Haloanilino Derivatives of Pyrimidines, Purines, and Purine Nucleoside Analogs: Synthesis and Activity against Human Cytomegalovirus

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2-Anilinopurines and 6-anilinopyrimidines bearing 3,4- or 3,5-dichloro substituents in the anilino ring inhibited virus-specific DNA synthesis by human cytomegalovirus (HCMV)-infected human embryonic lung (HEL) cells in culture. In general, active compounds had moderate to low selectivity for viral vs host cell DNA synthesis. Nucleoside and acyclonucleoside analogs of 2-(3,5-dichloroanilino)purines inhibited both HCMV and cellular DNA synthesis at similar concentrations. 2-Amino-4-chloro-6-(3,5-dichloroanilino)pyrimidine and several related compounds inhibited HCMV growth in yield reduction assays at concentrations that were nontoxic to HEL cells.

Substituted 6-anilinopyrimidines and 2-anilinopurines are potent and selective inhibitors of DNA polymerases (reviewed in ref 1). Appropriately substituted 6-anilinouracils and N^2-phenylguanines act competitively with dGTP, and the corresponding 6-anilinoisocytosines and 2-anilinoadenines act competitively with dATP. The mechanism of inhibition, established in detail for bacterial DNA polymerase III[2] and eukaryotic DNA polymerase α,[3] involves base pairing of the inhibitors with pyrimidines in the DNA template and interaction of the anilino group and its substituents with a unique site on each enzyme. Thus, the uracil and guanine inhibitors mimic dGTP by their ability to base pair with dC, and the isocytosines and adenines mimic dATP by their ability to base pair with dT.[4]

The advent of immunosuppressive therapy and the spreading of acquired immunodeficiency syndrome (AIDS) have significantly increased the incidence of opportunistic infections by microorganisms. Human cytomegalovirus (HCMV) infections are prominent causes of retinitis in AIDS and other immunosuppressed patients, and drugs currently licensed for use in HCMV retinitis, i.e., ganciclovir and phosphonoformate, possess serious toxicities.[5] Other pathogenic human herpesviruses (herpes simplex virus types 1 and 2, varicella zoster virus) respond to acyclovir and other nucleoside analogs by virtue of the drugs' "activation" by the virus-encoded thymidine kinases (TK).[6] HCMV does not apparently encode a TK that will phosphorylate modified nucleosides; rather, an enzyme with homology to protein kinases has been suggested to phosphorylate ganciclovir.[7,8] Thus, attention has been drawn to the study of nucleotide analogs that bypass the requirement for virus-specific activation, for example, nucleoside phosphonates.

Our observations that anilino derivatives of pyrimidines and purines can be tailored to selectively inhibit DNA polymerases and that such compounds inhibit the growth of cells that utilize these enzymes for DNA replication and cell division prompted us to target the HCMV DNA polymerase for the design of analogous compounds that would selectively inhibit viral replication in host cells. We began by screening various guanine and adenine derivatives available in our laboratory for inhibition of HCMV-specific DNA synthesis in a dot–blot hybridization assay and for inhibition of the HCMV DNA polymerase *in vitro*. On the basis of the discovery of lead compounds, we have synthesized and tested related purine nucleoside analogs and pyrimidine bases. We have identified potential anti-HCMV compounds that do not, however, appear to act by inhibiting the viral DNA polymerase.

Chemistry

(Haloanilino)pyrimidines and -purines. The candidate uracil and isocytosine derivatives shown in Table 4 were synthesized by reaction of the corresponding 6-chloropyrimidines with the haloanilines in refluxing 2-methoxyethanol as previously described.[9] Use of equimolar amounts of the aniline and its hydrochloride salt with 6-chloroisocytosine in refluxing 2-methoxyethanol under nitrogen has improved considerably the recoverable yields of 6-anilinoisocytosines. The 4-chloropyrimidines **35** and **36** were prepared by heating the anilines with 4,6-dichloro-2-aminopyrimidine in 2-methoxyethanol.

2-(Haloanilino)purines were synthesized generally as described.[10] The guanine derivatives **1** and **2** were prepared by reacting the anilines with 2-bromohypoxanthine in refluxing 2-methoxyethanol. Treatment of the guanines with thionyl chloride in DMF, followed by stirring in concentrated ammonium hydroxide (to deformylate, see below), gave the 6-chloropurines **5** and **6**. Ammonolysis of the 6-chloropurines with ammonia in methanol gave the adenine derivatives **3** and **4**. The conditions for synthesis of all products and their properties are summarized in Table 1.

2-(3,5-Dichloroanilino)purine Nucleoside Analogs. We selected the 3,5-dichloro substitution pattern for nucleoside analog syntheses because the corresponding bases had the greatest selectivity of antiviral versus cytotoxic activity (see below). A common starting material, 2-(3,5-dichloroanilino)-6-chloropurine (**6**), was used

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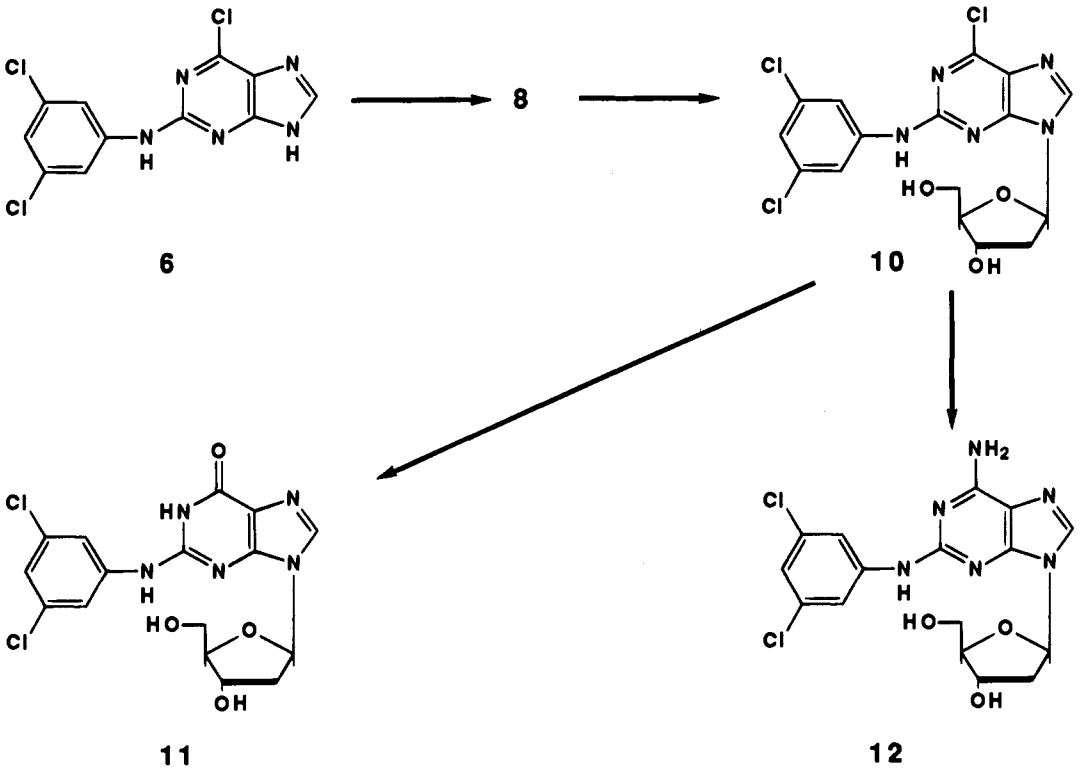
Table 1. Purines and Pyrimidines Synthesized[a]

compd	rx time (h)	yield (%)	mp (°C)	cryst solv	formula[b]	^{1}H NMR[c] 2-NH	8-H	6-NH	5-H	2-NH$_2$
2	30	71	>350	DMF	$C_{11}H_7N_5OCl_2 \cdot {}^1/_3H_2O$	9.04	7.86			
3	5	85	257–259	50% EtOH	$C_{11}H_8N_6Cl_2 \cdot H_2O$	9.20	7.89			
4	5	23	262–266	EtOH	$C_{11}H_8N_6Cl_2 \cdot {}^1/_3H_2O$	9.30	7.95			
5	16	69	286–289	DMF/H_2O	$C_{11}H_6N_5Cl_3 \cdot {}^1/_4H_2O$	10.14	8.31			
6	18	67	293–297	EtOH	$C_{11}H_6N_5Cl_3 \cdot {}^1/_3$EtOH	10.24	8.35			
22	18	47	336–338	75% HOAc	$C_{10}H_7N_3O_2ClF$			8.33	4.63	
23	60	44	337–339	HOAc	$C_{10}H_7N_3O_2ClF$			8.62	4.83	
24	20	63	331–334	75% HOAc	$C_{10}H_7N_3O_2F_2 \cdot H_2O$			8.35	4.68	
25	18	22	325–326	75% DMF	$C_{10}H_7N_3O_2Cl_2 \cdot {}^1/_4H_2O$			8.55	4.77	
26	4	44	349–351	75% HOAc	$C_{10}H_7N_3O_2F_2 \cdot 3/4H_2O$			8.67	4.91	
27	8	58	337–340	75% HOAc	$C_{10}H_7N_3O_2ClF \cdot {}^1/_8H_2O$			8.77	4.96	
28	2	55	274–276	HOAc	$C_{10}H_8N_4OCl_2 \cdot {}^1/_3H_2O$			8.94	4.85	6.50
29	4	40	242–244	25% EtOH	$C_{10}H_8N_4OClF \cdot {}^1/_2H_2O$			8.86	4.80	6.52
30	2.5	85	325–327	25% EtOH	$C_{10}H_8N_4OClF$			8.85	4.79	6.54
31	6	75	289–291	H_2O	$C_{10}H_8N_4OF_2$			8.83	4.79	6.48
32	6	56	278–280	75% EtOH	$C_{10}H_8N_4OCl_2 \cdot 1.5H_2O$			9.03	4.87	6.56
33	2.5	40	320–322	25% EtOH	$C_{10}H_8N_4OF_2$			9.08	4.86	6.44
34	2	44	293–296	75% EtOH	$C_{10}H_6N_4OClF \cdot {}^1/_4H_2O$			9.04	4.86	6.57
35	15	88	216–218	50% EtOH	$C_{10}H_7N_4Cl_3$			9.59	6.01	6.89
36	4	70	209–212	67% EtOH	$C_{10}H_7N_4Cl_3 \cdot {}^1/_3H_2O$			9.64	6.03	6.91

[a] Reaction conditions are given in the Experimental Section. [b] All agreed to within ±0.4% of calculated values except the following: **3**, calcd for C 42.19, found 42.75; **4**, calcd for N 27.91, found 27.32; **24**, calcd for H 3.53, found 3.02; **35**, calcd for N 19.34, found 18.77. [c] Spectra were obtained in Me_2SO-d_6, and all remaining ^{1}H resonances, and ^{19}F resonances where applicable, were as expected.

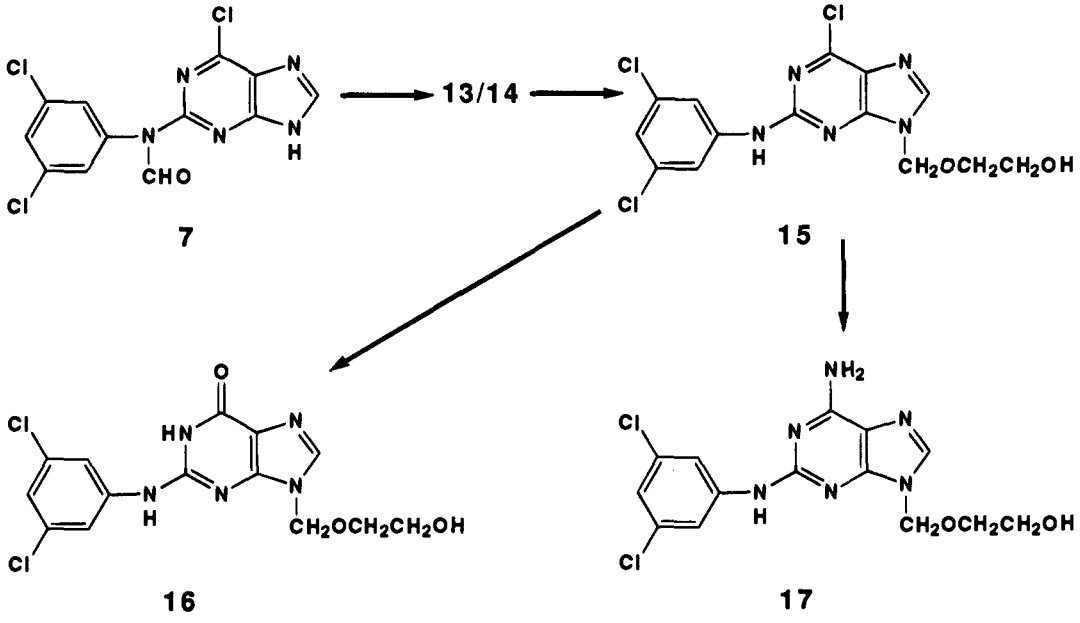
Scheme 1



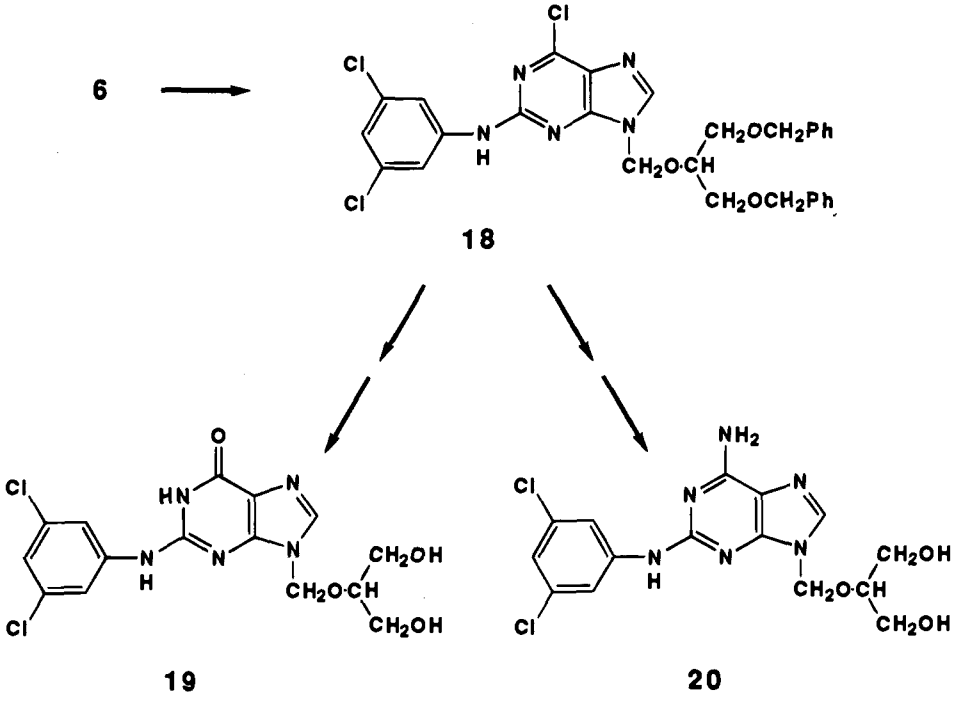
for these preparations, and the reactions were modeled on similar glycosylations and alkylations carried out in this and other laboratories.[10,11] The sodium salt of **6** was prepared with sodium hydride in acetonitrile and reacted with 2-deoxy-3,5-di-*p*-toluyl-α-D-ribofuranosyl chloride (Scheme 1) to give major 9–β (**8**) and minor 7–β (**9**) glycosylation products. Both products were β anomers based on the pseudotriplets observed for H-1′ in their ^{1}H NMR spectra and consistent with the established stereoselectivity of the sodium salt glycosylation reaction.[11] In this and the alkylation reactions described below, the predominant products were the 9-isomers, distinguished from the 7-isomers by characteristic ^{1}H NMR spectra—H-8 and N-CH resonances are typically downfield in 7- vs 9-isomers in related purine nucleoside analogs.[10,11] After deprotection of **8** to give the 6-chloro deoxyribonucleoside **10**, hydrolysis and ammonolysis yielded, respectively, the deoxyguanosine and deoxyadenosine derivatives **11** and **12** (Scheme 1).

Reaction of the sodium salt of **6** with (2-acetoxyethoxy)methyl bromide in acetonitrile gave low yields of alkylation products. However, higher yields were obtained when the N^2-formyl derivative of **6**, i.e., compound **7**, was used; the N^2-formyl 9-alkylated product **14** was isolated in 32.5% yield (Scheme 2), together with a small amount of the 7-isomer and the nonformylated 9-isomer **13**. The identity of **14** was based on the similarity of its ^{1}H NMR spectrum with that of **7**. In

Scheme 2



Scheme 3



both cases the downfield singlets (δ 9.75 and 9.68, respectively) were not exchangeable with D_2O, and the chemical shifts of H-2,6 and H-4 in the dichlorophenyl ring were reversed from the assignments in the nonformyl forms, **13** and **6**, respectively (see the Experimental Section). Deprotection of **14** to the 6-chloro acyclonucleoside **15**, followed by hydrolysis and ammonolysis, gave, respectively, the acycloguanosine and acycloadenosine derivatives **16** and **17** (Scheme 2).

Treatment of the sodium salt of **6** with [1,3-bis(benzyloxy)-2-propoxy]methyl chloride in DMF yielded 44% of the 9-alkylated intermediate **18**. Hydrolysis and ammonolysis of this compound, followed by debenzylation of the intermediates with boron trichloride in dichloromethane, gave, respectively, the guanine and adenine derivatives **19** and **20** bearing the 9-(1,3-dihydroxy-2-propoxy)methyl side chain characteristic of ganciclovir (Scheme 3).

Results

Several substituted N^2-phenylguanines and 2-anilinoadenines were tested for their effects on HCMV-specific DNA synthesis in cultures of virus-infected human embryonic lung (HEL) cells. A DNA hybridization assay in 96-well microtiter plates was used employing ^{32}P-labeled cloned viral DNA as probe to detect and quantitate viral DNA collected on nitrocellulose filters.[12] The assay was validated by testing known inhibitors of HCMV DNA replication under identical conditions. Both ganciclovir and phosphonoformate inhibited viral DNA synthesis with IC_{50} values (Table 2) close to those measured by plaque or yield reduction assays.[13,14] An

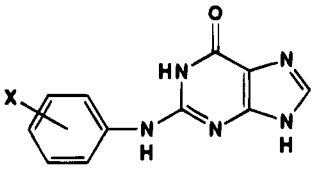
Table 2. Activity of 2-(Dichloroanilino)purines against HCMV and Cellular DNA Synthesis

compd	IC_{50} (μM)[a] HCMV DNA synth (dot–blot assay)	HEL cell DNA synth ([^{3}H]TdR incorp)
1	12	60
2	33	400
3	5	3.4
4	8	40
ganciclovir	0.5	7.0
phosphonoformate	15.5	nt

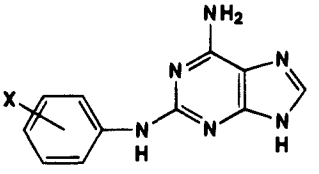
[a] IC_{50} values are concentrations which gave 50% inhibition of HCMV DNA synthesis in HCMV-infected HEL cells and 50% inhibition of [^{3}H]thymidine incorporation into the DNA of uninfected HEL cells. Details of assays are in the Experimental Section. nt, not tested.

initial estimate of cytotoxicity was obtained by measuring the effects of the compounds on cumulative [^{3}H]-thymidine uptake into the DNA of uninfected HEL cells during 24 h in culture.

N^2-Phenylguanine, 2-anilinoadenine, and several monosubstituted derivatives had no effect on HCMV DNA synthesis at 200 μM, the highest concentration tested (data not shown). Several (dihalophenyl)guanines, most notably 3,4-dichloro (**1**) and 3,5-dichloro (**2**) derivatives, did inhibit viral DNA synthesis with IC_{50} values of 12 and 33 μM, respectively (Table 2). The adenine derivatives **3** and **4** were even more potent, with IC_{50} values of 5 and 8 μM, respectively. However, the adenines appeared less selective than the guanines. This is especially apparent for the 3,4-dichloro substitution pattern, where the guanine derivative **1** was 5-fold more inhibitory to viral than host cell DNA synthesis but the adenine derivative **3** showed no selectivity (Table 2).



1, X=3,4-Cl_2



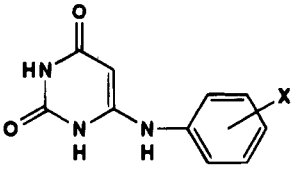
3, X=3,4-Cl_2

2, X=3,5-Cl_2

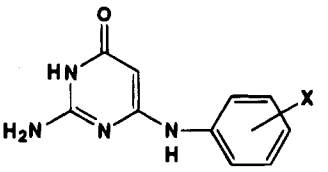
4, X=3,5-Cl_2

The 3,5-dichloro derivatives **2** and **4** were more selective than the 3,4-dichloro derivatives as inhibitors of HCMV DNA synthesis (Table 2). Therefore, we chose to prepare and test several purine nucleoside analogs with the 2-(3,5-dichloroanilino) substituent. Guanine and adenine derivatives substituted at the 9-position with 2-deoxyribofuranosyl, (2-hydroxyethoxy)methyl, and (1,3-dihydroxy-2-propoxy)methyl groups were prepared. In addition, we tested the 6-chloropurine synthetic intermediates **10** and **15** in the assays. The results of testing of these compounds (Table 3) showed little enhancement of anti-HCMV activity resulting from addition of the nucleoside or acyclonucleoside side chains to the bases, but higher apparent cytotoxicity was observed in most cases. Whereas the guanine derivatives (**11**, **16**, and **19**) inhibited both measures equally, the adenine derivatives (**12**, **17**, and **20**) were considerably more active as inhibitors of thymidine incorporation into HEL cell DNA than against viral DNA synthesis. The 6-chloro compounds (**10** and **15**) were nearly equally inhibitory to viral and host DNA synthesis. These results suggest that antiviral activity of most nucleoside analogs may be a result of cytotoxic effects (see Discussion).

Pyrimidine Derivatives. The inhibitory activity and moderate selectivity of the 2-(dihaloanilino)purine bases against HCMV DNA synthesis, and the lack of enhancement of that activity in the nucleoside analogs, suggested that structure–activity relationships to define the optimal anti-HCMV substituents could be obtained with simple base derivatives. Because the appropriately substituted pyrimidines were equivalent to the corresponding purines, at least for inhibition of DNA polymerases,[1] we prepared several series of 6-(polyhaloanilino)-pyrimidines, *viz.*, uracils, isocytosines, and 2-amino-4-chloropyrimidines. Effects of selected dihalo-substituted pyrimidine derivatives against viral and host DNA synthesis are summarized in Table 4. The 3,4-dichloro (**21**) and 3,5-dichloro (**25**) derivatives of 6-anilinouracil were selective inhibitors of viral DNA synthesis, with IC_{50} values of 40 and 25 μM, respectively. Difluoro or chlorofluoro derivatives of 6-anilinouracils were generally inactive. In contrast, all dihalo-substituted 6-anilinoisocytosines, e.g., **28** and **32**, showed activity against HCMV DNA synthesis, although with less selectivity than the uracils. Additional isomers of the (dihaloanilino)- and several (trihaloanilino)uracils and isocytosines, did not show enhanced activity or selectivity compared with the prototype dichloro analogs (data not shown). The 2-amino-4-chloropyrimidines **35** and **36** were the most potent inhibitors of viral DNA synthesis and had selectivities of about 3-fold and 6-fold, respectively, for viral vs host cell DNA synthesis.



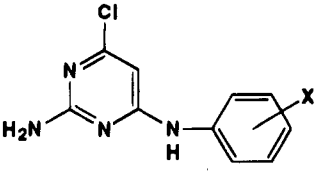
21, X=3,4-Cl_2



28, X=3,4-Cl_2

25, X=3,5-Cl_2

32, X=3,5-Cl_2

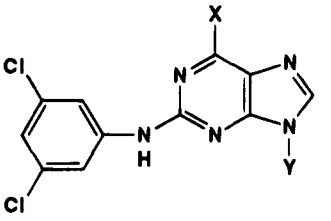


35, X=3,4-Cl_2

36, X=3,5-Cl_2

Antiviral Activity in Yield Reduction Assays. Anti-HCMV activity of representative compounds that inhibited HCMV DNA synthesis was measured in a yield reduction assay.[13] In this assay virus titers resulting from incubation of HCMV in HEL cells were determined by end-dilution titration in fresh HEL cell cultures. Sonicated aliquots of the first cultures were

Table 3. Effect of 2-(3,5-Dichloroanilino)purine Nucleoside Analogs on HCMV and HEL Cell DNA Synthesis



compd	X	Y	IC_{50} (μM)[a] HCMV DNA synth (dot–blot assay)	IC_{50} (μM)[a] HEL cell DNA synth ([^{3}H]TdR incorp)
		Guanines		
11	oxo	2-deoxy-β-D-ribofuranosyl	15.1	13
16	oxo	-$CH_2OCH_2CH_2OH$	15.2	14
19	oxo	-$CH_2OCH(CH_2OH)_2$	45	41.7
		Adenines		
12	NH_2	2-deoxy-β-D-ribofuranosyl	8.6	0.5
17	NH_2	-$CH_2OCH_2CH_2OH$	7.9	1.4
20	NH_2	-$CH_2OCH(CH_2OH)_2$	47.9	5.0
		6-Chloro Compounds		
10	Cl	2-deoxy-β-D-ribofuranosyl	7.9	3.4
15	Cl	-$CH_2OCH_2CH_2OH$	12.5	14.4

[a] Measured as described in the footnote to Table 2.

Table 4. Effect of 6-(Haloanilino)pyrimidines against HCMV and HEL Cell DNA Synthesis

compd	substituents	IC_{50} (μM)[a] HCMV DNA synth (dot–blot assay)	IC_{50} (μM)[a] HEL cell DNA synth ([^{3}H]TdR incorp)
	6-Anilinouracils		
21	3,4-dichloro	40	>200
22	3-chloro 4-fluoro	140	>200
23	3-fluoro 4-chloro	>200	>200
24	3,4-difluoro	>200	>200
25	3,5-dichloro	25	100
26	3,5-difluoro	>200	>200
27	3-chloro 5-fluoro	>200	>200
	6-Anilinoisocytosines		
28	3,4-dichloro	25	32
29	3-chloro 4-fluoro	20	110
30	3-fluoro 4-chloro	70	165
31	3,4-difluoro	115	>200
32	3,5-dichloro	28	95
33	3,5-difluoro	63	>200
34	3-chloro 5-fluoro	>200	>200
	2-Amino-4-chloropyrimidines		
35	3,4-dichloro	9.1	31
36	3,5-dichloro	4.7	32

[a] Measured as described in the footnote to Table 2.

added in several dilutions to fresh HEL cells, and after incubation for 14 days, the virus titers, i.e., the highest dilution where virus infection resulted in enumerable plaques, were read.

Data for representative dichloroanilino compounds and ganciclovir are displayed in the plot of Figure 1. The results are summarized in Table 5 as the concentration of compounds that inhibited virus yield by 90% (IC_{90}). Ganciclovir inhibited virus yield in this assay with an average IC_{90} of 2.1 ± 0.8 μM (n = 3), compared with the reported value of 1 μM.[13] The dichloroanilino compounds tested had direct anti-HCMV activity in the yield reduction assay, and the IC_{90} values closely paralleled their relative potencies from the dot–blot hybridization assays. In particular, 2-amino-4-chloro-6-(3,4-dichloroanilino)pyrimidine (**35**) inhibited virus yield with IC_{90} = 8.7 ± 1.3 μM (n = 2), a concentration that was not strongly inhibitory to thymidine incorporation by uninfected HEL cells (Table 4).

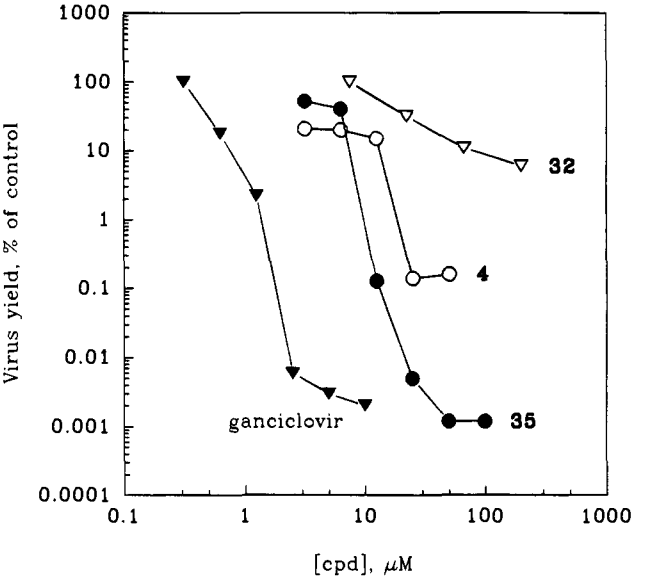


Figure 1. HCMV yield reduction assays. Virus yields from HCMV-infected HEL cell cultures incubated in the presence of compounds were determined on fresh HEL cell cultures as described in the Experimental Section: ▼, ganciclovir; ●, **35**; ○, **4**; ▽, **32**.

Table 5. HCMV Yield Reduction Assays

compd	IC_{90} (μM)[a]
2	56
4	16.8
25	69
32	91
35	8.7
36	18.0
ganciclovir	2.1[b]

[a] Concentration that reduced HCMV yield by 90% relative to control cultures without drugs (see the Experimental Section). Results of duplicate assays were typically ±(15–20)% of reported values. [b] ±0.8 μM (n = 3).

Table 6. Comparison of the Effects of (Dichloroanilino)pyrimidines and -purines on HCMV DNA Polymerase and HCMV DNA Synthesis in Virus-Infected Cells

	IC_{50} (μM)	
compd	DNA polymerase[a]	DNA synthesis[b]
1	330	12
4	250	5
2	140	33
21	45	40
25	65	25
28	360	25
32	175	28
35	150	9.1

[a] Isolated from HCMV-infected HEL cells and assayed with activated DNA and [^{3}H]dTTP, dCTP, dATP, and dGTP. Assay conditions are described in the Experimental Section. IC_{50}s are the average values from duplicate experiments. [b] From Tables 2 and 4.

Results of the yield reduction assays were corroborated for several compounds in a standard plaque reduction assay. Compound **35**, for example, gave IC_{50} = 8.9 μM in the plaque reduction assay compared with a value of 2.6 μM for ganciclovir.

Evaluation of HCMV DNA Polymerase as a Target for (Dihaloanilino)purines and -pyrimidines. We isolated and characterized the HCMV-specific DNA polymerase for the purpose of targeting N^2-phenylguanines, 2-anilinoadenines, and related compounds against this enzyme. The viral enzyme, isolated following reported procedures,[15,16] was shown to be distinct from a typical eukaryotic DNA polymerase α by its stimulation by 100 mM potassium chloride and its lack of reactivity toward a monoclonal antibody to DNA polymerase α (see the Experimental Section). In addition, the viral polymerase was more sensitive to inhibition by phosphonoformate and ganciclovir triphosphate but less sensitive to N^2-(*p*-*n*-butylphenyl)dGTP than DNA polymerase α.

Although numerous compounds tested, including the dichloroanilino derivatives of purines and pyrimidines, had weak inhibitory activity against HCMV DNA polymerase, their potencies did not ultimately correlate with anti-HCMV activity found in the dot–blot hybridization assays (Table 6). Moreover, kinetic analysis of several compounds indicated that inhibition was noncompetitive with respect to the base-analogous 2′-deoxyribonucleoside 5′-triphosphate, in contrast to the competitive kinetics expected from the precedents for related DNA polymerase inhibitors.[1] With the possible exception of the uracil derivatives **21** and **25**, antiviral potency was greater than polymerase inhibitory potency for all compounds shown in Table 6.

Discussion

(Haloanilino)purines and -pyrimidines inhibit HCMV DNA synthesis in infected cell cultures but with little selectivity compared to their effects on cellular DNA synthesis (Tables 2 and 4). Compounds with 3,4- and 3,5-dihalo substitution patterns were more active than those with other substitution patterns. Several derivatives such as **35** and **36** had antiviral activity in HCMV yield reduction assays (Table 5) and plaque reduction assays at concentrations that were not strongly cytotoxic. Nucleoside analogs of (3,5-dichloroanilino)purines did not have increased antiviral activity but, especially for the adenine derivatives, were strongly inhibitory to cellular DNA synthesis (Table 3).

The most potent anti-HCMV compounds in yield reduction assays were dichloroanilino derivatives of adenine and 2-amino-4-chloropyrimidine (Table 5), and effective concentrations were not strongly cytotoxic. Interestingly, simple dichloropyrimidines have been shown to have antiviral activity against unrelated RNA and DNA viruses including poliovirus, herpes simplex virus type 1, and vaccinia virus.[17] One of the most potent compounds, 2-amino-4,6-dichloropyrimidine, was found to inhibit assembly of poliovirus proteins into procapsids, but did not inhibit viral RNA synthesis. 2-Amino-4,6-dichloropyrimidine was effective in treatment of herpes keratitis in rabbits.[17]

Antiviral activity of the dihaloanilino compounds is unlikely to result from inhibition of the HCMV DNA polymerase (Table 6). However, the findings that identical compounds inhibit the helicase–primase from the related herpes simplex type 1 virus and possess moderate inhibitory activity toward viral DNA synthesis in HSV1-infected cells (see the following paper) lead us to suggest that a HCMV-specific helicase–primase may be the target for these compounds. We have identified sequences in the HCMV genome that are homologous to the corresponding HSV1 helicase and primase genes. Cloning and expression of the putative HCMV helicase-primase genes are in progress in order to evaluate this enzyme as a novel target for anti-HCMV compounds.

HEL cell proliferation studies showed that, for most active compounds, inhibition of cell growth and [^{3}H]-thymidine incorporation occurred with approximately the same IC_{50} (data not shown). At IC_{50} concentrations, the drug effect was cytostatic because cell viability was not decreased after prolonged exposure to compounds, as measured by inability of cells to take up trypan blue. Compound **36**, for example, reduced viability of HEL cells by only 15% after incubation for 7 days in the presence of 50 μM drug. As a preliminary measure of *in vivo* toxicity, mice were treated daily for 7 days with intraperitoneal injections of **26** (52 mg/kg) or **36** (28 mg/kg) as suspensions in poly(ethylene glycol) (PEG 6000). No signs of toxicity or weight loss were observed with either drug during the treatment period.

Experimental Section

General. Melting points were determined on a Mel-temp apparatus and are uncorrected. NMR spectra were taken on Bruker AC200 or Varian Unity 300 instruments. ^{1}H chemical shifts are reported relative to internal tetramethylsilane (δ = 0), and ^{19}F chemical shifts were measured relative to $CFCl_3$ (δ = 0). Analyses for C, H, and N were done by the Microanalysis Laboratory, University of Massachusetts, Amherst, MA, and agree to within ±0.4% of calculated values unless otherwise noted. 3-Chloro-5-fluoroaniline was prepared from 1-chloro-3-fluorobenzene as described.[18] All other chemicals and reagents were available from commercial sources. Preparative HPLC employed a Waters model 6000 pump and differential refractometer detector and a Altex 200 injector.

Purine and Pyrimidine Bases. Reaction times, yields, and properties of anilinopurine and -pyrimidine bases are summarized in Table 1. N^2-(3,5-Dichlorophenyl)guanine (**2**) was prepared by heating 2-bromohypoxanthine and 3,5-dichloroaniline in 2-methoxyethanol at reflux.

The 6-chloropurines **5** and **6** were prepared by reaction of the guanines with thionyl chloride in dry DMF at room temperature. The solution was poured into 10% sodium bicarbonate, and the precipitate was collected and stirred in

concentrated ammonium hydroxide for 3 h. After neutralization with glacial acetic acid, the suspension was filtered to give the products. 2-(*N*-Formyl-3,5-dichloroanilino)-6-chloropurine (**7**) was obtained when the ammonolysis step above was omitted. Crystallization from ethanol afforded 59% of **7**, mp >350 °C: NMR (Me_2SO-d_6) δ 9.68 (s, 1H, CHO), 8.55 (s, 1H, H-8), 7.70 (t, 1H, Ph H-4, J = 1.8 Hz), 7.52 (d, 2H, Ph H-2,6 J = 1.8 Hz). Anal. ($C_{12}H_6N_5OCl_3 \cdot H_2O$) C,H; N: calcd, 19.59; found, 19.15.

2-Anilinoadenines **3** and **4** were obtained by ammonolysis of **5** and **6**, respectively. Solutions of the 6-chloropurines in methanol saturated with ammonia were heated to 120 °C in a bomb. After dilution with water, the products were collected by filtration. 6-Anilinouracils (**22**–**27**) were synthesized by heating 6-chlorouracil with 2 equiv of the appropriate aniline in 2-methoxyethanol at reflux. After dilution with water and chilling, the crude products were collected by filtration. 6-Anilinoisocytosines (**28**–**34**) were synthesized by heating 6-chloroisocytosine with equimolar amounts of the appropriate aniline and the aniline hydrochloride. The mass was mixed with water, and the crude product was filtered. Compounds **35** and **36** were prepared by heating 2-amino-4,6-dichloropyrimidine with the anilines in 2-methoxyethanol at reflux.

2-(3,5-Dichloroanilino)-6-chloro-9-(2-deoxy-3,5-di-*p*-toluyl-β-D-ribofuranosyl)purine (8) and Its 7-Isomer 9. A suspension of 2-(3,5-dichloroanilino)-6-chloropurine, **6** (1.50 g, 4.77 mmol), and sodium hydride (50% in mineral oil; 240 mg, 5.0 mmol) in dry acetonitrile (200 mL) was stirred at room temperature for 30 min. 2-Deoxy-3,5-di-*p*-toluyl-α-D-ribofuranosyl chloride (1.90 g, 4.89 mmol) was added in small portions during 20 min, and stirring was continued for 10 min. The mixture was diluted with chloroform (200 mL) and filtered through Celite. The filtrate was evaporated to dryness and the residue chromatographed on a silica gel column with chloroform as eluent. All fractions containing the major components were combined and the solvents removed *in vacuo*. The two major products were separated by HPLC on a silica gel column (2.5 × 50 cm, Rainin) employing 7% acetone in toluene as mobile phase. The first product was crystallized from chloroform/methanol (4:1) to give 1.10 g (46%) of the 9-isomer **8**, mp 230–231 °C: NMR ($MeSO_2$-d_6) δ 10.43 (s, 1H, 2-NH), 8.56 (s, 1H, H-8), 7.95, 7.77, 7.37, 7.25 (d, 2H each, Tol-H), 7.85 (d, 2H, 2-Ph H-2,6, J = 1.8 Hz), 7.08 (t, 1H, 2-Ph H-4, J = 1.8 Hz), 6.54 (t, 1H, H-1′), 5.81 (m, 1H, H-3′), 4.63 (m, 1H, H-4′), 4.86–4.49 (m, 2H, H-5′,5″), 2.89 (m, H-2′,2″), 2.41, 2.36 (s, 3H, CH_3). Anal. ($C_{32}H_{26}N_5O_5Cl_3 \cdot 0.5\ H_2O$) C,H,N.

The second product was crystallized from chloroform/methanol (4:1) to give 0.29 g (12%) of the 7-isomer **9** as off-white crystals, mp 118–120 °C: NMR (Me_2SO-d_6) δ 10.28 (s, 1H, 2-NH), 8.95 (s, 1H, H-8), 7.95, 7.75, 7.38, 7.27 (d, 2H each, Tol-H), 7.92 (s, 2H, 2-Ph H-2,6), 7.13 (s, 1H, 2-Ph H-4), 6.77 (t, 1H, H-1′), 5.72 (m, 1H, H-3′), 4.65 (m, 1H, H-4′), 4.55 (m, 2H, H-5′,5″), 3.18 (m, 1H, H-2′), 2.93 (m, 1H, H-2″), 2.41, 2.35 (s, 3H, CH_3). Anal. ($C_{32}H_{26}N_5O_5Cl_3 \cdot 0.5\ H_2O$) C,H,N.

2-(3,5-Dichloroanilino)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine (10). A mixture of **8** (600 mg, 0.75 mmol) and sodium methoxide in methanol (1 M, 0.5 mL) was added to methanol (20 mL) and stirred at room temperature for 4 h. The solution was brought to pH 5 with acetic acid and evaporated to dryness. The residue was chromatographed on a silica gel column (2 × 30 cm). Elution with 10% methanol in chloroform provided the product which was crystallized from aqueous methanol to give 340 mg (87%) of **10**, mp 216–217 °C: NMR (Me_2SO-d_6) δ 10.40 (s, 1H, 2-NH), 8.57 (s, 1H, H-8), 7.85 (d, 2H, Ph H-2,6), 7.15 (d, 1H, Ph H-4), 6.32 (dd, 1H, H-1′), 5.33, 4.90 (OHs), 4.39 (m, 1H, H-3′), 3.87 (m, 1H, H-4′), 3.60–3.37 (m, 2H, H-5′,5″), 2.79 (m, 1H, H-2′), 2.36 (m, 1H, H-2″). Anal. ($C_{16}H_{14}N_5O_3Cl_3 \cdot 0.25H_2O$) C,H; N: calcd, 16.09; found, 15.58.

***N*²-(3,5-Dichlorophenyl)-2′-deoxyguanosine (11).** 2-Mercaptoethanol (0.6 mL) and sodium methoxide (1 M in methanol, 2.0 mL) were added to a solution of **10** (200 mg, 0.46 mmol) in ethanol (30 mL). After heating at reflux for 60 h, an equal volume of water was added and the solution was brought to pH 5 with acetic acid. After storing in the freezer the colorless precipitate was isolated by filtration and crystallized from water to give 130 mg (68%) of **11**, mp >350 °C: NMR (Me_2SO-d_6) δ 10.92 (s, 1H, H-1), 9.18 (s, 1H, 2-NH), 8.10 (s, 1H, H-8), 7.69 (s, 2H, Ph H-2,6), 7.24 (s, 1H, Ph H-4), 6.19 (t, 1H, H-1′), 4.35 (m, 1H, H-3′), 3.85 (m, 1H, H-4′), 3.50 (m, 2H, H-5′,5″), 2.69 (m, 1H, H-2′), 2.29 (m, 1H, H-2″). Anal. ($C_{16}H_{15}N_5O_4Cl_2 \cdot 0.5\ H_2O$) C,H,N.

2-(3,5-Dichloroanilino)-2′-deoxyadenosine (12). A solution of **10** (90 mg, 0.21 mmol) in methanol saturated with ammonia (18 mL) was heated in a bomb at 120 °C for 10 h. The solvent was removed *in vacuo*, and flash chromatography of the residue on silica gel (10% methanol in chloroform) afforded 60 mg (70%) of **12** as a colorless solid, mp 194–196 °C: NMR (Me_2SO-d_6) δ 9.38 (s, 1H, 2-NH), 8.13 (s, 1H, H-8), 7.92 (s, 2H, Ph H-2,6), 7.16 (s, 2H, NH_2), 6.98 (s, 1H, Ph H-4), 6.25 (t, 1H, H-1′), 5.27 (d, 1H, 3′-OH), 4.87 (t, 1H, 5′-OH), 4.38 (m, 1H, H-3′), 3.83 (m, 1H, H-4′), 3.54 (m, 2H, H-5′,5″), 2.74 (m, 1H, H-2′), 2.27 (m, 1H, H-2″). Anal. ($C_{16}H_{16}N_6O_3Cl_2$) C, H, N.

2-(3,5-Dichloroanilino)-6-chloro-9-[(2-acetoxyethoxy)methyl]purine (13) and 2-(*N*-Formyl-3,5-dichloroanilino)-6-chloro-9-[(2-acetoxyethoxy)methyl]purine (14). A suspension of **7** (1.3 g, 3.8 mmol) and sodium hydride (50% in mineral oil; 93 mg, 3.88 mmol) in dry acetonitrile (150 mL) was stirred at room temperature for 30 min. (2-Acetoxyethoxy)methyl bromide (765 mg, 3.88 mmol) was added in portions during 20 min, and stirring was continued for 2 h. The mixture was diluted with chloroform (160 mL) and filtered through Celite. The filtrate was evaporated to dryness, and the residue was chromatographed on a silica gel column in 5% methanol in chloroform. Fractions containing the major components were combined, the solvent was removed *in vacuo*, and the mixture was separated by HPLC on a silica gel column (2.5 × 50 cm) using 7% acetone in toluene as mobile phase. The first product was crystallized from chloroform/methanol to give 22 mg (1.3%) of **13** as colorless crystals, mp 195–197 °C: NMR δ 10.46 (s, 1H, 2-NH), 8.53 (s, 1H, H-8), 7.91 (s, 2H, Ph H-2,6), 7.16 (s, 1H, Ph H-4), 5.62 (s, 2H, 9-CH_2), 4.10, 3.80 (m, 2H, OCH_2), 1.92 (s, 3H, CH_3). Anal. ($C_{16}H_{14}N_5O_3Cl_3$) C,H,N.

The *N*-formyl derivative **14** (565 mg, 32.5%), obtained after evaporation of the solvent, was crystallized from EtOH/H_2O, mp 125–127 °C. NMR δ 9.75 (s, 1H, CHO), 8.72 (s, 1H, H-8), 7.71 (s, 1H, Ph H-4), 7.52 (s, 2H, Ph H-2,6), 5.57 (s, 2H, 9-CH_2), 4.00, 3.67 (m, 2H, OCH_2), 1.94 (s, 3H, CH_3). Anal. ($C_{17}H_{14}N_5O_4Cl_3 \cdot H_2O$) C,H,N.

A third product, presumably the *N*-formyl 7-isomer (77 mg, 4.4%), precipitated upon concentration of the solvents: NMR δ 9.69 (s, 1H, CHO), 8.95 (s, 1H, H-8), 7.72 (s, 1H, Ph H-4), 7.50 (s, 2H, Ph H-2,6), 5.80 (s, 2H, 9-CH_2), 4.06, 3.67 (m, 2H, OCH_2), 1.91 (s, 3H, CH_3).

2-(3,5-Dichloroanilino)-6-chloro-9-[(2-hydroxyethoxy)methyl]purine (15). A mixture of **14** (350 mg, 0.76 mmol) and methanolic sodium methoxide (1 M, 0.5 mL) in methanol (20 mL) was stirred at room temperature for 1 h. Acetic acid was added to bring the solution to pH 5. After evaporation of the solvent to dryness, the residue was chromatographed on a silica gel column (2 × 30 cm). Elution with 20% isopropyl alcohol in chloroform provided the product which was retrieved by evaporation and crystallized from ethanol/hexane to give **15** as fine crystals (192 mg, 65%), mp 245–246 °C: NMR δ 10.46 (s, 1H, 2-NH), 8.53 (s, 1H, H-8), 7.92 (s, 2H, Ph H-2,6), 7.16 (s, 1H, Ph H-4), 5.61 (s, 2H, 9-CH_2), 3.62, 3.51 (m, 2H, OCH_2). Anal. ($C_{14}H_{12}N_5O_2Cl_3$) C,H,N.

***N*²-(3,5-Dichlorophenyl)-9-[(2-hydroxyethoxy)methyl]guanine (16).** A mixture of **15** (55 mg, 0.14 mmol), 2-mercaptoethanol (1.5 mL), and methanolic sodium methoxide (1 M, 2.0 mL) in anhydrous ethanol (20 mL) was heated at reflux for 65 h. An equal volume of water was added, and the solution was brought to pH 5 with acetic acid. After storing in the freezer for 40 h, the crystalline product was collected by filtration and washed with water to give 32 mg (61%) of **16**, mp >350 °C: NMR δ 10.93 (s, 1H, H-1), 9.20 (s, 1H, 2-NH), 8.02 (s, 1H, H-8), 7.75 (s, 2H, Ph H-2,6), 7.23 (s, 1H, Ph H-4),

5.47 (s, 2H, 9-CH_2), 4.67 (s, 1H, OH), 3.59–3.49 (m, 4H, 2 × OCH_2). Anal. ($C_{14}H_{13}N_5O_3Cl_2 \cdot 0.5H_2O$) C,H,N.

2-(3,5-Dichloroanilino)-9-[(2-hydroxyethoxy)methyl]-adenine (17). A solution of **15** (60 mg, 0.15 mmol) in methanol saturated with ammonia (20 mL) was heated in a bomb at 120 °C for 10 h. The solvent was removed under reduced pressure, and flash chromatography of the residue on silica gel with 10% methanol in chloroform gave 40 mg (74%) of **17** as a colorless solid, mp 226–227 °C: NMR δ 9.44 (s, 1H, 2-NH), 8.07 (s, 1H, H-8), 7.99 (s, 2H, Ph H-2,6), 7.18 (s, 2H, NH_2), 6.99 (s, 1H, Ph H-4), 5.49 (s, 2H, 9-CH_2), 4.66 (t, 1H, OH), 3.58, 3.50 (m, 2H, OCH_2). Anal. ($C_{14}H_{14}N_6O_2Cl_2$) C,H,N.

2-(3,5-Dichloroanilino)-6-chloro-9-[[1,3-bis(benzyloxy)-2-propoxy]methyl]purine (18). Sodium hydride (240 mg of 50% suspension in mineral oil, 5 mmol) was added to a solution of 2-(3,5-dichloroanilino)-6-chloropurine, **6** (1.3 g, 4.14 mmol), in dry *N,N*-dimethylformamide (DMF) (30 mL) at room temperature. After stirring for 45 min, a solution of [1,3-bis-(benzyloxy)-2-propoxy]methyl chloride (1.4 g, 4.5 mmol) in dry DMF (25 mL) was added during 25 min, and the reaction mixture was stirred at room temperature for 1.5 h. Chloroform (100 mL) was added, and the resulting suspension was filtered through Celite. The Celite was washed with chloroform (2 × 25 mL), and the combined filtrates were dried by passing through phase separation paper (Whatman 1-PS). After removal of solvent at reduced pressure, the orange oil was flash chromatographed on silica gel with chloroform. Fractions containing the major component were combined, and the residue obtained after removal of solvent was purified by HPLC on a silica gel column (2.5 × 50 cm, Rainin) employing 10% acetone in toluene as mobile phase. Evaporation of fractions containing the major peak under reduced pressure gave 1.1 g (44%) of **18** as a colorless solid, mp 86–88 °C: NMR δ 10.41 (s, 1H, 2-NH), 8.50 (s, 1H, H-8), 7.89 (s, 2H, Ph H-2,6), 7.12–7.26 (m, 10H, BnH), 7.09 (s, 1H, Ph H-4), 5.71 (s, 2H, 9-CH_2), 4.37 (s, 4H, $BnCH_2$), 4.11 (m, 1H, CH), 3.43–3.50 (m, 4H, O-CH_2-C). Anal. ($C_{29}H_{26}N_5O_3Cl_3 \cdot {}^1/_4H_2O$) C,H,N. Minor products were not isolated.

N^2-(3,5-Dichlorophenyl)-9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine (19). A solution of **18** (1.0 g, 1.47 mmol) in methanol (20 mL) containing 0.8 mL of 2-mercaptoethanol and 8 mL of 1 N sodium methoxide in methanol was heated at reflux for 30 h. After concentration of the solution to 2 mL and dilution with water (10 mL), the resulting precipitate was collected by filtration and crystallized from methanol to give 0.50 g (52%) of N^2-(3,5-dichlorophenyl)-9-[[1,3-bis(benzyloxy)-2-propoxy]methyl]guanine as a colorless solid, mp 138–141 °C: NMR δ 10.94 (s, 1H, H-1), 9.19 (s, 1H, 2-NH), 8.00 (s, 1H, H-8), all other resonances as expected.

A solution of boron trichloride in dichloromethane (3.5 mL of 1 M) was added slowly to a solution of this intermediate (0.35 g, 0.6 mmol) in dichloromethane (18 mL) at −70 °C under a nitrogen atmosphere. The temperature was not allowed to exceed −55 °C. After the solution had stirred for 1 h, excess triethylamine was added and the mixture was evaporated under reduced pressure. The residual orange solid was crystallized from 33% methanol (decolorizing carbon) and recrystallized from water to give 0.11 g (46%) of **19** as a colorless solid, mp >350 °C: NMR δ 10.91 (s, 1H, H-1), 9.19 (s, 1H, 2-NH), 8.00 (s, 1H, H-8), 7.73 (s, 2H, Ph H-2,6), 7.22 (s, 1H, Ph H-4), 5.55 (s, 2H, 9-CH_2), 4.56 (t, 2H, OH), 3.60 (m, 1H, CH), 3.44–3.50 (m, 4H, O-CH_2-C). Anal. ($C_{15}H_{15}N_5O_4Cl_2 \cdot {}^1/_4H_2O$) C,H,N.

2-(3,5-Dichloroanilino)-9-[(1,3-dihydroxy-2-propoxy)-methyl]adenine (20). A solution of **18** (400 mg, 0.67 mmol) in methanol saturated with ammonia (20 mL) was heated in a bomb at 120 °C for 10 h. The solvent was removed under reduced pressure, and flash chromatography of the residue on silica gel with 10% methanol in chloroform provided 360 mg (93%) of 2-(3,5-dichloroanilino)-9-[[1,3-bis(benzyloxy)-2-propoxy]methyl]adenine as a pale yellow solid, mp 145–147 °C. NMR δ 9.42 (s, 1H, 2-NH), 8.04 (s, 1H, H-8), 7.24 (b s, 2H, NH_2), all other resonances as expected.

A solution of boron trichloride in methylene chloride (2 mL of 1 N) was added to a solution of this intermediate (200 mg, 0.34 mmol) in methylene chloride (20 mL) at −60 °C under argon at such a rate that the temperature did not exceed −45 °C. After stirring for 1 h excess triethylamine was added, and the solvent was evaporated under reduced pressure. The orange residue was flash chromatographed on silica gel in 10% methanol in chloroform to give 60 mg (44%) of **20** as a colorless solid, mp 206–208 °C: NMR δ 9.42 (s, 1H, 2-NH), 8.04 (s, 1H, H-8), 7.98 (s, 2H, Ph H-2,6), 7.16 (b s, 2H, NH_2), 6.98 (s, 1H, Ph H-4), 5.58 (s, 2H, 9-CH_2), 4.57 (t, 2H, 2OH), 3.60 (m, 1H, CH), 3.48 (m, 4H, O-CH_2-C). Anal. ($C_{15}H_{16}N_6O_3Cl_3$) C,H,N.

Isolation of HCMV DNA Polymerase. Following a reported procedure,[15] HEL cells were infected with HCMV (strain AD169) at a multiplicity of infection (MOI) of 2. After 3 days in culture, the cells (10 g) were lysed in 30 mL of 0.2 M potassium phosphate, pH 7.2, containing 1 mM dithiothreitol, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 2 mM benzamidine, and 1 mM aminoacetonitrile, for 10 min on ice. After sonication (10 × 10 s) the high speed supernatant (60000*g*, 1 h) was dialyzed (20 mM potassium phosphate buffer, pH 7.2, containing 20% glycerol and 2 mM 2-mercaptoethanol) and adsorbed to a phosphocellulose column (2 × 20 cm). Proteins were eluted with a gradient of 20–400 mM potassium phosphate. Two peaks of DNA polymerase activity, at 0.16–0.28 and 0.30–0.35 mM buffer, were detected. Enzyme activity in the first peak was inhibited about 3-fold, but that in the second, larger peak was stimulated about 2-fold, by 200 mM potassium chloride. The latter peak was passed through a second identical phosphocellulose column and, with a sodium chloride gradient, through a DEAE–cellulose column. The peak fractions containing enzyme activity, eluting at 0.12–0.18 M sodium chloride, were dialyzed as above and concentrated by dialysis against 20 mM potassium phosphate, pH 7.2, containing 50% glycerol and 1 mM DTT. Aliquots were stored at −70 °C.

DNA Polymerase Assays. The standard reaction for HCMV DNA polymerase was done in 40 mM Tris-HCl, pH 7.8, containing 8 mM $MgCl_2$, 100 mM KCl, 4 mM 2-mercaptoethanol, 0.1 mM each of dATP, dGTP, dCTP, and [^{3}H]dTTP (0.5 μCi/mmol), 10 μg of activated calf thymus DNA, and the enzyme fraction (2 μL) in a final volume of 40 μL. Reactions were incubated at 37 °C for 60 min. The activity of the enzyme was linear for 2 h (data not shown). DNA polymerase α from calf thymus was isolated and assayed as described.[19] Procedures for isolation and counting of acid-precipitable material have been described.[4]

Antiviral Assay Techniques. Dot–Blot Hybridization Assay. Compounds were screened for antiviral activity by a DNA hybridization assay[12] in 96-well microtiter plates. Human embryonic lung (HEL) cells were seeded in minimal Eagle's medium (MEM) plus 10% fetal calf serum (FCS) in 200 μL volumes and infected with HCMV AD169 at about 1 plaque forming unit (pfu)/cell. After 60–90 min, the medium was removed and replaced by fresh medium containing drugs or drug diluent (DMSO). Contents of each pair of wells were diluted 3-fold to result in four to six concentrations of each drug in duplicate wells. After incubation at 37 °C in air plus 5% CO_2 for 48–96 h, the cells were lysed by incubation of the contents of each well with 100 μL of a solution of 3% sodium dodecyl sulfate (SDS), 3 mg/mL pronase, and 50 mM EDTA in 50 mM Tris-HCl, pH 7.4, for 1 h at 37 °C. Four microliters of each of the supernatants was spotted on a replica nitrocellulose sheet, and the sheet was dried and laid successively on filter papers saturated with (1) 0.5 N sodium hydroxide, 1.5 M sodium chloride, (2) 1 M Tris-HCl, pH 6.5, 0.5 M sodium chloride, (3) 0.5 mM Tris-HCl, pH 7.5, 1.5 M sodium chloride; the sheet was then baked in an oven at 80 °C for 1 h. The sheet was prehybridized in a plastic freezer bag in 50% formamide in 0.75 M sodium chloride and 75 mM sodium citrate (SSC), 5× Denhardt's solution (0.1% aqueous Ficoll, polyvinylpyrrolidone and bovine serum albumin), and 0.5 mg/mL salmon sperm DNA, for 10 min at 42 °C. The paper was hybridized by soaking in a solution containing cloned HCMV DNA (*Eco*RI F fragment of HCMV strain AD169, a gift from Bernhard Fleckenstein[20]) labeled to high specific activity by nick-translation with [^{32}P]dCTP as described.[21] After washing

and drying, the paper was subjected to autoradiography for visualization. For quantitation of viral DNA, the spots were cut from the paper and counted in scintillation fluid, or the counts were read directly in a Betascope analyzer.

Plaque Reduction Assay. HEL cells were distributed as above into 24-well plates. After infection for 1 h with 5–15 pfu/well HCMV AD169, a Methocel overlay mixed with medium and drug was added and the plate was incubated at 37 °C for 1 week. A second Methocel overlay was then added and incubation continued for 1 week. The cultures were mixed with methanol and stained with Giemsa, and the number of plaques was read.

Yield Reduction Assay. In this assay, based on that of Pritchard et al.,[13] HEL cells were grown in 96-well microtiter plates as described above and infected with 0.1 pfu/cell HCMV AD169. After 30 min, cells were washed two times with medium and different dilutions of compounds or compound diluent (DMSO) in medium were added to the wells. After incubation at 37 °C for 7 days, cells were scraped and the suspensions were transferred to tubes and sonicated for 6 × 10 s. The virus titers were determined by end-dilution titration in fresh HEL cultures. Aliquots of the sonicated cultures were added in triplicate to fresh HEL cultures in a 96-well plate, and the contents of the first wells were serially diluted 3-fold or 5-fold into the next wells. After incubation for 14 days, the virus titers, i.e., the highest dilution where virus infection resulted in enumerable plaques, were read.

Cytotoxicity Assay Techniques. [^{3}H]Thymidine Incorporation. HEL cells were plated in 96-well plates to obtain about 1/3 confluent monolayers in Dulbecco's modified Eagle's medium (DMEM) plus 10% FCS. After incubation at 37 °C for 24 h, serial dilutions of test compounds in DMSO or DMSO alone were added to duplicate wells and [^{3}H]thymidine (1 μCi/mL) was added to each well. Following incubation for 24 h, the cells were lysed by addition of 0.4 M NaOH to each well. Macromolecules were precipitated with 5% trichloroacetic acid and collected on GF/C filters. The filters were washed with 96% ethanol and placed in scintillation fluid for counting.

Cell Proliferation and Viability. HEL cells were plated and incubated with serial dilutions of test compounds in DMSO or DMSO alone for 24 or 72 h as described above. Cells were detached by trypsinization, stained with 0.5% trypan blue, and counted using a hemocytometer.

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