they are not prodrugs of a diphosphate. These pyrophosphate diesters were first degraded into monophosphate derivatives by cellular pyrophophatase. These were then phosphorylated all the way to the corresponding triphosphates to exert biological activity (21).

In the present study, the above generalization well discussed in the literature was specifically addressed in conjunction with the cellular delivery/generation of N_3CDP and N_3UDP that are potent inhibitors of a key enzyme involved in *de novo* DNA synthesis, ribonucleoside diphosphate reductase. N_3Cyd has been known to inhibit 3T6 cell growth via inhibition of ribonucleotide reductase (9). It was subsequently shown that the phosphorylation of N_3Cyd requires deoxycytidine kinase, which is also responsible for phosphorylation of a number of therapeutically important nucleoside analogs such as Ara-C and dideoxycytidine (22).

Substrate specificity of deoxycytidine kinase is rather restricted to cytosine, adenine, and guanine bases, excluding uracil-containing nucleoside analogs (23). The other three principal enzymes involved in cellular phosphorylation also show their own stringent substrate specificity. Thymidine kinase 1 (EC 2.7.1.21) and 2 can phosphorylate several uridine derivatives but show a narrowly defined specificity on the ribose moiety (23). Deoxyguanosine kinase (EC 2.7.1.113) mainly phosphorylates purine derivatives (23). When the effect of the azidonucleosides on cell growth was evaluated, N_3Urd failed to show any significant activity up to 1.0 mM. On the other hand, as shown in Table I, N_3Cyd showed more effective inhibition of cell growth against 3T6 cells with higher deoxycytidine kinase activity than CHO cells. These observations are all in agreement with our speculation that N_3Cyd is phosphorylated with relative ease while N_3Urd is not.

The azidonucleosides should be phosphorylated twice to become biologically active. The first phosphorylation step was examined by correlating the deoxycytidine kinase level with the activity of the azidonucleoside. In investigating the second step, from monophosphate to diphosphate, an experimental condition was adopted under which not only a nucleoside but also the monophosphate and the diphosphate can access the cellular enzymes. The permeabilized cell system adopted in the present study provided an opportunity to investigate the intracellular effects of a membrane-impermeable charged agent on the reductase under a condition that perhaps best mimics the cellular environment. A permeabilized cell system allows the charged nucleotides to access cellular kinases as well as ribonucleotide reductase without significantly affecting normal cellular function.

As shown in Fig. 1, the azidonucleoside monophosphates introduced into the permeabilized cell demonstrated an equally active inhibition of the reductase as the corresponding diphosphates, indicating facile phosphorylation by nucleoside monophosphate kinases (24). These findings unambiguously establish that the cell growth inhibition observed was due to inhibition of the reductase caused by the diphosphates generated from corresponding monophosphates.

A logical extension of the above analysis is to develop a means of delivering azidonucleoside monophosphate across the cell membrane. With such a strategy one could circumvent the rate-limiting first phosphorylation step. In this study, to improve the cellular entry of azidonucleoside monophosphate, the negative charges on the phosphate group were masked with pivaloyloxyalkyl groups which has been well characterized (25). Being

a neutral lipophilic compound, the prodrug $(POM)_2$-N_3UMP could enter the cell most likely via a passive diffusional process. Once inside, the masking groups could be cleaved by non-specific esterase(s). It will be rapidly followed by expulsion of formaldehyde, producing mono-derivatized species $(POM)_1$-N_3UMP. The latter undergoes the same biotransformation once again to result in N_3UMP. The monophosphate will be then phosphorylated to its diphosphate (N_3UDP), inhibiting the reductase in the intact CHO cells.

Initial hydrolysis of $(POM)_2$-N_3UMP produces, in addition to the parent nucleoside monophosphate, trimethylacetic acid and formaldehyde as byproducts. The former is apparently innocuous and can diffuse out of the cell (26). Formaldehyde generated from the same type of pro-moiety showed little toxicity to isolated cells and tissues (27).

Since N_3UMP generated from $(POM)_2$-N_3UMP is a substrate analog of thymidylate synthase, it could be possible that the biological activity observed with the prodrug might be due to inhibition of thymidylate synthase. The enzyme activity determined as described elsewhere (28) was independent of N_3UMP (data not shown), supporting that the cytotoxicity demonstrated by the prodrug is mainly due to inhibition of ribonucleotide reductase. There are still other potential mechanisms contributing to this cytotoxicity, including interference of DNA synthesis by the nucleoside triphosphate. The relative importance of reductase inhibition by N_3UDP derived from $(POM)_2$-N_3UMP in the overall cytotoxicity observed is being further assessed.

As shown in Fig 4, the membrane-permeable $(POM)_2$-N_3UMP does indeed show the activity independent from deoxycytidine kinase activity. In this context, it is noteworthy that the IC_{50}, value of this derivative is lower than that of N_3Cyd even in the cell transfected with deoxycytidine kinase gene (Fig. 4c).

With the prodrug of nucleoside monophosphate, other additional and possibly significant advantages might be realized. A high and continuous dose of Ara-C induces drug resistance caused by mutation within deoxycytidine kinase gene (29). The resistant cell thus derived could become sensitive to a prodrug of the monophosphate. Secondly, by masking the negative charges, enhanced stability of nucleoside monophosphate may be resulted. Nucleoside monophosphates are readily dephosphorylated in extracellular fluid by non-specific phosphohydrolases (30). In addition, the glycosidic bond of nucleoside monophosphate prodrug would be more stable than that of the corresponding nucleoside which has a free 5′-OH group (31). The improved stability of the glycosidic bond becomes more relevant considering that a nucleoside can be degraded into its base by pyrimidine-specific nucleosidase (32).

In summary, the present study presents evidence that phosphorylation of N_3Urd and N_3Cyd 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.

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