Synthesis and Antitumor Evaluation of Bis[(pivaloyloxy)methyl] 2′-Deoxy-5-fluorouridine 5′-Monophosphate (FdUMP): A Strategy To Introduce Nucleotides into Cells

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The bis[(pivaloyloxy)methyl] [PIV_2] derivative of 2′-deoxy-5-fluorouridine 5′-monophosphate (FdUMP) was synthesized as a potential membrane-permeable prodrug of FdUMP. The compound was designed to enter cells by passive diffusion and to revert to FdUMP after removal of the PIV groups by hydrolytic enzymes. The most convenient preparation of PIV_2FdUMP was by condensation of 2′-deoxy-5-fluorouridine (FUdR) with PIV_2 phosphate in the presence of triphenylphosphine and diethyl azodicarboxylate (the Mitsunobo reagent). PIV_2FdUMP was stable in the pH range 1.0–4.0 ($t_{1/2}$ > 100 h). It was also fairly stable at pH 7.4 ($t_{1/2}$ = 40.2 h). In 0.05 M NaOH solution, however, it was rapidly degraded ($t_{1/2}$ < 2 min). In the presence of hog liver carboxylate esterases, PIV_2FdUMP was converted quantitatively to the mono-[(pivaloyloxy)methyl] [PIV_1] analogue PIV_1FdUMP. After a 24 h incubation, only trace amounts of FdUMP (1–3%) were observed, indicating that PIV_1FdUMP is a poor substrate for carboxylate esterases. In mouse plasma, PIV_2FdUMP was rapidly metabolized, first to PIV_1FdUMP and then to FdUMP. With continued incubation, FUdR was formed, presumably due to further catabolism of FdUMP by plasma phosphatases or 5′-nucleotidases. Since PIV_1FdUMP is a poor substrate for carboxylate esterase, the cleavage of the second PIV group is most likely mediated by plasma phosphodiesterases. The rate of degradation of PIV_2FdUMP in the presence of acid and alkaline phosphatase, 5′-nucleotidase, or spleen phosphodiesterase was the same as that in buffer controls, indicating that the compound is not a substrate for these nucleotide catabolizing enzymes. The concentration of PIV_2FdUMP and its 3′-*O*-acetyl ester (PIV_2 3′-O-Ac-FdUMP) required to inhibit the growth of Chinese hamster ovary (CHO) cells in vitro to less than 50 cells per colony was 5×10^{-6} M, the same as that required for 5-fluorouracil (FU). Both nucleotide prodrugs showed the same growth-inhibitory potency against a mutant CHO cell line that was 20-fold resistant to FU (CHO/FU). Administered intraperitoneally at optimal dosage for 5 consecutive days, PIV_2FdUMP and PIV_2 3′-*O*-Ac-FdUMP were as effective as FU at prolonging the life spans of mice bearing intraperitoneally implanted P388 leukemia. Both prodrugs retained full therapeutic activity against a P388 subline resistant to FU. Collectively, these data indicate that PIV_2FdUMP and PIV_2 3′-*O*-Ac-FdUMP are effective membrane-permeable prodrugs of FdUMP.

Introduction

5′-Mononucleotides play an important role in cellular metabolism, particularly as precursors for the biosynthesis of nucleic acids. However, the utility of such compounds as chemotherapeutic agents is limited. First, they are charged at physiologic pH and poorly penetrate into cells.[1,2] Second, they are susceptible to rapid degradation to the corresponding nucleosides in tissues by nonspecific phosphohydrolases, such as phosphatases and nucleotidases.[3,4]

To overcome these limitations, numerous attempts have been made to synthesize prodrugs of 5′-mononucleotides. The most frequent approach has been to prepare neutral lipophilic phosphotriesters that might penetrate cells by passive diffusion and revert, by chemical or enzymatic hydrolysis, to the parent ionic nucleotides. For example, simple alkyl or aryl esters of 6-mercaptopurine riboside 5′-phosphate (6-MPRP),[5,6] 2′-deoxy-5-fluorouridine 5′-phosphate,[7,8] and cyclic AMP[9] have been investigated. From a therapeutic point of view, these approaches have not been successful, presumably because the triesters failed to revert intracellularly to the parent nucleotides.

To overcome this problem, we have investigated the potential of neutral bis[(acyloxy)methyl] organophosphates as membrane-permeable prodrugs of the parent ionic phosphates.[10,11] The anticipated mechanism of reversion of the bis[(acyloxy)methyl] derivatives to the parent ionic compounds is shown in Scheme 1. After entering cells by passive diffusion, one of the (acyloxy)-methyl groups of **1** should be cleaved by nonspecific carboxylates esterases to generate the hydroxymethyl analogue, **2**. This intermediate should be inherently chemically labile and spontaneously dissociate with elimination of one molecule of formaldehyde to yield the corresponding mono[(acyloxy)methyl] phosphodiester, **3**. Repetition of this sequence with the second (acyloxy)-methyl group should generate the parent dianionic phosphate, **5**. Alternatively, **3** might be converted directly to **5** by the action of phosphodiesterases.

We have previously reported the application of this strategy to a number of nucleoside 5′-monophosphates including 2′-deoxy-5-fluorouridine 5′-monophosphate (FdUMP),[12,13] 2′,3′-dideoxyuridine 5′-monophosphate,[14] thymidine 5′-monophosphate,[15] 3′-azidothymidine 5′-

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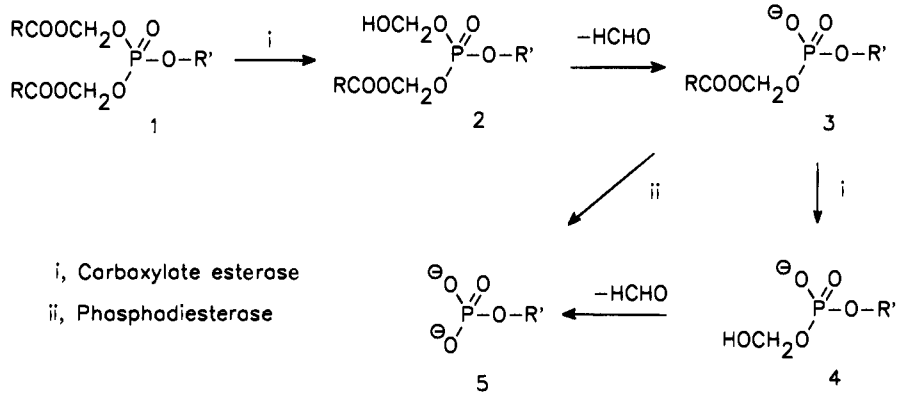
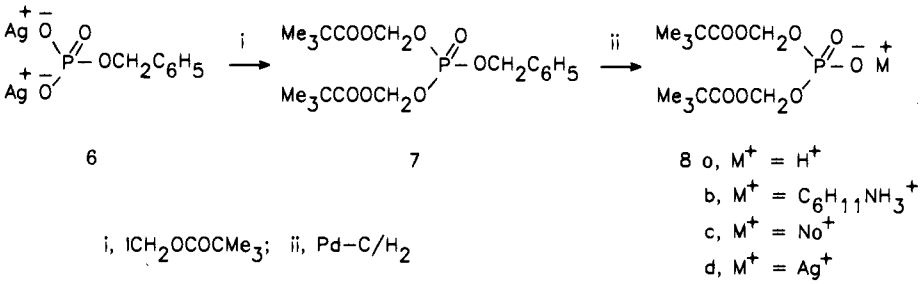
Scheme 1



Scheme 2



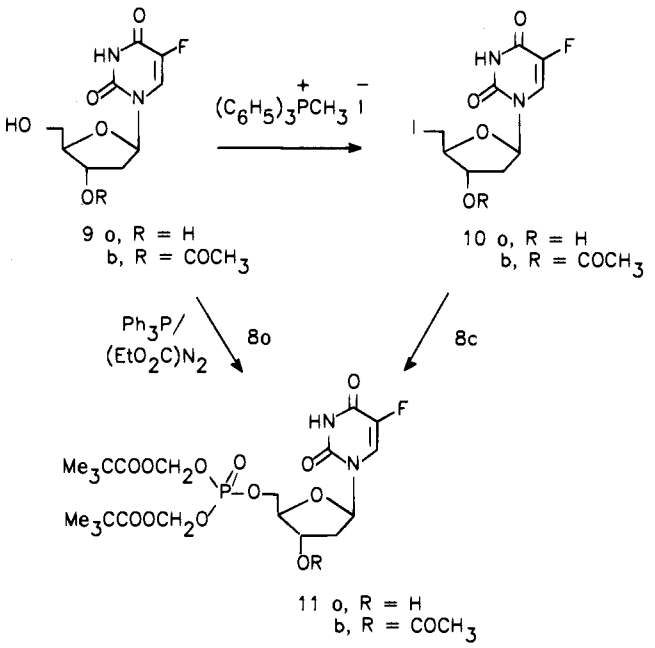
monophosphate,[16] and nucleoside phosphonates.[11] This strategy has been adopted by others to enhance the membrane permeability of cAMP[17] and phosphonoformate[18] and to facilitate the oral absorption of PMEA.[19,20] A number of alternative approaches to facilitate the entry of 5′-mononucleotides into cells, based upon the use of phosphotriester prodrugs, have recently been described.[21-26]

In this paper, we provide a detailed account of the chemical and biological properties of bis([pivaloyloxy)-methyl] [PIV_2] FdUMP (**11a**, Scheme 3).

Results

Synthesis. To avoid synthetic problems associated with the presence of the free 3′-OH group in **11a**, we selected the 3′-*O*-acetyl derivative, **11b** as a model compound to explore synthetic routes to PIV_2FdUMP (**11a**). Our first approach to the synthesis of **11b** was to react silver bis[(pivaloyloxy)methyl] phosphate (**8d**) with 3′-*O*-acetyl-2′,5′-dideoxy-5′-iodo-5-fluorouridine (**10b**) in toluene. **8d** was prepared from disilver benzyl phosphate as shown in Scheme 2. The free acid, **8a**,[10] prepared by catalytic hydrogenation of bis[(pivaloyloxy)-methyl] benzyl phosphate (**7**), was converted first to the cyclohexylammonium salt, **8b**, and then by passage through a strong cation-exchange resin (Na^+ form), to the sodium salt, **8c**. The silver salt, **8d**, was obtained by treatment of **8c** with $AgNO_3$ in aqueous solution. Reaction of **8d** with **10b** in toluene (Scheme 3) afforded a complex mixture of products from which the target phosphotriester **11b** was isolated in 16% yield. Efforts to improve the yield of **11b** by modifying the reaction conditions (temperatures and solvents) were unsuccessful. An alterative synthetic route to **11b** was attempted (Scheme 3) by direct condensation of 3′-*O*-acetyl-2′-deoxy-5-fluorouridine (3′-*O*-Ac-FUdR) (**10b**) with **8a** in dimethylacetamide in the presence of triphenylphosphine and diethyl azodicarboxylate (the Mitsunobo

Scheme 3

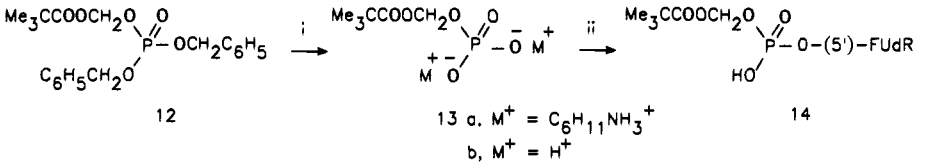


reagent).[27] Although the reaction was slow (6 days), **11b** was obtained in 66% yield. The unsubstituted triester, **11a**, was isolated in 57% yield when the same condensation reaction was attempted between **8a** and FUdR (**9a**). This synthetic route proved the most satisfactory for preparing **11a**, since it avoided problems associated with the use of 3′-OH protecting groups.

Solubility of 11a in Aqueous Buffer. The maximum solubility of **11a** in 0.05 M phosphate buffer, pH 7.4, determined by stirring the compound with the buffer for 2 h at ambient temperature, was 3.6 mg/mL.

Partition Coefficient of 11a. The partition coefficient (*P*) of **11a** between 1-octanol and 0.05 M potassium buffer, pH 7.4 (i.e., [1-octanol/buffer]), was 15.1 ($\log P = 1.18$).

Scheme 4[a]



[a] (i) Pd–C/H_2; (ii) FUdR/Ph_3P/$EtO_2CN{=}NCO_2Et$.

Table 1. Half-lives[a] (h) of **11a** in Aqueous Media under Different pH Conditions

buffer	half-life (h)
0.20 M HCl/KCl solution, pH 1.0	142
0.05 M sodium acetate buffer, pH 4.0	172
0.05 M potassium phosphate buffer, pH 7.4	40.2
0.05 M Tris-HCl buffer, pH 9.0	21.6
0.005 M NaOH, pH, 11.7	0.37
0.05 M NaOH, pH 12.6	0.03

[a] Half-lives represent the average of triplicate determinations.

Stability Studies of 11a. 1. Aqueous Media. 11a was minimally degraded ($t_{1/2}$ > 100 h) when stirred at ambient temperature in aqueous media at pH 1 and 4 (Table 1). It was also fairly stable at pH 7.4 and 9.0. In 0.05 N NaOH solution, however, it was rapidly degraded ($t_{1/2}$ < 2 min).

2. Enzyme Studies. a. Esterases. 11a was designed to be converted to FdUMP in the presence of carboxylate esterase. To examine this degradation pathway, a solution of **11a** in phosphate buffer, pH 7.4 (0.1 μmol in 1 mL), was incubated with hog liver carboxylate esterase at 37 °C for 24 h. In the presence of 0.3 units of the enzyme, **11a** was degraded with a half-life of 4 h. The half-life was reduced to 1.9 h in the presence of 1.0 unit of the enzyme and to 0.25 h in the presence of 2.5 units of the enzyme. The progressive decrease in the concentration of **11a** was accompanied by a progressive increase in the concentration of PIV_1-FdUMP (**14**). No other product was detected during the first 7 h of incubation. However, a small amount (1–3%) of FdUMP was observed after 24 h incubation.

b. Phosphohydrolases. 11a was incubated for 2 h at 37 °C with a 10-fold unit excess of alkaline phosphatase (EC 3.1.3.1), 5′-nucleotidase (EC 3.1.3.5), phosphodiesterase I (EC 3.1.4.1), and crude snake venom in 0.1 M Tris buffer, pH 8.0. The rate of degradation of **11a** under these conditions was the same as that in buffer alone (data not shown), indicating that the compound was not a substrate for these nucleotide-catabolizing enzymes.

c. Mouse Plasma. 11a was degraded with a half-life of less than 5 min in mouse plasma (25% in 0.05 M phosphate buffer, pH 7.4) at 37 °C (Figure 1). Fifteen minutes after the start of the incubation, unchanged **11a** could not be detected. PIV_1FdUMP was formed rapidly and reached a peak concentration at 15 min. Thereafter, PIV_1FdUMP levels declined with a half-life of 72 min. This was accompanied by a progressive increase in FdUMP levels. The concentration of FdUMP plateaued at 3 h and then gradually decreased with a half-life of 11.3 h. FUdR was also detected in the incubation mixture. Although present at comparatively low concentration during the first few hours, it constituted the major metabolite in the 24 h time sample. In contrast to the rapid degradation of **11a** in normal mouse plasma, the half-life of the compound in boiled plasma was 13.3 h; FdUMP was not detected at any time during the 24 h incubation period. In 100% mouse plasma (data not shown), the degradation kinetics of **11a** wre qualitatively similar to that in 25% plasma. However, the concentration of FdUMP peaked at 30–60 min and then declined with a half-life of 7.4 h.

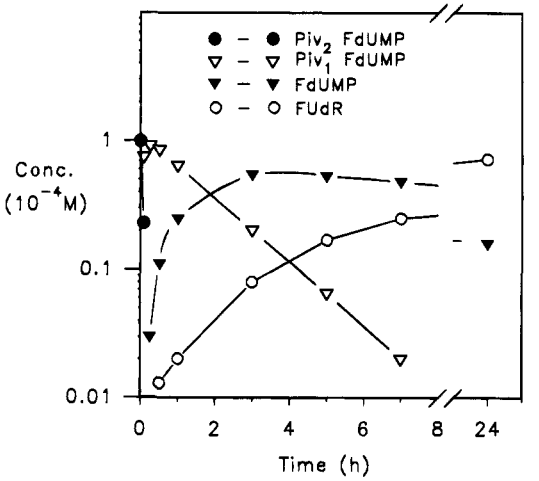


Figure 1. Degradation of PIV_2 FdUMP in mouse plasma–0.05 M phosphate buffer, pH 7.4 (1:3), at 37 °C.

Table 2. Growth Inhibitory Effects of **11a** and **11b** on CHO[a] Cells in Culture

	IC_{50}[b] (μM)		
cell line	FU	11a	11b
CHO	5	5	5
CHO/FU	100	5–10	5–10

[a] CHO = Chinese hamster ovary. [b] The minimum drug concentration that gives rise to colonies containing fewer than 50 cells after 7 d incubation at 37 °C.

Cell Growth Inhibitory Studies. 11a and **11b** were screened against Chinese hamster ovary (CHO) cells and a subline with 20-fold resistance to FU (CHO/FU) (Table 2). The minimal concentration of these compounds required to reduce cell growth to less than 50 cells per colony was 5×10^{-6} M, the same concentration as required for FU. **11a** and **11b** were as effective at inhibiting the growth of the FU-resistant cell line as the wild-type cells.

Antitumor Screening. The antitumor activities of **11a** and **11b** were determined against P-388 leukemia and a subline with acquired resistance to FU (FU/P-388) (Table 3). Administered intraperitoneally (ip), daily, for 5 consecutive days at optimal dosage, **11a** and **11b** were as effective as FU or FUdR at prolonging the life span of BDF_1 mice bearing the wild-type tumor. **11a** and **11b** were equally effective against the FU-resistant tumor. FUdR, at optimal dosage, was only marginally active. Animals in the FU/P-388 group that received FU alone died before saline-treated controls, presumably due to the combined effects of drug toxicity and lack of drug efficacy.

Table 3. Effect of **11a** and **11b** on the Survival of BDF_1 Mice Implanted Intraperitoneally with P-388 Leukemia[b] Sensitive (P-388/O) or Resistant (P-388/FU) to FU

compd	dose, mg/kg, qd, 1–5	P-388/O MST[c] (days)	P-388/O % ILS[d]	P-388/O % wt[e] change	P-388/FU MST[c] (days)	P-388/FU % ILS[d]	P-388/FU % wt[e] change
saline (0.9%)	(0.2 mL)	11	–	18	11	–	13
emulphor vehicle	(0.2 mL)	11	–	17	11	–	16
FU[f]	40				9	−18	−18
	30	17	55	−15	9	−18	−15
	20	16	45	−5	10	−9	−6
FUdR[f]	150	20	82	−5	13	18	−10
	100	19	73	−4	13	18	−10
	67	16	45	−2	12	9	−6
11a[g]	335	19	73	−12	18	64	−13
	223	16	45	−11	18	64	−12
	149	15	36	3	17	55	−9
11b[g]	360	20	82	−15	16	45	−13
	240	16	45	−15	16	45	−12
	160	15	36	−9	15	36	−9

[a] Six mice per group, average weight 22 g. [b] 1×10^{-6} cells inoculated intraperitoneally on day 0. [c] Median survival time (MST) of 15 mice used as controls was 11 days. [d] Percentage increase in life span of treated animals (T) compared with controls (C) was determined by the formula: $(T/C - 1) \times 100$. [e] The average precentage weight change on day 5 was taken as a measure of drug toxicity. [f] FU and FUdR were administered in 0.9% saline. [g] **11a** and **11b** were formulated in EtOH–Emulphor 620–0.9% saline (1:1.5:7.5; v/v/v); see the Experimental Section.

Discussion

The objective of this study was to prepare PIV_2-FdUMP and to evaluate its potential as a membrane-permeable prodrug of FdUMP. The most convenient synthetic route to PIV_2FdUMP was by direct condensation of FUdR with PIV_2 phosphate in the presence of the Mitsunobo reagent. Although the reaction was slow, the target compound was generated in moderate yield. Particular advantages of this approach were that PIV_2-FdUMP could be generated under mild neutral conditions, and the need for protection and subsequent deprotection of the 3′-OH protective group was avoided. This synthetic route has proved generally useful for the preparation of other bis(acyloxymethyl) nucleotides in the deoxyuridine series.[14–16] The stability of PIV_2-FdUMP in aqueous media over the pH range 4.0–7.4 indicates that it is well suited to formulation in neutral, or near neutral, aqueous vehicles. Moreover, the excellent stability of the compound in aqueous acid at pH 1 suggests that PIV_2 nucleotides may be suitable for oral administration. Indeed, bis(acyloxymethyl) derivatives have been used to enhance the oral bioavailability of PMEA.[19,20]

Under basic conditions, PIV_2FdUMP proved unexpectedly susceptible to degradation. The mechanism by which this occurs is uncertain because nucleophilic attack on the PIV phosphate group is possible at three different sites, namely, the carbonyl group, the phosphoryl group, and the methylene group. In fact, each of these reactions may occur simultaneously. The facility with which PIV_2FdUMP is hydrolyzed under basic conditions is paralleled by its susceptibility to degradation in the presence of carboxylate esterases. Interestingly, PIV_1FdUMP was the only product observed after incubation of PIV_2FdUMP with hog liver carboxylate esterase for 7 h at 37 °C. The formation of only trace amounts of FdUMP after 24 h incubation indicates that the second (pivaloyloxy)methyl group is much more resistant to enzyme-mediated cleavage than the first. This finding is consistent with reports that negatively charged compounds tend to be poor substrates for carboxylate esterase.[28]

The rate of degradation of PIV_2FdUMP was particularly rapid in mouse plasma; 15 min after the start of the incubation, none of the starting material could be detected. PIV_1FdUMP was initially formed and gradually gave way to FdUMP. The comparatively slow rate of hydrolysis of the second ester group ($t_{1/2}$ = 72 min) compared to the first ($t_{1/2}$ = < 15 min) suggests the involvement of enzymes other than carboxylate esterases. The most likely candidate is 5′-nucleotide phosphodiesterase (EC 3.1.4.1.), an enzyme system abundant in mammalian tissues[29] and known to catalyze the hydrolysis of a wide range of naturally occurring and synthetic phsophodiesters.[30] The progressively increasing concentration of FUdR in the plasma incubates is presumably due to dephosphorylation of FdUMP by phosphatases or 5′-nucleotidases.

The observation that PIV_2FdUMP and PIV_2 3′-*O*-Ac-FdUMP were equally cytotoxic to CHO cells and a mutant resistant to FU is consistent with the interpretation that both compounds are membrane-permeable prodrugs of FdUMP. Additional evidence in support of prodrug action is that both compounds were as effective as FU at prolonging the life span of mice bearing P-388 leukemia and retained full therapeutic efficacy against a P-388 mutant resistant to FU. The dosages of **11a** and **11b** required for optimal activity were much greater than that of FU, but this was due mainly to the 4-fold difference in molecular weights (554 and 596 for **11a** and **11b**, respectively, vs 130 for FU). On a molar basis, the dosages of **11a** and **11b** are about 2.5-fold greater than that of FU and approximately the same as that of FUdR.

Despite the strong circumstantial evidence that **11a** and **11b** are membrane-permeable prodrugs of FdUMP, proof of this mechanism of action is difficult to establish because (a) the precise biochemical mechanisms of resistance in the FU-resistant cell lines have not been established and (b) fluorinated nucleotides have multiple potential sites of antiproliferative action, including inhibition of DNA synthesis and impairment of RNA processing and function. However, we previously reported other evidence in support of the proposed prodrug mechanism. Thus, PIV_2FdUMP effectively inhibits DNA synthesis in mouse LM fibroblasts that are genetically devoid of thymidine kinase (LM tk^-) and therefore unable to directly convert FUdR to FdUMP.[13] The blockade of DNA synthesis induced in these cells by aminopterin[13] can be overcome by PIV_2 TMP. PIV_2-FdUMP has also been shown to inhibit thymidylate synthase in a mutant L1210 cell line that is resistant to FUdR because of the deletion of tk[31]. Finally, we have shown that PIV_2ddUMP gives rise to ddUMP, ddUDP, and ddUTP in CEM lymphocytes,[14] a leukemic cell line that intrinsically lacks the capacity to anabolize ddU to ddUMP.

Conclusion

Collectively, our findings with CHO cells in vitro and the P-388 mouse tumor model in vivo strongly suggest that both PIV_2FdUMP and PIV_2 3′-*O*-Ac-FdUMP act as membrane-permeable precursors of FdUMP. This par-

ticular prodrug strategy is not limited to nucleotides but can be used to promote the entry of any ionic organophosphate or organophosphonate into cells. Several potential therapeutic applications of the strategy may be envisaged. One is to overcome resistance to established antitumor and antiviral agents due to the deletion of activating kinases. Another is to aid in the design of new structural types of therapeutic agents that are not substrates for activating kinases. However, the susceptibility of the prodrugs to rapid degradation by plasma esterases constitutes a potential weakness of the approach. This shortcoming probably compromises the therapeutic potential of PIV nucleotide prodrugs when administered systemically. However, PIV prodrugs appear well-suited to local application, such as may be achieved by site- or organ-specific intravenous infusion, or by topical administration. Indeed, the efficacy of PIV_2FdUMP and PIV_2 3′-*O*-Ac-FdUMP against the P-388 mouse model constitutes an example of site-specific administration since the drugs and the tumor were both administered into the peritoneal cavity. We are currently exploring structural modifications of PIV nucleotides that render them more resistant to degradation by plasma enzymes.

Experimental Section

Nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR, and ^{31}P NMR) were recorded at ambient temperature on an IBM-Bruker Model NR/200 AF spectrometer in the Fourier transform mode, in $CDCl_3$, using tetramethylsilane as an internal standard. Some low-resolution ^{1}H NMR spectra were recorded, as noted, on a Varian Associates T-60 A spectrometer. Mass spectra were obtained on a Finnegan Model 3300 quadrupole spectrometer in the chemical ionization mode using methane as the reagent gas. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN; where indicated only by the symbols of elements, the results were within ±0.4% of the theoretical values. Melting points were determined on a Hoover capillary apparatus and are uncorrected. All reactions were carried out in dry glassware and were protected from atmospheric moisture. Solvents were dried over freshly activated (300 °C/4 h) molecular sieves (type 4A). Reactions with silver salts were conducted in dry glassware in the dark and were protected from atmospheric moisture. The silver salts were dried in vacuo over P_2O_5 before use. The homogeneity of the products was determined by ascending TLC on silica-coated glass plates (silica gel 60 F 254, Merck) using mixtures of $CHCl_3$–MeOH (typically 1–10% MeOH) as the eluting solvent. Chromatograms were visualized under a UV lamp (254 nm) or by placing the air-dried plates in a tank of I_2 vapor. Compounds containing a (pivaloyloxy)methyl group were identified by spraying the plates with a 0.25% solution of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) in 0.5 N NaOH solution, and heating them in an oven at 85 °C for 5 min. The liberated formaldehyde reacted with Purpald reagent to form purple spots against a white background. Preparative separations were performed by flash chromatography on silica gel (Merck, 230–400 mesh) using mixtures of EtOAc/hexane as eluent. All chemical reagents were purchased from Aldrich Chemical Co., Milwaukee, WI.

N.B. The synthesis of bis[(pivaloyloxy)methyl] hydrogen phosphate[10] (**8a**) was previously described, in brief, as part of a general synthesis of bis[(acyloxy)methyl] phosphates. The following is a detailed description of the synthesis of **8a** from phosphorous acid.

Disilver Benzyl Phosphate (6).[32] Phosphorus acid (dried over P_2O_5) (25.0 g, 0.30 mol) was dissolved in a solution of benzyl alcohol (500 mL) and Et_3N (150 mL, 33.75 mmol). Solid I_2 (116 g, 0.45 mol) was added, in 10 g portions, with cooling in an ice bath at 5 °C. The mixture was stirred for 30 min. It was then poured into acetone (2000 mL), and an excess of cyclohexylamine (100 mL) was added. The precipitate which formed was collected by filtration, washed with acetone, and recrystallized from aqueous EtOH (H_2O, 200 mL; EtOH, 1100 mL) containing 1% (v/v) of cyclohexylamine. The yield was 69.0 g (78%). The mother liquor was evaporated to dryness and the residual solid was recrystallized to give a further 17 g of product. The total yield of cyclohexylammonium benzyl phosphate was 86.0 g (97%); mp 229–233 °C (lit.[10] mp 233–234 °C). A solution of this product (50.0 g, 0.13 mol) in distilled H_2O (500 mL) was passed through a column (5 × 50 cm) of Bio-Rad AG 50W × 12 cation-exchange resin (200–400 mesh, 2.3 mequiv per mL of resin bed) in the Na^+ form. The resin was washed with 3 column volumes of distilled H_2O. The combined effluents were concentrated to ca. 300 mL on a rotary evaporator under reduced pressure at <40 °C and then added, dropwise, to a rapidly stirred solution of $AgNO_3$ (48.3 g, 0.29 mol) in H_2O (300 mL). The copious white precipitate that formed was filtered and washed sequentiallly with distilled H_2O, EtOH, and Et_2O and air-dried. The product was stored in vacuo over P_2O_5. The yield was 48.9 g, 94%. Anal. ($C_7H_7O_4PAg_2$) C, H, Ag.

Iodomethyl Pivaloate. Chloromethyl pivaolate (45.0 g, 43 mL, 0.3 mol) was added, dropwise, over 30 min with vigorous stirring at room temperature under a dry N_2 atmosphere, to a solution of anhydrous NaI (90.0 g, 0.60 mol; dried at 100 °C under vacuum in a drying pistol) in dry acetone (300 mL). A white precipitate formed, and the initially colorless mixture progressively darkened. After 24 h at room temperature, dry hexane (300 mL) was added, and the precipitated salts were filtered and washed with hexane. The filtrates were combined and concentrated under reduced pressure. The residue was triturated with hexane (300 mL), and the solution was filtered to remove additional precipitated salts. The filtrate was washed successively with 5% $NaHSO_3$ solution (150 mL) and H_2O (3 × 300 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure (30 mmHg) at <30 °C to give a light yellow liquid. This liquid turned dark brown when distilled under reduced pressure; the major boiling fraction (68–69 °C/4 mm) was heavily contaminated with I_2, indicating partial decomposition. Since the initial reaction product was over 95% pure as evidenced by NMR and TLC analysis, it was used in subsequent reactions without further purification. The yield was 56.2 g (78%). The compound was stored in a tightly stoppered flask contained inside a vacuum desiccator which was maintained at −10 °C. Although initially pale yellow, the compound progressively darkened on storage over a period of several months. Aged samples were purified as follows: A solution of the compound in 5 volumes of toluene was washed successively with 5% $NaHSO_3$ solution (1 vol) and H_2O (3 vol). The organic layer was separated, dried over anhydrous Na_2SO_4, and filtered. The filtrate was used directly in subsequent reactions. The concentration of iodomethyl pivaloate in solution was determined by comparing the integral of the NMR methylene proton resonances with that of the methyl proton resonances of toluene. ^{1}H NMR (60 MHz, $CDCl_3$): δ 5.87 (s, 2 H, CH_2), 1.17 (s, 9 H, $C(CH_3)_3$). Anal. ($C_6H_{11}IO_2$) C, H.

Bis[(pivaloyloxy)methyl] Benzyl Phosphate (7). A solution of iodomethyl pivaloate (79.5 g, 0.32 mol) in dry toluene (200 mL), was added, dropwise, over 30 min to a suspension of finely divided disilver benzyl phosphate (51.0 g, 0.13 mol) in toluene (250 mL). An exothermic reaction ensued. The mixture was stirred at room temperature for 5 h and then filtered to remove silver salts. The filtrate was washed successively with 5% $NaHSO_3$ solution (1 × 100 mL) and H_2O (3 × 50 mL), dried over anhydrous Na_2SO_4, and evaporated. The residual colorless oil was taken up in the minimum of EtOAc and chromatographed on a column of silica using EtOAc–hexane as eluent. Fractions containing **7**, as evidenced by TLC, were combined and evaporated. **7** was obtained as a colorless, viscous oil. It was dried in vacuo at room temperature for 24 h. The yield was 31.7 g (58%). ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.33 (s, 5 H, C_6H_5), 5.63 (d, 4 H, OCH_2O, J = 14 Hz), 5.10 (d, 2 H, $C_6H_5CH_2$, J = 8 Hz), 1.20 (s, 18 H, $C(CH_3)_3$). Mass spectrum: *m/z* (ion, relative intensity)

417 (MH^+, 2), 387 ($MH^+ - CH_2O$, 14), 357 ($MH^+ - 2\ CH_2O$, 100). Anal. ($C_{19}H_{29}O_8P$) C, H.

Bis[(pivaloyloxy)methyl] Hydrogen Phosphate (8a). A solution of **7** (25.0 g, 76.7 mmol) in cyclohexane (400 mL) was hydrogenated over 5% Pd–C (1.25 g) at a pressure of 30 psi. After 1 h, an additional 1.25 g of catalyst was added and hydrogenolysis was continued for a further 1 h. The catalyst was filtered, and cyclohexylamine (about 8.8 mL) was added to neutralize the liberated acid. The solution was concentrated under reduced pressure to about one half the original volume and stored overnight at 5 °C. White crystals of cyclohexylammonium bis[(pivaloyloxy)methyl] phosphate (**8b**) separated. Yield: 14.3 g (56%). Mp: 121–122 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 7.60–8.26 (br s, 3 H, NH_3^+), 5.57 (d, 4 H, OCH_2O, J = 13 Hz), 2.66–3.33 (br s, 1 H, NC-H), 0.83–2.40 (m, 10 H, methylene groups of the cyclohexylamine ring) 1.20 (s, 18 H, $C(CH_3)_3$). Anal. ($C_{18}H_{36}NO_8P$) C, H, N. A solution of **8b** (13.0 g, 30.6 mmol) in H_2O (200 mL) was passed through a column (25 × 2.5 cm) of Biorad cation-exchange resin (AG 50W × 12; H^+ form; 200–400 mesh; 2.3 mequiv per mL of resin bed) under a slight back pressure of N_2. The resin was washed with 3 column volumes of distilled H_2O, and the combined effluent was frozen in a dry ice–acetone bath and lyophilized. The amorphous white powder which remained was dried in vacuo over P_2O_5 for 24 h. Yield: 9.9 g (99%). Mp: 68–69 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 9.38 (br s, 1 H, POH), 5.63 (d, 4 H, OCH_2O, J = 13 Hz), 1.20 (s, 18 H, $C(CH_3)_3$). MS: m/z 327 (MH^+). Anal. ($C_{12}H_{23}O_8P$) C, H.

Bis[(pivaloyloxy)methyl] Phosphate Sodium Salt (8c). An aqueous solution of **8b** (2.0 g, 4.7 mmol) in distilled H_2O (30 mL) was passed through a column of Bio-Rad AG 50W-X12 cation-exchange resin (200–400 mesh; 2.3 mequiv per mL of resin bed; 20 mL resin) in the Na^+ form. The resin was washed with 3 column volumes of distilled H_2O, and the combined effluents were frozen and lyophilized to give **8d** as a white hygroscopic solid (1.1 g, 67%). ^{1}H NMR (D_2O): δ 5.37 (d, 4 H, OCH_2O, J = 13 Hz), 1.07 (s, 18 H, $C(CH_3)_3$).

Bis[(pivaloyloxy)methyl] Phosphate Silver Salt (8d). A solution of $AgNO_3$ (0.27 g, 1.58 mmol) in H_2O (2 mL) was added with stirring to a solution of **8c** (0.50 g, 1.44 mmol) in H_2O (3 mL). A pale yellow precipitate formed. The mixture was maintained at 4 °C overnight and then filtered. The product was washed with ice-cold water (2 × 1 mL) and dried in vacuo over P_2O_5 for 24 h (460 mg, 74%). A sample for elemental analysis was recrystallized from benzene. Pale yellow needles, mp 175–180 °C dec. ^{1}H NMR ($CDCl_3$): δ 5.72 (d, 4 H, OCH_2O, J = 13 Hz), 1.20 (s, 18 H, $C(CH_3)_3$). Anal. ($C_{12}H_{22}O_8PAg$) C, H, Ag.

2′,5′-Dideoxy-5′-iodo-5-fluorouridine (10a). This compound was prepared from 2′-deoxy-5-fluorouridine as described by Cook et al.[33]

3′-*O*-Acetyl-2′,5′-dideoxy-5′-iodo-5-fluorouridine (10b). Methyltriphenylphosphonium iodide (1.5 g, 3.32 mmol) was added to a solution of 3′-*O*-acetyl-2′-deoxy-5-fluorouridine[34] (634 mg, 2.2 mmol) in dry DMF (10 mL). The mixture was stirred for 2 h at room temperature. MeOH (10 mL) was added, and after 75 min, the solution was evaporated. The residual oil was dissolved in EtOAc (100 mL), and the solution was washed successively with aqueous 5% sodium thiosulfate (100 mL) and H_2O (3 × 100 mL). The organic layer was dried over Na_2SO_4 and then evaporated to give a white solid which was recrystallized from EtOAc–hexane to form white needles. Mp: 128–129 °C. Yield: 600 mg (68%). TLC: $CHCl_3$–MeOH, 95:5, R_f = 0.56. NMR (CD_3OD): δ 7.83 (d, 1 H, H-6, J = 6 Hz), 6.10 (t, 1 H, H-1′), 5.0–5.2 (m, 1 H, H-3′), 3.9–4.0 (m, 1 H, H-4′), 3.4–3.5 (m, 2 H, H-5′), 2.2–2.3 (m, 2 H, H-2′) 1.97 (s, 3 H, $OCOCH_3$). Anal. ($C_{11}H_{12}FIN_2O_5$) C, H, N.

Bis[(pivaloyloxy)methyl] 2′-Deoxy-5-fluorouridine 5′-Phosphate (11a). Method 1. **8d** (324 mg, 0.7 mmol) and 2′,5′-deoxy-5′-iodo-5-fluorouridine[30] (**10a**) (203 mg, 0.5 mmol) were suspended in dry toluene (50 mL) contained in a 250-mL round-bottom flask equipped with a mechanical stirrer, a H_2O condenser, and a dropping funnel. The mixture was heated at 100 °C for 5 h and then cooled to room temperature. The silver salts were filtered and washed with MeOH, and the combined filtrates were evaporated to dryness. The residue was taken up in the minimum of $CHCl_3$ and chromatographed on a column of silica (45 g) using $CHCl_3$–MeOH (95:5, v/v) as eluent; 10-mL fractions were collected. Fractions containing **11a** appeared as a dark quench when viewed under short-wavelength UV light on silica gel 60 F-254 TLC plates and gave a positive reaction with the Purpald spray reagent. The compound was isolated as a colorless oil. Yield: 45 mg (15%). UV: λ_{max} (H_2O) 268 (ϵ 7838). ^{1}H NMR ($CDCl_3$): δ 7.69 (d, 1 H, H-6, J = 6 Hz), 6.26 (t, 1 H, H-1′), 5.61–5.75 (m, 4H, OCH_2O), 4.50–4.57 (m, 1 H, H-3′), 4.32–4.37 (m, 2 H, H-5′), 4.04–4.07 (m, 1 H, H-4′), 2.22–2.45 (m, 2 H, H-2′), 1.25 (s, 18 H, $C(CH_3)_3$). MS: m/z 555 (MH^+). Anal. ($C_{21}H_{32}FN_2O_{12}P$) C, H, N.

Method 2. **8a** (980 mg, 3.0 mmol), 2′-deoxy-5-fluorouridine (**9a**) (492 mg, 2.0 mmol), and triphenylphosphine (0.79 g, 3.0 mmol) were dissolved in dimethylacetamide (5 mL) contained in a 50-mL round-bottom flask equipped with a dropping funnel. A solution of diethyl azodicarboxylate (0.52 g, 3.0 mmol) in dimethylacetamide (3 mL) was added with stirring, and the reaction mixture was heated at 60 °C for 5 days under a N_2 atmosphere. The dimethylacetamide was evaporated under reduced pressure. Toluene (25 mL) was added, and the solution was again evaporated; this was twice repeated. The contents of the flask were maintained under high vacuum for 24 h to remove residual dimethylacetamide. The remaining product was taken up in the minimum of $CHCl_3$ and chromatographed on a column of silica (40 g) using $CHCl_3$–MeOH (95:5) as eluent; 10 mL-fractions were collected and monitored for the presence of **11a** as described in method 1. The product, obtained as a colorless oil, was chromatographically and spectrally identical with that obtained by method 1. Yield, 635 mg (57%).

Bis[(pivaloyloxy)methyl] 3′-*O*-Acetyl-2′-deoxy-5-fluorouridine 5′-Monophosphate (11b). Method 1. A suspension of **8d** (574 mg, 1.27 mmol) and **10b** (420 mg, 1.06 mmol) in toluene (50 mL) was heated at 100 °C for 5 h as described for **11a** (method 1). The silver salts were filtered and washed with MeOH. The filtrates were combined and evaporated to dryness. The residue was taken up in chloroform (50 mL), and the solution was washed with H_2O (2 × 50 mL), dried over anhydrous Na_2SO_4, and evaporated. The residue was taken up in the minimum of $CHCl_3$ and chromatographed on a column of silica (70 g) using EtOAc/hexane (7:3) as eluent; 10 mL fractions were collected and monitored for the presence of **11b** by TLC on silica using the Purpald spray reagent. **11b** was obtained as a colorless oil. Yield: 94.3 mg (16%). UV: λ_{max} (H_2O) 268 (ϵ 8566). ^{1}H NMR ($CDCl_3$): δ 7.78 (d, 1 H, H-6, J = 6 Hz), 6.36 (t, 1 H, H-1′), 5.70 (d, 4 H, OCH_2O, J = 14 Hz), 5.31 (m, 1 H, H-3′), 4.21-4.42 (m, H-4′ and H-5′), 2.36 (m, 2 H, H-2′), 2.03 (s, 3 H, CH_3CO), 1.21 (s, 18 H, $C(CH_3)_3$). MS: m/z 597 (MH^+). Anal. ($C_{23}H_{34}FN_2O_{13}P$) C, H, N.

Method 2. **8a** (2.12 g, 6.5 mmol) was condensed with 3′-*O*-acetyl-2′-deoxy-5-fluorouridine (**10b**) (1.24 g, 4.3 mmol) in the presence of triphenylphosphine (2.57 g, 9.8 mmol) and diethyl azodicarboxylate (1.71 g, 9.8 mmol) in dimethylacetamide solution (15 mL) as described for **11a** (method 1). The product, isolated as a colorless oil after chromatography of the crude reaction mixture on a column of silica (70 g), was chromatographically and spectrally identical to that obtained by method 1. Yield, 1.69 g (66%).

Mono[(pivaloyloxy)methyl] Dibenzyl Phosphate (12). A solution of chloromethyl pivaloate (3.18 mL, 22 mmol) in dry toluene (10 mL) was added, with stirring over 10 min, to a suspension of finely divided silver dibenzyl phosphate (8.0 g, 20 mmol) in toluene (100 mL). The reaction mixture was refluxed with stirring for 24 h. The silver salts were removed by filtration, and the filtrate was evaporated to dryness. The residue was taken up in Et_2O (200 mL) and the solution was washed successively with 5% $NaHSO_3$ solution (100 mL) and H_2O (3 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The colorless viscous oil which remained was dried in vacuo at room temperature for 24 h. Yield: 7.6 g (95%). ^{1}H NMR ($CDCl_3$): δ 7.31 (s, 10 H, C_6H_5), 5.60 (d, 4 H, J_{PH} = 14 Hz, $P(O)OCH_2O$), 5.07 (d, 4 H, J_{PH} = 8 Hz, $OCH_2C_6H_5$), 1.18 (s, 9 H, $(CH_3)_3$). Anal. ($C_{20}H_{25}O_6P$) C, H.

Mono[(pivaloyloxy)methyl] Phosphate, Dicyclohexylammonium Salt (13a). A solution of **12** (6.0 g, 16.6 mmol) in EtOAc (200 mL) was shaken for 15 min under a hydrogen atmosphere at 35 psi in the presence of 5% Pd-on-C (300 mg). Additional catalyst (300 mg) was added, and the hydrogenation was continued for a further 30 min. The solution was filtered through a bed of powdered cellulose, and cyclohexylamine (approximately 3.8 mL) was added slowly, with stirring, to the filtrate until a drop of the solution gave a pH of 7.0 when tested against wet universal pH paper. The solution was concentrated under reduced pressure at room temperature to about half of its original volume and then stored overnight at 0 °C. The white crystalline mass that separated was filtered and washed with EtOAc–hexane (1:4, v/v). It was recrystallized from EtOAc–hexane. Yield: 4.3 g. (68%). ^{1}H NMR ($CDCl_3$): δ 5.34 (d, 2 H, J_{PH} = 13 Hz, P(O)OCH_2O), 1.19–1.89 (m, 20 H, cyclohexyl-H), 1.16 (s, 9 H, $C(CH_3)_3$). Anal. ($C_{18}H_{39}N_2O_6P$) C, H, N.

Mono[(pivaloyloxy)methyl] Hydrogen Phosphate (13b). A solution of **13a** (12.0 g, 31.7 mmol) in H_2O (50 mL) was applied to a column (25 × 2.5 cm) of Biorad cation-exchange resin (AG 50W × 12; H^+ form; 200–400 mesh; 2.3 mequiv per mL of resin bed) under a slight back-pressure of nitrogen. The resin was washed with 3 column volumes of distilled H_2O, and the combined effluent was frozen in a dry ice/acetone bath at −78 °C and lyophilized. The colorless oil that remained was dried in vacuo for 24 h over P_2O_5. The yield was 6.5 g (97%). The compound was stored frozen at −78 °C. ^{1}H NMR ($CDCl_3$): δ 9.48 (s, 1 H, OH), 5.59 (d, 2 H, J_{PH} = 14 Hz, P(O)-OCH_2O), 1.23 (s, 9 H, $C(CH_3)_3$).

Mono[(pivaloyloxy)methyl] 2′-Deoxy-5-fluorouridine 5′-Phosphate (14). Mono[(pivaloyloxy)methyl] hydrogen phosphate (212 mg, 1.0 mmol) was dissolved in anhydrous pyridine (3 mL), and the solution was evaporated to dryness. This was twice repeated. The residue was taken up in pyridine (2 mL), and 2′-deoxy-5-fluorouridine (100 mg, 0.41 mmol) and dicyclohexylcarbodiimide (413 mg, 2.0 mmol) were added. The mixture was stirred at room temperature for 4 days. The precipitated dicyclohexylurea was filtered, and the pyridine was removed under reduced pressure. The residue was taken up in H_2O (15 mL), and the solution was stirred for 1 h at room temperature. It was then filtered to remove additional dicyclohexylurea, concentrated to approximately 3 mL, and applied to a column (20 × 1.5 cm) of cation-exchange resin (AG 50W-X8, 200–400 mesh) in the H^+ form. The column was washed with distilled H_2O (200 mL), and the solution was lyophilized. The gummy residue was purified on a column of silica using $CHCl_3$–MeOH (8:2) as eluent. Yield, 94 mg (53%). The product was stored frozen at −78 °C. ^{1}H NMR (CD_3OH): δ 7.8 (d, 1 H, H-6), 5.35–5.45 (d, 2 H, P(O)OCH_2O, J_{PH} = 13 Hz), 4.03–4.21 (m, 3 H, H-4′ and H-5′), 3.40–3.51 (m, 1 H, H-3′), 2.20–2.21 (m, 2 H, H-2′), 1.05 (s, 9 H, $C(CH_3)_3$). Anal. ($C_{15}H_{22}FN_2O_{10}P{\cdot}H_2O$) C, H, N.

Solubility of 11a in Aqueous Buffer. Potassium phosphate buffer (0.05 M), pH 7.4 (3 mL), was added to **11a** (15.2 mg) in a round-bottom flask, and the mixture was stirred at room temperature for 2 h. The contents of the flask were transferred to a 10-mL centrifuge tube and centrifuged at 2000 rpm for 10 min. The supernatant was decanted and passed through a 0.22 μm Millipore filter to remove any remaining particulate matter. The concentration of **11a** in solution was determined by UV absorption using the measured value of 7838 for the molar extinction coefficient (ϵ) at a wavelength of 268 nm.

Partition Coefficient. 1-Octanol (3 mL; previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.4) was added to a solution of **11a** (6.6 mg) in 0.05 M potassium phosphate buffer, pH 7.4 (3 mL; previously equilibrated with 1-octanol), contained in a 15-mL capped centrifuge tube. The mixture was agitated for 5 min on a Vortex shaker and then centrifuged at 2000 rpm for 5 min. The octanol and aqueous layers were separated, and the concentration of **11a** in each was determined by UV spectrometry as described above.

Stability Studies. 1. Aqueous Media. Aliquots of a stock solution of **11a** in H_2O (10^{-3} M) were diluted with various buffers to a final concentration of 10^{-4} M. These solutions were stirred at room temperature. At selected time intervals (typically, 2, 4, 8, 12, 24, 30, 50, 100 h) aliquots (20 μL) were removed and were analyzed immediately for parent drug by HPLC on a μBondapak C-18 reverse phase column (20 cm × 4.6 mm, i.d.) (Waters Associates, Milford, MA) using MeOH–0.01 M potassium phosphate, pH 7.0 (6:4), as mobile phase at a flow rate of 1.5 mL/min. Eluted compounds were monitored with a variable-wavelength UV detector set at 268 nm and 0.01 AUFS sensitivity. Peak areas were quantitated electronically as a function of time using a Hewlett-Packard Model 3390 integrator. The retention times of **11a** and FUdR were 6.05 and 1.1 min, respectively. FdUMP and mono[(pivaloyloxy)-methyl] [PIV_1] FdUMP were analyzed on a SAX ion-exchange column (Whatman) using 0.02 M ammonium phosphate buffer, pH 3.5, as mobile phase. The retention times of FdUMP and PIV_1 FdUMP were 5.33 and 5.85 min, respectively.

2. Enzyme Studies. a. Esterases. Porcine liver carboxylate esterase (EC 3.1.1.1) was obtained from Sigma Chemical Co., St. Louis, MO, and used as received. The specific activity of the preparation was 200 units/mg of protein, where 1 unit is defined as the amount that will hydrolyze 1.0 μmol of ethyl butyrate to butyric acid and EtOH per minute at pH 8.0 and 25 °C. **11a** was dissolved in 0.05 M phosphate buffer, pH 7.4, at a concentration of 10^{-4} M. One-milliliter aliquots of this solution contained in a 5.0-mL screw-capped glass vial were incubated at 37 °C in the absence or the presence of the enzyme. At selected time intervals (typically 2, 4, 8, 12, 24, 30, 50, 100, and 250 h in the absence of enzyme), aliquots (40 μL) were removed and analyzed immediately for parent drug by HPLC as described above. For the enzyme studies, 3, 10, and 25 units of esterase per μmol of substrate were used. A control reaction was run using 25 units of enzyme that had previously been boiled for 10 min. To begin the reaction, the enzyme was added to 0.2 mL of the drug solution contained in 1.5-mL microcentrifuge tubes that were preequilibrated at 37 °C. At intervals of 0.25, 0.5, 1, 3, 5, 7, and 24 h, the vials were removed from the water bath, and 3 volumes (0.6 mL) of cold MeOH were added to deactivate the enzyme. The vials were agitated on a Vortex shaker for 20 s and then centrifuged for 5 min at 2000 rpm. Aliquots (40 μL) of the clear supernatant were analyzed by HPLC for parent compound and metabolites as described above. The half-lives were determined by linear least-square regression analysis of the pseudo-first-order reactions.

b. Phosphohydrolases. **11a** (0.7 μmol) in H_2O (137 μL) was added to the following enzyme solutions maintained at 37 °C in a shaking water bath, and the mixtures were incubated for 2 h: (a) 5′-nucleotidase (*Crotalus adamanteus*) (20 μL, 2.25 units/mL) in 0.1 M Tris-HCl buffer–0.01 mM $MgCl_2$, pH 8.0 (200 μL); (b) alkaline phosphatase (*Escherichia coli*) (20 μL, 2.18 units/mL) in 0.1 M Tris-HCl buffer, pH 8.0 (200 μL); (c) phosphodiesterase I (*Crotalus adamanteus*) (20 μL, 1.69 units/mL) in 0.1 M Tris-HCl buffer–0.01 mM $MgCl_2$, pH 8.0 (200 μL), and (d) snake venom (*Crotalus adamanteus*) (20 μL, 1.25 mg/mL) in 0.1 M Tris-HCl buffer–0.01 mM $MgCl_2$, pH 8.0 (200 μL). Immediately after the addition of **11a**, and at 30, 60, and 120 min thereafter, aliquots (50 μL) of the reaction mixture were withdrawn and added to 100 μL of MeOH in a centrifuge tube. The mixtures were agitated on Vortex mixer for 20 s and then centrifuged at 2000 rpm for 5 min. The supernatants were analyzed by HPLC as described above.

c. Mouse Plasma. Blood was obtained from female BDF_1 mice (Timco Co., Houston, TX). The sample were centrifuged at 2000*g* for 10 min at 4 °C, and the plasma supernatants were removed by aspiration. The reaction was started by adding 20 μL of a stock solution (2×10^{-2} M) of **11a** in EtOH to plasma (1980 μL) contained in a 5 mL vial, such that the final concentration of **11a** was (2×10^{-4} M). The sample was agitated for 15 s on a Vortex shaker to ensure thorough mixing and then immersed in a water bath at 37 °C. Samples (100 μL) were withdrawn at 0, 5, 15, 30, 60, 180, 360, and 1440 min and diluted with 4 volumes of MeOH. The mixture was agitated on a Vortex shaker for 1 min and then centrifuged at 10 000 rpm for 10 min to sediment precipitated protein. The supernatants were analyzed for **11a**, FdUMP, and FUdR as

described above. A control experiment was conducted using mouse plasma that had been boiled for 10 min, and then homogenized.

Cytotoxicity Studies. Chinese hamster ovary (CHO) cells were dispersed into a series of 35-mm plastic dishes (approximately 200 cells/dish) and allowed to form clones in the presence of drug at the following concentrations: 0, 0.5, 1.5, 5, 10, 50, and 100 μM. After incubation for 7 d in humified CO_2 at 37 °C, the media were decanted and the clones were fixed with 10% formaldehyde for 10 min and stained with 0.1% crystal violet. The minimum inhibitory drug concentration was the lowest tested that resulted in the formation of colonies containing fewer than 50 cells.

Antitumor Screening. Mice weighing 20–23 g were obtained from Jackson Laboratories, Madison, WI. Murine leukemia P-388 either sensitive or resistant to FU (P-388/0 and P-388/FU) were obtained from the National Cancer Institute Tumor Repository. The tumors were maintained by weekly passage in female DBA/2 mice. For antitumor screening, 1×10^6 cells were inoculated intraperitoneally into male BDF_1 mice. FU and FUdR were formulated in 0.9% saline solution. However, **11a** and **11b** were insufficiently soluble in saline at the dosages administered and were formulated with the aid if Emulphor 620, a polyoxyethylated vegetable oil (GAF Corporation, New York). This was accomplished by first preparing a stock solution of **11a** or **11b** in EtOH. Emulphor 620 (1.5 vol) was then added, and the solution was thoroughly mixed on a Vortex shaker. Finally, 0.9% saline solution (7.5 vol) was added, with stirring, so that each individual dose was contained in 0.2 mL of vehicle. The drugs were administered ip daily for 5 consecutive days beginning 24 h after tumor implantation. Animals were observed until the time of death. Antitumor activity was determined by comparing the median survival time of the treated animals (T) with that of saline-treated controls (C), and was expressed as the percentage increase in life span (%ILS) where %ILS = $(T/C - 1) \times 100$.

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