

## Inhibition of Herpes Simplex Virus Type 1 Helicase–Primase by (Dichloroanilino)purines and -pyrimidines

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Herpes simplex virus type 1 (HSV1) encodes a heterotrimeric helicase–primase comprised of the products of three of the seven DNA replication-specific genes. Several dihalo-substituted derivatives of *N*<sup>2</sup>-phenylguanines and 2-anilinoadenines weakly inhibited the intrinsic DNA-dependent NTPase activity of the HSV1 helicase–primase, and these compounds inhibited the DNA-unwinding activity of the enzyme. The primase activity of the enzyme was strongly inhibited by 3,4- and 3,5-dichloroanilino derivatives of adenine and 2-aminopyrimidines. These compounds and nucleoside analogs of 2-(3,5-dichloroanilino)purines inhibited viral DNA synthesis in HSV1-infected HeLa cells in culture but also inhibited cellular DNA synthesis, likely as a result of inhibition of cellular primase and/or DNA polymerases.

Herpes simplex virus type 1 (HSV1) encodes a heterotrimeric helicase–primase comprised of the products of the DNA replication-specific genes *UL5*, *UL8*, and *UL52*.<sup>1</sup> Overexpression of the products of these genes in insect cells yielded enzyme identical to that obtained from HSV1-infected CV-1 cells.<sup>2</sup> More recently, it has been shown that the products of the *UL5* and *UL52* genes form a stable subassembly of the HSV1 helicase–primase that retains all of the enzymatic activities present in the three-subunit holoenzyme.<sup>3</sup> The *UL8* gene product has been shown to increase primase activity.<sup>4</sup>

The helicase of the HSV1 helicase–primase is a DNA-dependent nucleoside triphosphatase (NTPase) that couples ATP or GTP hydrolysis to the unwinding of double-stranded DNA.<sup>5</sup> The primase activity synthesizes short oligoribonucleotides that can serve as primers for subsequent DNA synthesis. Therefore, the HSV1 helicase–primase contains the requisite activities that place it at the viral replication fork.

Because of the central role that the helicase–primase plays in HSV1 replication, we sought to identify inhibitors of each associated enzyme activity. Such compounds could serve both as probes of the catalytic sites of the enzymes and as leads in the development of antiviral drugs. 6-Anilino-pyrimidines and 2-anilino-purines have been found to selectively inhibit DNA polymerases of prokaryotic and eukaryotic origin (reviewed in ref 6). Related *N*<sup>2</sup>-phenylguanines and their 2'-deoxyribonucleosides are potent inhibitors of HSV thymidine kinases.<sup>7</sup> Inhibition by these analogs involves

competitive binding at dNTP sites (for DNA polymerases) or nucleoside binding sites (for thymidine kinases). We, therefore, screened representative analogs as inhibitors of the DNA-dependent NTPase and DNA primase activities of HSV1 helicase–primase. Among these were base and nucleoside analogs described in the preceding paper.<sup>8</sup> We report herein that several dihaloanilino derivatives of pyrimidines and purines weakly inhibit the NTPase and helicase activities but potently inhibit the primase activity of the viral enzyme. Weak activity against HSV1 DNA synthesis in cell cultures is accompanied by cytotoxicity that may be a result of inhibition of cellular primase and/or DNA polymerases.

### Results

**DNA-Dependent NTPase Inhibition.** The HSV1 helicase–primase hydrolyzes ATP or GTP in the presence of activated DNA, resulting in the formation of the nucleoside 5'-diphosphate and orthophosphate. The enzyme binds ATP and GTP with low affinity ( $K_m = 2$  mM) and couples hydrolysis of the nucleotides to helicase activity.<sup>5</sup> In order to screen potential inhibitors, we used a colorimetric orthophosphate production assay to monitor ATPase or GTPase activity, as described in detail in the Experimental Section.

Among several series of 2-anilino-purines and 6-anilino-pyrimidines, we found that several dihaloanilino derivatives inhibited the DNA-dependent NTPase activity of HSV1 helicase–primase. Table 1 lists representative results from screening of a series of *N*<sup>2</sup>-phenylguanines for inhibition of DNA-dependent ATPase activity of the three-subunit enzyme. The unsubstituted and several monosubstituted derivatives were inactive or weakly inhibitory at 400  $\mu$ g/mL, but 4-bromo (**4**) and 3-chloro (**6**) derivatives had moderate activity. 3,4-Dihalo (**9–11**) and 3,5-dichloro (**12**) derivatives strongly inhibited the enzyme. Other polysubstituted compounds, e.g., dialkyl derivatives, and *N*<sup>2</sup>-benzylguanines were generally inactive (data not shown). Inhibition by the dihalo compounds did not depend on the source or subunit structure of the helicase–primase.

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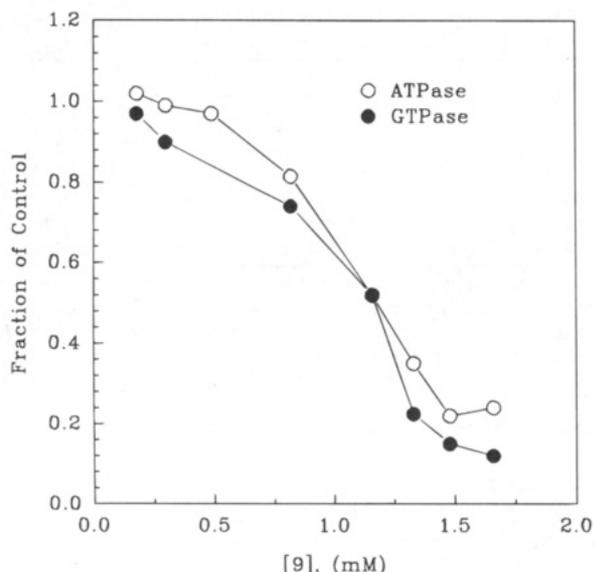
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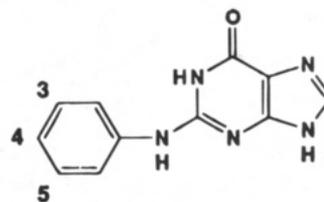
**Figure 1.** Inhibition of NTPase by **9**. Dose-response curves for NTPase inhibition by *N*<sup>2</sup>-(3,4-difluorophenyl)guanidine (**9**) are plotted, in which ATP (○) or GTP (●) was used as substrate.

The compounds were equally effective as inhibitors of the three-subunit enzyme isolated from virus-infected cells or the baculovirus expression system and of the two-subunit baculovirus-expressed enzyme (data not shown). Inhibition was independent of the NTP used in the assay. These results, summarized in Figure 1 for *N*<sup>2</sup>-(3,4-difluorophenyl)guanidine (**9**), show that the compounds inhibited ATP and GTP hydrolysis equally.

To study the dependence of inhibition on the heterocyclic component, other chloroanilino derivatives of purines and pyrimidines were tested. The results in Table 2 indicate that 3,4- and 3,5-dichloroanilino derivatives of purines and 2-aminopyrimidines, but not uracils, were approximately equally inhibitory against the DNA-dependent GTPase, although with IC<sub>50</sub> values close to 1 mM. (Solubility limitations prevented testing of the uracils **15** and **18** beyond 2 mM.) Inhibition did require the appropriate heterocyclic component, however, because 3,4-dichloroaniline and 3,4-dichloroacetanilide inhibited DNA-dependent GTPase by less than 15% at 1 mM.

**DNA-Unwinding (Helicase) Inhibition.** We next determined if inhibitors of the DNA-dependent NTPase

**Table 1.** Screening of *N*<sup>2</sup>-Phenylguanines (400 μg/mL) against DNA-Dependent ATPase Activity of HSV1 Helicase-Primase



compd	substituents	inhibition (%) <sup>a</sup>	ref for synthesis
<b>1</b>	—	0	9
<b>2</b>	4-methyl	0	9
<b>3</b>	4-fluoro	5	10
<b>4</b>	4-bromo	40	7
<b>5</b>	4-hydroxy	17	7
<b>6</b>	3-chloro	60	7
<b>7</b>	3-ethyl	42	9
<b>8</b>	3-(trifluoromethyl)	13	7
<b>9</b>	3,4-difluoro	73	7
<b>10</b>	3,4-dichloro	98	7
<b>11</b>	3-chloro 4-fluoro	96	7
<b>12</b>	3,5-dichloro	70	8
<b>13</b>	3,4-trimethylene	0	11

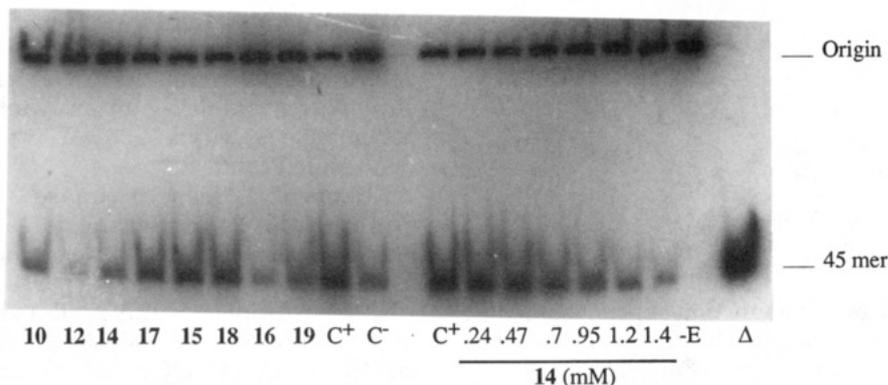
<sup>a</sup> Assays were done with the three-subunit enzyme from HSV1-infected CV-1 cells. Orthophosphate release was measured as described in the Experimental Section.

**Table 2.** Potencies of (Dichloroanilino)purines and -pyrimidines as Inhibitors of DNA-Dependent GTPase of Two-Subunit HSV1 Helicase-Primase

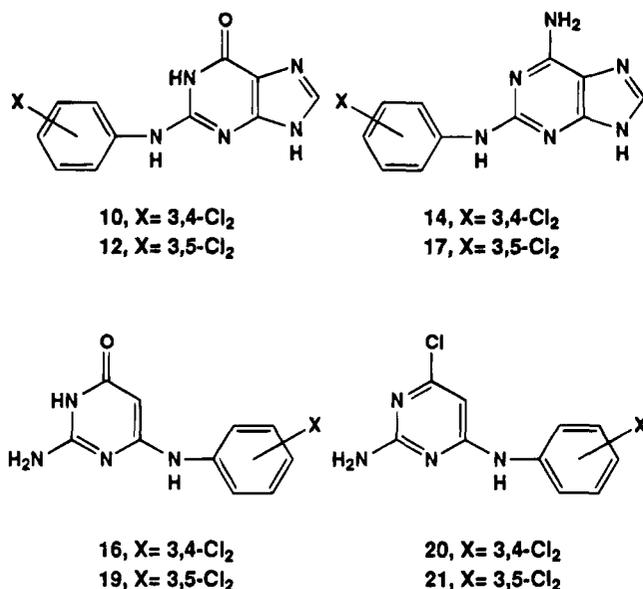
compd	IC <sub>50</sub> (mM) <sup>a</sup>
<b>10</b>	0.88
<b>14</b>	0.85
<b>15</b>	>2
<b>16</b>	0.85
<b>12</b>	0.91
<b>17</b>	0.92
<b>18</b>	>2
<b>19</b>	0.77

<sup>a</sup> Derived from plots of percent inhibition of orthophosphate release vs log(concentration of each compound), measured as described in the Experimental Section.

activity of HSV1 helicase-primase would also inhibit the DNA-unwinding (helicase) activity of the enzyme. Assays involved displacement of a labeled 45-mer oligodeoxyribonucleotide annealed to M13 circular DNA but containing a single-stranded 3' tail.<sup>5</sup> Polyacrylamide gel electrophoresis and autoradiography were used to distinguish annealed and free 45-mer (see the Experimental Section). The results in Figure 2 clearly



**Figure 2.** Inhibition of DNA-unwinding (helicase) activity. Reactions of HSV1 helicase-primase with <sup>32</sup>P-labeled 45-mer annealed to M13 DNA in the presence of ATP were done as described in the Experimental Section. From left to right: first eight numbered lanes, compounds present at 1 mM; C<sup>+</sup>, control assay with DMSO (compound diluent); C<sup>-</sup>, control assay without DMSO; 0.24–1.4, millimolar concentrations of **14** present during assay; -E, no enzyme; Δ, sample heated at 95 °C for 10 min.

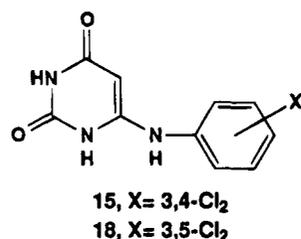


**Table 3.** Screening of 2-Anilinopurines and 6-Anilinopyrimidines against the Primase Activity of HSV1 Helicase-Primase

compd (200 $\mu\text{g/mL}$ )	inhibition (%) <sup>a</sup>
	Guanines
<b>10</b>	89
<b>12</b>	94
	Adenines
<b>14</b>	94
<b>17</b>	93
	Uracils
<b>15</b>	30
<b>18</b>	46
	2-Aminopyrimidines
<b>16</b>	44
<b>19</b>	34
<b>20</b>	98
<b>21</b>	92

<sup>a</sup> Primase activity was measured in the coupled assay with *E. coli* DNA polymerase I as described in the Experimental Section.

show that the NTPase inhibitors at 1 mM also inhibited unwinding of the double-stranded DNA. As depicted for 2-(3,4-dichloroanilino)adenine, **14**, the degree of inhibition was concentration-dependent (Figure 2). The uracil derivatives **15** and **18**, which weakly inhibited the NTPase (Table 2), were ineffective in the unwinding assay.



**Primase Inhibition.** The function of primases is to catalyze polymerization of *ribo*-NTPs on unprimed, single-stranded DNA templates. For our inhibitor screening, amplification of the incorporation signal by the HSV1 helicase-primase was done in an assay in which ATP incorporation on poly(dT) is coupled to radiolabeled dATP incorporation by *Escherichia coli*

**Table 4.** Inhibition of Primases and DNA Polymerases by Dichloroanilino Compounds

compd	IC <sub>50</sub> ( $\mu\text{M}$ )			
	HSV1		calf thymus	
	primase <sup>a</sup>	DNA polymerase <sup>b</sup>	primase <sup>c</sup>	DNA polymerase $\alpha^d$
<b>10</b>	59	>200	>100	46
<b>14</b>	13	>200	>100	85
<b>20</b>	8.7	>200	28	>1000

<sup>a</sup> Coupled primase-DNA polymerase I assay with the two-subunit recombinant HSV1 helicase-primase. <sup>b</sup> Direct assay with activated DNA and 25  $\mu\text{M}$  dNTPs including [<sup>3</sup>H]dTTP. <sup>c</sup> Coupled primase-DNA polymerase I assay with calf thymus DNA polymerase  $\alpha$ -primase. <sup>d</sup> Direct assay with activated DNA and 25  $\mu\text{M}$  dNTPs lacking the expected competitor substrate. Details of assay procedures are in the Experimental Section.

DNA polymerase I (Klenow) because of the low specific activity of the viral primase. In this assay label incorporation occurs only after primase-dependent oligoribonucleotide primer synthesis by the primase. As a control, compounds were tested for inhibition of DNA polymerase I in a direct assay with oligo(dT):poly(dA) and 10  $\mu\text{M}$  [<sup>3</sup>H]dTTP; none of the active compounds described below was found to inhibit this reaction by greater than 20% at 200  $\mu\text{M}$ .

Using the three-subunit helicase-primase isolated from HSV1-infected CV-1 cells, testing of the same series of compounds as that used for NTPase screening revealed that the dihaloanilino derivatives of purines and selected 2-aminopyrimidines also inhibited primase activity. Table 3 summarizes results of primase inhibition by several 3,4- and 3,5-dichloroanilino compounds. Both the guanine (**10**, **12**) and adenine derivatives (**14**, **17**) were highly active, and, among pyrimidines, only 2-amino-4-chloro derivatives (**20**, **21**) were highly active.

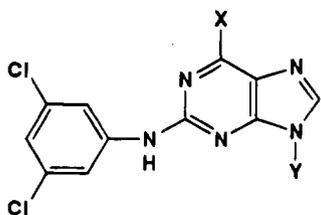
Dose-response studies of a representative series of 3,4-dichloroanilino compounds (Table 4) revealed that primase activity is considerably more sensitive to these compounds than is the NTPase activity associated with the helicase (Table 2). The most potent primase inhibitor, 6-(3,4-dichloroanilino)-2-amino-4-chloropyrimidine (**20**), inhibited the enzyme with IC<sub>50</sub> = 8.7  $\mu\text{M}$ , a potency 100-fold greater than that for helicase inhibition. When these compounds were tested under the same conditions on a eukaryotic primase, that associated with calf thymus DNA polymerase  $\alpha$ -primase, **20** inhibited the primase activity with IC<sub>50</sub> = 28  $\mu\text{M}$ , only severalfold weaker than against the viral enzyme, but the purines **10** and **14** were inactive at 100  $\mu\text{M}$  (Table 4). In contrast, both **10** and **14** were moderately potent inhibitors of the DNA polymerase activity of DNA polymerase  $\alpha$ -primase, but **20** was inactive at 1 mM (Table 4). None of these compounds significantly inhibited HSV1 DNA polymerase activity at 200  $\mu\text{M}$ , the highest concentration tested (Table 4).

**Effects of Helicase-Primase Inhibitors on HSV1 and Cellular DNA Synthesis.** To determine if the helicase-primase inhibitors had antiviral activity, we used a dot-blot hybridization assay to detect HSV1 DNA in virus-infected HeLa cells (see the Experimental Section). Results of these assays are listed in Table 5 together with the effects of the compounds on [<sup>3</sup>H]-thymidine incorporation in uninfected HeLa cells. The dichloroanilino compounds weakly inhibited viral DNA synthesis but were more inhibitory to DNA synthesis in uninfected HeLa cells.

**Table 5.** Effects of Dichloroanilino Compounds on HSV1-Specific and Cellular (HeLa) DNA Synthesis

compd	DNA synthesis IC <sub>50</sub> (μM) <sup>a</sup>	
	HSV1	HeLa
10	>200	250
12	>200	>200
14	75	15
17	69	21
20	62	25
21	45	23
acyclovir	1.9	>200

<sup>a</sup> IC<sub>50</sub> values are concentrations which gave 50% inhibition of HSV1 DNA synthesis in virus-infected HeLa cells and 50% inhibition of [<sup>3</sup>H]thymidine incorporation into the DNA of uninfected HeLa cells. Details of the assays are in the Experimental Section.

**Table 6.** Effects of (3,5-Dichloroanilino)purine Nucleoside Analogs on HSV1-Specific and Cellular (HeLa) DNA Synthesis

compd	X	Y	DNA synthesis IC <sub>50</sub> (μM) <sup>a</sup>	
			HSV1	HeLa
22	oxo	2-deoxyribofuranosyl	190	32
23	oxo	-CH <sub>2</sub> OCH <sub>2</sub> CH <sub>2</sub> OH	14.4	23.4
24	oxo	-CH <sub>2</sub> OCH(CH <sub>2</sub> OH) <sub>2</sub>	91	22.9
25	NH <sub>2</sub>	2-deoxyribofuranosyl	49	0.4
26	NH <sub>2</sub>	-CH <sub>2</sub> OCH <sub>2</sub> CH <sub>2</sub> OH	12.9	2.5
27	NH <sub>2</sub>	-CH <sub>2</sub> OCH(CH <sub>2</sub> OH) <sub>2</sub>	61.6	5.5
28	Cl	2-deoxyribofuranosyl	23	2.7
29	Cl	-CH <sub>2</sub> OCH <sub>2</sub> CH <sub>2</sub> OH	5.0	7.4
acyclovir			1.9	>200

<sup>a</sup> IC<sub>50</sub> values are concentrations which gave 50% inhibition of HSV1 DNA synthesis in virus-infected HeLa cells and 50% inhibition of [<sup>3</sup>H]thymidine incorporation into the DNA of uninfected HeLa cells. Details of the assays are in the Experimental Section.

2-Deoxyribonucleoside derivatives and several acyclonucleoside analogs of the 3,5-dichloropurines, assayed in the same manner, were more potent inhibitors of cellular than viral DNA synthesis (Table 6), with the exception of the 9-[(2-hydroxyethoxy)methyl] derivatives **23** and **29**.

## Discussion

Dihaloanilino derivatives of purines and pyrimidines were found to inhibit the NTPase, DNA-unwinding, and primase activities associated with the HSV1 helicase-primase. NTPase and DNA unwinding were inhibited by 3,4- and 3,5-dichloroanilino derivatives of purines and 2-aminopyrimidines, but at rather high concentrations (IC<sub>50</sub>s ca. 0.7–0.9 mM; Table 2, Figure 2). In contrast, the (dichloroanilino)purines and 2-amino-6-chloropyrimidines were more potent inhibitors of primase activity, as measured in a coupled assay. Preliminary mechanistic studies showing that NTPase inhibition is noncompetitive with DNA but of mixed kinetics with respect to NTP (data not shown) may suggest that inhibitors occupy one or both NTP sites on the helicase subunit. Indeed, we found that several *N*<sup>2</sup>-phenylguanosine 5'-triphosphates stimulated orthophosphate release in the NTPase assay, likely as a

consequence of their being substrates for the enzyme (data not shown). This could only occur if the modified nucleotides bound to the NTP site on the helicase subunit.

Primase activity of the viral enzyme appeared to be more sensitive to inhibition by the same family of compounds that inhibited the helicase. One possible mechanism is that allosteric inhibition of primase occurs as a consequence of binding of the compounds at one or both NTP sites on the helicase subunit, although this could not explain the potent inhibition of the calf thymus primase by the pyrimidine derivative **20** (IC<sub>50</sub> = 28 μM). Primase inhibition of the calf thymus enzyme is not simply a consequence of inhibition of the DNA polymerase α subunit because **20** was inactive at 1 mM in an assay of the DNA polymerase activity (Table 4). Furthermore, **20** inhibited the primase activity of the calf thymus enzyme in a direct oligoribonucleotide synthesis assay<sup>12</sup> with IC<sub>50</sub> = 50 μM (Robert D. Kuchta, personal communication).

(Dichloroanilino)pyrimidines and -purines, and nucleoside analogs of the latter, were nonselective inhibitors of viral DNA synthesis in HSV1-infected HeLa cells (Tables 5 and 6). Potent inhibition of cellular primase by the pyrimidines may account for the effect of **20** and **21** on HeLa DNA synthesis. In contrast, the effect of the guanine (**10**, **12**) and adenine (**14**, **17**) analogs may result from their direct effect on one or more replicative DNA polymerases (for example, see Table 4). That inhibition was a direct effect on DNA synthesis is supported by observations that **14** inhibited HeLa cell "DNA synthesis" irrespective of the labeled precursor, i.e., [<sup>3</sup>H]thymidine or [<sup>32</sup>P]orthophosphate (unpublished results). Indeed, compound **14** has been shown to inhibit both replicative DNA polymerases α and δ from calf thymus.<sup>13</sup> Incubation of HeLa cells with IC<sub>50</sub> concentrations of (dichloroanilino)guanines (**10**, **12**), -adenines (**14**, **17**) and -2-aminopyrimidines (**20**, **21**) for 72 h did not reduce cell numbers by more than 10% compared to control cultures nor result in significantly reduced viability of cells as measured by trypan blue uptake assays.

The same subset of compounds reported in this paper as inhibitors of HSV1 helicase-primase was found to inhibit DNA synthesis and growth of the related human cytomegalovirus (HCMV) (see preceding paper). We have hypothesized that a putative helicase-primase may be the target for the anti-HCMV activity, and we have begun efforts to clone and express the genes for this enzyme. The generally greater selectivity of dichloroanilino compounds for HCMV vs cellular DNA synthesis may suggest that HCMV helicase-primase will be more sensitive to inhibitors than the HSV1 helicase-primase. Availability of the HCMV enzyme and comparative structure-activity studies of analogs of the inhibitors identified in these papers on viral and cellular primases may lead to new targets and lead compounds for antiherpetic therapy.

## Experimental Section

All compounds were synthesized as described in the preceding paper<sup>8</sup> unless otherwise noted in Table 1. All inhibitor assays were done in duplicate, and results reported in Tables 1–6 are the average of at least two independent experiments.

**Enzyme Isolation and Assays.** The HSV1 helicase-primase used for drug screening was the three-subunit enzyme

isolated from HSV1-infected CV-1 cells as described.<sup>1</sup> Recombinant baculovirus-expressed two-subunit enzyme used for certain experiments was isolated as described.<sup>3</sup> DNA polymerase  $\alpha$ -primase from calf thymus was isolated by immunoprecipitation<sup>14</sup> and assayed as described.<sup>15</sup> Recombinant HSV1 DNA polymerase (Crute et al., in press) was assayed as described.<sup>16</sup>

*E. coli* DNA polymerase I, Klenow fragment (Boehringer Mannheim Inc.), was assayed in a mixture of 30 mM Tris·HCl (pH 7.5) and 10 mM Mg(OAc)<sub>2</sub> with 100  $\mu$ M oligo(dT):poly(dA) and 10  $\mu$ M [<sup>3</sup>H]dTTP (1250 cpm/pmol). After incubation for 10 min at 30 °C, workup and processing of acid-precipitable counts were done as described.<sup>15</sup>

**DNA-Dependent NTPase.** Assay mixtures (50  $\mu$ L) contained 1 mM ATP, 2  $\mu$ g of activated DNA, and 15 units of helicase-primase. Inhibitors were dissolved in DMSO and diluted into assay mixtures to give 5% DMSO. After incubation for 20 min at 34 °C, formation of orthophosphate was determined by the addition of 0.75 mL of acidic ammonium molybdate solution containing malachite green. After 5 min color development was stopped by the addition of 0.1 mL of 34% sodium citrate, and the absorbance at 650 nm was read. One unit of DNA-dependent ATPase hydrolyzes 1 nmol of ATP/h under these conditions.

**DNA Helicase.** Reaction mixtures (25  $\mu$ L) contained 10  $\mu$ M (as nucleotide) [5'-<sup>32</sup>P]-45-mer oligodeoxyribonucleotide annealed to M13 circular single-stranded DNA, 3 mM ATP, and 250 ng of two-subunit recombinant HSV1 helicase-primase. DMSO, alone or as compound diluent, was present at 10%. After incubation for 10 min at 34 °C, samples were processed, electrophoresed through a 15% polyacrylamide gel, and subjected to autoradiography as described.<sup>5</sup>

**Primase.** For the enzyme isolated from HSV1-infected CV-1 cells, assays (30  $\mu$ L each) contained 50 mM Tris·HCl (pH 8.7), 3.5 mM MgCl<sub>2</sub>, 10% glycerol, 100  $\mu$ g/mL bovine serum albumin (BSA), 1 mM ATP, 13  $\mu$ M [<sup>3</sup>H]dATP (10 Ci/mmol), 40  $\mu$ M (dT)<sub>6000</sub>, 2.5 units of primase, and 1 unit of *E. coli* DNA polymerase I, Klenow fragment. After incubation for 60 min at 34 °C, reactions were quenched by addition of 10  $\mu$ L of 0.5 M EDTA, and polymeric DNA was collected by centrifugation through G50 fine (Pharmacia Inc.) into scintillation fluid and counted. Assays employing the two-subunit recombinant HSV1 enzyme were identical except that 1 unit of primase, 50  $\mu$ M [<sup>3</sup>H]dATP (2500 cpm/pmol), and 100  $\mu$ M (dT)<sub>6000</sub> were used in a volume of 10  $\mu$ L. One unit of primase catalyzes the incorporation of 1 pmol of dATP/h under these conditions. Primase activity of calf thymus DNA polymerase  $\alpha$ -primase was assayed as described for the recombinant HSV1 enzyme except that 100  $\mu$ M [<sup>3</sup>H]dATP (500 cpm/pmol) was used in a 30 min assay. Isolation and counting of trichloroacetic acid-precipitable material from the latter assays were done as described.<sup>15</sup> In all cases inhibitor solutions in DMSO were diluted into assay media to result in 0.5% DMSO in each assay.

**Virology. Infection and Propagation of HSV1 in HeLa Cells.** Stocks of HSV1 strain KOS were grown in HeLa cells in Dulbecco's modified Eagles medium (DMEM) with 2% fetal calf serum (FCS). Semiconfluent cells were infected at a multiplicity of infection of 0.1–0.2 plaque forming unit (pfu) per cell. Cell-free virus was prepared from the cultures when the viral cytopathic effect reached 90%. HSV1 stocks were then titered on HeLa cells using the dot-blot hybridization assay (see below).

**Dot-Blot Assay for HSV1 DNA Synthesis.** HeLa cells (2  $\times$  10<sup>4</sup> cells/well) were plated in 96-well plates in DMEM containing 10% FCS. After incubation for 24 h at 37 °C, cells were infected by addition of 30  $\mu$ L of HSV1 (5  $\times$  10<sup>6</sup> pfu/mL) to each well. Virus was allowed to adsorb for 90 min after which unadsorbed virus was removed by aspiration of medium. Serial dilutions of compounds in DMEM with 2% FCS were added to duplicate wells. After 24 h at 37 °C, the supernatants were removed and the cells were lysed by addition of 50  $\mu$ L of 0.4 M NaOH and 10 mM EDTA to each well and incubation

for 30 min at 80 °C. An equal volume of 6 $\times$  SSC (0.9 M NaCl, 0.09 M sodium citrate) was added to each well, and the contents of each well were transferred to a Magnagraph nylon filter (Micron Separations Inc.) using a 96-well Milliblot apparatus (Millipore).

The filter was hybridized with a <sup>32</sup>P-labeled HSV1 DNA fragment (*Eco*RI-*Xba*I gC), labeled using the random priming radiolabeling method.<sup>17</sup> Filters were first prehybridized in 50% formamide containing 0.25 M sodium phosphate (pH 7.0), 0.25 M NaCl, 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA at 42 °C for 1 h. Radiolabeled probe in the same buffer was added, and the filter was incubated at 42 °C overnight. The filter was washed successively, at room temperature for 15 min, in each of the following: 2 $\times$  SSC/0.1% SDS, 0.5 $\times$  SSC/0.1% SDS, 0.1 $\times$  SSC/0.1% SDS; it was finally washed at 60 °C for 15 min in 0.1 $\times$  SSC/0.1% SDS. The dried filter was first counted in a Betascope (Betagen) for 1 h for quantitation and then exposed to Kodak X-omatic X-ray film for 24 h at -70 °C for visualization.

**Cytotoxicity Assays. [<sup>3</sup>H]Thymidine Incorporation.** HeLa cells (3  $\times$  10<sup>6</sup> cells/well) were plated in 24-well plates in DMEM plus 10% FCS. After incubation at 37 °C for 24 h, serial dilutions of compounds or diluent (DMSO) were added to duplicate wells. The cells were incubated a further 24 h at 37 °C, and [<sup>3</sup>H]thymidine (1  $\mu$ Ci/mL) was added to each well. After 3 h at 37 °C the supernatants were removed, and the cells were lysed by addition of 0.5 mL of 0.4 M NaOH to each well and incubation for 30 min at 42 °C. Macromolecules in the lysate were precipitated with 5% trichloroacetic acid, collected on GF/C filters, and placed in Omnifluor scintillation fluid for counting.

**Cell Proliferation and Viability.** HeLa cells (3  $\times$  10<sup>6</sup> cells/well) were plated and exposed to compounds as in the thymidine incorporation assay. At 24 and at 72 h following compound addition, the cells were detached by treatment with 0.25% trypsin in phosphate-buffered saline, stained with 0.5% trypan blue, and counted using a hemocytometer.

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