Structure−Activity Relationships of (*S,Z*)-2-Aminopurine Methylenecyclopropane Analogues of Nucleosides. Variation of Purine-6 Substituents and Activity against Herpesviruses and Hepatitis B Virus

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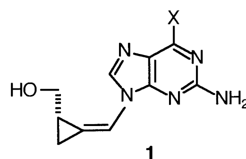
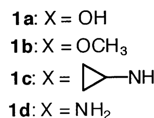
Received November 19, 2002

A series of 13 new (*S,Z*)-2-aminopurine methylenecyclopropane analogues was synthesized, and their antiviral activity was investigated. The nucleophilic displacement of chlorine of 2-amino-6-chloropurine derivative **5** with allyl-, propargyl-, cyclopropylmethyl-, isopropyl-, benzyl-, cyclohexyl-, and 2-hydroxyethylamine gave N^6-alkyl compounds **2a**, **2b**, **2c**, **2d**, **2e**, **2f**, and **2g**. A similar reaction of **5** with allyl, cyclopropylmethyl, propyl, or pentyl alcohol catalyzed by K_2CO_3 afforded O^6-alkyl analogues **3a**, **3c**, **3h** and **3i**. Propane- and pentanethiol furnished S^6-alkyl compounds **4h** and **4i**. The N^6-alkyl derivatives **2a**, **2b**, O^6 analogues **3a**, **3c**, **3h**, **3i**, and S^6 compounds **4h**, **4i** which were highly effective in all CMV assays and exhibited the lowest cytotoxicity in proliferating HFF cells appear to be good candidates for in vivo assays. Activity of new analogues against HSV-1 or HSV-2 was restricted to BSC-1 and Vero cultures. Compounds **2c**, **2b**, **3a** and **3h** were effective against EBV in one of two assays (Daudi or H-1). Analogues **3a** and **4i** were the most active anti-VZV agents whereas compounds **3h**, **3i**, and **4h** inhibited the replication of HBV in a micromolar concentration range.

In recent years, we have developed a new series of analogues of nucleosides where the ribofuranose moiety is replaced by a methylenecyclopropane unit. The purine analogues of *Z* (cis) configuration exhibit a broad spectrum antiviral activity with especially prominent anti-CMV and anti-EBV effects.[1,2] Subsequent studies have shown that a potent inhibition of replication of CMV is associated with the *S*-(+) enantiomers of 2-aminopurine analogues such as compounds[3,4] **1a**, **1b**, and **1c**. The enantioselectivity profiles of activity against viruses other than CMV, e.g., EBV, are less clear-cut. Investigations of racemic analogues **1a** and **1c** in a murine model of CMV infection have indicated that both compounds are effective.[5] Cyclopropylamino analogue **1c** was more potent, and very recent studies have shown that efficacy of the *S*-(+) enantiomer **1c** in SCID mice infected with HCMV was equal or superior to ganciclovir.[6]





In view of these results, it was of interest to investigate antiviral potency of (*S*)-(+)-2-aminopurine methylenecyclopropanes with modified substituents in the position 6. Three types of analogues were considered relevant. In the first group, the cyclopropylamino function of **1c** was replaced by an alkylamino group of a similar or different size (compounds **2a**−**g**). Because 6-methoxypurine analogue **1b** is an effective antiviral agent,[4] compounds **3a**, **3c**, **3h**, and **3i** where the methyl group was replaced by higher alkyl residues were also included. Last but not least, 6-alkylthio analogues **4h** and **4i** were also among our targets. The aim of investigation was 2-fold: (i) to study effects of variation of the purine 6-substituents on antiviral activity against CMV and other viruses and (ii) to facilitate selection of new anti-CMV agents for in vivo assays. This is also the first study of structure−activity relationships (SAR) of (*S,Z*)-2-aminopurine methylenecyclopropane analogues.

Synthesis

Recently, we have elaborated an efficient approach for synthesis[7] of (*S*)-(+)-2-amino-6-chloropurine methylenecyclopropane **5**. The latter compound served as a convenient starting material for all analogues of this study. Reaction of **5** with an excess of an appropriate primary amine in ethanol gave readily the expected analogues **2a**−**g** in 75−88% yield (Scheme 1). Analogues **3a**, **3c**, **3h**, and **3i** were also obtained from **5** using the corresponding alcohol and K_2CO_3 either without a solvent or in the presence of 1,2-dimethoxyethane (compounds **3c** and **3i**). The yields ranged from 79 to 85%. This method avoiding the use of NaH is more advantageous than the previously used procedure for synthesis of O^6-alkyl analogues of carbovir.[8] A similar

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10.1021/jm0205245 CCC: \$25.00 © 2003 American Chemical Society
Published on Web 03/15/2003

Scheme 1

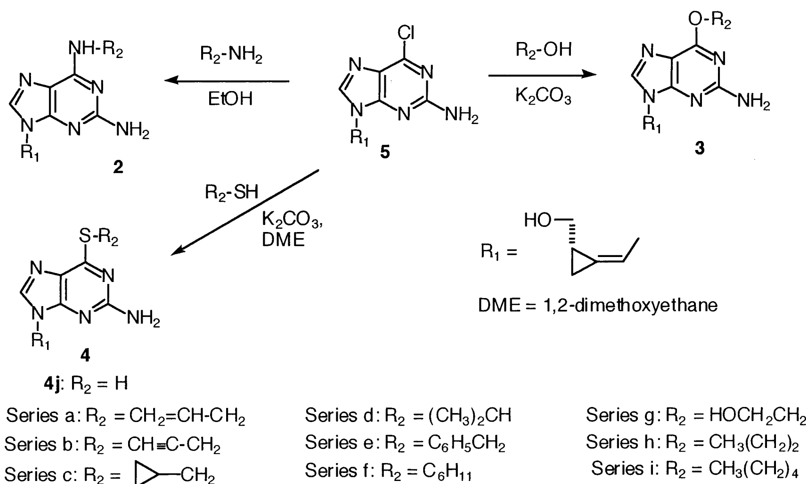

Table 1. Inhibition of HCMV, MCMV, HSV-1, and HSV-2 Replication by (*S,Z*)-(+)-2-Amino-6-substituted Purine Methylenecyclopropanes

	HCMV/HFF[a]		EC_{50}/CC_{50} (μM)			
compound	Towne[e]	AD169[f]	MCMV/MEF[a]	HSV-1/BSC-1[b]	HSV-1/Vero[c]	HSV-2/Vero[c,d]
1a (ref[4])	2.5	2.6/390(257)	0.39/>429	45	50/90	>50
1b (ref[4])	2.5	2.0/>404(15.8)	0.24/351	20	23/98	16
1c (ref[4])	1.8	1.9/367(213)	0.15/367	20	15/70	22
2a	1.8[g]	2.8/>368(>368)	0.33/>368	4	18/>100	21
2b	3.5[g]	2.6/>370(306)	0.37/>370	6	20/>100	50
2c	3.8	52/>349	1.7/279	2.5	25/>100	>50
2d	3.8	>365/>365[h]	NT[k]	15	38/>100	50
2e	>100	>310/>310[h]	NT	25	>50/>100	>50
2f	>100	>318/>318[h]	NT	15	>50/72	>50
2g	39[g]	>72/>362	11.9/>362	>100	>50/>100	>50
3a	1.2	2.1/>366(220)	0.29/>366	1.5	7/26	8
3c	0.32	1.7/>348(112)	0.35/278	7	8/41	12
3h	0.40[g]	2.0/363(69.4)	<0.11/363	1	4/32	10
3i	0.21	1.4/178(41.9)	1.45/224	7	3/87	4
4h	0.22	3.0/>343(283)	1.4/>343	1	9/>100	10
4i	0.32	1.5/>313(215)	1.5/171	7	10/26	14
4j	37	45/388	>16/64	>100	>50/29	>50
control	4.1[i]	5.1/>392[i]	2.1/>392[i]	0.15[j]	13.5/>200[j]	32.3[j]

[a] Plaque reduction assay run in duplicate. [b] ELISA results from quadruplicates for HSV-1 ELISA, EC_{50} data only. CC_{50}'s in growing KB cells were >100 μM in all cases. [c] Plaque reduction assay: cytotoxicity was determined in CEM cells. [d] EC_{50} data only, for CC_{50}'s see HSV-1. [e] Visual cytotoxicity was scored on uninfected HFF cells not affected by virus in the HCMV plaque reduction assay. The CC_{50} values were >100 μM in all cases. [f] Cytotoxicity byneutral red uptake. The CC_{50}'s for proliferating cells (in parentheses) are given only for analogues with EC_{50} <10 μM in both HCMV assays. [g] Average of duplicate experiments. [h] Cytopathic effect (CPE) inhibition assay. [i] Ganciclovir. [j] Acyclovir [k] NT = not tested.

reaction of **5** with 1-propane- or 1-pentanethiol gave the *S*-alkyl derivatives **4h** and **4i** in 72 and 75% yield, respectively. The 6-thio analogue **4j** was prepared as described.[9]

Antiviral Activity

The viruses included in this investigation were as follows: HCMV, MCMV, HSV-1, HSV-2, EBV, and HBV. The results with HCMV (two strains) and MCMV are summarized in Table 1. It is clear that regardless of the heteroatom attached to the position 6 of the purine ring, a number of compounds have been identified that are equally or more effective than ganciclovir or "parent" analogues **1a**, **1b**, or **1c**. Submicromolar efficacy of oxygen or thio analogues **3c**, **3h**, **3i**, **4h**, and **4i** in Towne strain of HCMV is of particular interest. This magnitude of inhibition was previously observed[1,2,4] only against murine CMV (see **1a**, **1b**, and **1c**). High potency of allyl analogue **2a** (isomeric with **1c**) and propargyl derivative **2b** is also noteworthy. In contrast, the cyclopropylmethyl derivative **2c** (homologue of **1c**) was only slightly active against AD169 strain of HCMV but was effective against the Towne strain of the virus and MCMV. Analogues **2e** and **2f** derived from bulkier amines were inactive. Compound **2g** with a polar N^6 substituent exhibited only a moderate effect. Thio analogue **4j** was devoid of a significant potency but the *S*-alkyl analogues **4h** and **4i** were potent anti-CMV agents. Interestingly, the *O*- and *S*-alkyl derivatives **3h**, **3i** and **4h**, **4i** are virtually equipotent against HCMV. This indicates that in both cases a lipophilic threshold of activity has not been reached with a five-carbon chain (pentyl). However, it is not clear whether a further extension of the chain can provide analogues with a superior potency.

Only *O*-alkyl derivatives **3h** and **3i** exhibited some cytotoxicity in proliferating HFF cells which was lower than that of the 6-methoxy analogue **1b**. On the basis of these results, any of the potent and noncytotoxic analogues from this group, e.g., **2a**, **2b**, **3a**, **3c**, **4h**, and

Table 2. Inhibition of EBV, HBV, and VZV Replication by (*S,Z*)-(+)-2-Amino-6-substituted Purine Methylenecyclopropanes

	EC_{50}/CC_{50} (μM)			
compound	EBV/ Daudi[a]	EBV/ H-1[b,c]	HBV/ 2.2.15[b,c]	VZV/ HFF[d]
1a (ref.[4])	2.3/>215[e] (<0.34)[c]	0.47	>10	55
1b (ref.[4])	3.8/146[e] (1.0)[c]	0.44	>10	10
1c (ref.[4])	6.2/176[e] (0.85)[c]	0.66	>10	12
2a	>184/>184	18.5	20	>368[f]
2b	>185/>185	~2	>10	>74
2c	0.56/>175 (7)[c]	>20	>20	10.8
2d	36.5/168	11	>10	>365[f]
2e	52/>155	>20	>20	>62[f]
2f	>159/>159	>20	>20	180[f]
2 g	>181/>181	>20	>20	>60[f]
3a	>183/>183	1.1	11	2.3
3c	29.2/112	12.6	12.6	8.4
3h	>182/>182	1.5	4.1	8.0
3i	23.4/>165	7.3	7.3	>66[f]
4h	>172/>172	6.3	5.1	>69[f]
4i	39.8/>157	>20	>20	2.5
4j	>8/35.3	>20	20	>80[f]
control	1.1/>222[g]	5[h]	0.02/>100[i]	1.5/>444[g]

[a] Viral capsid antigen immunofluorescence (VCA) enzyme-linked immunosorbent assay (ELISA). [b] EC_{50} data, for CC_{50}'s in CEM cells see Table 1. [c] DNA hybridization assay. [d] Plaque reduction assay, EC_{50} data only. For CC_{50}'s see Table 1. [e] Viral capsid antigen immunofluorescence (VCA-IF) assay. [f] Cytopathic effect (CPE) inhibition assay. [g] Acyclovir. [h] Ganciclovir. [i] Lamivudine (3TC).

4i, appears suitable as a good candidate for in vivo studies. It is noteworthy that favorable effects of *O*[6]-alkyl substitution on anti-HIV activity were absent[8] in the corresponding analogues of carbovir. In contrast, *N*[6]-cyclopropyl, *N*[6]-allyl, and *O*[6]-cyclopropylmethyl analogues of 2′-deoxy-4′-thioguanosine (related to compounds **1c**, **2a**, and **3c**) maintained a high anti-HBV and anti-HCMV potency of the parent compound.[10] Interestingly, *O*[6]-methyl and other alkyl derivatives of penciclovir were inactive in vitro against HSV-1 and -2.[11] Investigation of their metabolism in mice after oral administration indicated a conversion to penciclovir although to a more limited extent than the 6-deoxypurine prodrug.[12] An enzymatic oxidative dealkylation was suggested as a possible metabolic pathway.

Potent effects of *N*-, *O*-. or *S*-alkyl substitution observed in CMV assays do not fully extend to other viruses. Thus, neither of the new analogues was effective against HSV-1 or HSV-2 in HFF culture, but activity in the micromolar range was noted in HSV-1/BSC-1 system with analogues **2a**, **2b**, **2c**, **3a**, **3c**, **3h**, **3i**, **4h**, and **4i** (Table 1) which were devoid of cytotoxicity in KB cells. All these analogues were significantly more potent than the "parent" compounds **1a**, **1b** or **1c**. These effects were to some extent paralleled in HSV-1 assays in Vero cells (see, e.g., compounds **3c** and **3h**), but some cytotoxicity increases (in CEM cells) were also apparent. Activity against HSV-2 largely reflected that found in HSV-1 assays with compound **3i** being the most efficacous (EC_{50} 4 μM).

Analogues **1a**, **1b**, and **1c** are potent anti-EBV agents (Table 2) in both Daudi and H-1 cell culture assays.[4] Only *N*-cyclopropylmethyl derivative **2c** (Daudi cells) surpassed their efficacy in VCA-IF assay but not in DNA hybridization assay. It was not effective in H-1 cells. In several other cases the anti-EBV activity was also limited to a single assay. Thus, analogues **2b**, **3a**, **3h,** and **4h** were active in H-1 cells but ineffective in Daudi cells. Compounds **1b** and **1c** are inhibitors of replication of VZV in HFF culture[4] (EC_{50} 10 and 12 μM, respectively). Potency of *O*-alkyl derivatives **3c** and **3h** is roughly equal (EC_{50} 8.0−8.4 μM), and *O*-allyl and *S*-pentyl analogues **3a** and **4i** are the most potent of the whole series with EC_{50} 2.3−2.5 μM. In case of *O*-alkyl derivatives, the lipophilic threshold was apparently reached with *O*-pentyl compound (EC_{50} of **3h** ≪ **3i**). In contrast, the *S*-pentyl derivative **4i** was active whereas the *S*-propyl analogue **4h** was devoid of potency. From the new analogues, only *O*-alkyl compounds **3h**, **3i**, and *S*-alkyl analogue **4h** exhibited significant activity against HBV with EC_{50} ranging between 4.1 and 7.3 μM.

It is possible that active N-, O-, and S-alkylated methylenecyclopropane analogues described herein are prodrugs of synguanol (**1a**) or the respective phosphorylated species thereof. Adenosine deaminase can be ruled out as a possible transformation agent because racemic compound **1d** is not a substrate.[2] It is also not clear whether compound **1c** and related *N*-alkyl derivatives described in this study can utilize mechanisms described for 2-amino-6-cyclopropylaminopurine analogue of PMEDAP[13] and abacavir.[14] The activation of *O*- and *S*-alkyl analogues may then follow still a different pathway.

Experimental Section

General Methods. See reference 1. UV spectra were measured in ethanol, and NMR spectra in CD_3SOCD_3. Mass spectrometry was performed in an electrospray ionization (ESI) mode on MICROMASS QUATTRO LC-MS in aqueous MeOH containing NaCl. Compound **5** was prepared as described[7]/optical purity (ee) 99.2%/.

(*S,Z*)-(+)-2-Amino-6-allylamino-9-[(2-hydroxymethyl)-cyclopropylidenemethyl]purine (2a). A mixture of compound **5** (503 mg, 2.0 mmol) and allylamine (680 mg, 12 mmol) in ethanol (20 mL) was stirred overnight at room temperature. Volatile components were evaporated in vacuo, and the residue was chromatographed on a silica gel column using CH_2Cl_2−MeOH (95:5) to give product **2a** (445 mg, 82%). Mp 168−170 °C; $[\alpha]^{20}_D$ 70.2° (*c* 0.45, DMF). UV λ_{max} 284 (ϵ 16600), 223 nm (ϵ 42700); ^1H NMR δ 1.16 (m, 1H) and 1.44 (t, 1H, $H_{3'}$), 2.07 (m, 1H, $H_{4'}$), 3.33 (m, 1H) and 3.67 (m, 1H, $H_{5'}$), 4.04 (bs, 2, NCH_2), 5.00−5.14 (t + dd, $=CH_2$ + OH), 5.91 and 5.97 (m and s, 3H, =CH and NH_2), 7.17 (d, 1H, J = 1.5 Hz, $H_{1'}$), 7.48 (bs, 1H, NH), 8.33 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 19.8 ($C_{4'}$), 42.4 (NCH_2), 63.7 ($C_{5'}$), 111.0 ($C_{1'}$), 113.6 ($C_{2'}$), 114.2 (C_5), 115.4 ($=CH_2$), 134.7 (C_8), 136.8 (=CH), 150.4 (C_4), 155.4 (C_2), 161.2 (C_6). ESI-MS 567 (2M + Na, 61.0), 295 (M + Na, 58.9), 273 (M + H, 100.0). Anal. ($C_{13}H_{16}N_6O$) C, H, N.

(*S,Z*)-(+)-2-Amino-6-propargylamino-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (2b). A mixture of compound **5** (190 mg, 0.75 mmol) and propargylamine (270 mg, 4.8 mmol) in ethanol (18 mL) was stirred at 50 °C for 1 h and then for 16 h at room temperature. After the workup (see compound **2a**), product **2b** was obtained (175 mg, 85%). Mp 192−194 °C; $[\alpha]^{20}_D$ 77.3° (*c* 0.40, DMF); UV λ_{max} 283 (ϵ 15000) and 222 nm (ϵ 41100); ^1H NMR δ 1.16 (m, 1H) and 1.45 (t, 1H, $H_{3'}$), 2.10 (m, 1H, $H_{4'}$), 3.02 (s, 1H, ≡CH), 3.29−3.35 (m, 1H, overlapped with H_2O) and 3.68 (m, 1H, $H_{5'}$), 4.19 (bs, 2H, NCH_2), 5.07 (t, 1H, J = 5.0 Hz, OH), 6.08 (bs, 2H, NH_2), 7.18 (d, 1H, J = 2.5 Hz, $H_{1'}$), 7.67 (bs, 1H, NH), 8.35 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 19.8 ($C_{4'}$), 29.3 (NCH_2), 63.7 ($C_{5'}$), 73.1 (≡CH), 83.0 (≡C), 110.9 ($C_{1'}$), 113.7 ($C_{2'}$), 114.4 (C_5), 135.1 (C_8), 150.3 (C_4), 154.8 (C_2), 161.1 (C_6). ESI-MS 563 (2M + Na, 75.9), 293 (M + Na, 100.0), 271 (M + H, 15.5). Anal. ($C_{13}H_{14}N_6O$) C, H, N.

(*S,Z*)-(+)-2-Amino-6-cyclopropylmethylamino-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (2c). A mixture of compound **5** (200 mg, 0.8 mmol) and cyclopropylmeth-

ylamine (360 mg, 5.0 mmol) in ethanol (15 mL) was stirred at 50 °C for 16 h. The usual workup (see analogue **2b**) gave compound **2c** (201 mg, 88%). Mp 180−182 °C; $[\alpha]^{20}_D$ 68.4° (*c* 0.28, DMF); UV λ_{max} 284 (ϵ 14900), 223 nm (ϵ 34900); ^{1}H NMR δ 0.22 (m, 2H) and 0.37 (m, 2H, CH_2, cyclopropyl), 1.08 and 1.15 (2 partially overlapped m, 2H) and 1.44 (dt, 1H, *J* = 8.4 and 1.6 Hz, 1H, CH-cyclopropyl + $H_{3'}$), 2.07 (m, 1H, $H_{4'}$), 3.26 overlapped with 3.30 (2m, 3H) and 3.69 (m, 1H, NCH_2 + $H_{5'}$), 5.10 (t, 1H, *J* = 5.2 Hz, OH), 5.97 (bs, 2H, NH_2), 7.19 (s, 1H, $H_{1'}$), 7.35 (bs, 1H, NH), 8.33 (s, 1H, H_8); ^{13}C NMR 4.0 (CH_2, cyclopropyl), 6.9 ($C_{3'}$), 12.0 (CH, cyclopropyl), 19.9 ($C_{4'}$), 44.5 (NCH_2), 63.7 ($C_{5'}$), 111.1 ($C_{1'}$), 113.6 ($C_{2'}$), 114.1 (C_5), 134.6 (C_8), 150.4 (C_4), 155.5 (C_2), 161.2 (C_6); ESI-MS 309 (M + Na, 54.2), 287 (M + H, 100.0). Anal. ($C_{14}H_{18}N_6O$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-isopropylamino-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (2d)**. A mixture of compound **5** (250 mg, 1.0 mmol) and isopropylamine (590 mg, 10 mmol) in ethanol (10 mL) was stirred at room temperature for 16 h. After the workup (see analogue **2b**), compound **2d** was obtained (215 mg, 80%). Mp 153−155 °C; $[\alpha]^{20}_D$ 67.8° (*c* 0.36, DMF); UV λ_{max} 284 (ϵ 14200), 223 nm (ϵ 32700); ^{1}H NMR δ 1.15 (d, 7H, *J* = 6.5 Hz) and 1.43 (t, 1H, *J* = 8 Hz, CH_3 + $H_{3'}$), 2.07 (m, 1H, $H_{4'}$), 3.34 (m, 1H) and 3.68 (m, 1H, $H_{5'}$), 4.37 (bs, 1H, CH of i-Pr), 5.10 (t, 1H, *J* = 5.0 Hz, OH), 5.95 (bs, 2H, NH_2), 7.01 (bs, 1H, NH), 7.18 (s, 1H, $H_{1'}$), 8.32 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 19.8 ($C_{4'}$), 23.3 (CH_3), 41.4 (CH of i-Pr), 63.7 ($H_{5'}$), 111.1 ($C_{1'}$), 113.5 ($C_{2'}$), 114.1 (C_5), 134.5 (C_8), 150.3 (C_4), 154.9 (C_2), 161.2 (C_6); ESI-MS 571 (2M + Na, 47.0), 297 (M + Na, 47.6), 275 (M + H, 100.0). Anal. ($C_{13}H_{18}N_6O$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-benzylamino-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (2e)**. A mixture of compound **5** (250 mg, 1.0 mmol) and benzylamine (645 mg, 6.0 mmol) in ethanol (15 mL) was stirred at 50 °C for 16 h. The usual workup (see analogue **2b**, chromatography in CH_2Cl_2−MeOH (97:3)) furnished compound **2e** (240 mg, 75%). Mp 133−135 °C; $[\alpha]^{20}_D$ 69.7° (*c* 0.35, DMF; UV λ_{max} 285 (ϵ 16200), 224 nm (ϵ 38300); ^{1}H NMR δ 1.16 (ddd, 1H) and 1.45 (bt, 1H, $H_{3'}$), 2.08 (bm, 1H, $H_{4'}$), 3.40 (m, 1H, overlapped with H_2O) and 3.67 (m, 1H, $H_{5'}$), 4.62 (s, 2H, NCH_2), 5.07 (t, 1H, *J* = 5.0 Hz, OH), 5.96 (bs, 2H, NH_2), 7.17−7.20 (m and s) and 7.25−7.30 (m, 6H, Ph + $H_{1'}$), 7.86 (bs, 1H, NH), 8.32 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 19.9 ($C_{4'}$), 43.2 (NCH_2), 63.7 ($C_{5'}$), 111.1 ($C_{1'}$), 113.6 ($C_{2'}$), 114.3 (C_5), 127.2, 127.9, 128.8, and 141.2 (Ph), 134.8 (C_8), 150.5 (C_4), 155.5 (C_2), 161.2 (C_6); ESI-MS 345 (M + Na, 58.9), 323 (M + H, 100.0). Anal. ($C_{17}H_{18}N_6O$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-cyclohexylamino-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (2f)**. A mixture of compound **5** (200 mg, 0.8 mmol) and cyclohexylamine (645 mg, 6.5 mmol) in ethanol (15 mL) was refluxed with stirring for 16 h. The workup followed the procedure described for analogue **2e** to give compound **2f** (205 mg, 82%). Mp 183−185 °C; $[\alpha]^{20}_D$ 81.2° (*c* 0.34, DMF; UV λ_{max} 285 (ϵ 16100), 223 nm (ϵ 35500). ^{1}H NMR δ 1.07−1.82 (cluster of m, 12H, $(CH_2)_5$ + $H_{3'}$), 2.06 (m, 1H, $H_{4'}$), 3.33 (m, 1H, partly overlapped with H_2O) and 3.68 (m, 1H, $H_{5'}$), 4.01 (bs, 1H, NCH), 5.10 (t, 1H, *J* = 5.2 Hz), 5.93 (bs, 2H, NH_2), 6.95 (bs, 1H, NH), 7.18 (s, 1H, $H_{1'}$), 8.31 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 19.9 ($C_{4'}$), 25.8, 25.9, 33.3, 48.6 (C_6H_{11}), 63.7 ($C_{5'}$), 111.1 ($C_{1'}$), 113.4 ($C_{2'}$), 114.1 (C_5), 134.5 (C_8), 150.5 (C_4), 154.8 (C_2), 161.2 (C_6); ESI-MS 337 (M + Na, 29.9), 315 (M + H, 100.0). Anal. ($C_{16}H_{22}N_6O$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-(2-hydroxy)ethylamino-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (2g)**. A mixture of compound **5** (200 mg, 0.80 mmol) and (2-hydroxyethyl)amine (250 mg, 4.1 mmol) in ethanol (15 mL) was stirred at 50 °C for 16 h. The workup followed the procedure described for **2b** to give after chromatography using CH_2Cl_2−MeOH (9:1) compound **2g** (171 mg, 77%). Mp 150−153 °C; $[\alpha]^{20}_D$ 76.8° (*c* 0.32, DMF); UV λ_{max} 283 nm (ϵ 15000), 223 nm (ϵ 37100). ^{1}H NMR δ 1.16 (m, 1H) and 1.44 (t, 1H, $H_{3'}$), 2.07 (m, 1H, $H_{4'}$), 3.30−3.53 (3m, overlapped with H_2O) and 3.68 (dd, 1H, *J* = 10.4 and 5.6 Hz, $(CH_2)_2$ + $H_{5'}$/, 4.82 (bs, 1H) and 5.09 (bs, 1H, OH), 5.98 (bs, 2H, NH_2), 7.13 (bs, 1H, NH), 7.18 (s, 1H, $H_{1'}$), 8.32 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 19.8 ($C_{4'}$), 43.0 (NCH_2), 60.8 (OCH_2), 63.7 ($C_{5'}$), 111.1 ($C_{1'}$), 113.7 ($C_{2'}$), 114.3 (C_5), 134.8 (C_8), 150.3 (C_4), 155.7 (C_2), 161.2 (C_6); ESI-MS 299 (M + Na, 100.0), 277 (M + H, 95.8). Anal. ($C_{12}H_{16}N_6O_2$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-allyloxy-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (3a)**. A mixture of compound **5** (200 mg, 0.8 mmol), K_2CO_3 (165 mg, 12 mmol), and allyl alcohol (5 mL) was stirred at 60−70 °C for 16 h. The workup followed the procedure described for **2b** to give compound **3a** (172 mg, 79%). Mp 163−165 °C; $[\alpha]^{20}_D$ 68.4° (*c* 0.23, DMF); UV_{max} 280 (ϵ 11600), 225 (ϵ 29900), 204 nm (ϵ 19700); ^{1}H NMR δ 1.19 (m, 1H) and 1.43 (m, 1H, $H_{3'}$), 2.10 (m, 1H, $H_{4'}$), 3.30 (m 1H, overlapped with H_2O) and 3.71 (m, 1H, $H_{5'}$), 4.94 (d, 2H, *J* = 6.0 Hz, CH_2O), 5.07 (t, 1H, *J* = 5.2 Hz, OH), 5.25 (dd, 1H, *J* = 7.5 and 1.8 Hz) and 5.40 (dd, 1H, *J* = 17.1 and 1.8 Hz, $=CH_2$), 6.11 (ddd, 1H, =CH), 6.53 (s, 2H, NH_2), 7.21 (d, 1H, *J* = 1.2 Hz, $H_{1'}$), 8.50 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 19.9 ($C_{4'}$), 63.6 ($C_{5'}$), 66.8 (CH_2O), 110.8 ($C_{1'}$), 114.0 ($C_{2'}$), 115.4 (C_5), 118.8 ($=CH_2$), 133.9 (=CH), 137.3 (C_8), 153.4 (C_4), 160.6, 160.7 (C_2, C_6); ESI-MS 569 (2M + Na, 100.0), 296 (M + Na, 48), 274 (M + H, 10). Anal. ($C_{13}H_{15}N_5O_2$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-cyclopropylmethoxy-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (3c)**. A mixture of compound **5** (150 mg, 0.6 mmol), K_2CO_3 (210 mg, 1.5 mmol), and cyclopropylmethanol (2 mL) in DME (7 mL) was stirred at 80 °C for 16 h. The workup followed the procedure described for analogue **2e** to give compound **3c** (142 mg, 82%). Mp 129−130 °C; $[\alpha]^{20}_D$ 74.5° (*c* 0.33, DMF); UV λ_{max} 280 (ϵ 10900), 224 (ϵ 28900), 204 nm (ϵ 20500); ^{1}H NMR δ 0.34 (d, 2H, *J* = 4.0 Hz) and 0.57 (d, 2H, *J* = 7.2 Hz, CH_2, cyclopropyl), 1.19 (m, 1H) and 1.47 (t, 1H, *J* = 8.4 Hz, $H_{3'}$), 1.29 (m, 1H, CH, cyclopropyl), 2.10 (m, 1H, $H_{4'}$), 3.30 (m, 1H, overlapped with H_2O) and 3.70 (m, 1H, $H_{5'}$), 4.23 (d, 2H, *J* = 7.2 Hz, OCH_2), 5.07 (t, 1H, *J* = 5.4 Hz, OH), 6.49 (s, 2H, NH_2), 7.21 (s, 1H, $H_{1'}$), 8.48 (s, 1H, H_8); ^{13}C NMR 4.0 (CH_2, cyclopropyl), 6.9 ($C_{3'}$), 10.7 (CH, cyclopropyl), 19.9 ($C_{4'}$), 63.6 ($C_{5'}$), 71.1 (CH_2O), 110.8 ($C_{1'}$), 114.1 ($C_{2'}$), 115.3 (C_5), 137.1 (C_8), 153.3 (C_4), 160.8, 161.1 (C_2, C_6); ESI-MS 597 (2M + Na, 13.7), 575 (2M + H, 28.6), 288 (M + H, 100.0). Anal. ($C_{14}H_{17}N_5O_2$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-propoxy-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (3h)**. A mixture of compound **5** (200 mg, 0.8 mmol), K_2CO_3 (166 mg, 12 mmol), and 1-propanol (6 mL) was stirred at 70−80 °C for 16 h. The workup followed the procedure described for analogue **2b** to give compound **3h** (188 mg, 85%). Mp 147−149 °C; $[\alpha]^{20}_D$ 68.3° (*c* 0.42, DMF); UV λ_{max} 280 (ϵ 12100), 225 (ϵ 31500), 205 nm (ϵ 21500); ^{1}H NMR δ 0.94 (t, 3H, *J* = 7.2 Hz, CH_3), 1.17 (m, 1H) and 1.44 (dt, 1H, $H_{3'}$), 1.74 (m, 2H, CH_2), 2.09 (m, 1H, $H_{4'}$), 3.30 (m, 1H) and 3.71 (m, 1H, $H_{5'}$), 4.34 (t, 2H, *J* = 6.8 Hz, CH_2O), 5.08 (t, 1H, *J* = 5.0 Hz, OH), 6.49 (s, 2H, NH_2), 7.20 (d, 1H, *J* = 1.5 Hz, $H_{1'}$), 8.47 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 10.9 (CH_3), 19.9 ($C_{4'}$), 22.5 (CH_2), 63.6 ($C_{5'}$), 68.0 (CH_2O), 110.8 ($C_{1'}$), 114.1 ($C_{2'}$), 115.2 (C_5), 137.1 (C_8), 153.3 (C_4), 160.8, 161.2 (C_2, C_6); ESI-MS 573 (2M + Na, 100.0), 298 (M + Na, 39.9), 276 (M + H, 7.8). Anal. ($C_{13}H_{17}N_5O_2$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-pentyloxy-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (3i)**. A mixture of compound **5** (150 mg, 0.6 mmol), K_2CO_3 (210 mg, 1.5 mmol), 1-pentanol (0.4 mL, 3.7 mmol), and DME (10 mL) was stirred at 70−80 °C for 16 h. The workup followed the procedure described for **2b**. Chromatography using CH_2Cl_2−MeOH (96:4) afforded compound **3i** (155 mg, 85%). Mp 135−137 °C; $[\alpha]^{20}_D$ 77.7° (*c* 0.26, DMF); UV λ_{max} 280 (ϵ 11500), 225 (ϵ 30300), 205 nm (ϵ 21700); ^{1}H NMR δ 0.87 (t, 3H, CH_3), 1.18 (m, 1H) and 1.46 (m, 1H, $H_{3'}$), 1.34/m, 4H, $(CH_2)_2$/, 1.73 (m, 2H, CH_2), 2.09 (m, 1H, $H_{4'}$), 3.31 (m, overlapped with H_2O) and 3.70 (m, 1H, $H_{5'}$), 4.38 (t, 2H, *J* = 6.6 Hz, CH_2O), 5.07 (t, 1H, *J* = 5.4 Hz, OH), 6.49 (bs, 2H, NH_2), 7.20 (s, 1H, $H_{1'}$), 8.47 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 14.6 (CH_3), 19.9 ($C_{4'}$), 22.6, 28.3 and 28.8 (CH_2), 63.6 ($C_{5'}$), 66.5 (CH_2O), 110.8 ($C_{1'}$), 114.1 ($C_{2'}$), 115.2 (C_5), 137.0 (C_8), 153.3 (C_4), 160.8, 161.2 (C_2, C_6); ESI-MS 629 (2M + Na, 24.6), 607 (2M + H, 74.9), 304 (M + H, 100.0). Anal. ($C_{15}H_{21}N_5O_2$) C, H, N.

***(S,Z)*-(+)-2-Amino-6-propylthio-9-[(2-hydroxymethyl)cyclopropylidenemethyl]purine (4h)**. The mixture of com-

pound **5** (125 mg, 0.5 mmol), K_2CO_3 (140 mg, 1.0 mmol), 1-propanethiol (750 mg, 10 mmol), and DME (6 mL) was stirred at 50 °C for 10 h. The workup followed the procedure described for **2b** give compound **4h** (105 mg, 72%). Mp. 163–164 °C; $[\alpha]^{20}_D$ 77.3° (*c* 0.39, DMF); UV λ_{max} 311 (ϵ 13400), 232 (ϵ 32400), 198 nm (ϵ 12000); ^{1}H NMR δ 0.98 (t, 3H, J = 7.5 Hz, CH_3), 1.18 (bt, 1H) and 1.46 (t, 1H, $H_{3'}$), 1.66 (m, 2H, CH_2), 2.12 (m, 1H, $H_{4'}$), 3.15 (d, J = 5.4 Hz, CH_2S), 3.26 (t + m, overlapped with H_2O) and 3.70 (m, 1H, $H_{5'}$), 5.06 (t, 1H, J = 5.1 Hz, OH), 6.58 (s, 2H, NH_2), 7.19 (s, 1H, $H_{1'}$), 8.54 (s, 1H, H_8); ^{13}C NMR 6.9 ($C_{3'}$), 13.9 (CH_3), 19.9 ($C_{4'}$), 23.4 (CH_2), 30.0 (CH_2S), 63.6 ($C_{5'}$), 110.5 ($H_{1'}$), 115.8 ($C_{2'}$), 124.4 (C_5), 138.0 (C_8), 150.0 (C_4), 160.4, 160.6 (C_2, C_6); ESI-MS 605 (2M + Na, 100.0), 314 (M + Na, 26.3), 292 (M + H, 9.6). Anal. ($C_{13}H_{17}N_5OS$) C, H, N, S.

(*S,Z*)-(+)-2-Amino-6-pentylthio-9-[(2-hydroxymethyl)-cyclopropylidenemethyl]purine (4i). The experiment was performed as described above with 1-pentanethiol instead of 1-propanethiol at 0.6 mmol scale (80 °C for 16 h). The workup followed the procedure described for analogue **3i** to give compound **4i** (141 mg, 75%). Mp 146–147 °C; $[\alpha]^{20}_D$ 82.2° (*c* 0.37, DMF); UV λ_{max} 311 (ϵ 12000), 232 (ϵ 28600), 199 nm (ϵ 13000); ^{1}H NMR δ 0.85 (t, 3H, J = 7.2 Hz, CH_3), 1.19 (m, 1H) and 1.47 (poorly resolved dt, 1H, $H_{3'}$), 1.25–1.40 (m, 4H, $(CH_2)_2$), 1.64 (m, 2H, CH_2), 2.09 (m, 1H, $H_{4'}$), 3.25–3.31 (m, 3H, CH_2S and 1/2 $H_{5'}$) and 3.70 (m, 1H, $H_{5'}$), 5.06 (t, 1H, J = 5.2 Hz, OH), 6.57 (bs, 2H, NH_2), 7.20 (d, 1H, J = 1.6 Hz, $H_{1'}$), 8.54 (s, 1H, H_8); ^{13}C NMR 7.0 ($C_{3'}$), 14.6 (CH_3), 19.9 ($C_{4'}$), 22.4, 28.1 and 29.6 (CH_2), 31.1 (CH_2S), 63.6 ($C_{5'}$), 110.5 ($C_{1'}$), 115.8 ($C_{2'}$), 124.4 (C_5), 138.0 (C_8), 150.0 (C_4), 160.4, 160.6 (C_2, C_6); ESI-MS 661 (2M + Na, 16.2), 639 (2M + H, 25.8), 320 (M + H, 100.0). Anal. ($C_{15}H_{21}N_5OS$) C, H, N, S.

Biological Assays. These assays were performed as detailed previously[1] with some modifications. The following procedures were employed:

Cell Culture Procedures. The routine growth and passage of KB, BSC cells, and human foreskin fibroblasts (HFF) or murine embryo fibroblasts 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 (FBS) for HFF cells. The sodium bicarbonate concentration was varied to meet the buffering capacity required. These and other adherent cells were passaged at 1:2 to 1:10 dilutions according to conventional procedures by using 0.05% trypsin plus 0.02% EDTA in a HEPES buffered salt solution.[15] Similar suspension culture conditions were employed for CEM cells.

A. HSV-1 ELISA. An enzyme-linked immunosorbent assay (ELISA)[16] was employed to detect HSV-1. Briefly, 96-well cluster dishes were planted with BSC-1 cells, and after overnight incubation at 37 °C, selected drug concentrations in triplicate and HSV-1 (KOS strain kindly provided by Dr. Sandra K. Weller, University of Connecticut) at a concentration of 100 pfu/well were added. Following incubation, medium was removed, plates were blocked and rinsed, and horseradish peroxidase conjugated rabbit anti-HSV-1 antibody was added. Following removal of the antibody-containing solution, plates were rinsed and then developed by adding 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.

B. HCMV, HSV-1, HSV-2, VZV, and MCMV Plaque Reduction Assays. 1. HCMV Plaque Assay. Plaque assay, Towne strain, plaque-purified HCMV (Dr. Mark Stinski, University of Iowa) was used to infect nearly confluent HFF in 24-well cluster plates at approximately 100 plate forming units (pfu) per cm^2 cell sheet using the procedures detailed earlier.[17] Following virus adsorption, compounds dissolved in growth medium containing methocel overlay were added to duplicate wells in four to eight selected concentrations. After incubation at 37 °C for 8–10 days, cell sheets were fixed and stained with crystal violet, and microscopic plaques were enumerated. 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.

2. HFF and MEF Cells. Two days prior to use, HFF or MEF cells were plated into six well plates; on the date of assay, drug was made up in MEM with 2% FBS and then serially diluted 1:5 in MEM using six concentrations of drug ranging from 100 μg/mL to 0.03 μg/mL. Twenty to 30 plaques of virus were added to each well in triplicate with 0.2 mL of media being added to drug toxicity wells, and MEM containing the various drug concentrations was added to appropriate wells in duplicate. For VZV, no antibody in the overlay was utilized. For the HSV assay, the cultures were incubated for 3 days. For VZV assays additional media was added on day 5 and incubated for a total of 10 days. MCMV assays had no additional media added, and they were incubated for 7 days. For VZV, HCMV, and MCMV assays, the cells were stained with 1.5% neutral red solution for MCMV and 5% for HCMV and VZV. Cells were incubated for 6 h, the liquid was removed, and plaques were enumerated microscopically. The results were compared with control wells, and EC_{50} values were calculated as described below.

3. Vero Cells. Vero cells[18] were seeded and incubated for 24 h at 37 °C until they were confluent. The media were then removed, and virus (100 μL, HSV-1, KOS strain or HSV-2, 333 strain) was added at 20 or 100 pfu per well.The dishes were incubated for 1 h, drug solutions were prepared with in RPMI + 2% dialyzed FBS + 0.1% methylcellulose (4000 cps), and appropriate amounts were added to the wells. The cultures were then incubated for 48 to 72 h, media were removed, and staining was performed with 0.8% crystal violet. The dishes were rinsed with water and allowed to dry before determining the plaque number.

C. HCMV, VZV Cytopathic Effect (CPE) Inhibition Assay. Low passage HFF cells were seeded into 96-well tissue culture plates 24 h prior to use. After incubation, medium was removed and 100 μL of MEM containing 2% FBS was added to all but the first row. In the first row, 125 μL of experimental drug was added in triplicate wells. Medium alone was added to both cell and virus control wells. The drug in the first row of wells was then diluted serially 1:5 throughout the remaining wells The appropriate virus concentration was added to each well, excluding cell control wells which received 100 μL of MEM. For VZV assays, the virus concentration utilized was 1000 pfu per well. For CMV (AD169) assays, the virus concentration added was 2500 pfu per well. The plates were then incubated for 10 days for VZV and 14 days for HCMV. After the incubation period, cells were stained with a crystal violet/ethanol/formaldehyde solution. Plates were rinsed with tap water until all excess stain was removed, allowed to dry for 24 h, and then read on a Bio-Tek Instruments Microplate reader at 630 nm.

D. EBV Assays. 1. Viral Capsid Antigen (VCA) ELISA in Daudi Cells. a. Preparation of Infected Cells. Daudi cells at 1 × 10^6 were incubated with EBV stock virus obtained from P3HR-1 cells (ATCC, Manassas, VA) at a concentration sufficient to infect 10% of the cells. After incubation at 37 °C for 1 h, the cells were rinsed and pelleted by centrifugation. Appropriate concentrations of drug were added to infected cells in culture tubes and incubated for 3 days at 37 °C. Cells were then spotted onto slides or into 96-well plates and fixed with acetone or acetic acid/ethanol.

b. ELISA. An ELISA was performed on fixed Daudi cells by incubation first with a monoclonal antibody to EBV viral capsid antigen (VCA) (Chemicon, Temecula, CA) followed by an incubation with horseradish peroxidase-labeled goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL). The colorimetric reaction was initiated by addition of *o*-phenylenediamine dihydrochloride in citrate buffer (pH 5.0) and hydrogen peroxide, and the reaction was stopped by the addition of 3 N sulfuric acid and read on a microplate reader (Bio-Tek Instruments, Winooski, VT). The EC_{50} value for each drug was extrapolated from the plot of drug concentration versus

average OD_{492} for each concentration of drug. Acyclovir (ACV) was used as a positive control.

c. In Situ EBV DNA Hybridization Assay (Daudi Cells). The Simply Sensitive Horseradish Peroxidase-AEC In Situ Detection System (Enzo Diagnostics, Farmingdale, NY) was used to monitor EBV DNA synthesis in the presence of antiviral compounds and was performed according to the manufacturer's instructions. Cells were counted using a light microscope at a 400× magnification. The EC_{50} value for each drug was extrapolated from the plot of drug concentration versus the percentage of positive cells.

2. EBV DNA Hybridization Assay (H-1 Cells). The H-1 cells were maintained in a logarithmic phase of growth for 2 days prior to the initiation of treatment. They were seeded in 24-well plates at the density of 2×10^5 cells/well in fresh medium (2 mL) with or without drug and inucbated at 37 °C for 5 days. After treatment with drug, the cells were pelleted and the inhibitory effect of drug on EBV DNA was determined by slot-blot analysis as described previously.[1]

F. HBV. Chronically infected 2.2.15 cells were kindly provided by G. Acs (Mount Sinai Medical Center, New York, NY). Cells were maintained in minimal essential medium (MEM) supplemented with fetal bovine serum and incubated at 37 °C in a moist atmosphere containing CO_2 (5%). Cells were inoculated in tissue culture plates at a density of 5×10^4 cells/mL MEME in 12-well dishes. Medium was changed every 3 days after inoculation. On day 6 and day 9 the compounds studied were added to the medium. The cells were incubated with various concentrations of compounds for 6 days. The cells were washed with PBS and then they were lysed with 0.5 mL of 1% SDS, 10 mM TRIS pH 7.6, 10 mM EDTA, and 400 mM NaCl. The lysates were treated with 100 μg/mL of proteinase K at 50 °C for 3 h, and then they were extracted with phenol (1:1). The upper layer was diluted with isopropyl alcohol (1:1), and then it was kept frozen at −20 °C overnight. The samples were centrifuged at 10000 rpm for 20 min, and the pellet was washed with 70% ethanol. It was resuspended in TE (10 mM TRIS pH 8.1, 1 mM EDTA pH 8), RNase A (100 μg/mL) was added, and the samples were incubated at 37 °C for 1 h. The upper layer was diluted with 0.625 mL of isopropyl alcohol plus 84 μL of 3 M sodium acetate, and it was kept frozen at −20 °C overnight. The samples were centrifuged at 10000 rpm for 20 min, and the pellets were washed with 70% ethanol. They were resuspended in water and digested with Hind III at 37 °C overnight. The DNA concentration was determined spectrophotometrically, and 10 μg/well was loaded onto 1% agarose gel followed by blotting onto Hybond-N^+ membrane (Amersham). The blot was hybridized with a ^{32}P-labeled HBV DNA probe, and it was washed twice with standard saline citrate (SSC, 0.1% SDS) at room temperature for 15 min and three times at 65 °C for 10 min. The bands were subjected to autoradiography, and the bands were quantitated by a scanning densitometer (Molecular Dynamics).

Cytotoxicity Assays. Several different assays were used to explore cytotoxicity of selected compounds depending on the nature of the antiviral assay.

A. Visual Cytotoxicity. Cytotoxicity produced in stationary HFF, MEF, CEM, or CEM-SS cells from suspension cultures was determined by microscopic inspection of cells not affected by the virus used in plaque assays.[16]

B. KB Cell Growth. 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.[19] Briefly, 96-well cluster dishes were planted with KB cells, and following an overnight incubation, test compound was added in triplicate at eight concentrations. Plates were incubated for 48 h rinsed, fixed with 95% ethanol, and stained with crystal violet. Acidified ethanol was added, and plates were read at 570 nm in a spectrophotometer designed to read 96-well ELISA assay plates.

C. Cell Proliferation Assay. Twenty-fours hour prior to assay, cells (HFF or Daudi) were seeded in 6-well plates at a concentration of 2.5×10^4 cells per well in MEM containing 10% FBS. On the day of the assay, drugs were diluted serially in MEM containing 10% FBS at increments of 1:5 covering a range from 100 to 0.03 mg/mL. For drugs that had to be solubilized in DMSO, control wells receive MEM containing 10% DMSO. The media from the wells were then aspirated, and 2 mL of each drug concentration was added to each well. The cells were incubated in a CO_2 incubator at 37 °C for 72 h. At the end of this time, the media−drug solution was removed, and the cells were washed. Trypsin−EDTA (0.25%, 1 mL) was added to each well and incubated until the cells started to come off the plate. The cell−media mixture was then pipetted up and down vigorously to break up the cell suspension, and 0.2 mL of the mixture was added to 9.8 mL of Isoton III and counted using a Coulter Counter. Each sample was counted three times with two replicate wells per sample. The CC_{50} values were calculated using a computer program.

D. Neutral Red Uptake. Twenty-four hours prior to assay, HFF cells were plated into 96-well plates, the media then were aspirated, media containing drug were added to the first row of wells and diluted serially 1:5, and plates were incubated for 7 days. At this time, the media−drug was removed, cells were washed, and 200 mL/well of 0.01% neutral red in PBS was added and incubated for 1 h. The dye was removed, and the cells were washed using a Nunc Plate Washer. After the wash was removed, 200 mL/well of 50% ethanol/1% aqueous acetic acid was added. The plates were placed on a rotating shaker for 15 min and the optical densities then read at 540 nm using a plate reader.

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 (EC_{50}) concentrations were calculated from the regression lines. Samples containing positive controls as shown in the tables were used in all assays.

Acknowledgment. Our thanks are due to L. M. Hrihorczuk from the Central Facility, Department of Chemistry, Wayne State University (D. M. Coleman, Director) for mass spectra, and Benjamin Wei and Richard Spalding for preparation of some intermediates. We also thank Caroll Hartline, Stephanie Williams, Julie M. Breitenbach, and Kathy Z. Borysko for expert performance of antiviral and cytoxicity assays. The work described herein was supported by U.S. Public Health Service program project PO1-AI46390 (J.C.D., E.R.K., and J.Z.), contract NO1-AI85347 (E.R.K.) from the National Institute of Allergy and Infectious Diseases and grant RO1-CA44358 (Y.C.C.), National Cancer Institute, National Institutes of Health, Bethesda, MD.

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JM0205245