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Mechanism of Selective Inhibition of Human Cytomegalovirus Replication by 1-β-D-Arabinofuranosyl-5-Fluorouracil

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Four kinds of 1-β-D-arabinofuranosyl-5-halogenouracil were examined for inhibition of human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) replication. 1-β-D-Arabinofuranosyl-5-fluorouracil (ara-FU) was the most effective against HCMV, whereas 1-β-D-arabinofuranosyl-5-bromouracil was the most effective against HSV-1 and HSV-2. The mechanism of action of ara-FU on HCMV replication was also studied. The dTTP pool size in human embryonic fibroblasts was increased 33-fold by HCMV infection. However, treatment with ara-FU decreased the size of the dTTP pool by approximately 50%. On the other hand, 1-β-D-arabinofuranosyl-5-fluorouracil-5′-triphosphate inhibited HCMV DNA polymerase activity competitively with dTTP. These results suggest that ara-FU acts as a bifunctional inhibitor of HCMV replication. Ara-FU is phosphorylated by cellular thymidine kinase to 1-β-D-arabinofuranosyl-5-fluorouracil-5′-monophosphate, which inhibits cellular thymidylate synthetase, which in turn decreases the dTTP pool size in infected cells. As the dTTP pool size is reduced, inhibition of viral DNA polymerase by 1-β-D-arabinofuranosyl-5-fluorouracil-5′-triphosphate becomes more efficient.

A number of compounds exhibit a highly selective inhibitory effect against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) (2). However, few inhibitors of human cytomegalovirus (HCMV) have been reported so far (1, 5, 6, 17). Since HCMV does not induce viral thymidine kinase, antiviral nucleoside analogs, which are dependent on conversion to their 5′-triphosphate forms by viral thymidine kinase, cannot inhibit HCMV replication. As a part of our design program of antiviral nucleosides, we examined the antiherpetic activities of 1-β-D-arabinofuranosyl-5-halogenouracils (5-halo-ara-Us) and other related compounds by using HSV-1, HSV-2, and HCMV. The analogs used here are recognized as typical model compounds of 1-β-D-arabinofuranosyluracil, which has an electron-withdrawing substituent on the 5-position of the uracil nucleus (12). 1-β-D-Arabinofuranosyl-5-fluorouracil (ara-FU) was the most effective against HCMV among the compounds examined. We discuss the mechanism of action of ara-FU on HCMV replication.

MATERIALS AND METHODS

Cells and viruses. The viruses used in in vitro assay systems were HSV-1 (HF), HSV-2 (186), and HCMV (Ad169). The cells were Vero cells for HSV-1 and HSV-2 and human embryonic fibroblasts (HEF) for HCMV.

Compounds. The following 5-halo-ara-Us and related compounds were synthesized in this laboratory: ara-FU (14), 1-β-D-arabinofuranosyl-5-chlorouracil, 1-β-D-arabinofuranosyl-5-bromouracil, 1-β-D-arabinofuranosylthymine, and 1-β-D-arabinofuranosyluracil (7). Their 5′-triphosphates were synthesized from the corresponding nucleosides by the method described previously (19). 5-Fluoro-2′-deoxyuridine (FUdR) was purchased from Heinrich Mack, Illertissen, Bayern, West Germany. [*methyl*-^{3}H]dTTP (80 Ci/mmol) and [8-^{3}H]dGTP (18 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Virus plaque assay. Monolayers of Vero cells or HEF in 24-well tissue culture plates (Linbro) were infected with 120 to 300 PFU of virus. After a 1-h adsorption period at 37°C, the cultures were overlaid with 2 ml of 0.5% agarose in Eagle minimum essential medium containing appropriate concentrations of inhibitors. After an incubation period of 24 h for HSV-1 and HSV-2 and 7 days for HCMV, the plaques were counted with a dissecting microscope at ×20 magnification after fixation with 5% aqueous formaldehyde and staining with 0.1% crystal violet.

Assay for DNA polymerase activity. Viral DNA polymerases were partially purified from Vero cells infected with HSV-2 and from HEF infected with HCMV. Purification of the DNA polymerases was carried out by methods described previously (10). The viral DNA polymerases used were almost completely separated from host-cell polymerases. The specific activity of HCMV polymerase was 1,500 U/mg of protein. The HSV-2 polymerase used was described previously (10). The standard mixture for the assay of viral DNA polymerases contained 50 mM Tris hydrochloride (pH 7.8), 4 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 mM ammonium sulfate, 70 μM concentrations of dATP, dGTP, dCTP, and [^{3}H]dTTP (1.7 to 3.4 μCi/nmol), 2.5 μg of activated salmon sperm DNA, and enzyme in 25 μl. Under these conditions, viral DNA polymerase activity could be measured selectively. Incubation was carried out at 37°C for 15 min. The reaction mixture was chilled, and 20-μl samples were transferred to DEAE-cellulose paper disks (DE-81; Whatman, Inc., Clifton, N.J.). Disks were washed with 5% Na_2HPO_4 six times, washed twice with ethanol, and dried. Remaining radioactivity was measured in a toluene scintillator in a liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). One unit of activity was defined as the amount that catalyzed the incorporation of 1 nmol of deoxyribonucleoside triphosphates into DNA in 1 h.

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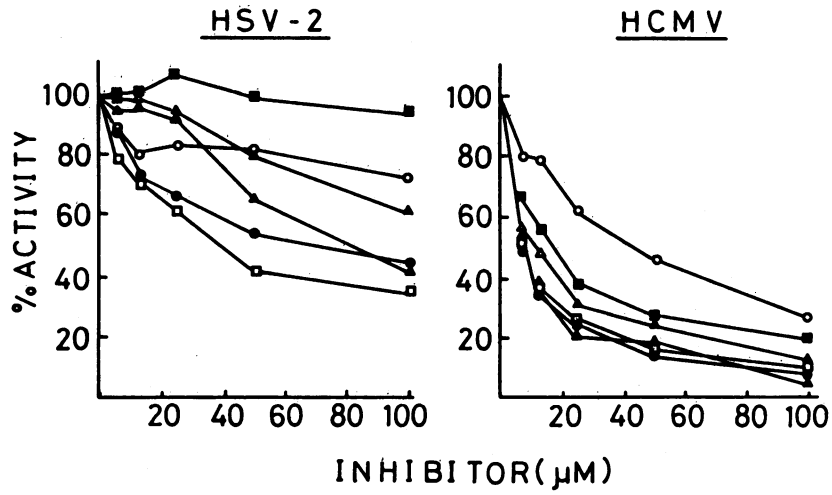

FIG. 1. Inhibition of DNA polymerases of HSV-2 and HCMV by 5-halo-ara-UTPs and related compounds. Symbols: ○, ara-FUTP; ●, 1-β-D-arabinofuranosyl-5-chlorouracil-5'-triphosphate; △, 1-β-D-arabinofuranosyl-5-bromouracil-5'-triphosphate; ▲, 1-β-D-arabinofuranosyl-5-iodouracil-5'-triphosphate; □, ara-TTP; ■, ara-UTP.

Determination of dTTP pool size. Quantitative analysis of the dTTP pool was performed by a previously described enzymatic procedure (11) with some modifications. Monolayers of HEF in 10-cm dishes were infected with HCMV at a multiplicity of infection of three. After adsorption at 37°C for 1 h, fresh medium containing nucleoside analogs was added. Cells were collected after incubation at 37°C for 72 h. Preparation of cell extracts was performed as described previously (16). The reaction mixture for the determination of dTTP concentration contained 50 mM Tris hydrochloride (pH 7.8), 4 mM $MgCl_2$, 0.5 mM dithiothreitol, 25 μg of native salmon sperm DNA, and 35 μM concentrations of dATP, dCTP, and [^{3}H]dGTP (3.4 μCi/nmol). Samples or dTTP standards and 0.063 U of DNA polymerase I Klenow fragment (P-L Biochemicals, Inc., Milwaukee, Wis.) were added to the mixture. The total volume of each reaction mixture was 50 μl. Incubation was carried out at 37°C for 30 min, and then the mixtures were chilled, and 40-μl samples were transferred to DE-81 paper disks. Measurement of radioactivity was performed by the method described above.

RESULTS

Antiviral activity in culture. Drug concentrations which reduced plaque numbers to 50% (ID_{50}) in the viruses tested are shown in Table 1. The ID_{50} values were calculated by plotting plaque formation versus concentration of the test compound. 1-β-D-Arabinofuranosyl-5-bromouracil was the most effective against HSV-1 (ID_{50}, 0.27 μM), and its ID_{50} value was similar to that of 1-β-D-arabinofuranosylthymine. Against HSV-2, all compounds showed relatively low activities when compared with the HSV-1 system. For HCMV, ara-FU was the most effective among the 5-halo-ara-Us tested, and its ID_{50} value was 3.8 μM. Thus, the order of efficacy against HCMV was different from that of the HSV systems.

TABLE 1. Antiviral activity of 5-halo-ara-Us and related compounds against herpesviruses in vitro

Compounds[a]	ID_{50} (μM) against:		
	HSV-1 (HF)	HSV-2 (186)	HCMV (Ad169)
ara-FU	2.6	133	3.8
ara-ClU	3.7	27	115
ara-BrU	0.27	1.9	35.6
ara-IU	14.6	104	111
ara-T	0.28	0.96	119
ara-U	103	382	40

[a] Abbreviations: ara-ClU, 1-β-D-arabinofuranosyl-5-chlorouracil; ara-BrU, 1-β-D-arabinofuranosyl-5-bromouracil; ara-IU, 1-β-D-arabinofuranosyl-5-iodouracil; ara-T, 1-β-D-arabinofuranosylthymine; ara-U, 1-β-D-arabinofuranosyluracil.

Inhibition of viral DNA polymerase activity by 5-halo-ara-UTPs. We synthesized 5-halo-ara-UTPs, 1-β-D-arabinofuranosylthymine-5'-triphosphate (ara-TTP), and 1-β-D-arabinofuranosyluracil-5'-triphosphate (ara-UTP) from the corresponding nucleosides by methods reported previously (19) and studied their inhibitory effects against HSV-2 and HCMV DNA polymerases. Inhibition of DNA polymerase activity in the presence of 5-halo-ara-UTPs is shown in Fig. 1. HCMV DNA polymerase was strongly inhibited by 5-halo-ara-UTPs. Figure 2 shows representative Lineweaver-Burk plots of the inhibition of DNA polymerases by the compounds tested. All compounds exhibited competitive inhibition of both polymerases with dTTP. The kinetic parameters calculated from the data in Fig. 2 are shown in Table 2. Inhibition of HCMV DNA polymerase by the compounds tested was greater than that of HSV-2 DNA polymerase. No significant correlation could be found for the K_i values of the compounds tested.

Utilization of ara-FUTP by HCMV DNA polymerase. The utilization of 1-β-D-arabinofuranosyl-5-fluorouracil-5'-triphosphate (ara-FUTP) as a substrate by HCMV DNA polymerase was studied. A concentration response experiment was performed with HCMV DNA polymerase and [^{3}H]dGTP as the labeled precursor. Activated DNA was used as a template primer. Incubation was at 25°C for 25 min. Figure 3 shows the effects of ara-FUTP and ara-TTP on the incorporation of [^{3}H]dGMP into DNA in vitro. In complete systems which contained dATP, dGTP, dCTP, and an appropriate concentration of dTTP as substrates, the incorporation of the labeled precursor by HCMV DNA polymerase increased in a manner dependent on the concentration of dTTP. In the absence of dTTP, ara-FUTP or ara-TTP was

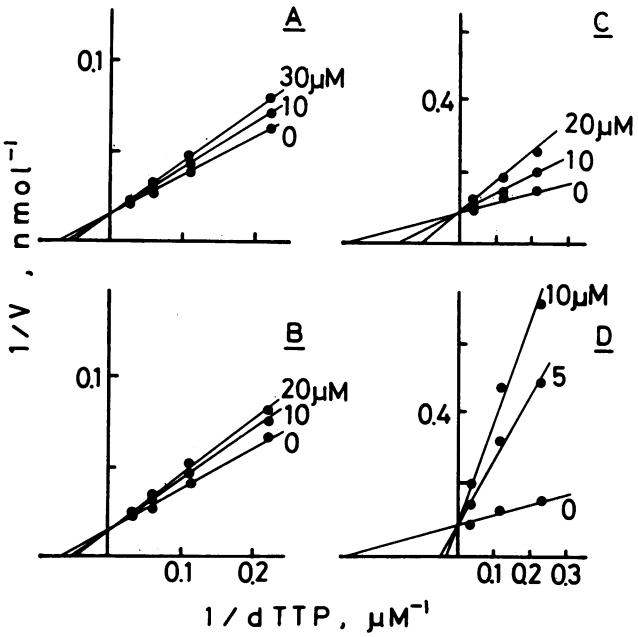

FIG. 2. Representative Lineweaver-Burk plots showing inhibition by ara-FUTP and ara-TTP. (A) ara-FUTP in HSV-2 system; (B) ara-TTP in HSV-2 system; (C) ara-FUTP in HCMV system; (D) ara-TTP in HCMV system. Added inhibitor concentrations are indicated in the graphs.

added to the mixture. No significant incorporation of [^{3}H]dGMP was observed under these conditions. Thus, neither compound could support DNA chain elongation and neither appeared to be utilized as a substrate for HCMV DNA polymerase in place of dTTP.

Effect of ara-FU on the size of the dTTP pool in HCMV-infected cells. 1-β-D-Arabinofuranosyl-5-fluorouracil-5′-monophosphate (ara-FUMP) is an irreversible inhibitor of thymidylate synthetase (9). To elucidate the mode of the inhibitory effect of ara-FU against thymidylate synthetase in cells infected with HCMV, we measured the size of the dTTP pool in HEF in the stationary phase (Table 3). In mock-infected cells in the stationary phase, the dTTP pool size was very small. In contrast, the dTTP pool size in infected cells increased 33-fold after virus infection. Treatment with ara-FU decreased the size of the dTTP pool to approximately 50%. Before this experiment, it was confirmed that the DNA polymerase I Klenow fragment was not inhibited by ara-FUTP.

In a separate experiment, treatment with FUdR produced a large reduction in the dTTP pool size in virus-infected cells (Table 3). However, with FUdR, the medium containing the compound had to be changed every 24 h to reduce the dTTP pool size. It should be noted that the half-life of FUdR in living cells is very short because the compound is a good substrate for thymidine phosphorylase (8), in contrast with ara-FU, which is quite resistant to this enzyme.

DISCUSSION

Four kinds of 5-halo-ara-Us were synthesized in this laboratory, and their 5′-triphosphates have been shown to inhibit cellular DNA polymerases and retroviral reverse transcriptase (12). In this study, we tested the antiviral activity of 5-halo-ara-Us against HSV-1, HSV-2, and HCMV in tissue culture. 1-β-D-Arabinofuranosyl-5-bromouracil was the most effective against HSV-1 and HSV-2. However, ara-FU was the most effective against HCMV among the compounds tested; its ID_{50} value was 3.8 μM. It has been reported that a few 5-halo-ara-Us inhibit HSV (13) and HCMV (15) replication. However, these studies did not examine the effect of ara-FU. Therefore, we studied the mechanism of action of ara-FU on HCMV replication with respect to viral DNA polymerase and dTTP pool size.

Although HCMV does not induce viral thymidine kinase, the virus enhances cellular thymidine kinase activity in virus-infected cells (3). On the other hand, we have reported that ara-FUMP is an irreversible inhibitor of thymidylate synthetase (9). Therefore, it was assumed that ara-FU inhibited both thymidylate synthetase and viral DNA polymerase after phosphorylation by the enhanced kinases to its 5′-monophosphate and 5′-triphosphate forms, respectively. Our results indicate that HCMV DNA polymerase activity is strongly inhibited by 5-halo-ara-UTPs, although ara-FUTP was somewhat weaker than other 5-halo-ara-UTPs. In another experiment, the dTTP pool size in HEF infected with HCMV was measured. The dTTP pool size in HCMV-infected cells was 33-fold greater than in mock-infected cells. Treatment with ara-FU, however, suppressed the increase in the dTTP pool size to approximately 50%. The results described above suggest that ara-FUMP and ara-FUTP are inhibitors of thymidylate synthetase and DNA polymerase, respectively, in HCMV-infected cells.

From these findings, it seems reasonable to assume that ara-FU is a bifunctional inhibitor (self-potentiator) of HCMV replication. Ara-FU is phosphorylated by cellular thymidine kinase, the activity of which is enhanced by HCMV infection. The resulting ara-FUMP inhibits cellular thymidylate

TABLE 2. Apparent kinetic constants for DNA polymerases

Compound[a]	HSV-2 DNA polymerase		HCMV DNA polymerase		Polymerase α	
	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m
dTTP	15.4 (K_m)		3.4 (K_m)		9.2 (K_m)[b]	
ara-FUTP	115	7.46	10	2.9	0.6[b]	0.07
ara-ClUTP	65	4.22	1.5	0.43	1.45[b]	0.16
ara-BrUTP	87	5.65	2.5	0.72	0.3[b]	0.03
ara-IUTP	62	4.03	2.0	0.58	0.53[b]	0.06
ara-TTP	74	4.80	1.0	0.29	0.3[c]	0.03
ara-UTP	93	6.04	2.4	0.70	0.5[b]	0.05

[a] Abbreviations: ara-ClUTP, 1-β-D-arabinofuranosyl-5-chlorouracil-5′-triphosphate; ara-BrUTP, 1-β-D-arabinofuranosyl-5-bromouracil-5′-triphosphate; ara-IUTP, 1-β-D-arabinofuranosyl-5-iodouracil-5′-triphosphate.

[b] Data from reference 12.

[c] Data from reference 12a.

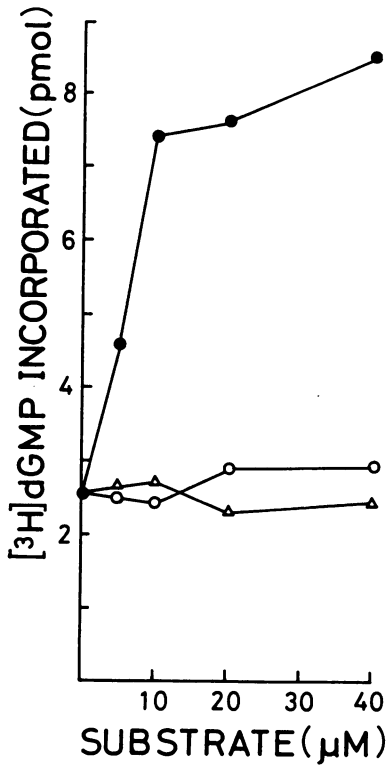

FIG. 3. Utilization of ara-FUTP and ara-TTP by HCMV DNA polymerase. Symbols: ●, dTTP; ○, ara-FUTP; △, ara-TTP.

synthetase, which in turn decreases the dTTP concentration. Viral DNA polymerase is inhibited by ara-FUTP. dTTP competes with ara-FUTP for DNA polymerase. The inhibitory effect of ara-FUTP against HCMV DNA polymerase was the lowest among all of the compounds tested (K_i, 10 μM), although ara-FU was the most effective agent against HCMV (ID_{50}, 3.8 μM). This apparent discrepancy could be partly explained by the reduced dTTP pool, which may enhance the inhibitory effect of ara-FUTP on viral DNA polymerase despite its large K_i value. There is a possibility that unknown factors produce a rather complex antiviral spectrum for these compounds (Table 1). However, little evidence on these factors, e.g., phosphorylation efficacy, is known at present. We confirmed that ara-FUTP does not replace dTTP in viral DNA synthesis. This indicates that ara-FUTP is not a substrate and is not incorporated into DNA. Thus, our results strongly suggest that ara-FU can act

TABLE 3. Effects of ara-FU and FUdR on dTTP pool size in HEF

Cell sample	dTTP (pmol/ 7×10^6 cells)
Mock infected	2.38
Mock infected plus ara-FU[a]	2.02
HCMV infected	78.8
HCMV infected plus ara-FU[a]	44.8
HCMV infected plus FUdR[b]	8.0

[a] In the presence of 74 μM ara-FU for 72 h.
[b] In the presence of 3.7 μM FUdR for 72 h. Eagle minimum essential medium containing FUdR was replaced with fresh medium every 24 h.

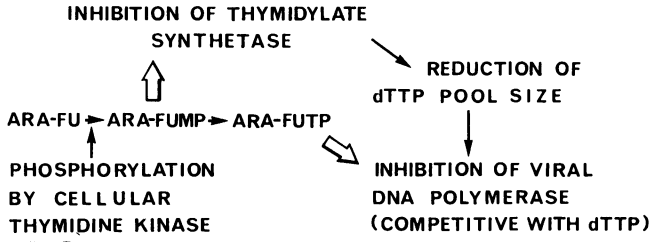

FIG. 4. Proposed inhibition scheme of ara-FU against HCMV. ⇨, Inhibition site.

as a bifunctional inhibitor, and the scheme of action presented in Fig. 4 explains why ara-FU is effective against HCMV at such a low concentration. In contrast, since other 5-halo-ara-Us cannot inhibit thymidylate synthetase, their antiviral activity against HCMV may be weaker than that of ara-FU.

Wingard et al. (18) reported that trifluorothymidine is active against HCMV replication. They suggested that the compound inhibits thymidylate synthetase and viral DNA polymerase after phosphorylation by cellular thymidine kinase to its monophosphate and triphosphate forms, respectively. It is also known (4) that trifluorothymidine-5'-monophosphate is incorporated into DNA. This suggests that trifluorothymidine has toxicity; thus, the compound should not be a selective inhibitor of HCMV. Moreover, since trifluorothymidine becomes a substrate for thymidine phosphorylase, like FUdR and other 2'-deoxy compounds, its half-life in cells may be short. However, arabinosyl nucleosides are resistant to thymidine phosphorylase (9). Concerning the toxicity of ara-FU, it has been reported that the compound shows weak toxicity in mice (14, 20). In this study also, no toxicity of ara-FU against HEF was observed at <500 μM. We propose that the design of a bifunctional inhibitor (self-potentiator), such as ara-FU, should be one of the ways to develop effective compounds against HCMV.

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