Synthesis and Anti-DNA Viral Activities *in Vitro* of Certain 2,4-Disubstituted-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine Nucleosides

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Several novel 2,4-disubstituted-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidines have been synthesized and evaluated for their anti-human cytomegalovirus (HCMV), anti-hepatitis B virus (HBV), and anti-herpes simplex virus (HSV) activities *in vitro*. These nucleosides were prepared starting from 2-amino-4-chloro-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**3**), which in turn was synthesized by direct glycosylation of the sodium salt of 2-amino-4-chloropyrrolo[2,3-*d*]pyrimidine (**1**) with 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranosyl bromide (**2**). Displacement of the 4-chloro group of **3** with OH, NH_2, NHOH, SH, and SeH nucleophiles furnished the corresponding nucleosides **6**–**8**, **12**, and **14**, respectively. The 3′-deoxygenation of 2-amino-4-chloro-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**4**) and subsequent amination gave 2,4-diamino-2′,3′-dideoxy derivative **19**. Catalytic hydrogenation of **3** followed by debenzoylation afforded 2-aminopyrrolo[2,3-*d*]pyrimidine nucleoside **23**. Among the compounds evaluated for their ability to inhibit the growth of HCMV (strain AD169) in MRC-5 cells using a plaque reduction assay, only **7** was significantly active *in vitro* with a 50% inhibitory concentration (IC_{50}) of 3.7 μg/mL (TI >125), whereas the IC_{50} value of ganciclovir (DHPG) was 3.2 μg/mL. Strain D16 of HCMV was more resistant to **7** (IC_{50} 11 μg/mL) than the AD169 strain. When **7** was tested in combination with DHPG, the resultant anti-HCMV activity was found to be moderately synergistic with no evidence of antagonism. Nucleoside **7** also reduced episomal HBV replication in human hepatoblastoma 2.2.15 cells with an IC_{50} of 0.7 μg/mL (TI >143). Development of cells harboring HBV which had become resistant to the drug was not observed with **7**. Compound **7** also exhibited significant activity against herpes simplex virus types 1 and 2 (IC_{50} of 4.1 and 6.3 μg/mL, respectively) in Vero cells.

Introduction

Immunocompromised individuals such as organ transplant recipients[1] and acquired immune deficiency syndrome (AIDS)[2] and burn[3] patients are susceptible to infection by human cytomegalovirus (HCMV). HCMV infection has been implicated as a leading cause of debilitating and often life- or sight-threatening conditions in these patients. In addition, intrauterine HCMV infections can cause serious malformation in infants.[4] The drugs currently approved for the treatment of HCMV infection are 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (ganciclovir, DHPG, Cytovene)[5,6] and the trisodium salt of phosphonoformic acid (foscarnet, PFA, Foscavir).[7] Use of foscarnet, however, has been associated with anemia, nephrotoxicity, and neutropenia.[8,9] Although ganciclovir is the drug of choice for the treatment of the above ailments, prolonged therapy with DHPG causes serious side effects, such as neutropenia[10] and bone marrow toxicity,[11] which limit its use. The emergence of drug-resistant HCMV strains[12,13] is also of considerable concern. Thus, there is still a need for a potent and safer drug than DHPG to treat HCMV infections alone or in combination with other antiviral agents.

In search of more effective, less toxic, and orally available nucleosides for the treatment of HCMV infection, it has been reported that certain pyrrolo[2,3-*d*]pyrimidine nucleosides are active *in vitro* against HCMV[14,15] but did not exhibit sufficient potency and selectivity, which further warranted modification of these nucleosides. The sugar modifications, specifically the addition of a fluorine atom "up" in the 2′-position, make certain purine nucleosides acid stable[16,17] and increase the metabolic stability by making them more resistant to hydrolysis by adenosine deaminase (ADA), as well as resistant to degradation by purine nucleoside phosphorylase (PNP).[18,19] Certain 5-substituted-2′-deoxy-2′-fluoroarabinosylpyrimidines, e.g., 5-iodo-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)cytosine (FIAC) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)thymine (FMAU), have emerged as potent antiviral agents active against herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) *in vivo*.[20,21] This potent activity coupled with enzymatic stability provided a good rationale for the synthesis of 2′-deoxy-2′-fluoroarabinosyl derivatives of pyrrolo[2,3-*d*]pyrimidines as anti-HCMV agents.

Hepatitis B virus (HBV) is a small, partially double-stranded DNA virus belonging to the hepadnaviridae, which includes woodchuck hepatitis virus,[22] ground squirrel hepatitis virus,[23,24] and duck hepatitis B virus (DHBV).[25] These viruses replicate their DNA genome by reverse transcription of an RNA intermediate.[26] HBV infection (acute or chronic) is one of the most prevalent viral diseases worldwide. The resulting liver diseases often lead to cirrhosis and/or hepatocellular carcinoma.[27–29] Although vaccination has been used for

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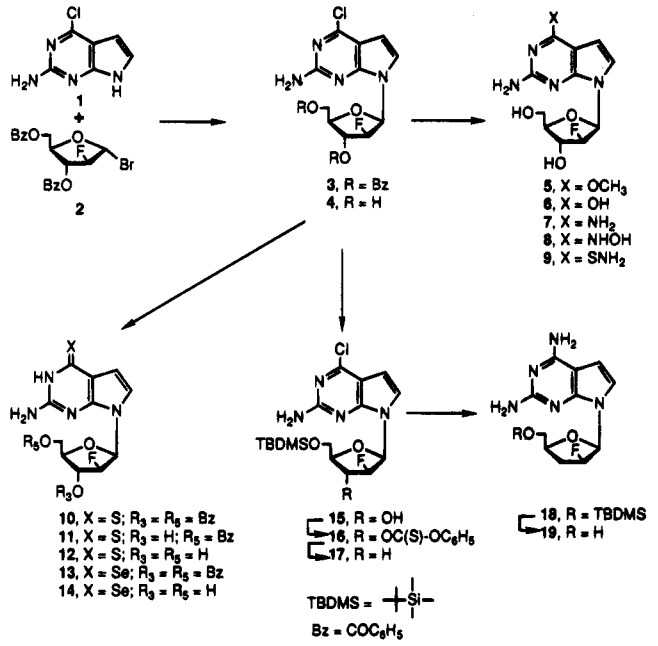
Scheme 1



the prevention of HBV infection, there are no effective chemotherapeutic agents available for the treatment of HBV infections without substantial cytotoxicity.[30-32] 2′,3′-Dideoxy-3′-thiacytidine (3TC) and 5-fluoro-2′,3′-dideoxy-3′-thiacytidine (FTC) were found to be potent and selective anti-HBV agents.[30,31] 3TC is currently undergoing phase II clinical trials against human HBV.[33] 9-[2-(Phosphonylmethoxy)ethyl]adenine (PMEA) has recently been shown to be active against DHBV *in vitro* and *in vivo*, awaiting further evaluation in patients.[34] It has been demonstrated that FIAC and its triphosphate were able to inhibit the replication of human and woodchuck hepatitis virus DNA polymerase[35] but exhibited severe mitochondrial toxicity.[36] In order to maintain the activity and selectivity of 2′-Arafluoro nucleosides, more modifications are in order. To this end, we have now synthesized certain novel Arafluoropyrrolo[2,3-*d*]pyrimidine nucleosides and evaluated them for efficacy against HBV in culture utilizing the human hepatoblastoma 2.2.15 cell line.[37,38] The synthesis and results of *in vitro* antiviral activities (anti-HCMV, -HBV, and -HSV) of these Arafluoropyrrolo[2,3-*d*]pyrimidine nucleosides are the subject of this paper.

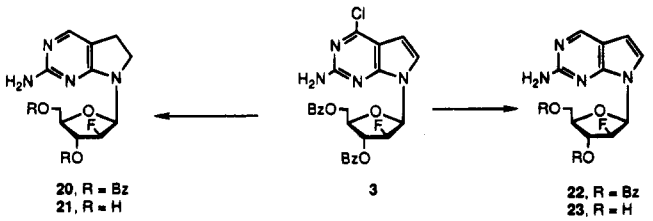
Chemistry

The novel 2,4-disubstituted-2′-deoxy-2′-Arafluoropyrrolo[2,3-*d*]pyrimidine nucleosides were prepared according to the route shown in Scheme 1. Glycosylation of the sodium salt of 2-amino-4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (**1**)[39] with 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranosyl bromide (**2**)[40] in dry CH_3CN gave 2-amino-4-chloro-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**3**) in 58% yield (based on the recovered base). Unlike the sodium salt glycosylation[41] of 4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine or 2-amino-5-bromopyrrole-3,4-dicarbonitrile with **2**, the sodium salt glycosylation of **1** with **2** was found to be stereospecific and gave only the β-anomer **3**. The anomeric configuration of **3** was assigned on the basis of ^1H NMR studies. The anomeric proton (C1′*H*) appeared as a doublet of doublets centered at δ 6.53 with $J_{H,F} = 16.24$ Hz and $J_{1',2'} = 3.56$ Hz, which is in agreement with the reported values of certain 2′-deoxy-2′-Arafluoro nucleosides.[17] Further, the ^1H NMR of **3** revealed C6*H* as a triplet with $J = 3.56$ Hz, which confirmed the β-configuration.[41] A nuclear Overhauser effect (NOE) experiment was also run to further substantiate the β-configuration of **3**. Irradiation of the 5′-proton produced enhancement (>5%) of both C3′*H* and C6*H* which established[42] the β-configuration for **3**. Debenzoylation of **3** with $MeOH/NH_3$ afforded the unprotected nucleoside **4** in a 93% yield, which on treatment with 0.5 N NaOMe in MeOH gave the corresponding 4-methoxy derivative **5**. The demethylation of **5** with iodotrimethylsilane in CH_3CN gave 2′-deoxy-2′-Arafluoro-7-deazaguanosine (**6**). Amination of **4** was accomplished under drastic conditions with $MeOH/NH_3$ (1:1) at 126 °C to give 2,4-diamino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**7**) in 80% yield without the formation of any detectable side products. No anomerization was observed under the reaction conditions employed. The anomeric proton of **7** resonated as a doublet of doublets and was centered at δ 6.37 with $J_{H,F} = 15.16$ Hz and $J_{1',2'} = 4.12$ Hz. The C6*H* appeared as a triplet and was centered at δ 6.84 with $J = 3.36$ Hz, and the pattern is comparable with that of **4**. Treatment of **4** with NH_2OH in the presence of Et_3N afforded the 4-hydroxylamino derivative **8**.

2-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-thione (**12**) was obtained *via* thiation of **3** with thiourea followed by debenzoylation. Thiation of **3** with thiourea in EtOH afforded 2-amino-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-thione (**10**) in a 92.6% yield. It was observed that use of a large excess of thiourea (8 equiv) resulted in the partial 3′-debenzoylation and gave a mixture of **10** and **11** (37%). Debenzoylation of **10** with $MeOH/NH_3$ afforded the 7-deazathioguanosine analog **12** in a 92% yield. The synthesis of 4-sulfenamide derivative **9** was accomplished starting from **12** by treatment with chloramine, generated *in situ* from sodium hypochlorite and NH_4OH at 0 °C. Our attempts to oxidize compound **9** with *m*-chloroperoxybenzoic acid at 0 °C to obtain the 2′-deoxy-2′-Arafluoro-7-deazasulfinosine were unsuccessful. An intractable reaction mixture of several components was obtained, from which the isolation of the desired product was difficult. The 4-selone derivative **13** was obtained by treatment of **3** with selenourea in absolute EtOH, which on subsequent debenzoylation with $MeOH/NH_3$ afforded 2-amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-selone (**14**).

The 3′-deoxy analog of **7**, viz. **19**, was prepared by deoxygenation of **4** *via* the conventional procedure.[43] The 5′-OH of **4** was protected with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) to obtain the 5′-*O*-TBDMS derivative **15**, which on treatment with phenyl chlorothionocarbonate in the presence of 4-(dimethylamino)pyridine (DMAP) gave the 3′-*O*-phenoxythiocarbonyl derivative **16** in a 55% yield. Catalytic reduction of **16** with *n*-Bu_3SnH in dry toluene in the presence of 2,2′-azobis(2-methylpropionitrile) (AIBN) gave the 3′-deoxy nucleoside **17** in a 90% yield, which on treatment with $MeOH/NH_3$ (1:1) at 120 °C gave the 2,4-diamino-3′-deoxy

Scheme 2



nucleoside **18**. The desilylation of **18** with tetraethylammonium fluoride in CH_3CN afforded the 2,4-diamino-3′-deoxy nucleoside **19**.

The synthesis of 2-amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]-pyrimidine (**23**) was accomplished *via* dehalogenation of **3** (Scheme 2). Catalytic reduction of **3** with 10% Pd/C in the presence of ammonium formate in MeOH gave 3′,5′-di-*O*-benzoylpyrrolo[2,3-*d*]pyrimidine nucleoside **22** in a 79% yield. It was observed that when **3** was heated under reflux with 10% Pd/C and ammonium formate in MeOH for only 30 min, compound **22** was formed, but when the reflux time was raised to 2 h, the hydrogenation of the C5 and C6 double bond in the pyrrolo[2,3-*d*]pyrimidine ring took place to give 5,6-dihydropyrrolo[2,3-*d*]pyrimidine derivative **20**. The synthesis of **22** was also accomplished by a direct catalytic hydrogenation of **3** in MeOH in the presence of 10% Pd/C at 50 psi. Debenzoylation of **20** and **22** with $MeOH/NH_3$ afforded the unprotected nucleosides **21** and **23**, respectively. Thus, we have accomplished the synthesis of a series of novel 2,4-disubstituted-2′-deoxy- and 2,4-disubstituted-2′,3′-dideoxy-2′-Arafluoropyrrolo[2,3-*d*]pyrimidines in overall good yields.

Results and Discussion

Antiviral Assays. Several of the 2,4-disubstituted-2′-deoxy-2′-Arafluoropyrrolo[2,3-*d*]pyrimidine nucleosides described above (compounds **4**–**7**, **9**, **12**, **14**, **19**, **21**, and **23**) were evaluated, in comparison with ganciclovir (DHPG), for their ability to inhibit the growth of HCMV in culture using a plaque reduction assay (Table 1). Within limits of biological variability, compound **7** was by far the most active nucleoside tested (IC_{50} of 3.7 μg/mL). The guanosine analog **6** had moderate activity (IC_{50} of 51 μg/mL), whereas the 2′,3′-dideoxy-2′-fluoro derivative **19** was at least 25-fold less active than compound **7** (Table 1). Since compound **6** is a partially deaminated version of **7**, it is unlikely that compound **6** is the active metabolite of **7**. The inactivity of **19** suggests that compound **7** is not a DNA polymerase (chain terminator) inhibitor. Therefore, the observed anti-HCMV activity of **7** may be due to its incorporation into the replicating viral DNA, which is subsequently inefficiently repaired.[17,44] To support this hypothesis, a timing experiment was performed in which drug was administered at various time points relative to infection of MRC-5 cells with HCMV (Towne) as described previously.[45] The results show that in a fashion similar to DHPG, compound **7** retained most of its antiviral activity when added to the infected cell culture up to 24 h postviral infection (Figure 1). This result suggests that **7** interferes with events leading up to and/or including viral DNA replication. In addition, the HCMV strain D16 which is reported to contain a mutation in the viral polymerase gene[46] is more resistant to compound **7** than the AD169 strain (Table 1).

Table 1. Anti-HCMV Activity of 2,4-Disubstituted-2′-deoxy-2′-Arafluoropyrrolo[2,3-*d*]pyrimidine Nucleosides

	anti-HCMV plaque reduction assay[a]	
compd	IC_{50} (μg/mL)[b]	TC_{50} (μg/mL)[c]
4	>100	32
5	>100	>100
6	51	>100
7	3.7	>100
7[d]	3.4	>1000
7[e]	11	>100
9	>100	>100
12	>50	>50
14	10	178
19	>100	>100
21	>100	32
23	16	>100
DHPG	3.2	>1000
DHPG[d]	4.4	>1000
DHPG[e]	52	>1000

[a] Plaque reduction assays performed in MRC-5 cells using the AD169 strain of HCMV. [b] Concentration required to reduce viral plaque production by 50%. Results shown are the averages of three or more experiments. [c] Visual cytotoxicity of stationary MRC-5 cells, median toxic concentration. [d] Plaque reduction and cytotoxicity experiments performed in the HFF cell line Hs68. [e] Plaque reduction experiments performed using the DHPG-resistant D16 strain of HCMV (viral polymerase mutant).

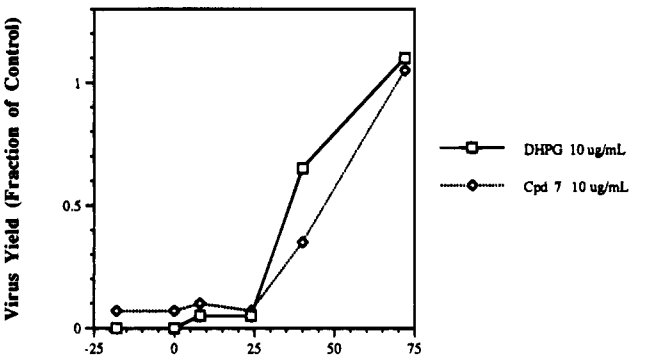


Figure 1. Effect of time of drug addition on the anti-HCMV inhibition profile of compound **7** and DHPG. At various times before, during, or postvirus infection, 10 μg/mL **7** or DHPG was added to MRC-5 cell cultures. Three days postinfection, the level of cell-associated virus was measured.[45,51] The values plotted are the averages of experiments performed in triplicate.

A study which examined the combined activity of compound **7** and DHPG was also performed using the HCMV assay format previously described.[45] The data from this experiment were plotted as a three-dimensional graph to produce a dose–response surface. Subsequent analysis of the data yielded a maximum synergistic activity when 4 μg/mL compound **7** was used with 2 μg/mL DHPG; however, at these same concentrations, a relatively similar degree of synergistic toxicity was noted (data not shown). Because of this overlap, the synergistic antiviral activity observed was probably due to a combined toxic effect of the two drugs.

Compounds **6**, **7**, and **19** were next evaluated for their ability to inhibit HSV-1 and HSV-2 in a cytopathic effect assay. The results from these assays are presented in Table 2. As was observed for the HCMV assays, compound **7** was a more efficacious inhibitor of HSV-1 and HSV-2 than either compound **6** or **19**.

Compounds **6**, **7**, and **19** were also evaluated for their ability to inhibit HBV production in 2.2.15 cells treated with various concentrations of each compound for 12

Table 2. Antiviral Activity of 2,4-Disubstituted-2′-deoxy-2′-Arafluoropyrrolo[2,3-*d*]pyrimidine Nucleosides

	IC_{50} value (μg/mL)			TC_{50} value (μg/mL)[a]		
compd	HSV-1[b]	HSV-2[b]	HBV[c]	Vero	MRC-5	2.2.15
6	ND	ND	60			>100
7	4.1	6.3	0.7	53	11.4	630
19	>100	>100	>30			149
ACV	0.7	1.1		255	299	ND
DHPG				270	>500	ND
ddC			2.1			64

[a] The concentration of drug required to reduce log phase cell growth by 50%. ND = not determined. [b] HSV-1 and HSV-2 plaque reduction assays were performed in Vero cells. Results shown are the averages of three or more experiments. [c] The concentration of drug required to reduce episomal HBV-DNA in 2.2.15 cells by 50%. The cytotoxicity was measured after 12 days of exposure to the drug as described in the methods section. Results shown are the averages of three or more experiments.

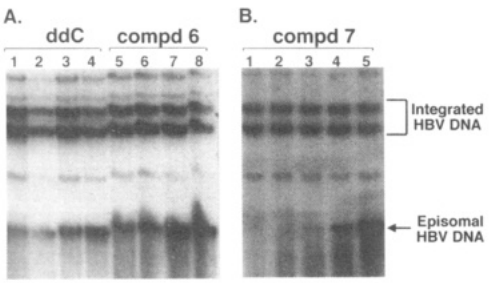


Figure 2. Effect of compound **7** on intracellular HBV-DNA. Confluent monolayers of 2.2.15 cells were cultured in medium with or without the indicated amount of compound **6** or **7** or ddC for 12 days. Total cellular DNA was then extracted and cleaved using the restriction endonuclease *Hin*dIII. Ten micrograms of digested DNA was then subjected to DNA blot hybridization analysis as described in the methods section. (A) DNA extracted from 2.2.15 cells treated with 100 (lanes 1 and 5), 50 (lanes 2 and 6), 25 (lanes 3 and 7), or 10 (lanes 4 and 8) μg/mL ddC or compound **6** is shown. (B) DNA extracted from 2.2.15 cells treated with 50, 25, 10, or 1 (lanes 1−4, respectively) μg/mL compound **7** are shown. In lane 5 (panel B) is the result obtained using untreated 2.2.15 cell DNA.

days. For this analysis, total intracellular DNA was digested with *Hin*dIII, which does not cleave the episomal HBV genome, and subjected to DNA blot analysis. The integrated and episomal HBV-DNA were easily separated using agarose gel electrophoresis which allowed for differential quantification.[47] As can be seen for cells treated with compound **6** or **7**, episomal HBV-DNA decreased in a dose-dependent manner, whereas both the amount and the restriction enzyme pattern of the integrated HBV genome were unaltered (Figure 2). There was also a dose-dependent decrease in the extracellular HBV-DNAs in cultures treated with **7** (data not shown). **7** (10 μg/mL) caused reduction of episomal HBV-DNA (Figure 2) and HBV-specific DNA in culture (data not shown) to undetectable levels by day 12 postdrug dosing. A compilation of the IC_{50} values for the three compounds is presented in Table 2. The results suggests that compound **7** was the most effective agent tested in inhibiting episomal HBV-DNA with an IC_{50} value of 0.7 μg/mL, while the IC_{50} value obtained using 2′,3′-dideoxycytidine (ddC) was 2.1 μg/mL (Table 2).

To test whether the episomal copies of the HBV genome had developed resistance to compound **7**, 2.2.15 cells (1×10^4) were initially exposed for 12 days to the culture medium containing compound **7** (10 or 50 μg/mL) followed by 12 days in drug-free medium and then a subsequent 12 day treatment in medium containing compound **7**. The intracellular episomal HBV-DNA levels were analyzed (by DNA blot analysis) at each time point. The result from this experiment showed that intracellular HBV-DNA levels were (a) undetectable after the first 12 days of drug treatment, (b) equal to untreated control following 12 days in drug-free medium suggesting that HBV production resumed when the drug was removed, and (c) reduced to the same levels in (a) when the drug was added to the culture described in (b) for an additional 12 days (data not shown). This result established that the HBV in 2.2.15 cells did not develop resistance to compound **7** during the initial 12 day treatment with compound **7**.

In conclusion, we have succeeded in synthesizing a series of 2,4-disubstituted-2′-deoxy-2′-Arafluoropyrrolo-[2,3-*d*]pyrimidine nucleosides in overall good yields. Analysis of the *in vitro* anti-HCMV (strain AD169) activity of these novel nucleosides showed that compound **7** was significantly active, and the activity was comparable to that of ganciclovir. However, compound **7** was 5-fold more potent against ganciclovir-resistant isolates. The strain D16 of HCMV was more resistant to **7** than the AD169 strain. None of the other analogs, including the 3′-deoxy derivative **19**, displayed any efficacious activity. Compound **7** in combination with ganciclovir showed a moderate synergistic effect as an anti-HCMV agent. Compound **7** was 3-fold more effective in reducing the episomal HBV levels in 2.2.15 cells with a good therapeutic index as compared to ddC and delayed the reemergence of episomal DNA for >12 days. Compound **7** also exhibited significant activity against HSV-1 and HSV-2 in culture, although it was less potent than acyclovir.

The key concern in antiviral drug development is selective toxicity. To address this issue, the nucleoside analogs were evaluated for their toxicity in static and growing MRC-5, growing Vero, and growing 2.2.15 cells as described in Virological Methods. The TC_{50} values (drug concentration required to inhibit cell proliferation by 50%) are presented in Tables 1 and 2. Compound **7**, the most active of all the compounds tested, was less toxic to all cell types except growing MRC-5 cells. This compound has also been evaluated in mice (T. Wallace et al., unpublished results). In this study female Balb/C mice were injected daily with compound **7** at 50 and 150 mg/kg (5 mice/group) for 50 days. On days 28 and 50, two mice from each group were sacrificed and histopathological analysis was performed. The daily physical observation and histopathology results revealed no adverse effect caused by compound **7**. The fact that *in vivo* toxicity data indicated that compound **7** was well tolerated in both acute and delayed toxicity studies overrides the significance of the moderate toxicity observed in the tissue culture study with growing MRC-5 cells. Although the preceeding observation is quite encouraging, further evaluation of the toxicity profile in other animals for compound **7** is warranted, especially in view of the recently observed mitochondrial toxicity[36] of FIAU.

Experimental Section

Melting points (uncorrected) were determined with a Thomas-Hoover capillary melting point apparatus. Elemental analyses were performed by Quantitative Technologies Inc., Whitehouse, NJ. The presence of solvent as indicated by elemental

analysis was verified by ^{1}H NMR spectroscopy. Thin layer chromatography (TLC) was performed on aluminum plates coated (0.2 mm) with silica gel$_{60}$ F_{254} (EM Science). Silica gel (EM Science; 230–400 mesh) was used for flash column chromatography. All solvents and chemicals used were reagent grade, and the solvent mixtures are in volumes. The detection of nucleoside components on TLC was by UV light and with 10% H_2SO_4 in MeOH spray followed by heating. Evaporations were conducted under diminished pressure with the bath temperature below 30 °C. Infrared (IR) spectra were recorded in KBr with a Perkin-Elmer 1420 IR spectrophotometer, and UV spectra were recorded with a Hewlett-Packard 8452 diode array spectrophotometer. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 400 MHz with a Brücker AM400 wide bore NMR spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane (TMS) as the internal standard (key: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, tt = triplet of triplets, q = quartet, m = multiplet, and br = broad).

2-Amino-4-chloro-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (3). To a suspension of 2-amino-4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (**1**)[39] (4.3 g, 25.4 mmol) in dry CH_3CN (350 mL) was added NaH (60% dispersion in mineral oil, 1.1 g, 28.2 mmol). The mixture was protected from moisture and stirred at ambient temperature for 1 h under an argon atmosphere. To this mixture was added slowly a solution of 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranosyl bromide (**2**)[40] (11.1 g, 27.5 mmol) in dry CH_3CN (50 mL). The reaction mixture was stirred at ambient temperature for 24 h. The mixture was filtered, and the filtrate was evaporated to dryness. The residue was purified on a flash silica gel column (5 × 25 cm) using hexane:EtOAc (7:3) as the eluent to give a white foam, which on crystallization from a mixture of CH_2Cl_2/hexane afforded 4.65 g (58.3%, based on 1.65 g of the recovered base) of **3** as white crystals: mp 168–169 °C; IR ν_{max} 3210 (NH_2), 1720 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.65 (q, 1 H, C4′*H*), 4.77 (m, 2 H, C5′H_2), 5.75 (tt, $J_{H,F}$ = 45.25 Hz, 1 H, C2′*H*), 5.85 (tt, $J_{H,F}$ = 12.75 Hz, 1 H, C3′*H*), 6.42 (d, $J_{H6,H5}$ = 3.84 Hz, 1 H, C5*H*), 6.53 (dd, $J_{H,F}$ = 16.24 Hz, $J_{1',2'}$ = 3.56 Hz, 1 H, C1′*H*), 6.83 (s, 2 H, NH_2), 7.22 (t, $J_{H6,H5}$ = 3.56 Hz, 1 H, C6*H*), 7.52–7.75 (m, 6 H, phenyl-*H*), 8.10 (m, 4 H, orthophenyl-*H*). Anal. ($C_{25}H_{20}FClN_4O_5$) C, H, N, F, Cl.

2-Amino-4-chloro-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (4). Compound **3** (0.8 g, 1.5 mmol) was added to MeOH/NH_3 (20 mL, saturated at 0 °C), and the mixture was stirred in a pressure bottle at room temperature for 24 h. The pressure bottle was cooled (0–5 °C) and opened carefully. The mixture was evaporated to dryness. The residue was coevaporated with MeOH (2 × 25 mL). The crude product was dissolved in MeOH (5 mL), adsorbed onto silica gel (10 g), and loaded on top of a prepacked dry silica gel column (3 × 30 cm). The column was eluted with CH_2Cl_2:MeOH (9:1), and the homogeneous product was isolated and crystallized from EtOH to afford 0.44 g (93%) of **4**: mp 166–168 °C; IR ν_{max} 3200–3350 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 318 (4.7), 262 (3.7), 236 (21.1), (pH 7) 316 (5.2), 262 (4.5), 234 (23.7), (pH 11) 316 (4.0), 262 (3.5), 236 (20.2); ^{1}H NMR (DMSO-d_6) δ 3.68 (m, 2 H, C5′H_2), 3.82 (q, 1 H, C4′*H*), 4.37 (tt, $J_{H,F}$ = 10.12 Hz, 1 H, C3′*H*), 5.17 (tq, 2 H, C5′O*H*, C2′*H*, after D_2O exchange resolved as tt, $J_{H,F}$ = 44.84 Hz, 1 H, C2′*H*), 5.91 (s, 1 H, C3′O*H*), 6.38 (d, $J_{H5,H6}$ = 4.04 Hz, 1 H, C5*H*), 6.44 (dd, $J_{H,F}$ = 12.16 Hz, $J_{1',2'}$ = 4.2 Hz, 1 H, C1′*H*), 6.76 (s, 2 H, NH_2), 7.28 (t, $J_{H6,H5}$ = 3.76 Hz, 1 H, C6*H*). Anal. ($C_{11}H_{12}FClN_4O_3$·0.5C_2H_5OH) C, H, N, F.

2-Amino-4-methoxy-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (5). A solution of **4** (0.5 g, 1.7 mmol) in 0.5 N NaOMe in MeOH (20 mL) was heated under reflux for 24 h. The reaction mixture was cooled in an ice bath and neutralized with 80% AcOH. The solvent was removed, and the residue was coevaporated with MeOH (2 × 20 mL) followed by toluene (2 × 15 mL). The dry residue was dissolved in MeOH (10 mL), adsorbed onto silica gel (10 g), loaded on top of a silica gel column (3 × 25 cm), and eluted with CH_2Cl_2:MeOH (9:1). The appropriate fractions were pooled and evaporated to give 0.4 g (73%) of **5** as white foam: mp 80–82 °C; IR ν_{max} 3300–3400 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$): (pH 1) 294 (13.1), 252 (7.25), 232 (26.4), (pH 7) 286 (12.1), 260 (17.4), 228 (25.0), (pH 11) 286 (11.2), 260 (16.1), 228 (24.9); ^{1}H NMR (DMSO-d_6) δ 3.61 (m, 2 H, C5′H_2), 3.78 (m, 1 H, C4′*H*), 3.92 (s, 3 H, OCH_3), 4.35 (tt, $J_{H,F}$ = 10.56 Hz, 1 H, C3′*H*), 5.10 (tq, 2 H, C5′O*H*, C2′*H*, resolved after D_2O exchange as tt, $J_{H,F}$ = 45.02 Hz, 1 H, C2′*H*), 5.85 (s, 1 H, C3′O*H*), 6.25 (br s, 2 H, NH_2), 6.28 (d, $J_{H5,H6}$ = 3.7 Hz, 1 H, C5*H*), 6.43 (dd, $J_{H,F}$ = 13.92 Hz, $J_{1',2'}$ = 3.96 Hz, 1 H, C1′*H*), 7.01 (t, $J_{H6,H5}$ = 3.28 Hz, 1 H, C6*H*). Anal. ($C_{12}H_{15}FN_4O_4$·0.5H_2O) C, H, N, F.

2-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (2′-Deoxy-2′-Arafluoro-7-deazaguanosine, 6). A solution of **5** (0.3 g, 1.0 mmol) in dry CH_3CN (50 mL) was stirred with iodotrimethylsilane (0.24 g, 1.2 mmol) for 1 h and then heated to reflux for 4 h. The reaction mixture was evaporated, and the residue was coevaporated with CH_3CN (2 × 15 mL). The crude product was purified by flash silica gel column (3 × 25 cm) chromatography using CH_2Cl_2:MeOH (92:8) as the eluent to give a white solid, which on crystallization from a mixture of CH_3OH/hexane afforded 0.26 g (92%) of **6**: mp 148–150 °C; IR ν_{max} 3300–3400 (OH, NH_2) , 1660 (C=O) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$): (pH 1) 284 (sh) (8.5), 260 (14.9), 220 (17.8), (pH 7) 286 (sh) (9.9), 260 (17.2), 226 (18.4), (pH 11) 282 (sh) (9.4), 260 (15.3), 224 (18.1); ^{1}H NMR (DMSO-d_6) δ 3.62 (m, 2 H, C5′H_2), 3.77 (q, 1 H, C4′*H*), 4.32 (tt, $J_{H,F}$ = 10.36 Hz, 1 H, C3′*H*), 5.03 (t, 1 H, C5′O*H*), 5.07 (tt, $J_{H,F}$ = 45.6 Hz, 1 H, C2′*H*), 5.87 (d, J = 4.42 Hz, 1 H, C3′O*H*), 6.25–6.31 (m, 4 H, C1′*H*, C5*H*, and NH_2), 6.85 (t, 1 H, C6*H*), 10.41 (br s, 1 H, N*H*). Anal. ($C_{11}H_{13}FN_4O_4$·0.25H_2O) C, H, N, F.

2,4-Diamino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-pyrrolo[2,3-*d*]pyrimidine (7). A suspension of **4** (0.6 g, 2.0 mmol) in a mixture of liquid NH_3 and MeOH (50:50, v/v, 35 mL) was stirred in a steel reaction vessel at 126 °C (oil bath temperature) for 5 days. The steel vessel was cooled to −20 °C and opened carefully. The mixture was evaporated to dryness, and the residue was coevaporated with MeOH (2 × 20 mL). The crude product was dissolved in MeOH (10 mL), adsorbed onto silica gel (15 g), and loaded on top of a prepacked dry silica gel column (3 × 28 cm). The column was flash eluted with CH_2Cl_2:MeOH (8:2), and the homogeneous product was isolated as a light brown foam: yield 0.45 g (80.2%); mp 118–120 °C; IR ν_{max} 3140–3450 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 300 (4.95), 261 (7.01), 231 (10.31), 210 (7.05), (pH 7) 286 (6.48), 261 (9.14), 230 (9.74), 210 (7.79), (pH 11) 281 (5.95), 261 (8.2), 228 (9.50), 211 (8.22); ^{1}H NMR (DMSO-d_6) δ 3.63 (m, 2 H, C5′H_2), 3.77 (q, 1 H, C4′*H*), 4.36 (tt, $J_{H,F}$ = 10.60 Hz, 1 H, C3′*H*), 5.04 (br tt, 2 H, C2′*H*, C3′O*H*, after D_2O exchange resolved as tt, $J_{H,F}$ = 45.2 Hz, 1 H, C2′*H*), 5.64 (s, 2 H, NH_2), 5.86 (d, J = 4.48 Hz, 1 H, C5′O*H*), 6.37 (dd, $J_{H,F}$ = 15.16 Hz, $J_{1',2'}$ = 4.12 Hz, 1 H, C1′*H*), 6.39 (d, $J_{H5,H6}$ = 3.68 Hz, 1 H, C5*H*), 6.59 (s, 2 H, NH_2), 6.84 (t, $J_{H6,H5}$ = 3.36 Hz, 1 H, C6*H*). Anal. ($C_{11}H_{14}FN_5O_3$·1.25H_2O) C, H, N, F.

2-Amino-4-(hydroxylamino)-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (8). To a solution of **4** (0.3 g, 1.0 mmol) in MeOH (30 mL), H_2O (5 mL), and Et_3N (3.5 mL) was added NH_2OH·HCl (0.21 g, 3 mmol), and the mixture was heated under reflux for 24 h. The reaction mixture was evaporated; the residue was coevaporated with MeOH (2 × 15 mL) and purified by flash silica gel column (3 × 25 cm) chromatography using CH_2Cl_2:MeOH (9:1) as the eluent to give 0.17 g (56%) of **8**: mp 125–128 °C; IR ν_{max} 3300–3500 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 298 (4.1), 266 (5.3), 232 (9.5), 208 (6.4), (pH 7) 292 (4.6), 266 (5.8), 228 (10.6), 210 (8.4), (pH 11) 288 (sh) (3.9), 264 (4.6), 222 (9.1), 212 (8.8); ^{1}H NMR (DMSO-d_6) δ 3.57 (m, 2 H, C5′H_2), 3.76 (m, 1 H, C4′*H*), 4.14 (br s, 1 H, NHO*H*), 4.31 (dd, $J_{H,F}$ = 17.44 Hz, 1 H, C3′*H*), 4.91 (d, 1 H, C5′O*H*), 5.13 (tt, $J_{H,F}$ = 48.96 Hz, 1 H, C2′*H*), 5.75 (t, 1 H, C5′O*H*), 6.13 (br s, 2 H, NH_2), 6.35 (dd, $J_{H,F}$ = 12.32 Hz, $J_{1',2'}$ = 3.84 Hz, 1 H, C1′*H*), 6.39 (s, 1 H, C5*H*), 6.92 (s, 1 H, C6*H*), 9.12 (br s, 1 H, N*H*OH). Anal. ($C_{11}H_{14}FN_5O_4$·0.5H_2O) C, H, N, F.

2-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4-sulfenamide (9). Sodium hypochlo-

rite (0.77 M, 5.25%, 4 mL, a freshly opened bottle of commercial bleach) was cooled to 0 °C (ice bath temperature), and to this solution was added a cold concentrated solution of NH_4OH (0.77 M, 5 mL cooled to 0 °C in an ice bath). The mixture was stirred at −5−0 °C for 15 min. To this mixture was added a solution of **12** (0.85 g, 2.8 mmol) in KOH (2 N, 2 mL), and the mixture was stirred at room temperature for an additional 1.5 h. The mixture was evaporated to dryness. The residue was coevaporated with MeOH (3 × 10 mL), dissolved in MeOH (10 mL), and adsorbed onto silica gel (6 g). After evaporation of MeOH, the silica gel was loaded on top of a prepacked (CH_2Cl_2) silica gel column (3 × 30 cm). The column was eluted with CH_2Cl_2:MeOH (9:1), and the appropriate fractions were pooled and evaporated to dryness to give 0.14 g (51.5%) of **9** as a light yellow foam: mp 120−122 °C; IR ν_{max} 3100−3450 (NH_2, OH) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 292 (9.9), 258 (sh) (8.1), 234 (15.6), (pH 7) 290 (8.9), 260 (12.0), 232 (14.6), 212 (10.5), (pH 11) 288 (10.1), 260 (13.2), 232 (14.9), 216 (11.6); ^1H NMR (DMSO-d_6) δ 3.60 (s, 2 H, S-NH_2), 3.77 (m, 3 H, C4′H, C5′H_2), 4.33 (tt, $J_{H,F}$ = 10.52 Hz, 1 H, C3′H), 4.97 (t, 1 H, C5′OH), 5.08 (tt, $J_{H,F}$ = 45.24 Hz, 1 H, C2′H), 5.89 (s, 1 H, C3′OH), 6.28 (br s, 3 H, C5H, NH_2, after D_2O exchange resolved as d, $J_{H5,H6}$ = 3.64 Hz, 1 H, C5H), 6.43 (dd, $J_{H,F}$ = 13.36 Hz, $J_{1',2'}$ = 4.12 Hz, 1 H, C1′H), 7.01 (t, $J_{H6,H5}$ = 3.32 Hz, 1 H, C6H). Anal. ($C_{11}H_{14}FN_5O_3S\cdot0.5H_2O$) C, H, N, F, S.

2-Amino-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-thione (10). A mixture of **3** (2 g, 3.9 mmol), thiourea (0.6 g, 7.8 mmol), EtOH (35 mL), and 2 drops of formic acid was heated to reflux for 3 h. The mixture was evaporated to dryness. The residue was dissolved in MeOH (10 mL), adsorbed onto silica gel (15.0 g), evaporated to dryness, and loaded on top of a prepacked silica gel column (3 × 28 cm). The column was eluted with CH_2Cl_2:MeOH (9:1). The appropriate fractions were collected and evaporated to give 1.85 g (92.6%) of **10** as a light yellow foam: mp 150−152 °C; IR ν_{max} 3100−3350 (NH_2), 1725 (C=O), 1265 (C=S) cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.63 (m, 3 H, C4′H, C5′H_2), 5.69 (tt, $J_{H,F}$ = 50.6 Hz, 1 H, C2′H), 5.77 (dd, $J_{H,F}$ = 18.32 Hz, 1 H, C3′H), 6.45 (m, 2 H, C1′H, C5H), 6.70 (br s, 2 H, NH_2), 6.98 (d, $J_{H6,H5}$ = 2.76 Hz, 1 H, C6H), 7.51−7.72 (m, 6 H, phenyl-H), 8.07 (m, 4 H, *o*-phenyl-H), 11.90 (s, 1 H, NH). Anal. ($C_{25}H_{21}FN_4O_5S\cdot0.5H_2O$) C, H, N, F, S.

It was observed that when compound **3** (1.85 g, 3.62 mmol) and an excess of thiourea (2.2 g, 29 mmol) were refluxed in EtOH (250 mL) in the presence of formic acid (8 drops) for 2 h, the 3′-*O*-benzoyl group was cleaved. Purification of the reaction mixture by flash silica gel column (3 × 25 cm) chromatography afforded 2-amino-7-(2-deoxy-2-fluoro-5-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-thione (**11**) (0.55 g, 37%): mp 110−112 °C; IR ν_{max} 3200−3400 (NH_2, NH), 1715 (C=O), 1275 (C=S) cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.02 (m, 1 H, C4′H), 4.62 (m, 3 H, C3′H, C5′H_2), 5.20 (tt, $J_{H,F}$ = 48.8 Hz, 1 H, C2′H), 6.14 (d, J = 4.8 Hz, 1 H, C3′OH), 6.40 (m, 2 H, C1′H, C5H), 6.70 (br s, 2 H, NH_2), 6.96 (t, J = 2.8 Hz, 1 H, C6H), 7.57 (m, 3 H, phenyl-H), 8.01 (d, 2 H, *o*-phenyl-H), 11.85 (br s, 1 H, NH). Anal. ($C_{18}H_{17}FN_4O_4S\cdot H_2O$) C, H, N, F, S.

2-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-thione (12). A solution of **10** (0.45 g, 0.9 mmol) in MeOH/NH_3 (25 mL, saturated at 0 °C) was stirred in a pressure bottle at room temperature for 18 h. The mixture was evaporated to dryness and coevaporated with MeOH (3 × 15 mL). The residue after purification by silica gel column (3 × 28 cm) chromatography using CH_2Cl_2:MeOH (9:1) as the eluent and subsequent crystallization from MeOH/hexane mixture gave 0.25 g (92%) of **12**: mp 146−148 °C; IR ν_{max} 3200−3400 (NH_2, OH) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 310 (10.5), 270 (10.1), 231 (11.1), 210 (8.2), (pH 7) 310 (10.5), 270 (10.6), 231 (11.3), 212 (8.4), (pH 11) 342 (10.4), 270 (10.1), 236 (11.2), 212 (8.6); ^1H NMR (DMSO-d_6) δ 3.61 (m, 2 H, C5′H_2), 3.8 (q, 1 H, C4′H), 4.34 (tt, $J_{H,F}$ = 10.36 Hz, 1 H, C3′H), 4.92 (s, 1 H, C5′OH), 5.10 (tt, $J_{H,F}$ = 45.28 Hz, 1 H, C2′H), 5.79 (br s, 1 H, C3′OH), 6.29 (dd, $J_{H,F}$ = 12.8 Hz, $J_{1',2'}$ = 4.4 Hz, 1 H, C1′H), 6.41 (d, $J_{H5,H6}$ = 3.6 Hz, 1 H, C5H), 6.60 (s, 2 H, NH_2), 7.03 (t, $J_{H6,H5}$ = 2.88 Hz, 1 H, C6H), 11.73 (br s, 1 H, NH). Anal. ($C_{11}H_{13}FN_4O_3S\cdot0.5H_2O$) C, H, N, F, S.

2-Amino-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-selone (13). A mixture of **3** (1.0 g, 2.0 mmol), selenourea (0.4 g, 3.5 mmol), and absolute EtOH (30 mL) was heated under reflux for 3 h. The yellow reaction mixture was cooled to room temperature and evaporated to dryness. The residue was dissolved in EtOH (15 mL), adsorbed onto silica gel (35 g), and loaded on top of a prepacked (dry) silica gel column (3 × 30 cm). The column was eluted with CH_2Cl_2:MeOH (96:4); the appropriate fractions were collected and evaporated to give a yellow solid which on crystallization from EtOH afforded 0.8 g (75%) of **13**: mp 126−128 °C; IR ν_{max} 3400−3500 (NH_2), 1725 (C=O) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 380 (2.4), 234 (25.5), 208 (16.5), (pH 7) 322 (4.5), 236 (25.1), 210 (17.0), (pH 11) 320 (4.0), 282 (sh) (3.5), 234 (25.1), 212 (18.3); ^1H NMR (DMSO-d_6) δ 4.60 (q, 1 H, C4′H), 4.75 (m, 2 H, C5′H_2), 5.73 (tt, $J_{H,F}$ = 48.8 Hz, 1 H, C2′H), 5.79 (tt, $J_{H,F}$ = 16.4 Hz, 1 H, C3′H), 6.46 (s, 2 H, NH_2), 6.65 (m, 2 H, C1′H, C5H), 7.13 (t, 1 H, C6H), 7.48−7.73 (m, 6 H, phenyl-H), 8.07 (m, 4 H, *o*-phenyl-H), 11.25 (br s, 1 H, NH). Anal. ($C_{25}H_{21}FN_4O_5Se\cdot C_2H_5OH$) C, H, N, F, Se.

2-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-4(3*H*)-selone (14). A solution of **13** (0.15 g, 0.27 mmol) in MeOH/NH_3 (30 mL, saturated at 0 °C) was stirred at room temperature in a pressure bottle for 16 h. The bottle was cooled and opened carefully, and the solvents were evaporated to dryness. The residue was coevaporated with MeOH (3 × 10 mL). The yellow product was dissolved in MeOH (4 mL), adsorbed onto silica gel (5 g), and loaded on top of a prepacked (dry) silica gel column (2 × 25 cm). The column was eluted with CH_2Cl_2:MeOH (9:1), and the homogeneous product on crystallization from MeOH/hexane gave 0.09 g (91%) of **14**: mp 186−188 °C; IR ν_{max} 3300−3450 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 376 (3.6), 294 (4.1), 236 (13.7), 214 (11.2), (pH 7) 320 (7.01), 236 (13.7), 214 (11.2), (pH 11) 320 (7.01), 236 (13.7), 214 (11.2); ^1H NMR (DMSO-d_6) δ 3.60 (m, 2 H, C5′H_2), 3.77 (q, 1 H, C4′H), 4.34 (dd, $J_{H,F}$ = 14.82 Hz, 1 H, C3′H), 4.91 (t, 1 H, C5′OH), 5.10 (dd, $J_{H,F}$ = 49.16 Hz, 1 H, C2′H), 5.80 (d, J = 4.56 Hz, 1 H, C3′OH), 6.43 (m, 3 H, NH_2, C1′H, resolved as dd after D_2O exchange, $J_{H,F}$ = 13.48 Hz, $J_{1',2'}$ = 3.62 Hz, 1 H, C1′H), 6.58 (d, $J_{H5,H6}$ = 3.72 Hz, 1 H, C5H), 7.15 (s, 1 H, C6H), 11.45 (br s, 1 H, NH). Anal. ($C_{11}H_{13}FN_4O_3Se\cdot CH_3OH$) C, H, N, F, Se.

2-Amino-4-chloro-7-[2-deoxy-2-fluoro-5-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]pyrrolo[2,3-*d*]pyrimidine (15). To a solution of **4** (2.6 g, 8.7 mmol) in dry DMF (50 mL) was added imidazole (4.7 g, 6.9 mmol). Under argon, *tert*-butyldimethylsilyl chloride (1.7 g, 11.3 mmol) was added, and the mixture was stirred under argon at 25 °C for 2 h. The reaction mixture was evaporated to dryness. The resulting solid was dissolved in CH_2Cl_2 (110 mL), washed with H_2O (150 mL), dried (Na_2SO_4), and evaporated to dryness. The residue was applied to a flash silica gel column (4 × 28 cm) and eluted with CH_2Cl_2:EtOAc (7:3) to afford 3.2 g (89.3%) of the desired product **15** (R_f = 0.43; CH_2Cl_2:EtOAc, 6:4) as a white foam: mp 120−122 °C; IR ν_{max} 3100−3500 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$): (pH 1) 318 (4.3), 264 (4.2), 236 (18.4), (pH 7) 316 (5.3), 262 (4.3), 236 (18.9), (pH 11) 316 (4.9), 262 (4.1), 236 (18.4); ^1H NMR (DMSO-d_6) δ 0.07 [s, 6 H, *t*-Bu-Si(CH_3)$_2$], 0.94 [s, 9 H, *t*-Bu-Si(CH$_3$)$_2$], 3.85 (m, 3 H, C5′H_2, C4′H), 4.36 (tt, $J_{H,F}$ = 15.08 Hz, 1 H, C3′H), 5.20 (tt, $J_{H,F}$ = 44.43 Hz, 1 H, C2′H), 5.97 (s, 1 H, C3′OH), 6.37 (d, J = 3.45 Hz, 1 H, C5H), 6.43 (dd, $J_{H,F}$ = 11.2 Hz, $J_{1',2'}$ = 4.48 Hz, 1 H, C1′H), 6.77 (br s, 2 H, NH_2), 7.21 (t, J = 2.36 Hz, 1 H, C6H). Anal. ($C_{17}H_{26}FClN_4O_3Si$) C, H, N, F, Cl.

The 2-amino-4-chloro-7-[2-deoxy-2-fluoro-3,5-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]pyrrolo[2,3-*d*]pyrimidine derivative (R_f = 0.69; CH_2Cl_2:EtOAc, 6:4) was obtained in 15.2% yield (characterized by ^1H NMR).

2-Amino-4-chloro-7-[2-deoxy-2-fluoro-3-*O*-(phenoxythiocarbonyl)-5-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]pyrrolo[2,3-*d*]pyrimidine (16). To a stirred solution of **15** (1.5 g, 3.6 mmol) and 4-(dimethylamino)pyridine (DMAP; 2.8 g, 23 mmol) in dry CH_3CN (40 mL) was added phenyl chlorothionocarbonate (1 mL, 4.7 mmol) dropwise under argon over a period of 5 min. The reaction mixture was stirred at room temperature under argon for 18 h. The solvent was

evaporated, and the residue was purified on a flash silica gel column (3 × 20 cm) using hexane:EtOAc (9:1) as the eluent to afford 1 g (55.4%) of **16** as a foam: mp 76–77 °C; IR ν_{max} 3350 (NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 316 (5.1), 262 (5.7), 236 (25.2), (pH 7) 316 (7.0), 262 (7.1), 236 (25.9), (pH 11) 316 (6.3), 262 (6.6), 234 (25.4); ^1H NMR (DMSO-d_6) δ 0.08 [s, 6 H, *t*-Bu-Si(CH_3)$_2$], 0.89 [s, 9 H, *t-Bu*-Si(CH_3)$_2$], 3.95 (m, 2 H, C5′H_2), 4.34 (m, 1 H, C4′*H*), 5.74 (tt, $J_{H,F}$ = 16.8 Hz, $J_{1',2'}$ = 47.32 Hz, 1 H, C2′*H*), 6.00 (tt, $J_{H,F}$ = 12.04 Hz, 1 H, C3′*H*), 6.44 (d, J = 3.8 Hz, 1 H, C5*H*), 6.55 (dd, $J_{H,F}$ = 16.8 Hz, $J_{1',2'}$ = 3.44 Hz, 1 H, C1′*H*), 6.78 (br s, 2 H, NH_2), 7.22–7.52 (m, 6 H, Ar-*H*, C6*H*). Anal. ($C_{24}H_{30}FClN_4O_4SSi$) C, H, N, F, S.

2-Amino-4-chloro-7-[2,3-dideoxy-2-fluoro-5-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]pyrrolo[2,3-*d*]pyrimidine (17). To a solution of **16** (0.7 g, 1.3 mmol) in dry toluene (35 mL) were added 2,2′-azobis(2-methylpropionitrile) (AIBN; 0.3 g, 1.8 mmol) and *n*-Bu_3SnH (3.0 mL), and the mixture was stirred at 80 °C for 3 h under argon. The solvent was evaporated, and the residue was purified by flash silica gel column (3 × 20 cm) chromatography using hexane:EtOAc (7:3) as the eluent to afford 0.45 g (90%) of **17** as a foam: mp 90–92 °C; IR ν_{max} 3200–3300 (NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 318 (10.3), 260 (8.5), 238 (16.9), (pH 7) 316 (11.84), 260 (9.4), 236 (17.1), (pH 11) 318 (10.5), 262 (8.6), 236 (16.8); ^1H NMR (DMSO-d_6) δ 0.7 [s, 6 H, *t*-Bu-Si(CH_3)$_2$], 0.88 [s, 9 H, *t-Bu*-Si(CH_3)$_2$], 2.23 (m, 1 H, C3′H_a), 2.60 (m, 1 H, C3′H_b), 3.82 (m, 2 H, C5′H_2), 4.16 (m, 1 H, C4′*H*), 5.46 (tt, $J_{H,F}$ = 42.72 Hz, 1 H, C2′*H*), 6.31 (dd, $J_{H,F}$ = 12.64 Hz, $J_{1'2'}$ = 3.76 Hz, 1 H, C1′*H*), 6.37 (d, J = 3.44 Hz, 1 H, C5*H*), 6.73 (br s, 2 H, NH_2), 7.26 (t, J = 3.24 Hz, 1 H, C6*H*). Anal. ($C_{17}H_{26}FClN_4O_2Si$) C, H, N, F, Cl.

2,4-Diamino-7-[2,3-dideoxy-2-fluoro-5-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]pyrrolo[2,3-*d*]pyrimidine (18). A mixture of **17** (0.53 g, 1.6 mmol), liquid NH_3 (15 mL), and MeOH (15 mL) was stirred in a steel reaction vessel at 120 °C for 5 days. The vessel was cooled to 0 °C and opened carefully. The solvent was evaporated; the residue was coevaporated with MeOH (3 × 25 mL) and purified by flash silica gel column (3 × 18 cm) chromatography using CH_2Cl_2:MeOH (96:4) as the eluent to afford 0.47 g (80%) of **18**: mp 82–84 °C; IR ν_{max} 3200–3400 (NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 292 (7.7), 266 (11.2), 232 (17.5), (pH 7) 290 (8.1), 266 (10.7), 230 (16.7), (pH 11) 288 (8.6), 264 (11.0), 230 (11.3); ^1H NMR (DMSO-d_6) δ 0.71 [s, 6 H, *t*-Bu-Si(CH_3)$_2$], 0.89 [s, 9 H, *t-Bu*-Si(CH_3)$_2$], 2.18 (m, 1 H, C3′H_a), 2.57 (m, 1 H, C3′H_b), 3.33 (m, 2 H, C5′H_2), 3.74 (m, 1 H, C4′*H*), 4.09 (m, 1 H, C3′*H*), 5.33 (tt, $J_{H,F}$ = 44.38 Hz, 1 H, C2′*H*), 5.56 (br s, 2 H, NH_2), 6.22 (dd, $J_{H,F}$ = 12.12 Hz, $J_{1'2'}$ = 3.36 Hz, 1 H, C1′*H*), 6.37 (d, J = 3.48 Hz, 1 H, C5*H*), 6.55 (br s, 1 H, NH_2), 6.85 (t, J = 3.04 Hz, 1 H, C6*H*). Anal. ($C_{17}H_{28}FN_5O_2Si$) C, H, N, F.

2,4-Diamino-7-(2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (19). Compound **18** (0.25 g, 0.66 mmol) was dissolved in dry CH_3CN (20 mL) and stirred under argon for 10 min. To this solution was added dry tetraethylammonium fluoride (0.5 g, 3.3 mmol), and stirring was continued under argon at room temperature for an additional 1 h. Water (2 mL) was added to the mixture, and the mixture was stirred for 20 min at room temperature. The solvent was evaporated, and the residue was coevaporated with MeOH (2 × 20 mL) and purified on a flash silica gel column using CH_2Cl_2:MeOH (88:12) as the eluent to afford 0.15 g (83.5%) of **19** as a white foam: mp 110–112 °C; IR (KBr) ν_{max} 3000–3400 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 300 (5.3), 264 (7.3), 232 (12.5), 210 (7.6), (pH 7) 290 (6.4), 264 (9.3), 230 (11.1), 214 (9.1), (pH 11) 288 (6.1), 264 (8.5), 228 (10.8), 217 (8.7); ^1H NMR (DMSO-d_6) δ 2.18 (m, 1 H, C3′H_a), 2.54 (m, 1 H, C3′H_b), 3.52 (m, 2 H, C5′H_2), 4.04 (q, 1 H, C4′*H*), 4.93 (br s, 1 H, C5′O*H*), 5.32 (tt, $J_{H,F}$ = 45.6 Hz, 1 H, C2′*H*), 5.54 (br s, 2 H, NH_2), 6.20 (dd, $J_{H,F}$ = 15.5 Hz, $J_{1'2'}$ = 3.44 Hz, 1 H, C1′*H*), 6.37 (d, J = 3.76 Hz, 1 H, C5*H*), 6.54 (br s, 2 H, NH_2), 6.87 (t, J = 3.2 Hz, 1 H, C6*H*). Anal. ($C_{11}H_{14}FN_5O_2$) C, H, N, F.

2-Amino-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)-5,6-dihydropyrrolo[2,3-*d*]pyrimidine (20). A mixture of **3** (0.2 g, 0.4 mmol), MeOH (15 mL), ammonium formate (0.04 g, 0.63 mmol), and 10% Pd/C (0.4 g) was heated under reflux for 2 h. The reaction mixture was filtered through a Celite pad, and the pad was washed with EtOH (2 × 10 mL). The combined filtrate and washings were evaporated, and the residue was purified by silica gel column (2 × 28 cm) chromatography using CH_2Cl_2:EtOAc (7:3) as the eluent to give 0.15 g (75%) of **20**: mp 89–91 °C; IR ν_{max} 3300–3450 (NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 280 (3.8), 232 (12.8), 214 (9.9), (pH 7) 300 (3.5), 232 (12.6), 208 (9.9), (pH 11) 304 (3.6), 232 (12.1), 210 (11.9); ^1H NMR (DMSO-d_6) δ 2.83 (m, 2 H, C5H_2), 3.73 (m, 2 H, C6H_2), 4.52 (q, 1 H, C4′*H*), 4.66 (m, 2 H, C5′H_2), 5.54 (tt, $J_{H,F}$ = 21.13 Hz, 1 H, C3′*H*), 5.60 (tt, $J_{H,F}$ = 45.23 Hz, 1 H, C2′*H*), 6.01 (s, 2 H, NH_2), 6.21 (dd, $J_{H,F}$ = 20.64 Hz, $J_{1',2'}$ = 3.34 Hz, 1 H, C1′*H*), 7.49–7.73 (m, 6 H, phenyl-*H*), 8.05 (m, 5 H, *o*-phenyl-*H* and C4*H*). Anal. ($C_{25}H_{23}FN_4O_5$) C, H, N, F.

2-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5,6-dihydropyrrolo[2,3-*d*]pyrimidine (21). Method A: A solution of **20** (0.1 g, 0.2 mmol) in MeOH/NH_3 (saturated at 0 °C, 20 mL) was stirred at room temperature in a pressure bottle for 24 h and then evaporated to dryness. The residue was coevaporated with MeOH (2 × 10 mL) and purified by flash silica gel column (2 × 25 cm) chromatography using CH_2Cl_2:MeOH (8:2) as the eluent. The homogeneous product on crystallization from a mixture of CH_2Cl_2/MeOH gave 0.06 g (97%) of **21**: mp 170–172 °C; IR ν_{max} 3350–3450 (NH_2, OH) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 278 (8.3), 236 (9.8), 216 (7.9), (pH 7) 300 (8.9), 240 (9.0), 214 (7.8), (pH 11) 300 (8.9), 240 (8.0), 216 (7.8); ^1H NMR (DMSO-d_6) δ 2.86 (m, 2 H, C5H_2) 3.64 (m, 4 H, C5′H_2, C6H_2), 3.81 (q, 1 H, C4′*H*), 4.17 (dd, $J_{H,F}$ = 14.8 Hz, 1 H, C3′*H*), 4.84 (d, 1 H, C5′O*H*), 5.07 (tt, $J_{H,F}$ = 44.64 Hz, 1 H, C2′*H*), 5.72 (s, 1 H, C3′O*H*), 6.01 (s, 2 H, NH_2), 6.10 (dd, $J_{H,F}$ = 14.4 Hz, $J_{1'2'}$ = 4.5 Hz, 1 H, C1′*H*), 7.58 (s, 1 H, C4*H*). Anal. ($C_{11}H_{15}FN_4O_3$·0.5CH_3OH) C, H, N, F.

Method B: A solution of **3** (0.2 g, 0.4 mmol) in MeOH (15 mL) was hydrogenated in the presence of 10% Pd/C (0.1 g) at room temperature at 50 psi for 12 h. The mixture was filtered through a Celite pad, and the pad was washed with EtOH. The combined filtrate and washings were evaporated, and MeOH/NH_3 (saturated at 0 °C, 20 mL) was added to the residue. The reaction mixture was stirred overnight. The mixture was evaporated to dryness, and the residue was coevaporated with MeOH (2 × 10 mL). Purification of the residue on a silica gel column (2 × 25 cm) using CH_2Cl_2:MeOH (8:2) as the eluent and subsequent crystallization from a mixture of CH_2Cl_2/MeOH afforded 0.085 g (75.6%) of **21**: mp 171–172 °C; identical in all respects with **21** prepared by method A.

2-Amino-7-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (22). A mixture of **3** (0.5 g, 1.0 mmol), MeOH (35 mL), ammonium formate (0.1 g, 1.6 mmol), and 10% Pd/C (0.45 g) was heated under reflux for 30 min. The reaction mixture was filtered through a Celite pad and the pad was washed with EtOH (2 × 15 mL). The combined filtrate and washings were evaporated, and the residue was purified by flash silica gel column (3 × 28 cm) chromatography using CH_2Cl_2:EtOAc (7:3) as the eluent. The homogeneous product was crystallized from MeOH/hexane to give 0.4 g (79%) of **22**: mp 110–112 °C; IR ν_{max} 3400–3450 (NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 332 (2.1), 268 (4.96), 238 (17.5), 210 (11.95), (pH 7) 316 (5.2), 262 (sh) (5.7), 236 (17.9), 208 (11.8), (pH 11) 314 (4.3), 264 (4.3), 236 (17.7), 210 (2.1); ^1H NMR (DMSO-d_6) δ 4.59 (t, 1 H, C4′*H*), 4.74 (m, 2 H, C5′H_2), 5.70 (tt, $J_{H,F}$ = 47.5 Hz, 1 H, C2′*H*), 5.80 (tt, $J_{H,F}$ = 14.69 Hz, 1 H, C3′*H*), 6.32 (s, 2 H, NH_2), 6.41 (d, $J_{H5,H6}$ = 3.78 Hz, 1 H, C5*H*), 6.63 (dd, $J_{H,F}$ = 17.83 Hz, $J_{1'2'}$ = 3.46 Hz, 1 H, C1′*H*), 7.13 (t, $J_{H6,H5}$ = 3.46 Hz, 1 H, C6*H*), 7.51–7.75 (m, 6 H, phenyl-*H*), 8.09 (m, 4 H, *o*-phenyl-*H*), 8.50 (s, 1 H, C4*H*). Anal. ($C_{25}H_{21}F_4N_4O_5$) C, H, N, F.

2-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (23). Compound **22** (0.15 g, 0.31 mmol) was stirred in a pressure bottle with MeOH/NH_3 (saturated at 0 °C) for 24 h. The volatile matters were removed. The residue was dissolved in MeOH (5 mL), adsorbed onto silica gel (5 g), and loaded on top of a prepacked silica gel column (3 × 15 cm). The column was eluted with CH_2Cl_2:MeOH (90:10), and the homogeneous product on

crystallization from a mixture of MeOH/CH_2Cl_2 afforded 0.08 g (94%) of **23**: mp 152 °C; IR ν_{max} 3200–3400 (OH, NH_2) cm^{-1}; UV λ_{max} (nm) ($\epsilon \times 10^{-3}$) (pH 1) 334 (4.5), 268 (7.6), 238 (20.9), (pH 7) 314 (10.5), 260 (9.1), 238 (21.8), (pH 11) 314 (9.0), 258 (8.0), 236 (21.4); ^1H NMR (DMSO-d_6) δ 3.65 (m, 2 H, C5′H_2), 3.79 (m, 1 H, C4′H), 4.35 (tt, $J_{H,F}$ = 10.4 Hz, 1 H, C3′H), 5.01 (s, 1 H, C5′OH), 5.11 (tt, $J_{H,F}$ = 44.84 Hz, 1 H, C2′H), 5.89 (s, 1 H, C3′OH), 6.31 (s, 2 H, NH_2), 6.48 (d, 1 H, C5H), 6.56 (dd, $J_{H,F}$ = 9.56 Hz, $J_{1',2'}$ = 4.32 Hz, 1 H, C1′H), 7.19 (t, 1 H, C6H), 8.45 (s, 1 H, C4H). Anal. ($C_{11}H_{13}FN_4O_3$·0.5CH_3OH) C, H, N, F.

Virological Methods. Cell lines, Viruses, and Materials. The routine growth and passage of Vero cells were performed in monolayer cultures using minimal essential medium (MEM) with either Hanks [MEM(H)] or Earle [MEM(E)] salts supplemented with 10% calf serum and pennstrep (100 U/mL penicillin G, 100 μg/mL streptomycin). Cultures of the diploid human foreskin fibroblast (HFF) cell line Hs68 and MRC-5 cells were routinely grown in MEM(E) supplemented with 10% fetal bovine serum (FBS). Cells were passaged at 1:2–1:10 dilutions according to conventional procedures by using 0.05% trypsin plus 0.02% EDTA in a HEPES-buffered salt (HBS) solution as previously described.[48,49] HFF and MRC-5 cells were passaged only at 1:2 dilutions. Ganciclovir (DHPG) was obtained from Syntex Research, Palo Alto, CA, and acyclovir (ACV) was obtained from Burroughs Welcome Co., NC. 2′,3′-Dideoxycytidine was purchased from Sigma Chemical Co., St. Louis, MO.

The Towne and AD169 strains of HCMV were obtained from the American Type Culture Collection (ATCC). HSV-1 strain 17 and HSV-2 strain HG52 were obtained from D. J. McGeoch (University of Glasgow). Stock preparations of HCMV, HSV-1, and HSV-2 were prepared as previously described.[45] The HBV-producing cell line 2.2.15, derived from HepG2 cells transfected with episomal HBV-DNA,[37,50] were generously provided by G. Acs (Mount Sinai School of Medicine). Prior to plating 2.2.15 cells, 24-well plates were coated with 150 μL of 0.1 mg/mL collagen (rat tail) type I (Sigma) reconstituted in 0.02 M acetic acid. Plates were coated with collagen and left uncovered under sterile conditions at room temperature overnight. The plates were rinsed twice with serum-free medium, just prior to use, to remove the residual acid.

Antiviral Assays. The effects of compounds on the replication of HCMV, HSV-1, and HSV-2 have been measured using plaque reduction and cytopathic effect (CPE) assays. The effect of compounds on episomal HBV production in 2.2.15 cells was measured using DNA hybridization analysis.

(a) HCMV Plaque Reduction Assays. Plaque reduction assays were employed to determine the anti-HCMV efficacy of the test compounds in culture. The assay used MRC-5 or HFF cells and the AD169 (Towne) or D16 strains of HCMV as described previously.[45]

(b) HCMV Timing Experiment. MRC-5 cells (10 000 cells/well) were plated in 96-well plates 1 day prior to use. Medium was removed, and then medium plus drug (100 μL) was added to the −18 h time point wells. All other wells received culture medium only. All compounds were tested in triplicate. Medium alone was added to cell control and virus control wells. The cells were then incubated at 37 °C for 18 h. The medium was removed, the cells were rinsed three times with fresh medium, and virus was added [1×10^3 plaque-forming units (PFU)/well] in a 50 μL vol to all but cell control wells. The virus was allowed to adsorb for 2 h at 37 °C. The virus-containing medium was then removed, and the cells were rinsed three times with medium further cultured in 100 μL of complete medium. Drug was then added back to the culture medium at various time points postinfection. The cells were continuously incubated for 3 days at 37 °C, at which time the medium was removed, the cells were rinsed twice with medium, and a final 100 μL of medium was added to each well. The plates were then placed at −70 °C and subjected to a single freeze-thaw cycle. The cell-associated virus was titered on MRC-5 cells.[45,48]

(c) HCMV Yield Assay. HCMV yield reduction assays were performed as previously described.[51] MRC-5 cells were plated in 96-well cluster dishes at a concentration of 12 500 cells/well and incubated overnight. The next day, the medium was shaken out and the cultures were inoculated with HCMV (Towne) at a multiplicity of infection (MOI) of 0.5–1 PFU/cell. After virus adsorption, virus inoculum was replaced with 0.2 mL of fresh medium containing test compounds. Plates were incubated at 37 °C for 3 days and then subjected to one cycle of freezing at −76 °C and thawing at 37 °C to disrupt the cells. Aliquots from each of the eight wells of a given column were transferred to the first column of a fresh 96-well monolayer culture of MRC-5 cells. The contents were mixed and serially diluted (1:3) across the remaining 11 columns of the secondary plate. Each column of the original primary plate was diluted across a separate plate in this manner. Cultures were incubated, plaques were enumerated, and titers were calculated as previously described.[45]

(d) HSV-1 and HSV-2 CPE Assays. Vero cells, incubated at 37 °C in a humidified 5% CO_2 atmosphere, were plated at 4 × 104 cells/well in a 96-well microtiter dish in 0.1 mL of culture medium 24 h before infection with virus at a MOI of 0.001. The virus was allowed to adsorb to the cells for 10 min at 37 °C. The virus-containing medium was then removed, and the cells were rinsed three times with fresh medium. A final 100 μL aliquot of fresh medium containing the various dilutions of test compounds was then added to each well. Plaques were observed 24 h postinfection, and the degree of CPE was scored 40–48 h postinfection. Acyclovir was used as a standard in all HSV assays.

(e) Combination Study. Drug combination studies were performed as previously described.[45]

(f) Cellular Cytotoxicity. Stationary uninfected HFF and MRC-5 cells were evaluated for visual cytotoxicity on the scoring basis of 0 (no visual cytotoxicity at 20-fold magnification) to 4 (cell sheet nearly destroyed). Cytotoxicity as measured by inhibition of cell growth was performed in Vero or MCR-5 cells using a CellTiter 96 aqueous nonradioactivity cell proliferation assay (Promega). Cytotoxicity assays were performed using a 4 day incubation of test compound with Vero cells plated at 5000 cells/assay. Each assay was performed in quadruplicate. The resultant data were averaged, graphed, and then used to calculate the TC_{50} (median toxic concentration) for the compound tested.

To determine the cytotoxicity of compounds in the HepG2 cell line, the viable cell number was determined by trypan blue staining, and cells were resuspended in MEM supplemented with 10% FBS (GIBCO). Eighty microliters of cell suspension (1.7×10^4 cells/well) was dispensed subconfluently onto collagen-coated 96-well microtiter plates. At this time, 20 μL of various concentrations of drug (or control) was added to appropriate wells in each plate. Each concentration was assayed in quadruplicate. The degree of cell proliferation was assayed immediately, on day 0, while other plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere as previously described.[45] The cell proliferation assay was performed again on days 4, 8, and 12. Fresh drug-containing medium was added to remaining cell cultures every second day. The average absorbance of the samples was graphed for each concentration, and the data obtained were used to calculate the TC_{50} (drug concentration necessary to inhibit cell proliferation by 50%) for each compound.

Data Analysis. Dose–response relationships were constructed by linear regression analysis of inhibition parameters derived in the preceding sections against log drug concentrations. Fifty percent inhibitory (IC_{50}) concentrations were calculated from the regression lines. Samples containing positive controls (ACV for HSV-1 or HSV-2, DHPG for HCMV, and ddC for HBV) were used in all assays. The analysis of the data obtained from the combination drug study was performed using the MacSynergy program as described previously.[52]

***In Vitro* Anti-HBV Assay.** The 2.2.15 cells were seeded (1×10^5 cells/well) in 300 μL of MEM supplemented with 10% FBS (GIBCO). Once the cells reached confluency (ca. 1 day), the culture medium was replaced every 2 days with medium containing various concentrations of the indicated drug. Twelve days postdosing with drug, the cells were harvested and the DNA was prepared as described below. At the same

time the culture medium was saved and assayed, using a slot-blot hybridization technique to detect the presence of extracellular HBV-DNA. The control wells were treated in the same fashion except that no drug was added to the culture medium.

Isolation and Characterization of HBV-DNA. Total intracellular DNA was extracted from 2.2.15 or HepG2 cells by lysing cells in 10 mM Tris·HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, and 1% SDS. The lysed cells were digested with 100 μg/mL proteinase K at 55 °C for 3 h and deproteinized by phenol:chloroform extraction. Nucleic acids were then isolated by precipitation in aqueous EtOH. The nucleic acid pellet was redissolved in 10 mM Tris·HCl (pH 7.5) and 1 mM EDTA (pH 8.0) and then treated with RNase A (100 μg/mL) and *Hin*dIII (New England BioLabs) at 37 °C for at least 2 h. Concentrated aqueous NH_4OAc was added to a final concentration of 0.4 M. The DNA samples were precipitated with 2 vol of aqueous EtOH and washed with 70% aqueous EtOH. The pellets were redissolved in 10 mM Tris·HCl/1 μM EDTA (pH 8.0) buffer. The DNA concentration was determined by spectrophotometric analysis at 260 nm, and the samples were stored at −20 °C until use.

For DNA blot hybridization analysis, 5−10 μg of *Hin*dIII-cleaved total intracelluar DNA was subjected to electrophoresis using a 0.8% agarose gel in 1× TAE buffer and transferred to a Zeta Probe membrane (Bio-Rad Laboratories) by capillary method.[53] As a molecular probe, a subgenomic fragment of the HBV-DNA was cloned into the pCRII TA cloning vector (Invitrogen) plasmid between the two *Eco*RI sites. The subgenomic HBV-DNA fragment was obtained using the polymerase chain reaction (PCR) technique to amplify a portion of the HBV genome (nucleotide positions 2134−143). Total DNA isolated from 2.2.15 cells was used as the PCR template with primer sets corresponding to nucleotide positions 2134−2151 (forward) and 143−126 (reverse). The resulting fragment was then inserted into pCRII TA. The 1200 bp fragment of HBV-DNA was cleaved out of the recombinant plasmid using *Eco*RI, separated away from plasmid DNA using a 0.8% agarose gel, and then purified from the gel using GENECLEAN II (Bio 101) as described by the manufacturer. The purified fragment was radiolabeled using [α-^{32}P]dATP (New England Nuclear) and the Rediprime random primer labeling kit (Amersham) to a specific activity of 2 × 10^9 cpm/μg. Prehybridization, hybridization, and washes were performed according to the Rapid-hyb kit (Amersham) instructions. Autoradiography of the filters was performed at −80 °C with Kodak X-ray film (Eastman Kodak). The filters were also exposed to a photoimaging plate and quantitated using a Fujix Bioimaging Analyzer System BAS 1000 instrument.

For slot-blot hybridization analysis, 1−2 μg of extracted DNA or 500 μL of culture medium was boiled for 10 min in 0.4 M NaOH and 10 mM EDTA and filtered through a S&S Nytran membrane (Schleicher and Schuell) using a Bio-Rad Minifold slot-blot apparatus according to the supplier's instructions. HBV-DNA probe preparation, hybridization conditions, and autoradiography were performed as described above for DNA blot hybridization analysis.

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