Synthesis and Antiproliferative and Antiviral Activity of 2′-Deoxy-2′-fluoroarabinofuranosyl Analogs of the Nucleoside Antibiotics Toyocamycin and Sangivamycin

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The glycosylation of 3,4-dicyano-2-[(ethoxymethylene)amino]pyrrole (**7**) with 2-deoxy-2-fluoro-α-D-*erythro*-pentofuranosyl bromide (**2**) furnished an anomeric mixture of nucleosides (**8a,b**). This mixture was separated, and the individual anomers were treated with methanolic ammonia to effect a concomitant deblocking and ring closure. This furnished both anomers of 2′-deoxy-2′-fluoro-ara-toyocamycin (**9a,b**). The cyano moiety of **9b** was converted to the carboxamide moiety to furnish 2′-deoxy-2′-fluoro-ara-sangivamycin (**10**) and to the thiocarboxamide moiety to furnish 2′-deoxy-2′-fluoro-ara-thiosangivamycin (**11**). The target compounds **10** and **11** showed similar antiproliferative activity against L1210 cells *in vitro*, with IC_{50}'s of 3 and 5 μM. Antiviral evaluation revealed a somewhat different pattern of activity. All analogs, both α and β anomers, were active against human cytomegalovirus (HCMV), albeit the β anomers were most active. The β anomers also were active against herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus (HIV). Compound **10** was most active in the series, ca. 10-fold more potent than **11**; IC_{50}'s for **10** ranged from 4 to 25 nM for HCMV, HIV, and varicella zoster virus (VZV) and from 30 to 500 nM for HSV-1. Even though compound **10** was cytotoxic, which will probably preclude its use as an antiviral drug (IC_{50}'s = 0.2–5.5 μM), the difference between cytotoxicity and activity against HCMV, HIV, and VZV was sufficient to indicate specific activity against a viral target.

Recent work in our laboratory on pyrrolo[2,3-*d*]-pyrimidine nucleosides has concentrated on the synthesis of sugar-modified analogs of the naturally occurring nucleoside antibiotics toyocamycin and sangivamycin as potential antiviral drugs.[1-6] We,[1,7] as well as others,[8-12] have previously synthesized the 2′-deoxy and the arabinofuranosyl analogs of sangivamycin and reported the inhibitory properties of these analogs on the replication of human cytomegalovirus *in vitro*[1] and *in vivo*.[12] This work prompted us to synthesize other closely related 2′-deoxy-2′-substituted arabinofuranosyl analogs of toyocamycin and sangivamycin for evaluation of their antiviral activities. We have successfully synthesized[4] the 2′-deoxy-2′-chloro-, 2′-deoxy-2′-bromo-, and 2′-deoxy-2′-iodoarabinofuranosyl analogs of sangivamycin and toyocamycin, using routes previously described[13-15] for the synthesis of similar arabinofuranosyl derivatives of adenosine and neoplanocin A. The antiviral evaluations of these compounds showed that the 2′-deoxy and arabinofuranosyl derivatives of sangivamycin were the most active against human cytomegalovirus. This activity generated considerable interest for us to synthesize and evaluate for antiviral activity the 2′-deoxy-2′-fluoro derivative of sangivamycin. However, the synthetic routes described above were not amenable[16,17,29] to the introduction of a fluoro substituent into the 2′ position of the sugar moiety. This prompted us to develop an alternative method for the synthesis of the 2′-deoxy-2′-fluoroarabinofuranosyl analog of sangivamycin. We report herein the synthesis of this sangivamycin derivative and several analogs along with their antiproliferative and antiviral activities.

Results and Discussion

Chemistry. The major impediment in the application of the methodology which we had previously used for the introduction of substituents in the 2′ position of preformed nucleoside derivatives was that the 5′ and 3′ positions, which required protection, were most conveniently blocked by means of a bifunctional silylating agent.[18] After blocking the 5′ and 3′ position of the free nucleoside, the 2′-hydroxyl group could be expediently sulfonylated to afford a leaving group which would be displaced with the desired nucleophile. In the case where the silyl ether bonds of the protecting group are fairly stable to the nucleophilic reagent, the reaction proceeds smoothly. On the other hand, when the nucleophile employed is fluoride ion, side reactions seriously impede the course of the reaction.

Thus, we chose to synthesize the fluoroarabinosides by the coupling of a fluorine-containing pentose moiety with a suitable heterocycle followed by an elaboration of this intermediate into the target compounds. The requisite sugar had been reported[19] as an intermediate in the synthesis of 9-(2-deoxy-2-fluoroarabinofuranosyl)-adenine and 9-(2-deoxy-2-fluoroarabinofuranosyl)guanine.[20] However, this reported synthesis[21] was long and

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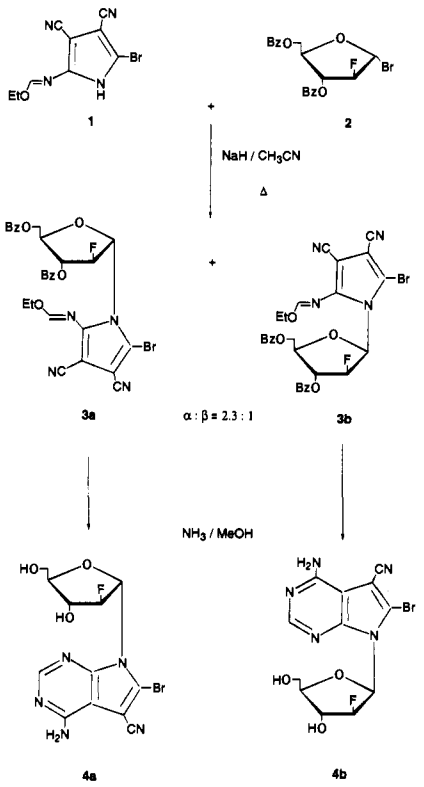
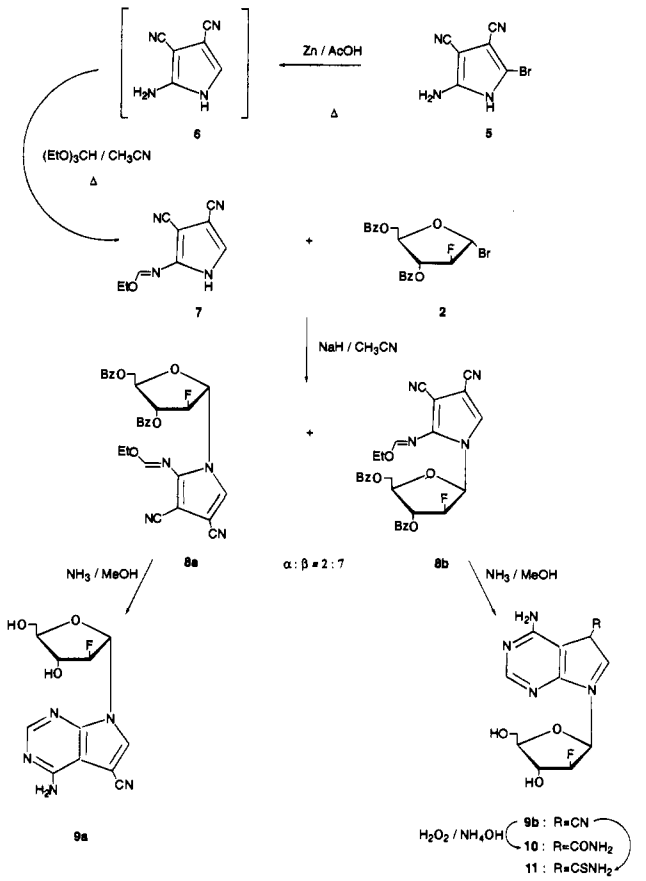
Scheme 1



Scheme 2



tedious. Recently, a very facile and straightforward approach, to a closely related sugar (**2**), has been reported,[22] and we chose this route for the synthesis of the pentose moiety. With the bromo sugar **2** in hand, we first tried to glycosylate the aglycone of toyocamycin using the sodium salt method. This method had been used previously to prepare acyclic analogs of toyocamycin and sangivamycin.[3] Unfortunately, this aglycone was very insoluble in acetonitrile, and the sodium salt could only be prepared in dimethylformamide. The glycosylation of this sodium salt with **2** in dimethylformamide did not proceed smoothly, and this method was abandoned. The next approach we investigated involved a glycosylation of the sodium salt of the pyrrole **1**, which had been previously reported[10,11] to be a useful intermediate in the synthesis of sugar-modified analogs of toyocamycin and sangivamycin (Scheme 1). Treatment of the sodium salt of **1** with **2** in acetonitrile, at reflux temperature, afforded a 2.3:1 anomeric mixture of nucleosides (**3a**,**b**) which was readily separated by silica gel chromatography. Treatment of each nucleoside with methanolic ammonia effected a simultaneous deblocking and ring closure to yield two compounds whose ^{1}H NMR spectra were very different yet whose UV spectra and elemental analyses were identical. From the analytical data, their structures were assigned as the α and β anomers of the 6-bromo derivatives **4a**,**b**.

The anomeric configuration could not be unequivocally assigned from the coupling constants of the 1' proton. Thus, the anomeric configuration of **4a**,**b** was assigned using the method reported for assigning the anomers of 9-(2-deoxy-2-fluoroarabinofuranosyl)adenine,[19] i.e., by forming the 5'-tosylate of each of the anomers and then observing the UV spectrum of a refluxing solution of each of the 5'-tosylates in *p*-dioxane. Each of the anomers (**4a**,**b**) was treated with 2 equiv of *p*-toluenesulfonyl chloride in pyridine, and the reaction was followed by TLC. Only one more mobile product was formed in either case, and after 5 h, the new products were isolated. The UV spectrum of each of these products exhibited the same absorption maximum (285 nm) as the starting materials. These compounds were dissolved separately in *p*-dioxane, and the individual solutions were heated at reflux. Whereas the UV spectrum of the tosylate solution derived from nucleoside **4a** remained unchanged after 24 h at reflux, the absorption maximum displayed in the UV spectrum of the solution with the tosylate derived from nucleoside **4b** shifted from 285 to 294 nm, indicative of the formation of a cyclonucleoside. TLC analyses also showed that no starting material was left, and the product did not migrate on the TLC plate. Thus, evidently the minor nucleoside **4b** could be assigned the β configuration, and the major nucleoside **4a** possessed the α configuration.

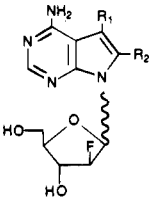
The poor yield of the desired β anomer prompted us to investigate an alternative route for the preparation of the desired β compound. We elected to remove the bromine substituent from the pyrrole[23] **1** on the premise that removal of the bromine moiety residing at the 2 position would decrease the acidity of the N1 proton, as well as reduce the steric hindrance toward alkylation at N1. This should lead to an increase in the rate and stereospecificity of the glycosylation reaction. We treated the pyrrole **5** with zinc dust in acetic acid at reflux to yield the intermediate desbromopyrrole **6** (Scheme 2). Without isolation, compound **6** was treated with triethyl orthoformate in acetonitrile, and the product was isolated by silica gel chromatography to yield the desired pyrrole aglycone **7** in a modest 22% yield. In contrast to the reaction of the sodium salt of **1** with the bromo

sugar **2**, a reaction of the sodium salt of **7** with **2** was rapid even at room temperature. This reaction yielded two glycosylated pyrroles (**8a,b**) in a 2:7 ratio. The anomeric mixture was resolved, and each anomer was separately treated with methanolic ammonia to yield the desired 2′-deoxy-2′-fluoro-ara-toyocamycin derivatives (**9a,b**). During the initial deblocking procedure, we noticed the formation of a second slower running product on TLC, which gave us a mixture of products. This slower moving product was assumed to be the 5-methylformimidate, which was formed by the addition of methanol to the cyano moiety.[10] It was subsequently found that by dissolving the crude product in water and treating the solution with a small amount of triethylamine, the methylformimidate could be converted back into the desired product.[24] This eliminated the need for a chromatographic separation of a reaction mixture and afforded a pure product.

The anomeric configurations of these isomers (**9a,b**) were assigned by comparing their ^{1}H NMR spectra with those of the 6-bromo derivatives **4a,b** which had been obtained previously, as well as to the spectra of the previously reported 2′-deoxy-2′-fluoroadenosine analogs. In the case of the 6-bromo derivatives, the coupling constants between H-1′, H-2′ and H-1′, F-2′ were smaller and greater, respectively, in the α anomer **4a** when compared to the corresponding coupling constants of the β isomer **4b**. On the basis of the assumption that this pattern would hold in the case of the anomers **9a,b**, we made a preliminary assignment of the anomeric configuration of **9b** as β and that of **9a** as α. Further support for this assignment was made by the observation that in the case of the 6-bromo derivatives **4a,b**, the 4′ and 2′ protons of the α anomer each appeared more than 0.6 ppm downfield when compared with the positions of the corresponding protons of the β anomer. The 4′ and 2′ protons of the nucleoside **9a**, which had been tentatively assigned the α configuration, also appeared ca. 0.4 ppm downfield when compared to the positions of the corresponding protons of the isomer **9b**. Since coupling between a fluorine substituent in the 2′-arabino configuration and the H-8 proton of the purine base of β purine nucleosides, but not of the α purine nucleosides, has been previously reported,[19,20] the observed splitting of the H-6 resonance in the ^{1}H NMR spectrum of **9b** and not in the spectrum of **9a** provided additional support for the anomeric assignment. The assignment was then chemically confirmed, as described previously for the 6-bromo derivatives by converting the two isomers **9a,b** into their 5′-tosylate derivatives followed by heating the solution of each individual tosylate in *p*-dioxane. As expected, the tosylate derived from nucleoside **9a** was stable for 6 h at reflux. However, the tosylate derived from **9b** reacted rapidly, under the same conditions, to provide a bathochromic shift of 9 nm in the absorption maximum of the UV spectrum of the *p*-dioxane solution.

This established that we had prepared the desired 2′-deoxy-2′-fluoro-ara-toyocamycin (**9b**). We then converted[25] the cyano moiety of **9b** into the carboxamide moiety with hydrogen peroxide in a solution of methanol and ammonium hydroxide. This provided the desired 2′-deoxy-2′-fluoro-ara-sangivamycin (**10**). We then converted the cyano moiety of **9b** into a thioamide group with dihydrogen sulfide to obtain 2′-deoxy-2′-fluoro-ara-thiosangivamycin (**11**).

Table 1. Antiproliferative Activity of α- and β-2′-Deoxy-2′-fluoroarabinosides of 5-Substituted and 5,6-Disubstituted 4-Aminopyrrolo[2,3-*d*]pyrimidines in L1210 Murine Leukemia *in Vitro*



compd	R_1	R_2	configuration[a]	screen GR[b] (% of control)	IC_{50}[c] (μM)
4b	CN	Br	β	21	66
4a	CN	Br	α	45	90
9b	CN	H	β	11	39
9a	CN	H	α	98	
10	$CONH_2$	H	β	9.0	3.2
11	$CSNH_2$	H	β	5.0	5.0

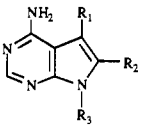
[a] Configuration of the glycosidic bond. [b] Screen GR, growth rate in the presence of the test compound at 100 μM. See the Experimental Section for definition. [c] IC_{50}, concentration required to decrease growth rate to 50% of control.

Antiproliferative Studies. The antitumor potential of this group of compounds was assessed by evaluating their capacity to slow proliferation of L1210 cells *in vitro*. As shown in Table 1, the target compounds **10** and **11** had significant antiproliferative activity. Their IC_{50}'s are comparable to the value of 5 μM obtained previously for arabinosylthiosangivamycin,[26] showing that replacement of a 2′-OH with a 2′-F (**11**) did not affect the potency of this compound. The stereochemistry of the glycosidic bond had the expected influence on antiproliferative potency. The α anomers (**4a** and **9a**) of the 5-CN derivatives were less potent than their respective β anomers (**4b** and **9b**), with **9a** completely lacking antiproliferative activity at 100 μM.

The 5 substituent played an important role in determining the antiproliferative potency of this series of compounds. The 5-CN derivative **9b** had an IC_{50} on the order of 10-fold higher than that of the 5-$CONH_2$ derivative **10** and the 5-$CSNH_2$ derivative **11**. This finding confirmed the correlation noted previously that a 5-$CSNH_2$ substituent conferred greater antiproliferative potency than a 5-CN among 2′-modified pyrrolo-[2,3-*d*]pyrimidine adenosine analogs, specifically, the 2′-NH_2-2′-deoxyarabinosyl and 2′-deoxy derivatives.[26]

Antiviral Studies. The antiviral potential of the compounds was investigated by examining their activity against selected herpes viruses and human immunodeficiency virus (HIV). Data in Table 2 establish that certain compounds have potent antiviral activity. All compounds, both α and β anomers, showed some activity against both human cytomegalovirus (HCMV) and HIV. The activity of the α anomers was surprising and was confirmed in replicate experiments. The activity against HCMV was investigated more extensively using yield reduction assays. In these assays the α anomers were only slightly active, indicating that activity in plaque assays might have involved inhibition of cell to cell spread of the virus and not reflecting inhibition of infectious virus production. The more potent activity of the β anomers against HCMV, particularly in the

Table 2. Antiviral Activity and Cytotoxicity of 2'-Fluoroarabinosylpyrrolo[2,3-*d*]pyrimidine Analogs



				50% or 90% inhibitory concentration (μM)					
				antiviral activity[a]					
				HCMV		HIV-1[c]	HSV-1[b]	cytotoxicity[d]	
compd	R_1	R_2	R_3	plaque	yield	RT	ELISA	visual	growth
4a	CN	Br	α-D-2-F-arabinosyl	3.2	>100	32	>100[e]	55	>150
4b	CN	Br	β-D-2-F-arabinosyl	12	7	12	>100	21	144
9a	CN	H	α-D-2-F-arabinosyl	16	100	>100	46	100	>100
9b	CN	H	β-D-2-F-arabinosyl	1.0	1.1	12	6.5	6.8	8.8
10	$CONH_2$	H	β-D-2-F-arabinosyl	0.025	0.075	0.02	0.47	5.5	1.9
11	$CSNH_2$	H	β-D-2-F-arabinosyl	0.22	1.5	0.07	8.7	3.1	3
zidovudine[f]						0.011 ± 0.0008		>100	
ganciclovir[g]				7.4 ± 6.5	1.6 ± 1.2		3.5 ± 2.1	>100	>100

[a] Assays were performed in duplicate as described in the text. Results from plaque, syncytium, and ELISA are reported as IC_{50}'s, those for yield reduction experiments as IC_{90}'s. [b] All compounds were assayed by ELISA in quadruplicate wells. [c] Assay for HIV-1 by measurement of RT activity in culture supernatant. [d] Visual cytotoxicity was scored on HFF cells at time of HCMV plaque enumeration for numbered compounds. For zidovudine scoring was on CEM cells at time of RT assay. (Results of visual cytotoxicity scoring in CEM cells for numbered compounds gave IC_{50}'s of >100, 33, >100, 33, 0.4, and 2 μM, respectively.) Cell growth inhibition was determined in KB cells in quadruplicate assays as described in the text. All results are presented as IC_{50}'s. [e] > indicates IC_{50} not reached at the noted (highest) concentration tested. [f] Mean ± standard deviation from four experiments. [g] Mean ± standard deviation from 108, 33, and 3 experiments, respectively, in which ganciclovir was used as a positive control.

Table 3. Antiviral Activity of 2'-Deoxy-2'-fluoroarabinosylsangivamycin (**10**)

	50% inhibitory concentration[a] (μM)	
virus and cell line	antiviral	cytotoxicity
Antiviral Activity		
HSV-1	0.03	
HSV-2	0.03	
VZV	0.003	
HIV-1[b]	0.0037 ± 0.0017	
Cytotoxicity		
human foreskin fibroblasts		
stationary cells, neutral red uptake		6.7
stationary cells, MTT assay		0.13
proliferating cells, MTT assay		0.16
CEM-SS cells		
visual inspection		0.4
[^{3}H]dThd uptake[c]		0.193 ± 0.074

[a] All assays performed in duplicate as described in the text. [b] Mean ± standard deviation from quadruplicate assays in the syncytial plaque assay using CEM-SS cells. [c] Mean ± standard deviation from triplicate assays.

yield reduction assay (Table 2), established their capacity to inhibit infectious virus production at micromolar and lower concentrations. Compounds **9b**, **10**, and **11** were the most potent with activity extending to HIV and possibly herpes simplex virus type 1 (HSV-1) (Table 2). The activity of **9b**, however, was not well separated from cytotoxicity (Table 2). Consequently, activity against HIV and HSV-1 may be only a manifestation of cytotoxicity for this compound. Additional experiments showed that it also inhibited [^{3}H]dThd incorporation into DNA (IC_{50} = 3.3 μM).

In contrast, the difference between cytotoxicity and activity of compounds **10** and **11** (particularly **10**) against both HCMV and HIV was sufficient to indicate specific antiviral activity in the low nanomolar range. Activity against HSV-1 was less apparent in an ELISA assay (Table 2) and could reflect cytotoxicity. More extensive examination of compound **10** revealed that it also was highly active against HSV-2 and varicella zoster virus (VZV) in a plaque assay (Table 3). Interestingly, it was also more active against HSV-1 in the plaque assay (Table 3) compared to weaker activity in the ELISA assay (Table 2). Because plaque assays measure more parameters needed for viral replication than an ELISA assay, the plaque results may reflect more closely the effect of the compound on replicating virus. Likewise, compound **10** also was more active against HIV in a syncytial plaque assay (Table 3) than in an assay which used reverse transcriptase (RT) activity to detect the virus (Table 2). In an additional series of experiments, compound **10** was much less active in cells persistently infected with HIV. In such cells, 100 nM **10** inhibited virus production by 23–50% in U1 cells and 0–32% in $H9III_B$ cells based on inhibition of p24 antigen production, RT activity, and infectious virus production measured in the syncytial plaque assay. We have reported previously that zidovudine (AZT) gave a similar pattern of potent inhibition in acutely infected cells and weaker activity in persistently infected cells (20–40% inhibition of the three parameters at 1 μM).[27] These data show that like AZT compound **10** does not inhibit processes which are needed only in persistently infected cells which already contain the integrated viral genome. Because RT activ-

ity is required for genome integration and not for virus production in persistently infected cells, the data suggest that compound **10** or a metabolite inhibits RT.

There may be another factor affecting the antiviral activity and cytotoxicity of compound **11**. In other studies we have shown that certain 7-substituted 4-aminopyrrolo[2,3-*d*]pyrimidine-5-thiocarboxamides, such as **11**, are converted to their corresponding 5-carbonitrile derivatives *in vitro*.[28] Although we did not examine compound **11** for this phenomenon, we expect it would undergo such a conversion in cell culture medium. Hence, the biological data for compound **11** may be the result of a mixture of 5-thioamide (**11**) and 5-carbonitrile (**9b**) derivatives. Because the nitrile analog **9b** is less active, the activity of **11** must be greater than we observed if conversion from the thioamide to the nitrile occurred.

Compounds **10** and **11** both are moderately potent cytotoxic agents in L1210 murine leukemic cells (Table 1), CEM human leukemic cells, HFF human foreskin fibroblasts, and KB human nasopharyngeal carcinoma cells (Tables 2 and 3). In L1210 cells, compound **10** is 1000-fold less potent than the parent ribosyl nucleoside antibiotic sangivamycin,[3] and compound **11** is 100-fold less potent than its ribosyl counterpart, thiosangivamycin.[26] These compounds appear to be more cytotoxic in CEM and KB cells suggesting that they might be of interest for cancer chemotherapy if they retained sufficient efficacy *in vivo* and also caused less host toxicity than the parent compounds.

The antiviral activity of most compounds was separated from cytotoxicity by ca. 10–100-fold. Compounds which had the most potent antiviral activity also were the most cytotoxic, but some of the less active and cytotoxic compounds such as **4b** and **9b** had the poorest differential between the two parameters (Table 2). In contrast, the most active and cytotoxic compound (**10**) had the best difference between activity against HCMV and HIV and cytotoxicity measured in our initial tests (Table 2). To better understand the cytotoxicity of compound **10**, other cytotoxicity tests were performed. Data in Table 3 show that the effect on human foreskin fibroblasts and KB cells measured by dye uptake was similar to visual effects on HFF cells (Table 2) and similar to effects on proliferating L1210 cells as well (Table 1). In contrast, measurement of more biochemical parameters such as mitochondrial dehydrogenase (MTT assay) and cell DNA synthesis ([^{3}H]dThd uptake) revealed more potent effects (IC_{50}'s = 0.13–0.19 μM, Table 3). To better understand the cytotoxicity of this compound, its effect on growing KB cells was determined in a traditional cell growth experiment. Data in Figure 1 show that little to no growth inhibition occurred up to 0.1 μM but that 1.0 μM blocked cell growth completely. Higher concentrations were clearly cytotoxic (Figure 1). Thus by all measures, cytotoxicity was not observed at concentrations which gave activity against HCMV, VZV, and HIV leading us to conclude that compound **10** could act against a specific viral function. Further structure–activity relationship studies among analogs of compound **10** are warranted.

Experimental Section

General Methods. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. The silica gel used for chromatography was silica gel 60 230–400 mesh (E. Merck, Darmstadt, West Germany). Thin layer chromatography (TLC) was performed on prescored SilicAR 7GF plates (Analtech, Newark, DE). Compounds were visualized by illuminating under UV light (254 nm) and spraying with 20% methanolic sulfuric acid followed by charring via a Bunsen burner. The following solvent designations are used throughout the manuscript: solvent system A, ethyl acetate/cyclohexane (1:4, v:v); solvent system B, ethyl acetate/cyclohexane (1:3, v:v); solvent system C, chloroform/methanol (9:1, v:v); solvent system D, chloform/methanol (4:1, v:v); solvent system E, chloroform/methanol (16:1, v:v); solvent system F, ethyl acetate/cyclohexane (1:2, v:v); solvent system G, ethyl acetate/methanol (9:1, v:v). All evaporations were carried out using a rotary evaporatory connected to a water aspirator pump. The water bath temperature was maintained between 40 and 50 °C. Determination of UV spectra was performed on a Hewlett-Packard 8450-A UV/vis spectrophotometer. IR spectra were obtained on a Nicolet 5 DXB Fourier transform spectrophotometer. ^{1}H NMR spectra were obtained using an IBM WP 270-SY spectrometer operating in the FT mode at 270 MHz. Where necessary, deuterium exchange and homonuclear decoupling experiments were used to obtain proton shift assignments of the ribose moiety.

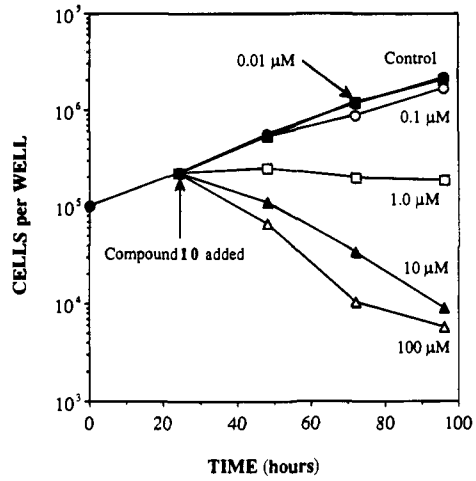


Figure 1. Effect of compound 10 on the growth of KB cells. Cells were planted as described in the text at 1 × 10^5 cells/well in 6-well cluster plates. Following one population doubling, medium was removed and replaced with medium containing compound **10** in the noted concentrations in duplicate. Cells were harvested at the times indicated and enumerated by a Coulter counter.

4-Amino-6-bromo-5-cyano-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (4b) and 4-Amino-6-bromo-5-cyano-1-(2-deoxy-2-fluoro-α-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (4a). To a solution of 2-bromo-3,4-dicyano-5-[(ethoxymethylene)amino]pyrrole[23] (1; 0.267 g, 1 mmol) in acetonitrile (25 mL) was added sodium hydride (0.040 g, 1 mmol, 60% oil dispersion). After the evolution of hydrogen ceased (ca. 15 min), a solution of 1-bromo-2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranose[22] (**2**; 0.464 g, 1 mmol) in acetonitrile (10 mL) was added, and the temperature was maintained at 80 °C for 4 h. After this time, the mixture was partitioned between ethyl acetate (50 mL) and water (50 mL), and the organic layer was washed with brine, dried over sodium sulfate, and evaporated. The residue was chromatographed on a silica gel column (25 mm × 300 mm) using solvent system A to afford two fractions as determined by TLC (solvent B). The fraction containing the faster moving product (R_f = 0.35, solvent system B) was evaporated to yield 0.23 g of a white foam: ^{1}H NMR ($CDCl_3$) δ 8.34 (s, 1H, CH=N), 8.10–7.30 (m, 10H, benzoyl), 6.44 (dd, 1H, H-1, $J_{1',2'}$ = 5.4 Hz, $J_{1,F}$ = 17.0 Hz), 6.10 (ddd, 1H, H-2', $J_{2',F}$ = 53.9 Hz), 5.86 (ddd, 1H, H-3', $J_{3',F}$ = 21.2 Hz) 4.80–4.60 (m, 3H, H-4', H-5'a,b), 4.20 (m, 2H, CH_2), 1.20 (t, 3H, CH_3).

This material was dissolved in methanolic ammonia (saturated at 0 °C), and the solution was kept in a sealed bottle at

room temperature for 16 h. The solution was then evaporated, the residue was triturated with acetonitrile, and the solid which had formed was collected by filtration to yield 0.073 g of the α anomer **4a**: mp 206–207 °C; ^{1}H NMR (DMSO-d_6) δ 8.25 (s, 1H, H-2), 7.07 (bs, 2H, NH_2), 6.26 (dd, 1H, H-1', $J_{1',2'}$ = 5.4 Hz, $J_{1',F}$ = 16.7 Hz), 6.08 (bs, 1H, 3'-OH), 6.05 (ddd, 1H, H-2', $J_{2',F}$ = 55.9 Hz), 4.93 (bs, 1H, 5'-OH), 4.47 (m, 1H, H-3'), 4.39 (m, 1H, H-4'), 3.56 (m, 2H, H-5'a,b); UV λ_{max} (nm) methanol, 285; IR (KBr) 2224 cm^{-1} (cyano); TLC R_f = 0.31, solvent system C. Anal. ($C_{12}H_{11}N_5O_3FBr$) C,H,N.

The fraction containing the slower running product (R_f = 0.20, solvent system B) was also evaporated to yield a crisp foam (0.1 g): ^{1}H NMR ($CDCl_3$) δ 8.36 (s, 1H, CH=N), 8.10–7.30 (m, 10H, benzoyl), 6.47 (dd, 1H, H-1', $J_{1',2'}$ = 4.9 Hz, $J_{1',F}$ = 17.5 Hz), 5.88 (ddd, 1H, H-3', $J_{3',F}$ = 19.7 Hz), 5.38 (ddd, 1H, H-2', $J_{2',F}$ = 52.1 Hz), 4.76 (ddd, 2H, H-5'ab), 4.41 (m, 3H, H-4', CH_2), 1.41 (t, 3H, CH_3).

This material was dissolved in methanolic ammonia (saturated at 0 °C), and the solution was kept in a sealed bottle at room temperature for 16 h. The solution was then evaporated, the residue was triturated with acetonitrile, and the solid which had formed was collected by filtration to yield 0.032 g of the β anomer **4b**: mp 220–223 °C; ^{1}H NMR (DMSO-d_6) δ 8.21 (s, 1H, H-2), 7.02 (bs, 2H, NH_2), 6.69 (dd, 1H, H-1', $J_{1',2'}$ = 6.2 Hz, $J_{1',F}$ = 11.2 Hz), 5.93 (d, 1H, 3'-OH), 5.29 (ddd, 1H, H-2', $J_{2',F}$ = 55.4 Hz), 5.00 (t, 1H, 5'-OH), 4.66 (ddd, 1H, H-3', $J_{3',F}$ = 23.3 Hz), 3.75 (m, 3H, H-4', H-5'a,b); UV λ_{max} (nm) methanol, 285; IR (KBr) 2228 cm^{-1} (cyano); TLC R_f = 0.31, solvent system C. Anal. ($C_{12}H_{11}N_5O_3FBr$) C,H,N.

Anomeric Confirmation of 4a,b. A solution of nucleoside **4a** (12 mg) in pyridine (0.4 mL) was treated with *p*-toluenesulfonyl chloride (20 mg). After ca. 5 h, water (3 mL) was added, and the precipitate was collected by filtration to afford a white solid (14 mg; R_f = 0.42, solvent system C; UV λ_{max} (nm) methanol, 285). A solution of this material (5 mg) in *p*-dioxane (2 mL) was heated at reflux for 18 h. TLC analysis of the solution (solvent system C), after this time, showed the presence of only starting material, and the UV spectrum of the solution was unchanged.

Likewise, a solution of nucleoside **4b** (12 mg) in pyridine (0.4 mL) was treated with *p*-toluenesulfonyl chloride (20 mg). After 5 h, water was added (5 mL), and the cloudy emulsion was extracted with ethyl acetate (3 × 5 mL). The combined extracts were washed with water (10 mL) and brine (10 mL), dried over sodium sulfate, and evaporated to yield a foam (11 mg; R_f = 0.47, solvent system C; UV λ_{max} (nm) methanol, 285). A solution of this foam (5 mg) was heated at reflux in *p*-dioxane for 1 h. TLC analysis (solvent system C) of the solution after this time revealed an absence of starting material and the presence of a new product at R_f = 0.0. The UV absorbtion maximum of the solution was observed at 294 nm.

3,4-Dicyano-2-[(ethoxymethylene)amino]pyrrole (7). A suspension of 2-amino-5-bromo-3,4-dicyanopyrrole[23] (**5**; 20.3 g, 96.2 mmol) and zinc dust (50 g, 765 mmol) in acetic acid (250 mL) was heated at reflux under argon for 1 h. The suspension was filtered, and the zinc bed was washed with acetic acid (2 × 50 mL). The combined filtrates were evaporated *in vacuo* to afford a thick syrup, and this syrup was coevaporated three times with a mixture of ethanol and toluene (1:1, 50 mL) to afford a dark foam. This foam was dissolved in hot ethyl acetate (50 mL), and the resulting warm slurry was filtered. The filtrate was evaporated, and the residue was dissolved in acetonitrile (250 mL). The solution was treated with triethyl orthoformate (20 mL, 120 mmol), and the mixture was heated at reflux for 0.5 h. After this time, the solution was evaporated, and the residue was coevaporated with toluene (2 × 50 mL). The residue was then triturated with warm ethyl acetate (250 mL), the suspension was filtered, and to the filtrtate was added a saturated aqueous solution of sodium bicarbonate (100 mL). The resulting emulsion was vacuum-filtered through a fritted glass funnel, and brine was added to the biphasic mixture. The organic layer was then separated and washed with brine (200 mL), dried over sodium sulfate, filtered, and evaporated. The residue was treated with solvent system C (100 mL), and the mixture was momentarily heated at reflux on a steam bath. The warm suspension was applied to a silica gel column (21 cm × 10 cm) which had been slurry packed in chloroform. The column was eluted with solvent system E, and the fraction containing the product (R_f = 0.45, solvent system E) was collected and evaporated. The residue was triturated with cyclohexane (50 mL), and the product was collected to afford 4.02 g (22%) of a light yellow solid: mp 120–121 °C; ^{1}H NMR (DMSO-d_6) δ 12.58 (bs, 1H, NH), 8.39 (s, 1H, CH=), 7.66 (s, 1H, H-5), 4.28 (q, 2H, CH_2), 1.29 (t, 3H, CH_3); UV λ_{max} (nm) (log ε) methanol, 274 (4.05), 213 (4.26); pH 1, 271 (4.05), 214 (4.22); pH 11, 281 (4.08), 247 (4.01), 230 (4.05); IR (KBr) 2213, 2226 cm^{-1} (cyano); TLC R_f = 0.45, solvent system E. Anal. ($C_9H_8N_4O$) C,H,N.

4-Amino-5-cyano-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (9b) and 4-Amino-5-cyano-1-(2-deoxy-2-fluoro-α-D-arabinofuranosyl)pyrrolo-[2,3-*d*]pyrimidine (9a). Sodium hydride (1.05 g, 50% oil dispersion, 22 mmol) was added to a solution of 3,4-dicyano-2-[(ethoxymethylene)amino]pyrrole (**7**; 3.76 g, 20 mmol) in acetonitrile (250 mL). The mixture was stirred for 0.5 h with the exclusion of moisture, and after this time, a solution of 1-bromo-2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranose (8.4 g, 20 mmol) in acetonitrile (125 mL) was added. The mixture was stirred at room temperature for 16 h, and the resulting suspension was evaporated to yield a thick paste. This paste was partitioned between ethyl acetate (500 mL) and water (250 mL). The organic layer was washed with brine (250 mL), dried over sodium sulfate, filtered, and evaporated to yield a foam. This foam was dissolved in solvent system F (100 mL), and the solution was applied to a silica gel column (10 cm × 35 cm) which had been slurry packed in solvent system B. The column was eluted first with solvent system B (1 L) and then with solvent system F. The fraction containing the faster running product (R_f = 0.32, solvent system B) was evaporated to afford 2.34 g (22%) of **9a** as a foam: ^{1}H NMR ($CDCl_3$) δ 8.47 (s, 1H, CH=N), 8.2–7.4 (3m, 10H, benzoyl), 7.29 (s, 1H, H-5), 6.27 (d, 1H, H-1', $J_{1',2'}$ < 1 Hz, $J_{1',F}$ = 14.0 Hz), 5.67 (dd, 1H, H-3', $J_{3',F}$ = 15.7 Hz), 5.35 (dd, 1H, H-2', $J_{2',F}$ = 48.1 Hz), 4.95 (m, 1H, H-4'), 4.67 (m, 2H, H-5'ab), 4.34 (q, 2H, CH_2), 1.38 (t, 3H, CH_3); UV λ_{max} (nm) (log ε) methanol, 282 (3.93), 274 (3.91), 226 (4.55); pH 1, 240 (4.45), 206 (4.44); pH 11, 284 (4.11), 234 (4.53); IR (KBr) 2228 cm^{-1} (cyano); TLC R_f = 0.32 solvent system B. Anal. ($C_{28}H_{23}N_4O_6F\cdot H_2O$) C,H,N.

This material (1.4 g, 2.6 mmol) was dissolved in methanolic ammonia (50 mL, saturated at 0 °C), and the solution was kept at room temperature in a pressure bottle for 16 h. The solution was then evaporated, and the residue was dissolved in water (50 mL). The aqueous solution was treated with triethylamine (5 mL), and the mixture was allowed to stand at room temperature for 4 h. The solid which had precipitated from the solution was collected and washed with ether (2 × 20 mL) to yield 0.56 g of the α anomer **9a** as a solid (74%): mp 228.5–229 °C; ^{1}H NMR (DMSO-d_6) δ 8.36, 8.24 (2s, 2H, H-2, H-6), 6.95 (bs, 2H, NH_2), 6.36 (dd, 1H, H-1', $J_{1',2}$ = 3.1 Hz, $J_{1',F}$ = 15.4 Hz), 5.98 (d, 1H, 3'-OH), 5.54 (ddd, 1H, H-2', $J_{2',F}$ = 52.1 Hz), 5.02 (t, 1H, 5'-OH), 4.34 (m, 1H, H-3'), 4.29 (m, 1H, H-4'), 3.53 (m, 2H, H-5'ab); UV λ_{max} (nm) (log ε) methanol, 279 (4.15), 229 (4.01), 210 (4.21); pH 1, 273 (4.07), 234 (4.21), 205 (4.09); pH 11, 278 (4.14), 231 (3.98); IR (KBr) 2228 cm^{-1} (cyano); TLC R_f = 0.64 solvent system C. Anal. ($C_{12}H_{12}N_5O_3F$) C,H,N.

Further elution of the column afforded a fraction containing the slower running product (R_f = 0.16, solvent system B) which upon evaporation yielded 7.34 g (69%) of **8b** as a solid: ^{1}H NMR ($CDCl_3$) δ 8.45 (s, 1H, CH=N), 8.1–7.4 (3m, 10H, benzoyl), 7.34 (d, 1H, H-5, $J_{5',F}$ = 2.1 Hz), 6.23 (dd, 1H, H-1', $J_{1',2}$ = 2.9 Hz, $J_{1',F}$ = 17.4 Hz), 5.63 (dd, 1H, H-3', $J_{3',F}$ = 16.0 Hz), 5.17 (dd, 1H, H-2', $J_{2',F}$ = 50.1 Hz), 4.73 (m, 2H, H-5'ab), 4.53 (m, 1H, H-4'), 4.33 (q, 2H, CH_2), 1.37 (t, 3H, CH_3); UV λ_{max} (nm) (log ε) methanol, 282 (3.93), 276 (3.93), 226 (4.57); pH 1, 241 (4.47), 208 (4.45); pH 11, 284 (4.13), 235 (4.54); IR (KBr) 2229 cm^{-1} (cyano); TLC R_f = 0.16, solvent system B. Anal. ($C_{28}H_{23}N_4O_6F$) C,H,N.

This material (7.2 g, 13.6 mmol) was suspended in methanol (50 mL), and methanolic ammonia (50 mL, saturated at 0 °C) was added. The solution was kept at room temperature in a pressure bottle for 16 h and then evaporated. The residue was dissolved in water (100 mL), the aqueous solution was treated

with triethylamine (10 mL), and the mixture was allowed to stand at room temperature for 1 h. After this time, the cloudy mixture was evaporated, the resulting syrup was triturated with acetonitrile (25 mL), and the solid which had precipitated was collected. The process of evaporation and trituration was repeated twice more, and the combined product was recrystallized from water/ethanol (4:1, v:v, 50 mL) to yield 1.72 g (43%) of the β anomer **9b**, mp 188–190 °C. An analytical sample was obtained by recrystallizing the material (1.05 g) from methanol (15 mL) to yield 0.91 g of material: mp 225.5–227.5 °C; ^{1}H NMR (DMSO-d_6) δ 8.33 (d, 1H, H-6, $J_{6,F}$ = 1.5 Hz), 8.22 (s, 1H, H-2), 6.95 (bs, 2H, NH_2), 6.56 (dd, 1H, H-1′, $J_{1',2'}$ = 4.5 Hz, $J_{1',F}$ = 13.3 Hz), 5.94 (d, 1H, 3′-OH), 5.17 (ddd, 1H, H-2′, $J_{2',F}$ = 52.7 Hz), 5.11 (t, 1H, 5′-OH), 4.36 (ddd, 1H, H-3′, $J_{3',F}$ = 18.7 Hz), 3.84 (m, 1H, H-4′), 3.66 (m, 2H, H-5′ab); UV λ_{max} (nm) (log ϵ) methanol, 278 (4.23), 229 (4.08), 208 (4.32); pH 1, 272 (4.12), 232 (4.24), 205 (4.11); pH 11, 277 (4.23), 231 (4.05); IR (KBr) 2223 cm^{-1} (cyano); TLC R_f = 0.57, solvent system C. Anal. ($C_{12}H_{12}N_5O_3F$) C,H,N.

Anomeric Configuration of 9a,b. To a solution of nucleoside **9a** (50 mg) in pyridine (1 mL) was added *p*-tolunesulfonyl chloride (90 mg). The solution was kept at room temperature for 4 h, and the mixture was then partitioned between water (20 mL) and ethyl acetate (20 mL). The organic layer was washed with water (3 × 20 mL), saturated aqueous sodium bicarbonate solution (20 mL), and brine (20 mL) and then dried over sodium sulfate, filtered, and evaporated to yield 59 mg of a foam (R_f = 0.72, solvent system G; UV λ_{max} (nm) methanol 278). A solution of this foam (20 mg) in *p*-dioxane (2.5 mL) was heated at reflux for 6 h. TLC (solvent system G) analysis of the solution after this time revealed only starting material, and the UV spectrum was unchanged.

To a solution of nucleoside **9b** (50 mg) in pyridine (1 mL) was added *p*-toluenesulfonyl chloride (90 mg). The solution was kept at room temperature for 4 h, and the mixture was then partitioned between water (20 mL) and ethyl acetate (20 mL). The organic layer was washed with water (3 × 20 mL), saturated aqueous sodium bicarbonate solution (20 mL), and brine (20 mL) and then dried over sodium sulfate, filtered, and evaporated to yield 78 mg of a foam (R_f = 0.61, solvent system G; UV λ_{max} (nm) methanol, 278). A solution of this foam (20 mg) in *p*-dioxane (2.5 mL) was heated at reflux for 6 h. TLC (solvent system G) analysis of the solution after this time revealed no starting material and one product with R_f = 0.0. The UV spectrum (methanol) showed a new absorbtion maximum at 287 nm.

4-Amino-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-5-carboxamide (10). To a stirred suspension of 4-amino-5-cyano-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**9b**; 0.292 g, 1 mmol) in water/methanol (2:1, v:v, 15 mL) was added concentrated ammonium hydroxide (0.15 mL) followed by three portions of hydrogen peroxide (30%, 0.2 mL) at 0.5 h intervals. The solution was stirred for an additional 1 h, and the solution was then evaporated using an ambient temperature water bath. The resulting syrup was coevaporated with ethanol (2 × 10 mL), and the residue was triturated with ethanol (10 mL). The resulting solid was crystallized from water (10 mL) with the aid of decolorizing carbon to afford 0.13 g (42%) of product **10**: mp 271 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.90 (bs, 2H, $CONH_2$), 8.10 (s, 1H, H-2), 8.08 (d, 1H, H-6, $J_{6,F}$ = 1.9 Hz), 7.38 (bs, 2H, NH_2), 6.58 (d, 1H, H-1′, $J_{1',2'}$ = 4.1 Hz, $J_{1',F}$ = 17.2 Hz), 5.95 (d, 1H, 3′-OH), 4.68 (ddd, 1H, H-2′, $J_{2',F}$ = 56.3 Hz), 4.98 (t, 1H, 5′-OH), 4.34 (ddd, 1H, H-3′, $J_{3',F}$ = 17.9 Hz), 3.84 (m, 1H, H-4′), 3.67 (m, 2H, H-5′ab); UV λ_{max} (nm) (log ϵ) methanol, 280 (4.17), 230 (4.01), 206 (4.32); pH 1, 275 (4.12), 226 (4.19), 204 (4.18); pH 11, 279 (4.17), 232 (4.00); IR (KBr) no cyano absorbtion observed; TLC R_f = 0.30, solvent system G. Anal. ($C_{12}H_{14}N_5O_4F$) C,H,N.

4-Amino-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine-5-(thiocarboxamide) (11). Hydrogen sulfide gas was bubbled through a solution of 4-amino-5-cyano-7-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**9b**; 0.292 g, 1 mmol) in pyridine (15 mL) containing triethylamine (0.1 mL) over a period of 2 h. The solution was evaporated, and the residue was triturated with ethanol (10 mL). The solid which formed was collected and crystallized from water/ethanol (3:1, v:v, 40 mL) to yield 0.16 g (49%) of the product 11 as a light yellow solid: mp 227.5–228.5 °C; ^{1}H NMR (DMSO-d_6) δ 9.65, 9.50 (2bs, 2H, $CSNH_2$), 8.13 (s, 1H, H-2), 7.97 (bs, 2H, NH_2), 7.85 (d, 1H, H-6, $J_{6',F}$ = 1.4 Hz), 6.59 (dd, 1H, H-1′, $J_{1',2'}$ = 4.3 Hz, $J_{1',F}$ = 15.4 Hz), 6.49 (d, 1H, H-3′, $J_{2',3'}$ = 5.8 Hz), 5.93 (d, 1H, 5′-OH), 5.14 (ddd, 1H, H-2′, $J_{2',F'}$ = 55.1 Hz), 5.03 (t, 1H, 5′-OH), 4.38 (ddd, 1H, H-3′, $J_{3',F'}$ = 18.2 Hz), 3.84 (m, 1H, H-4′), 3.65 (m, 2H, H-5′ab); UV λ_{max} (nm) (log ϵ) MeOH, 285 (4.11), 246 (4.06), 207 (4.44); pH 1, 291 (4.14), 241 (4.26), 205 (4.41); pH 11, 281 (4.18), 244 (4.11); IR (KBr) no cyano absorbtion; TLC R_f = 0.37, solvent C. Anal. ($C_{12}H_{12}N_5O_2S$) C,H,N.

***In Vitro* Antiproliferative Studies.** The *in vitro* cytotoxicity against L1210 was evaluated as described previously.[30] L1210 cells were grown in static suspension culture using Fischer's medium for leukemic cells of mice with 10% heat-inactivated (56 °C, 30 min) horse serum. The growth rate is defined as the number of cell doublings in the treated cultures as a percent of the number in the control culture and was calculated from determinations of cell number at 0, 48, and 96 h in the presence of various concentrations of the test compound. Control population-doubling time was 12 h. When the growth rate in the treated cultures decreased during the experiment, the rate used was that between 48 and 96 h. The IC_{50} is defined as the concentration required to decrease the growth rate to 50% of the control.

***In Vitro* Antiviral Studies. Cell Culture Procedures.** The routine growth and passage of KB and HFF cells was performed in monolayer cultures using minimal essential medium (MEM) with either Hanks salts [MEM(H)] or Earle salts [MEM(E)] supplemented with 10% calf serum or 10% fetal bovine serum (HFF cells). The sodium bicarbonate concentration was varied to meet the buffering capacity required. 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 solution. Similar suspension culture conditions were employed for CEM cells.

Virological Procedures. The Towne strain, plaque-purified isolate P_o of HCMV was kindly provided by Dr. Mark Stinski, University of Iowa, IA. The KOS strain of HSV-1 was used in most experiments and was provided by Dr. Sandra K. Weller, University of Connecticut, CT. Stock HCMV was prepared by infecting HFF cells at a multiplicity of infection (MOI) of <0.01 plaque-forming units (PFU)/cell as detailed previously.[1] High-titer HSV-1 stocks were prepared by infecting KB cells at an MOI of <0.1 also as detailed previously.[1] Virus titers were determined using monolayer cultures of HFF cells for HCMV and monolayer cultures of BSC-1 cells for HSV-1 as described earlier.[31] U1 and H9III$_B$ cells persistently infected with HIV-1 and strain III$_B$ of HIV-1 were obtained and propagated as described previously.[27]

Antiviral Assays: HCMV. Both plaque and yield reduction assays were used. HFF cells in 24-well cluster dishes were infected with ca. 100 PFU of HCMV/well of cell sheet using the procedures detailed above. Following virus adsorption, compounds dissolved in growth medium were added to duplicate wells in four to eight selected concentrations. After incubation at 37 °C for 7–10 days, cell sheets were fixed and stained with crystal violet and microscopic plaques enumerated as described above. Drug effects were calculated as a percentage of reduction in number of plaques in the presence of each drug concentration compared to the number observed in the absence of drug. For yield reduction assay, the procedure devised by us[31] was used. HFF cells were planted as described above in 96-well cluster dishes and incubated overnight, the medium was removed, and the cultures were inoculated with HCMV at a MOI of 0.5–1 PFU/cell. After virus adsorption, inoculum was replaced with 0.2 mL of fresh medium containing test compounds. The first row of 12 wells was left undisturbed and served as virus controls. Each well in the second row received an additional 0.1 mL of medium with test compound at 3 times the desired final concentration. The contents of the 12 wells were mixed by repeated pipetting and then serially diluted 1:3 along the remaining wells. In this manner, six compounds could be tested in duplicate on a

single plate with concentrations from 100 to 0.14 μM. Plates were incubated at 37 °C for 7 days and subjected to one cycle of freezing and thawing; 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 HFF cells. 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 described.[31]

HSV-1. An ELISA assay developed by us[32] was employed to detect HSV-1. Ninety-six-well cluster dishes were planted with 10 000 BSC-1 cells/well in 200 μL/well MEM(E) plus 10% calf serum. After overnight incubation at 37 °C, selected drug concentrations in quadruplicate and HSV-1 at a concentration of 100 PFU/well were added. Following a 3 day incubation at 37 °C, medium was removed, plates were blocked and rinsed, and horse radish peroxidase-conjugated rabbit anti-HSV-1 antibody was added. Following removal of the antibody-containing solution, plates were rinsed and then developed by adding 150 μL/well of a solution of tetramethylbenzidine as substrate. The reaction was stopped with H_2SO_4, and absorbance was read at 450 and 570 nm. Drug effects were calculated as a percentage of the reduction in absorbance in the presence of each drug concentration compared to absorbance obtained with virus in the absence of drug.

Other Herpes Viruses. The activity of compound **10** against other herpes viruses was determined by plaque assay. The sensitivity of HSV-1 (E-377 strain), HSV-2 (MS strain), and VZV (Ellen strain) to compounds was determined in HFF cells by a plaque reduction assay. Confluent cell monolayers in 6-well plates were inoculated with 20–50 PFU of the appropriate virus and incubated at 37 °C and 90% humidity for 1 h. Serial 5-fold dilutions of each drug were prepared in 2× concentrated MEM(E) and mixed with an equal volume of 1% agarose, and 2 mL of the mixture was added to the monolayer cultures. An additional overlay mixture without drug (1 mL) was added on days 3 and 6 for VZV. At the appropriate time (HSV, 3 days; VZV, 10 days) monolayers were stained with neutral red and plaques enumerated either visually or with the aid of a stereodissecting microscope. Drug-treated cultures were compared to untreated control cultures, and IC_{50} values were calculated using a dose–effect analysis software program (Elsevier-Biosoft, Cambridge, U.K.).

HIV. Two separate assays were employed to evaluate the activities of compounds in cells acutely infected with HIV. The syncytial plaque assay performed as previously described by us[27] was used to measure the effect of compounds on HIV-1. Briefly, CEM-SS cells rendered adherent to substrate by means of poly(L-lysine) were infected with HIV-1 strain III_B, in the presence and absence of selected concentrations of compounds. Syncytial plaques were enumerated at 30-fold magnification 7 days postinfection. The second assay measured the presence of HIV in supernatants of CEM cells infected with strain III_B by the amount of RT activity. Cells were grown, infected, and incubated in the presence of seven concentrations (one-half $\log_{10}$ dilutions) beginning at a 1 or 100 μM concentration of the compounds to be assayed. Procedures and the RT assay were performed as detailed previously by White et al.[33] Techniques for determination of activity of test compounds in cells persistently infected with HIV, including assays for p24 antigen, have been detailed previously.[27]

Cytotoxicity Assays. Several different assays were used to explore cytotoxicity of selected compounds using methods we have detailed previously. (i) Cytotoxicity produced in stationary HFF cells was determined by microscopic inspection of cells not affected by the virus used in plaque assays.[1] (ii) The effect of compounds during two population doublings of KB cells was determined by crystal violet staining and spectrophotometric quantitation of dye eluted from stained cells as described earlier.[34] Briefly, 96-well cluster dishes were planted with KB cells at 3000–5000 cells/well. After overnight incubation at 37 °C, test compound was added in quadruplicate at six to eight concentrations. Plates were incubated at 37 °C for 48 h in a CO_2 incubator, rinsed, fixed with 95% ethanol, and stained with 0.1% crystal violet. Acidified ethanol was added, and plates were read at 570 nm in a spectrophotometer designed to read 96-well ELISA assay plates. (iii) The MTT dye assay also was used to determine the effect of compound **10** on selected cells lines. Briefly, cells were grown as described above, and compound **10** was added in one-half $\log_{10}$ dilutions. Plates were incubated for one to three population doublings in a humidified atmosphere of 5% CO_2 in air. Viable cells were measured with the tetrazolium salt MTT. Plates were read at a wavelength of 570 nm on a plate reader, and the percent inhibition per drug concentration was measured as test over control and expressed in percent. (iv) Inhibition of DNA synthesis was measured by determining the effect of compounds on the uptake of [^{3}H]dThd into total acid-precipitable DNA as detailed earlier.[27]

Data analysis. Dose–response relationships were constructed by linearly regressing the percent inhibition of 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 (acyclovir for HSV-1, ganciclovir for HCMV, and 2-acetylpyridine thiosemicarbazone for cytotoxicity) were used in all assays.

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