Synthesis and Biological Activity of Aromatic Amino Acid Phosphoramidates of 5-Fluoro-2′-deoxyuridine and 1-β-Arabinofuranosylcytosine: Evidence of Phosphoramidase Activity

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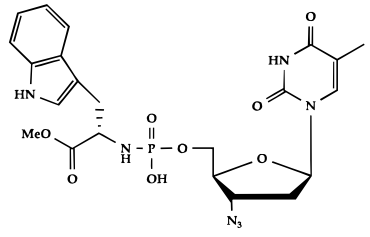
The amino acid phosphoramidate diesters of FUdR (**2**) and Ara-C (**6**), 5-fluoro-2′-deoxy-5′-uridyl *N*-(1-carbomethoxy-2-phenylethyl)phosphoramidate (**5a**), 5-fluoro-2′-deoxy-5′-uridyl *N*-(1-carbomethoxy-2-indolylethyl)phosphoramidate (**5b**), 1-β-arabinofuranosylcytosine 5′-*N*-(1-carbomethoxy-2-phenylethyl)phosphoramidate (**8a**), and 1-β-arabinofuranosylcytosine 5′-*N*-(1-carbomethoxy-2-indolylethyl)phosphoramidate (**8b**), were synthesized and tested for their antitumor activity against L1210 mouse lymphocytic leukemia cells and CCRF-CEM human T-cell lymphoblastic leukemia cells. Ara-C phosphoramidates **8a,b** were found to be inactive at a concentration of 100 μM, while the FUdR conjugates **5a,b** exhibited IC_{50} values within a range of 0.30−0.40 μM. Stability studies revealed that >99% of the phosphoramidates remained intact after incubation for >2 days in 20% calf or 20% human serum. Intracellular thymidylate synthase (TS) inhibition studies revealed that treatment of L1210 and CCRF-CEM cells with **5a** or **5b** resulted in significant inhibition of TS in intact and permeabilized cells, while treatment of L929 TK^- cells with these compounds did not result in inhibition of TS activity in intact cells. However, permeabilization of L929 TK^- cells enhanced the activity of **5a,b** toward intracellular TS by 900- and 1500-fold, respectively. In addition, incubation of cell-free extracts of CEM cells with radiolabeled **5b** resulted in the rapid production of FUdR 5′-monophosphate and a lag in the generation of FUdR. Consequently, it is proposed that the metabolism of the phosphoramidate diesters of FUdR in proliferating tissue proceeds through two separate enzymatic steps involving P−N bond cleavage by an unknown phosphoramidase followed by P−O bond cleavage by phosphatases such as 5′-nucleotidase.

Introduction

Several purine and pyrimidine base and nucleoside analogs are important weapons in the anticancer and antiviral chemotherapeutic arsenal. The biological activity of most of these analogs requires intracellular metabolism to 5′-mononucleotides by kinase-mediated phosphorylation. The development of drug resistance due to decreased nucleotide kinase activity has limited the effectiveness of these agents. In order to overcome the problem of drug resistance, prodrug approaches have been developed to deliver phosphorylated nucleoside analogs as neutral derivatives into the cell. These prodrugs must be converted intracellularly to the corresponding nucleotides. Several attempts have been made to deliver the 5′-monophosphate of 5-fluoro-2′-deoxyuridine (FUdR) to tumor cells via phosphoramidate derivatives.[1−5] In all but one report, the activity of FUdR phosphoramidate triesters, rather than phosphoramidate diesters, was investigated, presumably because phosphoramidate diesters would be charged and therefore less likely to diffuse across the cell membrane. The lack of observable cytotoxicity associated with the phosphoramidate diester, 5-fluoro-2′-deoxy-5′-uridyl *N*-(1-carboxy-3-methylethyl)phosphoramidate, supported this assumption.[2] In addition, although cyclic phosphoramidate derivatives of FUdR are rapidly converted in cell culture to the corresponding phosphoramidate diesters, the associated activity was shown to result from extracellular conversion to the parent nucleoside.[6]

Recently, our laboratory has reported the chemical synthesis and biological activity of a series of aromatic amino acid phosphoramidate di- and triesters of 3′-azido-3′-deoxythymidine (AZT).[7,8] One of these derivatives, AZT 5′-*N*-(1-carbomethoxy-2-indolylethyl)phosphoramidate, **1**, was found to be 8-fold more active than the parent nucleoside and at least 10-fold less toxic.



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Surprisingly, when the diester derivatives were incubated in fetal calf serum at pH 7.2, 37 °C, for 6 days, no degradation to the corresponding 5′-monophosphate or nucleoside was observable. Preliminary mechanistic studies demonstrated that **1** is internalized by lymphocytes to the same extent as AZT.[7] However, in contrast to peripheral blood mononuclear cells (PBMCs) incubated with AZT, little or no free nucleoside and nearly 4-fold more phosphorylated AZT were observed in PBMCs incubated with **1**.[7]

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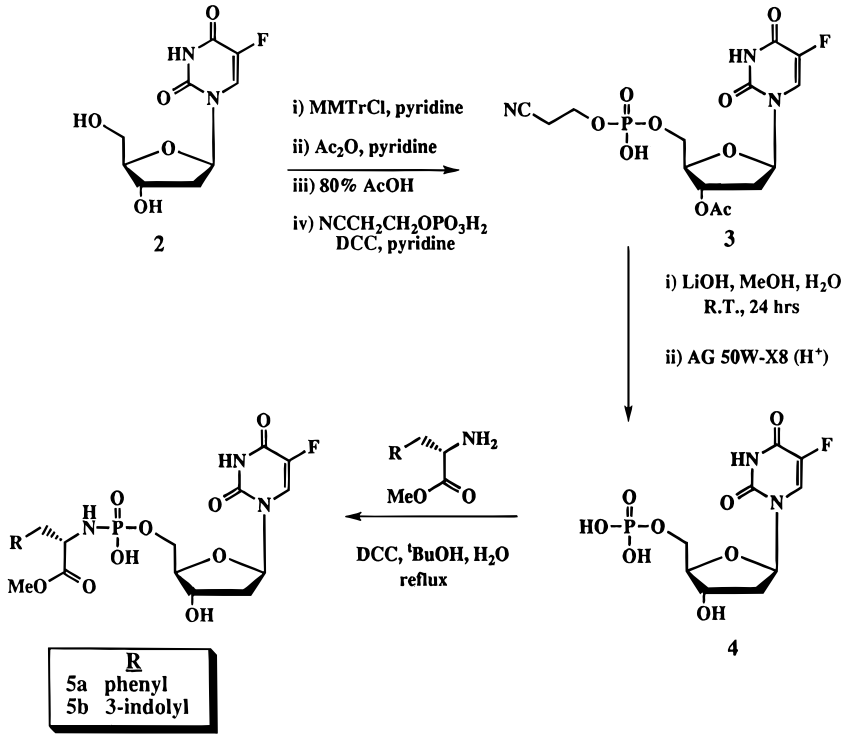
Scheme 1



The behavior of AZT phosphoramidates in human PBMCs led us to propose that aromatic amino acid phosphoramidate diesters may be useful for the delivery of phosphorylated FUdR and 1-β-arabinofuranosylcytosine (Ara-C) to neoplastic tissues. In this report we describe the sythesis of a series of phenylalanine and tryptophan phosphoramidate diesters (**5a,b** and **8a,b**) of FUdR (**2**) and Ara-C (**6**), respectively, and the study of their ability to inhibit the growth of murine and human leukemia cell lines. In addition, mechanistic studies conducted to determine the extra- and intracellular behavior of these compounds are also described.

Chemistry

The synthetic protocol developed for constructing phosphoramidates of FUdR was based on a procedure by Moffatt and Khorana, in which they describe the dicyclohexylcarbodiimide (DCC)-mediated coupling of adenosine 5′-monophosphate to *p*-anisidine.[9] Initial attempts to synthesize FUdR 5′-monophosphate directly from FUdR, employing phosphorus oxychloride in triethyl phosphate, also resulted in 3′-OH phosphorylation.[10,11] Therefore, it was necessary to protect the 3′-OH in order to effect selective phosphorylation at the 5′-OH (Scheme 1). The 5′-OH of FUdR was first protected as the monomethoxytrityl derivative (MMTrCl in pyridine) and the 3′-OH as the acetate (acetic anhydride in pyridine).[12,13] Deprotection of the 5′-*O*-MMTr-3′-*O*-AcFUdR with 80% acetic acid yielded 3′-*O*-AcFUdR. Phosphorylation of the 5′-OH was accomplished by treating 3′-*O*-AcFUdR dissolved in pyridine with 2-cyanoethyl phosphate in the presence of DCC to yield 3′-*O*-AcFUdR 5′-(2-cyanoethoxy)phosphate, **3**.[12,13] Removal of both the acetyl and cyanoethyl moieties was accomplished by treatment with LiOH in methanol and water. The resulting FUdR 5′-monophosphate, **4**, was subsequently purified by ion-exchange chromatography (H^+).

Once the FUdR 5′-monophosphate was obtained, the methyl esters of phenylalanine and tryptophan were coupled to the phosphorylated nucleoside in a straightforward manner by minor modification of the method of Moffat and Khorana.[9] Typically, FUdR 5′-monophosphate, the amino acid methyl ester, and DCC were dissolved in *tert*-butyl alcohol and water. After refluxing for 4 h, the 5′-amino acid phosphoramidates of FUdR, **5a,b**, were isolated in yields ranging from 63% to 72%, respectively.

In contrast to FUdR, the 5′-monophosphate (**7**) of Ara-C was prepared directly by treatment with phosphorus oxychloride in triethyl phosphate (Scheme 2).[10,11,14] The coupling of **7** with the methyl esters of phenylalanine or tryptophan was carried out as described above for FUdR, affording the corresponding phosphoramidates **8a,b** in yields of 63% and 58%, respectively.

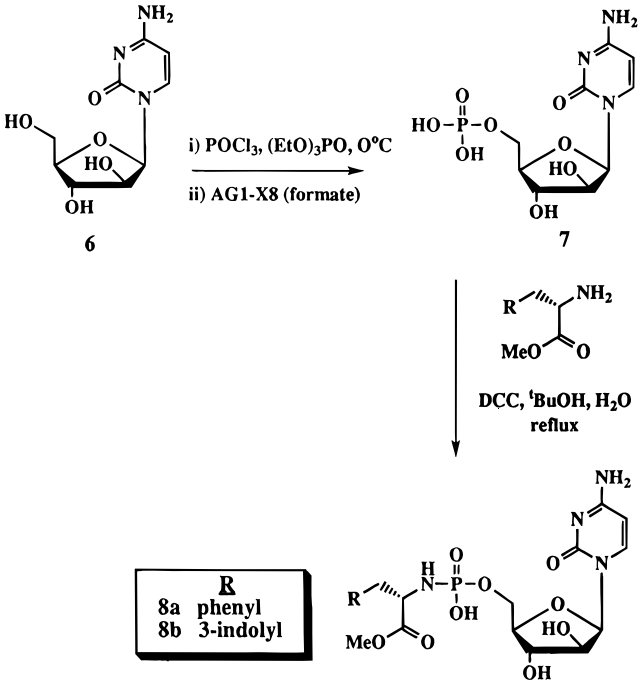
Before testing the biological activity of the phosphoramidate diesters, the stability of these compounds in culture media and human serum was determined. The rates of decomposition of the phosphoramidates **5a,b** and **8a,b** in serum were determined by incubating each compound at a concentration of 100 μM in 20% fetal calf or 20% human serum, pH 7.2, at 37 °C followed by analysis of the remaining phosphoramidates by reverse-phase HPLC at 14, 24, 40, and 60 h.

The rate of decomposition for the four phosphoramidates was shown to be negligible over 2.5 days, ranging from 4.0×10^{-10} to 0.2×10^{-10} mol/h in fetal calf serum and from 2.3×10^{-10} to 0.2×10^{-10} mol/h in human serum. Typically, >99% of the added phosphoramidate remained intact after incubation in culture media or human serum for 2.5 days. Consequently, unlike 5′-phosphorylated nucleosides, the phosphoramidates are not rapidly degraded by endogenous blood phosphohydrolases or phosphorylases.

Table 1. *In Vitro* Cytotoxicity of Phosphoramidate Diesters

compd	cell line	IC_{50}, μM
2	L1210	0.012 ± 0.006
	CCRF-CEM	0.002 ± 0.002
	CEM-TK$^-$	66.3 ± 12.0
	L929 TK$^-$	7.7 ± 1.9
4	L1210	0.015 ± 0.001
	CCRF-CEM	0.0055 ± 0.003
	L929 TK$^-$	6.8 ± 1.8
5a	L1210	0.41 ± 0.1
	CCRF-CEM	0.32 ± 0.01
	CEM-TK$^-$	86.5 ± 6.0
	L929 TK$^-$	200 ± 14
5b	L1210	0.40 ± 0.02
	CCRF-CEM	0.32 ± 0.12
	CEM-TK$^-$	58.1 ± 15.0
	L929 TK$^-$	240 ± 28
6	CCRF-CEM	0.090 ± 0.035
7	CCRF-CEM	0.100 ± 0.023
8a	CCRF-CEM	135 ± 6
8b	CCRF-CEM	195 ± 13

Scheme 2



Results and Discussion

Biological Activity. The inhibitory activities of **5a,b** and **8a,b** on the murine leukemia cell line L1210 and human leukemia cell line CCRF-CEM were determined and are given in Table 1. In each case, the cells were treated with the compounds for 48 h and the number of remaining viable cells was determined with a trypan blue dye exclusion assay.[15] The IC_{50} values for the FUdR-containing compounds **5a,b** were similar in CCRF-CEM and L1210 cells, ranging from 0.32 to 0.41 μM, respectively. Little observable dependence on either the indolyl or benzyl side chain was detected. Nevertheless, **5a,b** were found to be 160-fold less cytotoxic than FUdR toward CCRF-CEM cells and 34-fold less cytotoxic toward L1210 cells.

The ascertain whether the antitumor activity of **5a,b** was due to their intracellular conversion to either the corresponding 5′-monophosphate or parent nucleoside, cytotoxicity studies were conducted with cell lines devoid of thymidine kinase (TK$^-$) activity. As can be seen in Table 1, **5a,b** are 29000−43000-fold less cytotoxic toward CEM-TK$^-$ cells relative to CEM cells, respectively, and 17000−21000-fold less cytotoxic toward the mouse fibroblast cell line L929 TK$^-$ when compared to L1210 cells. Therefore, because thymidine kinase is necessary for the activity of **5a,b**, these compounds must ultimately be converted intracellularly to FUdR and not FUdR 5′-monophosphate. However, the transient intracellular generation of the nucleoside monophosphate, which is quickly dephosphorylated to the parent nucleoside, cannot be ruled out by these experiments.

Table 2. Inhibition of Cellular Thymidylate Synthase Activity in Intact and Permeabilized Cells

		IC_{50}, μM		
compd	cell line	intact	permeabilized	*I/P*
2	L1210	0.043 ± 0.027	59 ± 35	0.0007
	L929 TK$^-$	7.3 ± 3.6	11 ± 4.9	0.66
4	L1210	0.017 ± 0.001	0.042	0.40
	L929 TK$^-$	>10	0.008 ± 0.004	>1250
5a	L1210	21 ± 9	5.0 ± 1	4.2
	CCRF-CEM	190 ± 92	3.1 ± 0.71	61.3
	L929 TK$^-$	>1000	1.1 ± 0.74	>909
5b	L1210	12.0 ± 8.5	2.3 ± 0.87	5.2
	CCRF-CEM	27 ± 9.9	0.89 ± 0.25	30.3
	L929 TK$^-$	>1000	0.67 ± 0.55	>1492

In contrast to the FUdR derivatives, the phosphoramidates of Ara-C, **8a,b**, were shown to be inactive, exhibiting IC_{50} values greater than 100 μM, although Ara-C displayed potent cytotoxicity. The difference in cell growth inhibitory activity between the FUdR and Ara-C phosphoramidates may be due either to preferential transport of aromatic amino acid phosphoramidates of uridine relative to cytosine or to the substrate specificity of the metabolizing enzyme or enzymes.

Metabolism Studies. To determine the mechanism of action of the two active FUdR phosphoramidates **5a,b**, we chose to directly measure *in situ* the inhibition of thymidylate synthetase (TS) activity in intact cells with a rapid and convenient tritium release assay (Table 2).[16] In addition, the inhibitory effect of the FUdR analogs on TS activity was assayed in permeabilized cells from normal and TK$^-$ mutant strains in order to assess the role of cellular uptake and phosphorylation on their antitumor activity.

Determination of the IC_{50} values in intact cells revealed that **5b** was 1.8- and 7.0-fold more effective than **5a** at inhibiting the cellular TS activity of L1210 and CCRF-CEM cells, respectively. Neither compound was capable of inhibiting TS activity in L929 TK$^-$ cells, which is consistent with the inability of these compounds to inhibit the growth of thymidine kinase deficient cells and implies that they are processed to FUdR. The IC_{50} values for **5a,b** were increased by at least 50- and 80-fold in L929 TK$^-$ cells compared to L1210 cells, respectively. A similar trend was also observed when the IC_{50} values for L929 TK$^-$ cells are compared to the IC_{50} values for CCRF-CEM cells, in which a 40- and 5-fold increase in the IC_{50} values was observed for **5a,b**, respectively.

To assess the significance of transport on the inhibitory activity of **5a,b** on intracellular TS, the effect of permeabilization with high molecular weight dextran sulfate on the activity of TS in L1210, CEM, and L929 TK$^-$ cells was determined as previously described.[17] Permeabilization resulted in an increase of 4.2- and 5.2-fold in the TS inhibitory activity in L1210 cells of **5a,b**,

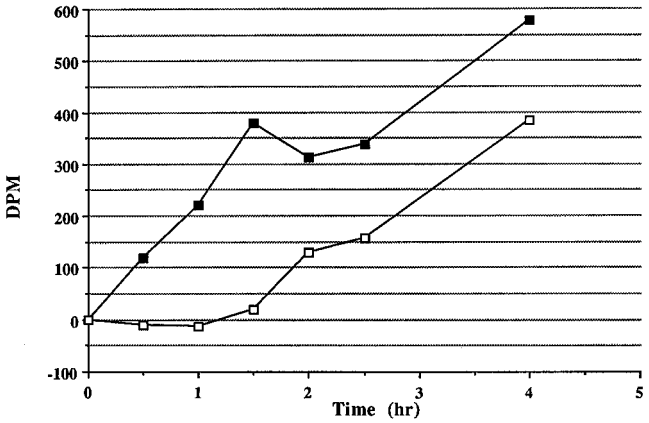


Figure 1. Production of FUdR 5′-monophosphate (■) and FUdR (□) from cell-free extracts of CEM cells incubated with radiolabeled **5b**. The radiolabeled compound was HPLC purified, and the DPM values were corrected for background counts as described in the Experimental Section.

respectively. For CCRF-CEM cells, an increase of 61- and 30-fold for **5a,b** was observed, respectively. Unexpectedly, permeabilization of L929 TK$^-$ cells resulted in a 900- and 1500-fold increase in the TS inhibitory activity observed for **5a,b**, respectively.

In order to directly observe the generation of FUdR 5′-monophosphate and FUdR by CEM cells, we synthesized 6-^{3}H-labeled **5b**. Upon incubation of 10 mM ^{3}H-labeled **5b** with CEM cell extracts, a time dependent generation of FUdR 5′-monophosphate and FUdR was observed (Figure 1). The production of FUdR lagged significantly behind the production of FUdR 5′-monophosphate. After 2 h, however, the rate of production of FUdR 5′-monophosphate (138 DPM/h) and FUdR (132 DPM/h) became nearly identical.

Mechanistic Implications. The results obtained are consistent with metabolism of **5a,b** to the corresponding 5′-monophosphate before conversion of FUdR. Although the cytotoxic response of **5a,b** maybe partially due to direct inhibition of thymidylate synthetase, this possibility seems unlikely given that the growth of TK$^-$ cells was not significantly inhibited. It is proposed that intracellular metabolism of the phosphoramidate diesters of FUdR proceeds through two separate enzymatic steps: one involving P−N bond cleavage by an unknown phosphoramidase followed by P−O bond cleavage by phosphatases such as 5′-nucleotidase.

The possible role of a lymphocytic phosphoramidase in the antiviral activity of amino acid phosphoramidate triesters and diesters of AZT and d4T has been suggested.[7,18,19] Production of d4T 5′-monophosphate, presumably by an endogenous phosphoramidase, from CEM and CEM-TK$^-$ cells incubated with radiolabeled d4T 5′-*N*-phenyl-*N*-(1-carbomethoxy-2-methylethyl)-phosphoramidate has been observed.[19] Similar studies have demonstrated that intact PBMCs and cell-free extracts of PBMCs are capable of converting 3′-fluoro-2′-deoxythymidine (FLT) 5′-*N*-(1-carbomethoxy-2-indolylethyl)phosphoramidate to FLT 5′-monophosphate (McIntee, E. J., Remmel, R. P., Schinazi, R. F., Abraham, T. W., and Wagner, C. R., manuscript in preparation). Although the isolation of ribonucleoside 5′-phosphoramidase from rabbit and rat liver has been reported, deoxyuridine and deoxythymidine 5′-*N*-amino acid phosphoramidates were shown not to be substrates for the liver-derived enzyme, suggesting that the phosphoramidate hydrolase activity detected in proliferating tissue may be of a different nature.[20,21] Characterization of the enzyme responsible for hydrolytic activity should shed light on its cellular function. We conclude that amino acid diester phosphoramidates of FUdR warrant further study as potential therapeutic agents and prototypes of nucleotide prodrugs.

Experimental Section

Materials. NMR (^{1}H and ^{31}P) spectra were recorded on a GE Omega-300 spectrometer. An external standard of 85% H_3PO_4 was used for all ^{31}P-NMR spectra. FAB mass spectra were obtained on a VG 7070E-HF mass spectrometer. Analytical TLC was performed on Analtech silica gel GHLF (0.25 mm) plates. Column chromatography was performed with grade 62, 60−200 mesh silica gel. Flash chromatography was performed with grade 60, 230−400 mesh Merck silica gel. Column chromatography of water soluble compounds on silica gel was performed using the following solvent gradient: $CHCl_3$:MeOH:H_2O (5:2:0.25), $CHCl_3$:MeOH:H_2O (5:3:0.5), and finally $CHCl_3$:MeOH:H_2O (5:4:1). Reverse-phase MPLC was performed at a pressure of 10−12 psi using an FMI lab pump equipped with a flow meter and pulse dampener. A C-18 column ($^3/_4$ in. × 18 in., 230−400 mesh) was used with a solvent flow rate of ∼1.5 mL/min. A solvent gradient of H_2O:CH_3CN (95:5) to H_2O:CH_3CN (80:20) was used. Pyridine was distilled from BaO and stored over 3 Å molecular sieves. All other solvents were reagent grade and used as received. 2-Cyanoethyl phosphate was prepared from the barium salt (Aldrich Chemical Co.) by ion-exchange on BioRad AG 50W-X8 (H^+) resin. Concentration under reduced pressure refers to solvent removal on a Buchi rotary evaporator. High vacuum refers to $<10^{-2}$ psi attained with a DuoSeal mechanical pump.

[6-^{3}H]-5-Fluoro-2′-deoxyuridine 5′-monophosphate diammonium salt (16.8 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA. CCRF-CEM cells are a human T-lymphoblastoid leukemia cell line that was obtained from American Type Culture Collection, Rockville, MD. CEM-TK$^-$ cells have less than 1% of wild-type thymidine kinase activity and were obtained from the NIH AIDS Research and Reference Reagent Program, Ogden BioServices Corp., Rockville, MD. Trypan blue stain (0.4%) in saline (0.85%) was purchased from GIBCO, Grand Island, NY.

5′-*O*-(Monomethoxytrityl)-5-fluoro-2′-deoxyuridine. FUdR (**2**; 500 mg, 2.03 mmol) was dissolved in dry pyridine (10 mL) under nitrogen. Monomethoxytrityl chloride (0.9419 g, 3.05 mmol) was added and the reaction mixture stirred at room temperature for 48 h. The reaction was quenched with methanol (5 mL), and then the mixture was concentrated to dryness under reduced pressure. Flash chromatography on silica gel, using chloroform/methanol (97/3) as the solvent, gave the product as a colorless, crystalline solid (1.05 g, 100% yield). ^{1}H-NMR ($CDCl_3$): δ 9.67 (1H, s, H3), 7.82 (1H, d, $J_{H\text{-}F} = 6$ Hz, H6), 7.50−6.82 (14H, m, aromatic H's), 6.30 (1H, t, H1′), 4.56 (1H, br s, H3′), 4.08 (1H, br d, H4′), 3.77 (3H, s, OCH_3), 3.40 (2H, m, H5′), 2.78 (1H, br s, 3′-OH), 2.48 (1H, m, H2′), 2.27 (1H, m, H2′).

3′-*O*-Acetyl-5-fluoro-2′-deoxyuridine. 5′-*O*-MMTrFUdR (800 mg, 1.54 mmol) was dissolved in dry pyridine (20 mL) under nitrogen. Freshly distilled acetic anhydride (1.45 mL, 15.4 mmol) was added and the reaction mixture heated at 100 °C (boiling water bath) for 4 h. Then ∼50 g of chipped ice was added and the reaction mixture stirred until all the ice had melted. The resulting mixture was extracted with chloroform (4 × 20 mL), and the combined extracts were dried over anhydrous $MgSO_4$. Concentration under reduced pressure followed by removal of excess pyridine under high vacuum gave 3′-*O*-acetyl-5′-*O*-(monomethoxytrityl)-5-fluoro-2′-deoxyuridine as a yellow oil.

The 3′-*O*-acetyl-5′-*O*-(monomethoxytrityl)-5-fluoro-2′-deoxyuridine was redissolved in 80% acetic acid (20 mL) and heated at 100 °C for 20 min. The reaction mixture was then evaporated to dryness under reduced pressure. Repeated evaporations from ethanol to remove traces of water and acetic

acid gave a light brown solid. Trituration with diethyl ether gave 3′-*O*-acetyl-5-fluoro-2′-deoxyuridine as an off-white powdery solid (356 mg, 80% yield). ^{1}H-NMR (CD_3OD): δ 8.23 (1H, d, $J_{H\text{-}F}$ = 6 Hz, H6), 6.25 (1H, t, H1′), 5.28 (1H, br d, H3′), 4.07 (1H, m, H4′), 3.79 (2H, m, H5′), 2.31 (2H, m, H2′), 2.06 (3H, s, CH_3CO).

3′-*O*-Acetyl-5-fluoro-2′-deoxyuridine 5′-(2-Cyanoethoxy)-phosphate (3). To a mixture of 2-cyanoethyl phosphate (120 mg, 0.795 mmol) in dry pyridine (5 mL) under nitrogen was added a solution of 3′-*O*-acetylFUdR (100 mg, 0.347 mmol) and DCC (572 mg, 2.78 mmol) in dry pyridine (5 mL). The reaction mixture was stirred at room temperature for 24 h and then diluted with water (20 mL) and stirred for another 1 h. The precipitate of 1,3-dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The residue was subjected to column chromatography on silica gel with a $CHCl_3$:MeOH:H_2O gradient (see Materials). The product was isolated as a colorless solid (136 mg, 93% yield). ^{1}H-NMR (D_2O): δ 8.05 (1H, d, $J_{H\text{-}F}$ = 6 Hz, H6), 6.30 (1H, t, H1′), 5.40 (1H, br d, H3′), 4.40 (1H, m, H4′), 4.15 (2H, t, OCH_2CH_2CN), 4.05 (2H, m, H5′), 2.85 (2H, t, CH_2CN), 2.55 (1H, dd, H2′), 2.40 (1H, dd, H2′), 2.10 (3H, s, CH_3CO). ^{31}P-NMR (D_2O): δ 0.63.

5-Fluoro-2′-deoxyuridine 5′-Monophosphate (4). 3′-*O*-Acetyl-5-fluoro-2′-deoxyuridine 5′-(2-cyanoethoxy)phosphate (**3**; 135 mg, 0.32 mmol) was dissolved in a mixture of methanol (5 mL) and 0.5 N aqueous LiOH (10 mL) and stirred at room temperature for 24 h. The reaction mixture was concentrated to dryness, introduced onto an ion-exchange column (BioRad AG 50W-X8, H^+), and eluted with water. Fractions containing the product were combined and extracted with diethyl ether (3 × 15 mL). The aqueous phase was then lyophilized to give the crude product as a colorless solid which was used in the next step without further purification. ^{1}H-NMR (D_2O): δ 7.95 (1H, d, $J_{H\text{-}F}$ = 6 Hz, H6), 6.20 (1H, t, H1′), 4.45 (1H, br m, H3′), 4.10 (3H, m, H4′, H5′), 2.35 (1H, m, H2′), 2.25 (1H, m, H2′). ^{31}P-NMR (D_2O): δ 0.78. HPLC: t_R 1.48 min.

5-Fluoro-2′-deoxy-5′-uridyl *N*-(1-Carbomethoxy-2-phenylethyl)phosphoramidate (5a). To the flask containing 5-fluoro-2′-deoxy-5′-uridine 5′-monophosphate (**4**) were added phenylalanine methyl ester (400 mg, 2.24 mmol), DCC (329 mg, 1.60 mmol), ^{t}BuOH (5 mL), and water (1 mL). A reflux condenser was attached to the flask and the reaction mixture heated in a boiling water bath for 4 h. After cooling to room temperature the solvents were removed under reduced pressure. The residue was resuspended in water (15 mL) and extracted with diethyl ether (4 × 15 mL). The aqueous phase was then lyophilized. The colorless solid that was obtained was subjected to column chromatography on silica gel using a $CHCl_3$:MeOH:H_2O gradient. The product was isolated as a colorless solid (99 mg, 63% for two steps). ^{1}H-NMR (D_2O): δ 7.92 (1H, d, $J_{H\text{-}F}$ = 6 Hz, H6), 7.25 (5H, m, Phe), 6.20 (1H, t, H1′), 4.38 (1H, m, H3′), 4.01 (1H, m, H4′), 3.90 (1H, dd, CHCO_2Me), 3.71 (2H, m, H5′), 3.63 (3H, s, CO_2CH_3), 2.95 (1H, dd, PheCH_2), 2.87 (1H, dd, PheCH_2), 2.30 (1H, m, H2′), 2.13 (1H, m, H2′). ^{31}P-NMR (D_2O): δ 7.08. FABMS: $[M + 1]^+$ 488.05. HPLC: t_R 5.59 min.[22]

5-Fluoro-2′-deoxy-5′-uridyl *N*-(1-Carbomethoxy-2-indolylethyl)phosphoramidate (5b). 3′-*O*-Acetyl-5-fluoro-2′-deoxyuridine 5′-(2-cyanoethoxy)phosphate (**3**; 121 mg, 0.29 mmol) was converted to 5-fluoro-2′-deoxyuridine 5′-monophosphate (**4**) as previously described and then coupled with tryptophan methyl ester (440 mg, 2.03 mmol) in the presence of DCC (299 mg, 1.45 mmol), ^{t}BuOH (5 mL), and H_2O (1 mL). Column chromatography was carried out on silica gel using a $CHCl_3$:MeOH:H_2O gradient, and the product was further purified by reverse-phase MPLC. The pure product was isolated as a colorless, crystalline solid (109 mg, 72% for two steps). ^{1}H-NMR (D_2O): δ 7.56 (1H, d, $J_{H\text{-}F}$ = 6 Hz, H6), 7.51 (1H, d, indole H4), 7.32 (1H, d, indole H7), 7.12 (1H, s, indole H2), 7.09 (1H, t, indole H6), 7.00 (1H, t, indole H5), 6.00 (1H, t, H1′), 4.22 (1H, m, H3′), 3.96 (2H, m, H4′, CHCO_2Me), 3.69 (2H, m, H5′), 3.65 (3H, s, CO_2CH_3), 3.12 (1H, dd, indoleCH_2), 2.97 (1H, dd, indoleCH_2), 2.09 (1H, m, H2′), 1.76 (1H, m, H2′). ^{31}P-NMR (D_2O): δ 7.20. FABMS: $[M + 1]^+$ 527.0. HPLC: t_R 6.07.[22]

1-β-Arabinofuranosylcytosine 5′-Monophosphate (7). Triethyl phosphate (20 mL) was placed in a dry flask and cooled to 0 °C in an ice bath under nitrogen. Distilled phosphorus oxychloride (0.82 mL, 8.83 mmol) was added, and then Ara-C (1.00 g, 4.11 mmol) was added in one portion. The reaction mixture was stirred at 0 °C for 22 h and then poured into a mixture of diethyl ether and petroleum ether (1:1, 200 mL). The suspension was extracted with water (4 × 50 mL), and the combined aqueous extracts were neutralized to pH 7 using concentrated ammonium hydroxide.

The aqueous phase was then applied onto an ion-exchange column (BioRad AG1-X8, $HCOO^-$, 40 g, 3 cm × 13 cm, prepacked in water). The column was eluted with water (400 mL) and then with 0.5 N formic acid (400 mL). The latter eluant was concentrated to dryness and treated with acetone. The colorless solid obtained was filtered and washed with acetone (803 mg, 60% yield). ^{1}H-NMR (D_2O): δ 8.15 (1H, d, H6), 6.25 (2H, two overlapping d, H5, H1′), 4.50 (1H, t, H3′), 4.25−4.05 (4H, m, H4′, H5′, H2′). ^{31}P-NMR (D_2O): δ 1.25. HPLC: t_R 1.58 min.

1-β-Arabinofuranosylcytosine 5′-*N*-(1-Carbomethoxy-2-phenylethyl)phosphoramidate (8a). As described above for **5a**, 1-β-arabinofuranosylcytosine 5′-monophosphate (250 mg, 0.774 mmol) was coupled with phenylalanine methyl ester (970 mg, 5.41 mmol) in the presence of DCC (797 mg, 3.87 mmol). The colorless solid that was obtained was subjected to column chromatography on silica gel using a $CHCl_3$:MeOH:H_2O gradient. The product was isolated as a colorless solid (237 mg, 63% yield). ^{1}H-NMR (D_2O): δ 7.72 (1H, d, J_{6-5} = 7.5 Hz, H6), 7.30−7.10 (5H, m, Phe), 6.14 (1H, d, $J_{1'-2'}$ = 5 Hz, H1′), 5.93 (1H, d, J_{5-6} = 7.5 Hz, H5), 4.34 (1H, dd, H3′), 4.06 (1H, dd, H4′), 3.87 (2H, br m, H2′, CHCO_2Me), 3.76 (2H, br s, H5′), 3.56 (3H, s, CO_2CH_3), 2.90 (2H, br d, PheCH_2). ^{31}P-NMR (D_2O): δ 7.00. FABMS: $[M + 1]^+$ 484.86, $[M + gly]^+$ 577.24. HPLC: t_R 8.03 min.[22]

1-β-Arabinofuranosylcytosine 5′-*N*-(1-Carbomethoxy-2-indolylethyl)phosphoramidate (8b). As described above for **5a**, 1-β-arabinofuranosylcytosine 5′-monophosphate (250 mg, 0.774 mmol) was coupled with tryptophan methyl ester (1.18 g, 5.42 mmol) in the presence of DCC (797 mg, 3.87 mmol). Column chromatography on silica gel followed by reverse-phase MPLC gave the product as a colorless, crystalline solid (233 mg, 58% yield). ^{1}H-NMR (D_2O): δ 7.57 (1H, d, J_{6-5} = 7.5 Hz, H6), 7.47 (1H, d, indole H4), 7.37 (1H, d, indole H7), 7.11 (2H, overlapping s and t, indole H2, H6), 6.97 (1H, t, indole H5), 5.98 (1H, d, H1′), 5.72 (1H, d, J_{5-6} = 7.5 Hz, H5), 4.29 (1H, dd, H3′), 4.00 (1H, dd, H4′), 3.91 (1H, dd, CHCO_2Me), 3.78 (3H, br m, H5′, H2′), 3.54 (1H, s, CO_2CH_3), 3.07 (2H, br d, indole CH_2). ^{31}P-NMR (D_2O): δ 7.05. FABMS: $[M + 1]^+$ 524.00. HPLC: t_R 8.82 min.[22]

[6-^{3}H]-5-Fluoro-2′-deoxy-5′-uridyl *N*-(1-Carbomethoxy-2-indolylethyl)phosphoramidate (5b). [6-^{3}H]-5-Fluoro-2′-deoxyuridine 5′-monophosphate diammonium salt (125 μCi, 16.8 Ci/mmol) was diluted with unlabeled FUdR 5′-monophosphate diammonium salt (61 mg) to a final specific activity of 0.74 mCi/mmol. Ion-exchange chromatography on BioRad AG 50W-X8 (1.5 cm × 15 cm, 100−200 mesh, H^+) and lyophilization gave a colorless solid. This was coupled to tryptophan methyl ester (257 mg, 1.18 mmol) in the presence of DCC (174 mg, 0.85 mmol) as described above for **5b**.

Purification was by column chromatography on silica gel ($CHCl_3$:MeOH:H_2O gradient) followed by reverse-phase HPLC purification on a 10 × 250 mm 10 μm Alltech Econosphere reverse-phase C8 preparative column. The HPLC system consisted of a Beckman System Gold 406 analog interface module, System Gold 166 programmable detector module, and two 110B solvent delivery modules, a Hewlett Packard 3393A integrator, and a Rheodyne manual injector. The compound was eluted by using a gradient of water (solvent A) and acetonitrile (solvent B). The gradient ran at 4.0 mL/min starting at 100% A and 0% B with a linear gradient shift to 50% A and 50% B over the first 8 min, where it ran isocratically for 2 min. From 10 to 12 min there was a linear gradient change back to 100% A. Column effluent was monitored at a wavelength of 255 nm, and the product was

collected from 5.7 to 7.7 min, yielding a colorless solid (20.3 mg, 23% yield, 0.76 mCi/mmol).

Metabolism of [6-^{3}H]-5-Fluoro-2′-deoxy-5′-uridyl *N*-(1-Carbomethoxy-2-indolylethyl)phosphoramidate (5b) in CEM Cell Lysates. CEM cells were separated from their culture medium by centrifugation (430*g*, 5 min, room temperature). The residue (about 100 μL, 1 × 10^8 cells) was resuspended in 1 mL of buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) and sonicated with a VirSonic 300 cell disrupter with microtip adapter (4 × 4 s bursts on ice). Lysate from ~8 × 10^6 cells was incubated with [6-^{3}H]-5-fluoro-2′-deoxy-5′-uridyl *N*-(1-carbomethoxy-2-indolylethyl)phosphoramidate (10 mM, 0.76 mCi/mmol) for indicated lengths of time at 37 °C. Enzymatic reactions were halted by quickly freezing the samples in dry ice and storing at −20 °C until assayed. Methanol was added to precipitate proteins and ensure termination of enzymatic reactions. Samples were then centrifuged (13200*g*, 15 min, 4 °C), and a 20 μL aliquot from the methanolic lysate was subjected to HPLC analysis on a 4.6 × 250 mm 5 μm Spherisorb reverse-phase C8 column that was protected by a 4.6 × 7.5 mm All-Guard Econosphere RP-C8 precolumn. The HPLC system was identical with the one described above. Metabolites were eluted by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B). The gradient ran at 1.5 mL/min using 99% A and 1% B isocratically for 4 min. From 4 to 9 min there was a linear gradient change from 99% A to 70% A. From 9 to 12 min there was a linear gradient change back to 99% A; 0.5 mL fractions were collected for the first 16 min, and each was mixed with scintillation cocktail (5 mL) and then counted on a Beckman LS-3801 liquid scintillation counter. Metabolite identification was based on retention times of coinjected cold synthesized standards of FUdR 5′-monophosphate, FUdR, and **5b**. The corresponding retention times for FUdR 5′-monophosphate, FUdR, and **5b** when monitored at 260 nm were 1.8, 5.4, and 10.5 min, respectively. Values are reported in DPMs of radioactivity corresponding to the respective retention times and the background DPMs from a 10 mM control sample subtracted out.

Cytotoxicity Assays. The purity of the compounds was evaluated by HPLC prior to biological testing. In brief, separation and quantitation of nucleoside and nucleotide impurities present in the compounds to be tested were performed on a 4.6 × 250 mm 5 μm Spherisorb reverse-phase C8 column. The HPLC system consisted of a Spectra-Physics SP8800 ternary HPLC pump and SP4600 integrator, a Kratos Spectraflow 757 absorbance detector, and a Rheodyne manual injector. The compounds were eluted by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B) and monitored at 255 nm. For the FUdR compounds **5a,b**, the gradient ran at 1.5 mL/min and changed linearly from 85% A to 72% A over the first 15 min. From 15 to 20 min there was a linear gradient change back to 85% A. The retention time for FUdR was 2.30 min. For the Ara-C compounds **8a,b**, the gradient ran at 1.5 mL/min and changed linearly from 90% A to 75% A over the first 15 min. From 15 to 20 min there was a linear gradient change back to 90% A. The retention time for Ara-C was 2.29 min. Relative amounts of impurities were determined by comparing the peak area of the nucleoside and nucleotide to the area of the compound being evaluated. Purity is expressed as a percent of total area.

The compounds were evaluated for their growth inhibitory activity with L1210, L929 TK$^-$, CEM, and CEM-TK$^-$ cells in culture. The CEM and CEM-TK$^-$ cells were maintained in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 0.5% human interleukin-2. The cells were cultured with and without compound at 37 °C in a 5% CO_2−95% air environment for 48 h, at which time portions were counted for cell proliferation and viability by the trypan blue dye exclusion method.[15]

Determination of Decomposition Rates in Serum. The stability of **5a,b** and **8a,b** was assessed in fetal bovine serum and human serum. In brief, 100 μM of the compound was incubated in a solution of either 20% heat-inactivated fetal bovine serum or 20% human serum in PBS (pH 7.4) at 37 °C for 14, 24, 40, and 60 h in triplicate. Cold HPLC grade methanol (288.8 μL) was added to precipitate proteins, and 3.75 μL of 10 mM d4T in methanol was added as an internal standard. Samples were then centrifuged (13200*g*, 10 min, 4 °C), and a 20 μL aliquot from the methanolic solution was subjected to HPLC analysis. Analysis of the remaining phosphoramidate was performed on a 4.6 × 250 mm 5 μm Spherisorb reverse-phase C8 column. The HPLC system was as described above. A standard curve (2−200 μM) based on area under the curve (AUC) was constructed for the compounds with d4T (50 μM) used as the internal standard. The compounds were eluted by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B) and monitored at 260 nm. For the FUdR compounds, the gradient ran at 1.5 mL/min and changed linearly from 85% A to 75% A over the first 10 min. From 10 to 15 min there was a linear gradient change to 60% A; then from 15 to 20 min the gradient was changed back to 85% A. For the Ara-C compounds, the gradient ran at 1.5 mL/min and changed linearly from 90% A to 80% A over the first 10 min. From 10 to 15 min there was a linear gradient change to 65% A; then from 15 to 20 min the gradient was changed back to 90% A. The concentration of the remaining phosphoramidate was determined from the standard curve. Decomposition rates were determined by linear plots of the remaining concentration of the phosphoramidates relative to time.

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(22) Although the purity of **5a,b** and **8a,b** was verified by NMR, HPLC, and MS analyses, their hydroscopic nature prevented the obtaining of satisfactory (<0.5% of theoretical) elemental analyses. Compounds were therefore judged pure by observation of a single peak using two different RP-HPLC methods.

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