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IMPROVED SYNTHESIS OF ZEBULARINE [1-(ß-D-RIBOFURANOSYL)-DIHYDROPYRIMIDIN-2-ONE] NUCLEOTIDES AS INHIBITORS OF HUMAN DEOXYCYTIDYLATE DEAMINASE

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The 2′-deoxy (**2a**) and 2′-ara-fluoro (**3a**) derivatives of zebularine [1-(β-D-ribofuranosyl)-dihydropyrimidin-2-one, **1a**] were phosphorylated in high yield to the 5′-nucleotides **2b** and **3b**, respectively, and characterized by HPLC, enzyme degradation, ^{1}H, ^{13}C and ^{31}P NMR, and high resolution mass spectral analysis. Their inhibitory activity against partially purified MOLT-4 deoxycytidylate deaminase (dCMPD) in the presence of the allosteric effector deoxycytidine triphosphate (dCTP) and Mg^{+2} ion was examined. Compounds **2b** and **3b** inhibited dCMPD with K_i values of 2.1×10^{-8} M and 1.2×10^{-8} M, respectively. The parent nucleotide, zebularine monophosphate **1b** was ineffective at concentrations > 100 μmol. The effect of the nucleosides, **1a–3a**, as well as tetrahydrouridine (THU) and 2′-deoxy THU (dTHU), on the cellular production of DNA precursors was examined in human MOLT-4 peripheral lymphoblasts. It was shown that **1a**, **2a** and **3a** all elevated intracellular dCTP and TTP levels in whole cells with the most powerful effect elicited by **1a**. The 2′-fluoro derivative **3a** was chemically phosphorylated much more cleanly and higher yield than **2a**, without the formation of diphosphorylated by-products. This compound was found to be infinitely less sensitive to acid-catalyzed degradation than **2a**. Since the substitution of fluorine for hydrogen had a slight potentiating effect on the dCMPD inhibitory activity while stabilizing the compound toward acid-catalyzed and enzymatic depyrimidination, compound **3b** emerges as a very attractive tool for the pharmacological modulation of pyrimidine deaminase activity.

KEY WORDS: dCMP deaminase, zebularine, MOLT-4, inhibition, deoxyribonucleotide pools, anti-cancer therapeutics

INTRODUCTION

The biosynthesis of pyrimidine deoxyribonucleotides (PydRN) in mammalian systems is tightly controlled by a series of steps which are subject to allosteric and "feedback" regulation.[1] A key player in the balanced production of the DNA precursors dCTP and TTP is deoxycytidylate deaminase (dCMPD), the enzyme which converts dCMP

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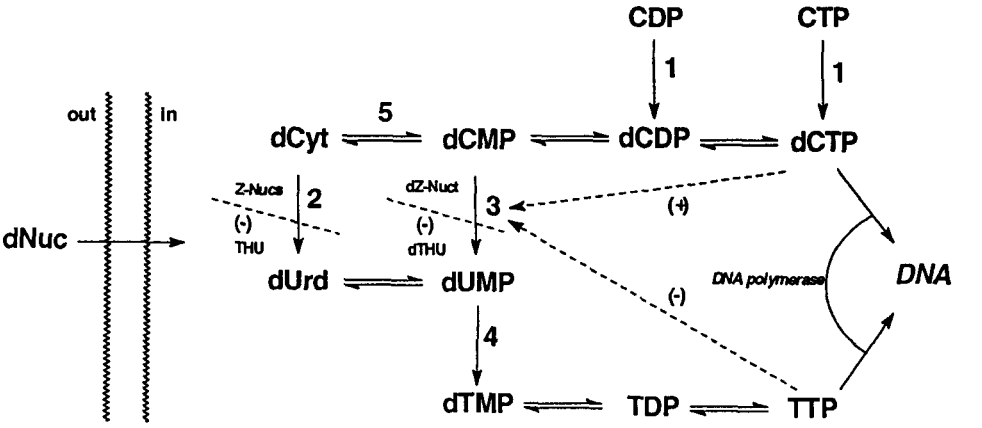
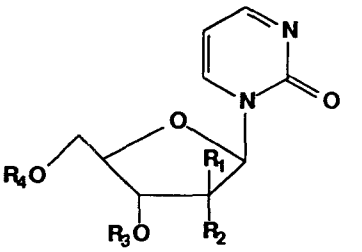


FIGURE 1 Pyrimidine nucleotide biosynthesis in mammalian cells. Wavy lines indicate cell membrane. Dotted arrows indicate allosteric inhibition (-) or activation (+) of dCMPD. Dotted lines indicate inhibition by the indicated compounds. Z-Nucs = zebularine nucleosides, dZ-Nuct = deoxyzebularine nucleotides. (1) Ribonucleotide reductase, (2) cytidine deaminase, (3) deoxycytidylate deaminase, (4) thymidylate synthase, (5) deoxycytidine kinase.

to dUMP, thus supplying a substrate for thymidylate synthase (TS, Figure 1). The activity of purified dCMPD from a variety of sources was invariably shown to be allosterically sensitive to the end-products of its pathway, i.e., dCTP (activation) and TTP (inhibition).[2-6] Several reports suggest that the relative amounts of these triphosphates regulate the activity of dCMPD in intact cells;[7,8] indeed, a recent study proposed that the operation of dCMPD in intact CCRF-CEM cells is linearly dependent on the dCTP:TTP ratio.[9] Abnormally high levels of dCMPD have been observed in tumor cells[10-12] implicating this enzyme as a potential point of intervention for anticancer therapy. Consequently, a variety of nucleoside analogues have been designed as regulators of PydRN metabolism *via* blockage of dCMPD; the most widely used of these inhibitors is 2′-deoxy-tetrahydrouridine-5′-monophosphate (dTHUMP).[13,14] More recently, 2′,2′-difluorodeoxycytidine has also been shown to allosterically inhibit dCMPD by way of its 5′-triphosphate in whole cells.[9] Inhibiton of dCMPD can also effectively potentiate the cytotoxicity of the antimetabolite nucleoside arabinofuranosylcytidine (ara-C), a drug whose phosphorylated metabolite is inactivated by dCMPD deamination.[14,15] In a variation on this approach, high tumor levels of dCMPD have been exploited to convert 5-fluoro-2′-deoxycytidine (FDC) to the toxic metabolite 5-fluoro-2′-deoxy-UMP intracellularly.[16] The cytidine deaminase (CDA) inhibitor, THU, when used in combination with FDC augmented the antitumor effects of the latter by preventing plasma deamination of FDC.[16]

While compounds of the THU family remain very potent modulators of deaminase function, the tetrahydrouridyl ring is extremely sensitive to mild acid-catalyzed breakdown. On the other hand, several of the pyrimidin-2-one family of nucleosides were



1a; $R_1 = R_3 = R_4 = H$, $R_2 = OH$,
1b; $R_1 = R_3 = H$, $R_2 = OH$, $R_4 = PO_3H_2$

2a; $R_1 = R_2 = R_3 = R_4 = H$
2b; $R_1 = R_2 = R_3 = H$, $R_4 = PO_3H_2$
2c; $R_1 = R_2 = H$, $R_3 = R_4 = PO_3H_2$

3a; $R_1 = F$, $R_2 = R_3 = R_4 = H$
3b; $R_1 = F$, $R_2 = R_3 = H$, $R_4 = PO_3H_2$

shown to have potent deaminase inhibitory activity while being much more resistant to degradation at low pH.[17,18] Zebularine (1-(β-D-ribofuranosyl)-dihydropyrimidin-2-one, **1a**), the parent member of this family, was originally synthesized by two groups[19,20] in the 1960's. Later the preparation of **1a**[21] and 2′-deoxyzebularine **2a**[18] was improved in our laboratory. Compound **1a** was originally found to strongly inhibit the growth of bacterial cells *in vitro*.[22] It was shown that the metabolite responsible for the cytotoxicity of **1a** was in fact the 2′-deoxy-5′-monophosphate **2b** which was formed intracellularly and inhibited bacterial TS.[23] We have demonstrated that **1a** is a potent inhibitor of human CDA[17] and have evaluated its *in vivo* antitumor activity alone and in combination with ara-C, which is also a substrate for CDA.[24] We also reported preliminary data on the preparation of **2b** and its inhibitory activity against human dCMPD.[18] Subsequently, a comprehensive examination of the properties of affinity-purified HeLa cell-derived dCMPD, including its inhibition by **2b**, was reported by Maley, *et al.*[25]

Compound **3a** was synthesized[24] as an acid-stable (to anomeric hydrolysis) analogue of **2a** akin to the approach taken with other acid-labile nucleoside therapeutics such as 2′-dideoxyadenosine.[26] Since nucleosides often retain their biological attributes upon fluorine-for-hydrogen substitution, the nucleotide **3b** was prepared and tested as an inhibitor of dCMPD. Herein we describe improved routes for the preparation of nucleotides **2b** and **3b**, their complete characterization by enzymatic and spectroscopic means, and their inhibitory potency against partially purified dCMPD from MOLT-4 cells. We propose that the fluorinated analogue **3b** is the most promising lead for future pharmacological studies on PydRN biosynthesis modulation based on its chemical stability and effective cellular metabolism profile.

MATERIALS AND METHODS

Chemicals

Chemical reagents were purchased from Aldrich Chemical Co. and dCMP and dCTP were obtained from Sigma. THU and dTHU were obtained from the Drug Synthesis and Chemistry Branch, NCI. Snake (*Crotalus durissus*) venom phosphodiesterase was purchased from Boehringer Mannheim. Snake venom 5′-nucleotidase and *Escherichia coli (E. coli)* alkaline phosphatase were purchased from Sigma. HPLC was performed on a Waters DeltaPrep 3000 system on either 10 μM, strong anion-exchange (SAX) or C18 stationary phases (25 cm columns, 4.6 mm i.d.) with the eluants and conditions described below. ^{1}H NMR spectra were run at 250 or 500 MHz on Bruker spectrometers (AC250 or AMX500),^{13}C NMR at 62.9 MHz (AC250) and ^{31}P NMR at 202 MHz (AMX500), all at room temperature using standard pulse sequences. The proton spectra were referenced to the particular NMR solvent (HDO = 4.65 ppm at 250 MHz), carbon spectra to internal dioxane-d_8 (66.5 ppm) and the phosphorous spectra to H_3PO_4 (0.0 ppm at 202 MHz). The ^{31}P NMR spectra were run without broadband proton decoupling. Negative ion mass spectra were obtained on a VG 7070E mass spectrometer operating at an accelerating voltage of 6 kV and a resolution of 2000. Glycerol was used as a sample matrix and ionization was effected by a beam of xenon atoms derived by charge-exchange neutralization of a 1.0–1.2 mA beam of xenon ions accelerated through 8.0–9.2 kV. Spectra were acquired under the control of a VG 11/250 J+ data system at a scan speed of 10 s/decade, and the background due to the glycerol matrix was automatically subtracted.

General Procedure for Phosphorylation of Zebularine Nucleosides

Compounds **2a** and **3a** were synthesized as described previously.[18,24] A solution of 0.25–0.35 mmol of the nucleoside in H_2O was freeze-dried in a lyophilization vial and accurately weighed . Trimethyl phosphate (0.5 ml) was added and the suspension was warmed to 30–35°C to effect dissolution. After being capped with a serum stopper and flushed with argon, this mixture was cooled to 0°C. Phosphorous oxychloride (0.5–0.7 mmol, Aldrich, 99.999%) was added slowly by syringe to the stirred solution. After stirring at 0°C for 20 min, a small aliquot (5–10 μl) was removed via a syringe and added to 0.3 ml of 1.0 M triethylammonium bicarbonate (pH 8.4). After 1 min, HPLC analysis (SAX-10 column, 0.01–0.7 M ammonium phosphate, native pH) of this solution revealed the presence of nucleotide(s) and residual starting nucleoside. After 45 min the reaction was usually 90% complete as judged by HPLC. One ml of triethylammonium bicarbonate was added to the cold reaction mixture and this was stirred at 0°C for 1 h. A small sample was analyzed by ion-exchange HPLC which showed that the major product, corresponding to the 5′-phosphate, eluted at 11 min. The reaction mixture was either separated by chromatography on Whatman 3MM paper (80:20 ethyl alcohol/water, eluant, overnight in the ascending mode) or injected directly onto a semi-preparative HPLC column. The major band from the paper chromatogram was removed with dilute ammonia, the particulates were

trapped on a 0.22 μm MILLEX-GS filter, and the solution was freeze-dried. Final purification involved HPLC on a 25 cm Whatman Partisil 10 SAX semi-preparative column using 10% methanol in 0.1 M NH_4HCOO (solvent A) or 10% methanol in 1.0 M NH_4HCOO (solvent B). Pure solvent A was run just long enough to elute contaminating nucleoside (6–7 min) followed by a jump to 100% solvent B for an additional 20 min. In this system the monophosphates eluted at 20–22 min. The volatile salts were removed by repeated lyophilization or by trituration of the freeze-dried powder with 70% ethanol/diethylether followed by centrifugation and removal of the pellet containing a high concentration of the nucleotide. A second HPLC was performed on reverse phase C18 silica with H_2O/CH_3OH 99:1. **2b**: ^{1}H NMR (D_2O) δ 9. 055 (dd, J = 2.3 and 6.4 Hz, 1H, H4), 8.592 (dd, J = 2.5 and 6.0 Hz, 1H, H6), 6.936 (t, J = 6.2 Hz, 1H, H5) 6.100 (t, J = 6.0 Hz, 1H, H1′), 4.358 (m, 1H, H3′), 4.095 (br dt, A of ABMX system, $J_{5',5''}$ = 11.8, $J_{5',4'}$ = 3.6, $^3J_{H5',P}$ = 2.9 Hz, 1H, H5′), 3.960 (ddd, B of ABMX system, $J_{5',5''}$ = 11.8, $J_{5',4'}$ = 8.0, $^3J_{H5'',P}$ = 2.9 Hz, 1H, H5″), 2.582 (ddd, J = 4.3, 6.5 and 14.5 Hz, 1H, H2′), 2.305 (dt, J = 5.8 and 14.5, 1H, H2″). ^{13}C NMR (D_2O) δ 167.19, 157.58, 146.56, 107.61, 88.85, 87.55 (d, $^2J_{C5',P}$ = 9.0 Hz), 70.94, 64.36 (d, $^3J_{C4',P}$ = 5.4 Hz), 41.00. ^{31}P NMR (D_2O) δ 5.927. FAB MS (negative ion): M – H = 292. **3b**: ^{1}H NMR (D_2O) δ 8.508 (dd, J = 2.7 and 4.4 Hz, 1H, H4), 8.423 (dd, J = 2.6 and 6.7 Hz, 1H, H6), 6.670 (dd, J = 4.4 and 6.7 Hz, 1H, H5), 6.200 (dd, J = 4.2 and 14.7 Hz, 1H, H1′), 5.227 (dt, J = 3.7 and 51.3 Hz, 1H, H2′), 4.391 (ddd, J = 3.2, 3.5 and 17.9 Hz 1H, H3′), 4.149 (m, 1H, H4′), 3.955 (m, 1H, H5′), 3.872 (m, 1H, H5″). ^{13}C NMR (D_2O, 1 drop dioxane-d_8) δ 181.60, 167.88, 157.04; 147.38, 107.22 (d, $^2J_{C1',F}$ = 8.9 Hz) 95.05 (d, $^1J_{C2',F}$ = 192.7 Hz), 86.91 (d, $^2J_{C3',F}$ = 14.8 Hz), 84.20 (dd, $^3J_{C4',F}$ = 8.1, $^3J_{C4',P}$ = 3.9 Hz), 73. 85 ($^2J_{C5',P}$ = 25.5 Hz). ^{31}P NMR (D_2O) δ 8.753. FAB MS (negative ion) M – H = 309.

Enzyme Purification

Partially purified dCMP deaminase (EC 3.4.3.12) was prepared as follows: Five hundred ml of logarithmically growing MOLT-4 human lymphoblasts (1×10^6 cells/ml) were collected by centrifugation at 1,500 ×g for 10 min, washed once with serum-free RPMI 1640 medium, recentrifuged and extracted with 1 ml of 70% methanol rendered 10 mM in Tris HCI, pH 8.4 and 10 mM in DTT. The resulting suspension was centrifuged at 4°C and 14,000×g for 15 minutes and the supernatant dialyzed against 1.01 of the homogenization buffer in 10 mm wide Spectra/por dialysis membranes with a 50,000 MW cutoff. Particulates in the dialyzed preparation were removed by centrifugation and the resultant methanol-soluble preparation of dCMP deaminase was used in the experiments described below. This dialyzed extract contained 0.25 mg/ml protein and for each enzymatic reaction 30 μl of this enzyme preparation was used. The enzyme so prepared was purified 20-fold over comparable extracts prepared in methanol-free buffers and exhibited a specific activity of 0.32 μmoles/min of dCMP deaminated/min/mg protein with dCMP and dCTP present at 200 and 7 μM respectively. Protein was measured using the Bio-Rad (Bradford) Protein Assay method.[27]

Enzyme Assay & Kinetic Studies

Kinetic measurements and enzymatic assays were performed on a Shimadzu 2101 UVPC by monitoring the decrease in absorbance at 280 nm. Enzyme reactions were performed with varying concentrations of dCMP at 37°C in 1.0 ml volumes which contained 20 mM TrisHCl, pH 8.0, 2 mM $MgCl_2$ with 7 μM dCTP, and for kinetic runs the indicated amounts of the zebularine analogs. Stock solutions were prepared in water and concentrations verified spectroscopically at 304 nm, employing the extinction coefficient of zebularine at pH 7.0 (ε = 6,041). The K_i and K_m values were obtained from weighted ($1/y^2$) data using EZ-FIT (Frank W. Perrella, E.I. Dupont de Nemours & Co., Glenolden, PA), an interactive curve-fitting program for the analysis of enzyme kinetic data.

Effect of inhibitors on deoxyribonucleotide (dRNT) pools in intact cells

To measure the *in situ* effect of the various inhibitors on dRNT pool sizes (and hence obtain an indirect measure of dCMPD activity), Molt-4 cells in exponential growth were incubated with 50 μM of compounds **1a, 2a, 3a,** THU, dTHU or saline for 16 h. Cells were collected by centrifugation at 2000×g for 5 min and the pellets were extracted with 60% methanol. After heating at 95°C for 2 min, the extracts were centrifuged at 12,500×g for 3 min; 200 μl of supernatant was treated with 10 μl of 10% $NaIO_4$ at pH 7.0 for 45 min to destroy ribonucleotides and the reaction product was analyzed directly (without the addition of cyclohexylamine[28]) using a SAX HPLC column as previously described.[29]

Acid Stability

The acid stability of the zebularine analogs was determined by adding the compounds to a pH 1.0 buffer solution (0.05 M KCI-HCI), pre-equilibrated to 3°C, to give a final concentration of 0.25 mM. At timed-intervals, 20 μl aliquots were removed and diluted with 480 μl of 0.1 M potassium phosphate buffer, pH 7.4. An aliquot of the diluted solution (50 μl) was injected onto a reverse phase HPLC column (Altex/Beckman ODS 5μ, 250×4.6 mm) using an AS 3000 autosampler with a Spectra-Physics P2000 binary pump and the chromatograms developed with a mobile phase of 3% CH_3CN in 0.01 M potassium phosphate buffer, pH 7.0 (flow rate, 1 ml/min). Time points were collected for 300 min at 37°C and peak areas were determined using a Spectra Physics SP4400 computing integrator following detection at 304 nm with a Gilson 116 UV detector.

RESULTS

Synthesis and Purification of Inhibitors

Compound **2a** was previously synthesized in this laboratory from **1a** by chemical deoxygenation of the 2′-hydroxyl group of a protected derivative.[18] The fluoro compound **3a** was prepared via glycosylation of a fluorinated sugar precursor with the

silylated form of the 2-oxo-pyrimidone base.[24] The synthesis of the nucleotides was carried out by methodology similar to that described by Wightman and Holy[30] for the preparation of **2b**, with the major exception being that the compounds were purified to homogeneity. Reaction conditions were varied to try to optimize the yield of the desired monophosphates and minimize by-product formation.

When **2a** was treated with $POCl_3$ in trimethylphosphate under argon at 0°C, reaction was almost immediate as judged by HPLC analysis on both C18 reverse phase and SAX columns. Two products were observed initially; the desired nucleotide and a product which was more strongly retained (~10 min) on SAX-10. The amount of this slower moving product increased with longer reaction times. Varying the molar amounts of reagent, the substrate concentration or the temperature had little effect on the formation of the undesired material. Optimum reaction times for the phosphorylation reactions were between 30 and 45 min. The fluorinated analogue **3a** also reacted quickly under the conditions although *no* by-products were detected by HPLC. At this point, paper chromatography was useful in separating the by-product from the desired **2b**, as well as for removal of excess solvent. Alternatively, the reaction mixture could be injected directly onto a SAX HPLC column and the desired compounds eluted very cleanly as judged by ^{1}H NMR analysis. The only contaminant at this point was the counter ion of the eluting buffer ($HCOO^-$) and in the case of **2b** some free nucleoside from hydrolysis of the 5′-phosphate group. The majority of the salt was removed by either, (1) repeated lyophilization from water or (2) trituration of the freeze-dried powder with mixtures of ethanol/ether, centrifugation, removal of the pellet and washing with ether (ammonium formate is freely soluble in ethanol and moderately soluble in diethyl ether). After each step, the material was analyzed by HPLC and ^{1}H NMR to evaluate purity. Representative ^{1}H NMR spectra for **2b** and **3b** are shown in Figure 2.

By-products and acid stability

The by-product from the phosphorylation of **2a** was shown to be the 3′, 5′-diphosphate derivative **2c** by mass spectral and enzymatic analysis (Figure 3). Treatment f a mixture of **2b** and the by-product with 5′-nucleotidase quantitatively converted **2b** to **2a** while reducing the intensity of the by-product peak (Figure 3D). Both compounds were unaffected by phosphodiesterase treatment (Figure 3C) but alkaline phosphatase from *E. coli* cleanly converted both peaks to nucleoside (Figure 3B). The by-product was formed concomitantly with the desired **2b** and hence its formation under these phosphorylation conditions was unavoidable. To the contrary, the 2′-fluoro derivative was phosphorylated on the 5′ position in almost quantitative yield and with no indication of a 3′, 5′-derivatized product. The use of other phosphorylating reagents which require basic conditions for reaction catalysis (i.e., phosphoramidites or protected chlorophosphates) was precluded by the instability of the zebularine family of nucleosides to alkaline condtions.[31] During the course of this study, we observed by ^{1}H NMR spectroscopy a degradative process, similar to that seen at high pH,[31] to occur under acidic conditions for the nucleotide **2b**. This process was much slower in the case of the fluorinated phosphate **3b**. Figure 4 shows the time-course of the disappearance

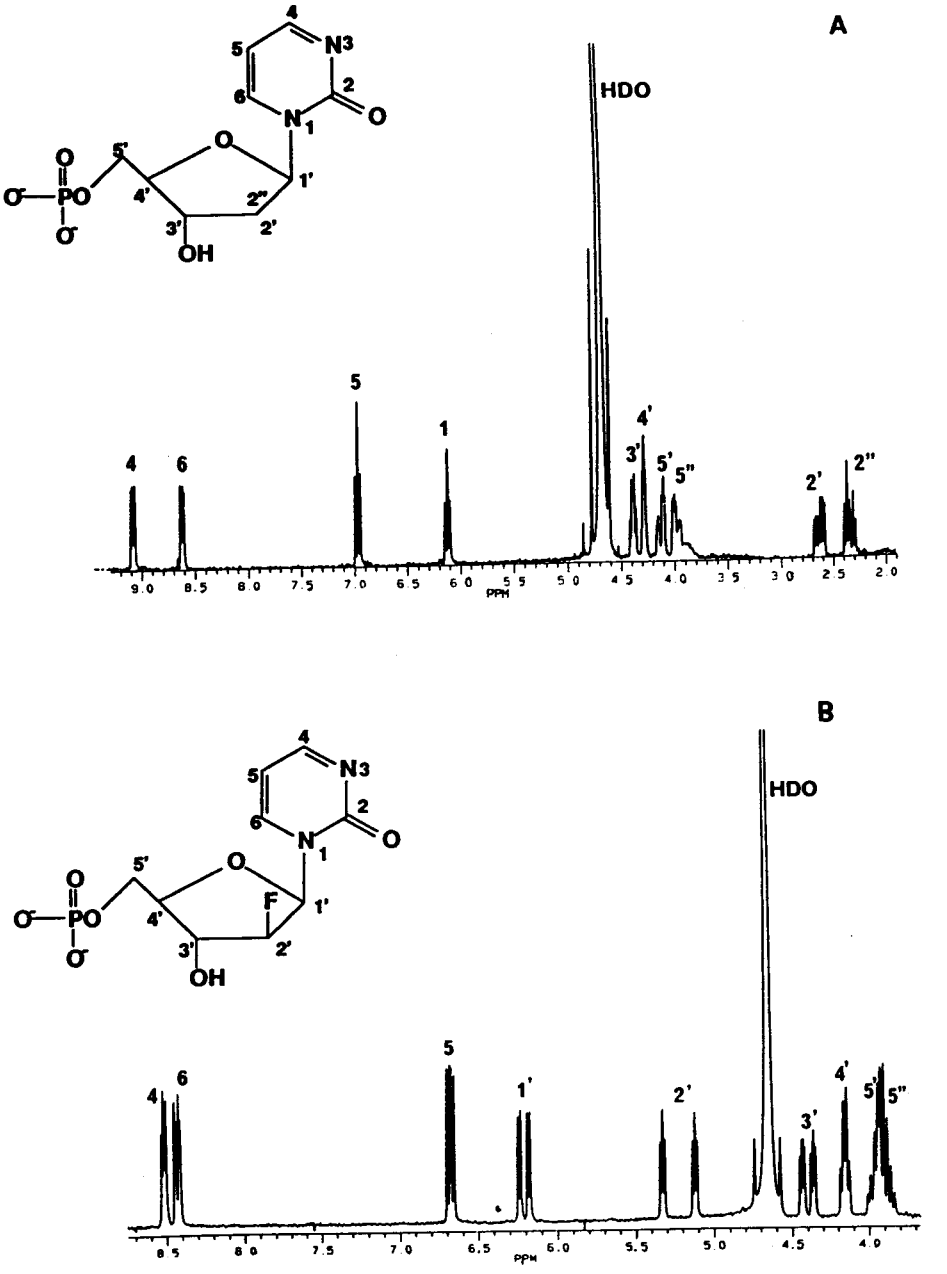
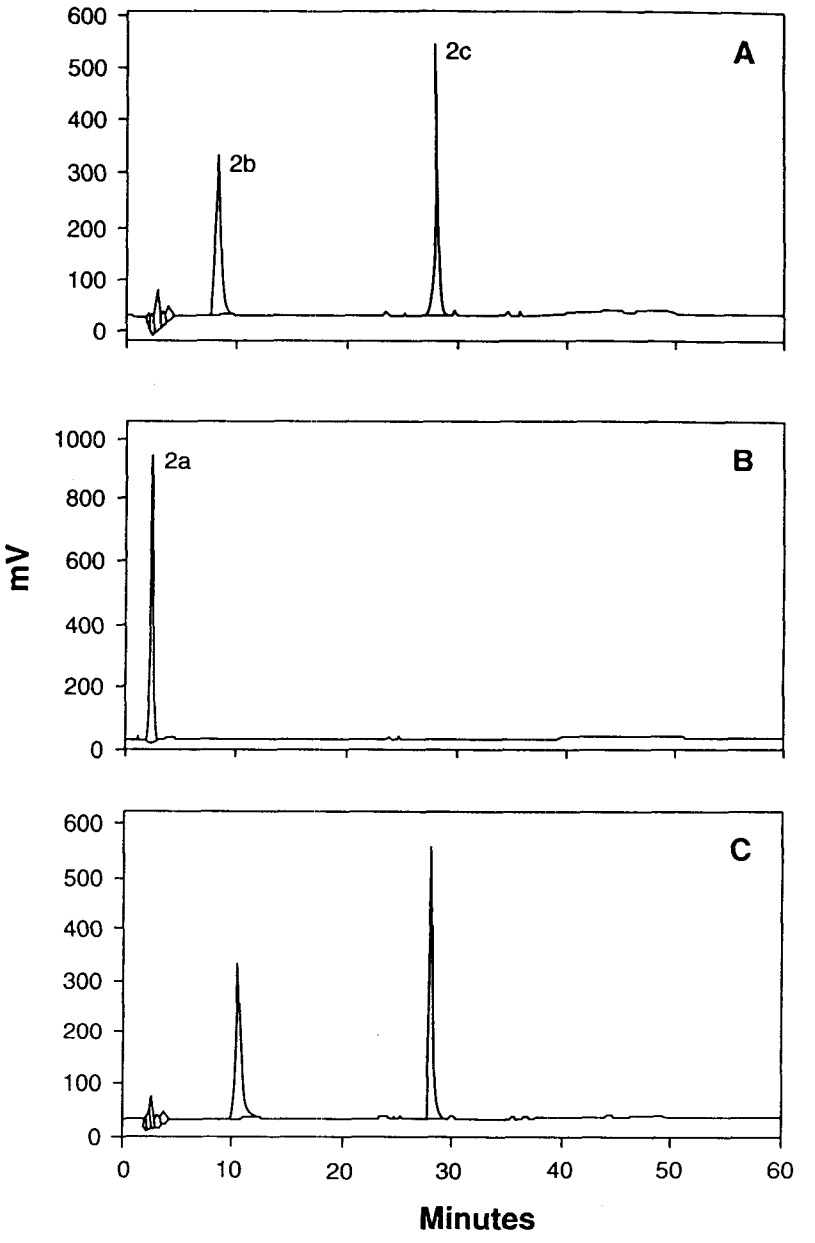


FIGURE 2 ^{1}H NMR spectra of **2b** (A) and **3b** (B) in D_20 at 250 MHz and 25°C. Assignments are shown above the appropriate resonances.



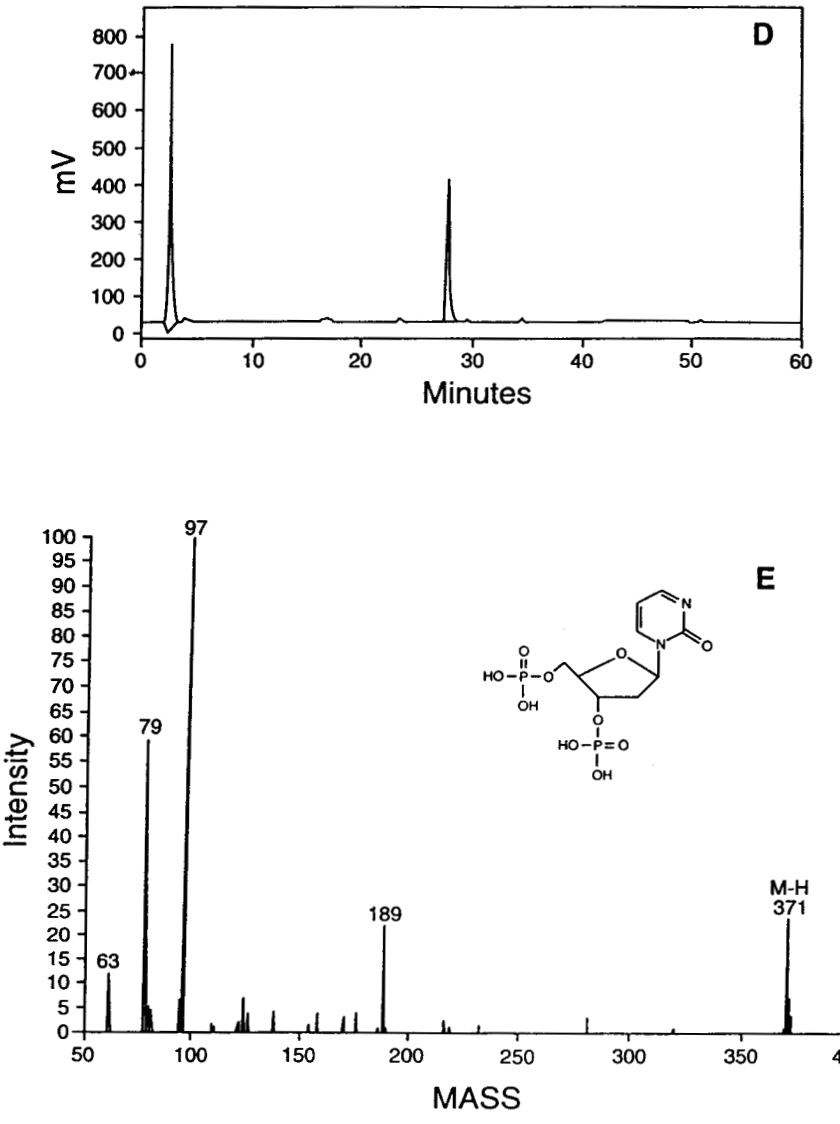


FIGURE 3 (A) SAX HPLC trace of the product mixture from the reaction of **2a** with $POCl_3$. (B) Product mixture after treatment with alkaline phosphatase, (C) phosphodiesterase, (D) 5′-nucleotidase. (E) Negative ion fast atom bombardment (FAB) mass spectrum of compound **2c**.

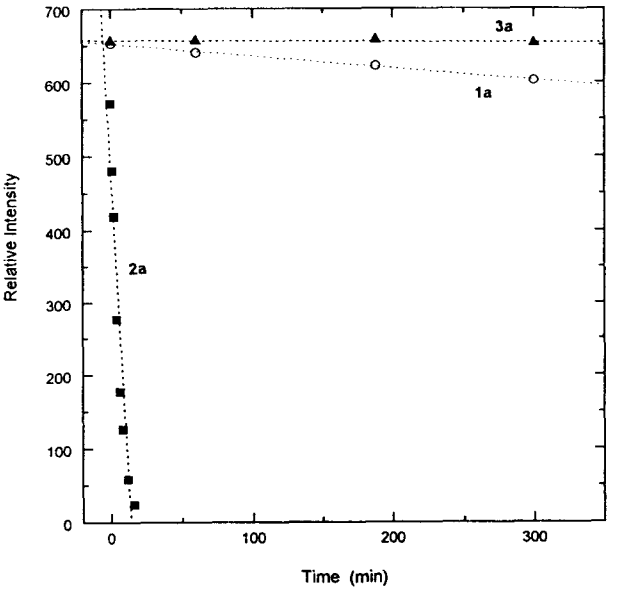


FIGURE 4 Time course of the disappearance of **1a** (○), **2a** (●) and **3a** (▲) at pH 1, 37°C.

of **1a**, **2a** and **3a** during incubation at pH 1.0 (KCl, HCl buffer). Deoxygenation at the 2′ position renders compound **2a** 700 times less stable to low pH than the parent riboside, whereas near absolute stability to the reaction conditions was imparted to **3** upon introduction of the fluorine atom.

Enzyme purification and kinetics

The dCMPD from MOLT-4 cells was purified ~30 fold by a simple procedure of extraction with buffered, reducing (DTT) methanol followed by dialysis against the homogenation buffer. Enzymatic reactions were run in the presence of Mg^{+2} ion and the allosteric effector dCTP, and are described in Materials and Methods. Although a quantitative measure of the activation of the enzyme by dCTP was not determined, the deamination velocity without the activator was prohibitively slow. The Michaelis-Menton binding constant (K_M) for the substrate dCMP with human MOLT-4 dCMPD was found to be 53±2 μM. This agrees well with the value of 58 μM recently determined by Maley *et al.*[25] for HeLa cell dCMPD. The Lineweaver-Burke plots showed lines with intercepts at a constant $1/V_{max}$ for increasing inhibitor concentrations, consistent with competitive inhibition for both **2b** and **3b** (Figure 5), respectively, while the K_i's were correspondingly 2.1×10^{-8} M and 1.2×10^{-8} M. Maley *et al.* found the K_i for **2b** (prepared from 2′-deoxyuridine-5′-monophosphate and sodium amalgam) against HeLa cell-derived dCMPD to be 1.2×10^{-8} M.[24] IC_{50} values were shown to be 129 nm and 65 nm for **2b** and **3b**, respectively, with 20 μM dCTP.

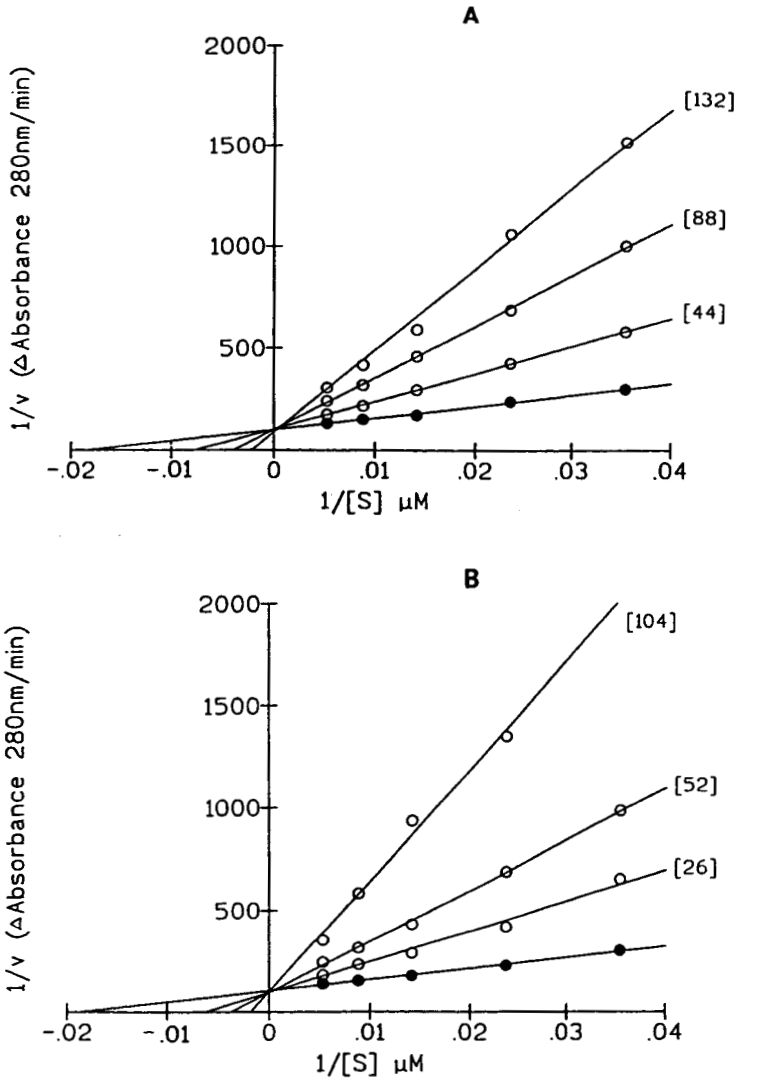


FIGURE 5 Double reciprocal plots (Lineweaver-Burk) of (A) **2b** and (B) **3b** with dCMPD at 0 inhibitor (●) concentration and varying inhibitor (○) concentrations (μM).

TABLE 1
Effect of THU, dTHU, **1a**, **2a** and **3a** on dNTP pools in Molt-4 cells

Inhibitor (50 μM)	Cellular dNTP pool (nM) TTP	dCTP	dATP	dGTP
Control	0.957(0.056)	0.126(0.012)	0.850(0.086)	0.238(0.056)
THU	1.041(0.018)	0.080(0.004)	0.651(0.033)	0.229(0.005)
dTHU	1.598(0.066)	0.714(0.022)	0.513(0.053)	0.246(0.023)
1a	2.198(0.063)	2.263(0.059)	0.583(0.023)	0.315(0.010)
2a	1.294(0.023)	0.914(0.044)	0.663(0.042)	0.169(0.051)
3a	1.345(0.092)	0.866(0.067)	0.552(0.118)	0.146(0.104)

Cells were incubated for 16 h with 50 μM. Intracellular dNTP concentrations were determined as indicated in Materials and Methods. Data are the average of three independent experiments with individual values varying by <10%. Numbers in ( ) are standard deviations.

Effect of dCMPD inhibitors on dRNT pools in whole cells

To examine the influence of the title compounds (**1a–3a**), along with THU and dTHU on cellular dCTP and TTP levels, whole MOLT-4 human peripheral lymphoblasts were incubated with the various inhibitors and the level of dRNT was quantitated by SAX HPLC. The results are illustrated in Table 1. Triplicate runs showed individual variations in dNTP levels to be < 10%. All of the compounds studied (except THU) elevated both dCTP and TTP levels relative to controls. TTP levels rose moderately (1.1–2.3 times) whereas dCTP levels increased by as much as 20 times in response to **1a** and were depleted slightly by THU (0.6 times). dATP and dGTP levels either remained constant or were slightly modified by the compounds employed

DISCUSSION

Two analogues of zebularine, **2a** and **3a** have been converted to their corresponding nucleotides and rigorously characterized by chromatographic and spectroscopic means. The observation that **2a** forms a 3′,5′-diphosphate derivative rapidly whereas **3a** does not suggests that the fluorine atom exerts some effect to render the 3′-OH group less accessible or less reactive to the phosphorous electrophile than the 2′-deoxy compound. In the course of our synthetic exploits regarding fluorinated nucleosides, we have at times encountered unusual reactivity of functional groups which seem to be influenced by the strong electronegative properties of the fluorine atom.[32] The basis for this type of reactivity modulation is the subject of further investigation.

In this work, we have shown that the purified monophosphates **2b** and **3b** were potent and competitive inhibitors of dCMPD from an extract of MOLT-4 human peripheral lymphoblasts with K_i values of 2.1×10^{-8} M and 1.2×10 M, respectively. These data are consistent with those recently reported for the inhibition of purified

HeLa-cell dCMPD by **2b** ($K_i = 1.2\times10^{-8}$ M).[25] The nearly identical inhibitory constants for **2b** with an affinity-purified enzyme and an enzyme from a comparatively crude preparation, may allow rapid screening of potential dCMPD inhibitor candidates with a minimum effort devoted to enzyme isolation. Although the extent of phosphatase activity was not determined in the protein preparation described here, it is most surely ineffective in degrading the nucleotides considering the values obtained from the kinetic studies.

In metabolism studies with whole MOLT-4 cells, it was shown that the 2′-deoxy compounds **2a** and **3a** elevated dCTP levels 8–10 times over control values which closely paralleled the effect with dTHU, both in our hands and others.[9] Very little modulation was seen with THU, as was expected since THU is a riboside. Surprisingly, the other riboside tested, compound **1a**, elicited a > 20 fold elevation in dCTP levels as shown by HPLC. The elevation of dCTP levels may be regarded as presumptive evidence for *in situ* inhibition of dCMPD by a cellular metabolite of the nucleosides employed, in all likelyhood the 5′-monophosphates. The increased production of dCTP could in turn activate dCMPD causing a weak elevation of the TTP pool size (1.1–2.4 times, see Table 1) to recover the loss due to blockage of dCMPD, and reestablish an equilibrium ratio of dCTP:TTP. Since it is well documented that dTHU is phosphorylated intracellularly[7,33] it is reasonable to assume that **2a** and **3a** may also be converted to **2b** and **3b** inside the cell; these metabolites will inhibit dCMPD, thus driving the *de novo* pathway toward production of dCTP (Figure 1). We attempted to quantify the activity of dCMPD in whole cells in the presence or absence of the inhibitors by calculating the dCMPD deaminase activity index as devised by Plunkett,[9] but the data were inconclusive (data not shown). Definitive proof of the mechanism(s) responsible for the pharmacology outlined above must await a comprehensive evaluation of this system.

The metabolic fate of compound **1a** is worth noting. By virtue of being a ribonucleoside, zebularine, presumably as the nucleoside-5′-diphosphate, must first be reduced at the 2′ position and further converted to **2b** to exert any negative effect on dCMPD since we showed that **1b** has no inhibitory activity against dCMPD even at 100 μM concentrations. The conversion of **1a** to **2b** has been documented to occur in *E. coli*, and this path was deemed responsible for the cytotoxic effects of **1a** towards the bacteria.[23] If this path is functional in MOLT-4, it follows that the dCTP pools should rise in response to inhibition of dCMPD by **2b** originating from **1a**. However, it is difficult to rationalize the more than 2-fold difference in dCTP pool size when comparing **1a** with **2a, 3a** or dTHU, assuming dCMPD inhibition is the cause of the dCTP pool elevation. Although zebularine is a powerful inhibitor of CDA, a mechanism involving blockage of cytidine nucleosides to uridine nucleosides is, in all probability, not operable here since there are very low to undetectable levels of CDA in many hematopoietic cell lines, including MOLT-4.[15,34] One possible explanation is that the uptake and/or intracellular phosphorylation of **1a** is much more efficient in MOLT-4 than the other inhibitors. A comprehensive evaluation of the metabolism of **1a** in these cells is underway in our laboratory.

The fluorinated compound **3a** was originally prepared as an intermediate in the synthesis of 2′-fluoro-2′,3-dideoxyzebularine.[35] We reasoned that **3a** might serve as

an acid-stable isostere of **2a** if it was accepted by dCMPD as its nucleotide. As was reported above, **3b** was about twice as potent as **2b** in inhibiting partially purified dCMPD while being infinitely more stable toward acid-catalyzed degradation. The fluorinated derivative is chemically phosphorylated much more cleanly and in higher yield than **2a**. This, along with the fact that **3a** seemed to inhibit dCMPD equally well in whole cells argues that compound **3a** is a more suitable candidate than **2a**, or dTHU, as a pharmacological tool for inhibiting dCMPD and advancing our understanding of the mechanisms involved in human PyrdRN biosynthesis. Based on the results of this study, it also seems evident that examination of the metabolism of the parent zebularine, may provide new insights into the mechanism of dRNT modulation in human cells.

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