0026-895X/87/030301-06$02.00/0

MOLECULAR PHARMACOLOGY, 31:301-306

A Proposed Mechanism for the Selective Inhibition of Human Cytomegalovirus Replication by 1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-fluorouracil

SATORU SUZUKI, HEMANT K. MISRA, LEONARD I. WIEBE, EDWARD E. KNAUS, and D. LORNE J. TYRRELL

Departments of Biochemistry (S.S., D.L.J.T.) and Medical Microbiology and Infectious Diseases (D.L.J.T.), and Faculty of Pharmacy (H.K.M., L.I.W., E.E.K.), University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received September 30, 1986; Accepted December 22, 1986

SUMMARY

The biological activities of 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-fluorouracil (2′F-ara-FU), 1-(3′-deoxy-3′-fluoro-β-D-arabinofuranosyl)-5-fluorouracil (3′F-ara-FU) and 1-β-D-arabinofuranosylthymine (ara-T) were compared in human cytomegalovirus (HCMV)-infected and noninfected human fibroblasts. 2′F-ara-FU inhibited HCMV plaque formation (ED_{50}, 16 μM for AD 169 strain) at lower concentrations than 3′F-ara-FU (ED_{50}, 100 μM for AD 169). These nucleoside analogues are expected to be phosphorylated to their 5′-phosphate forms by cellular thymidine kinase in HCMV-infected cells. The thymidine kinase activities in the virus-infected and noninfected cells were compared. Cellular thymidine kinase was increased in the virus-infected cells and showed better phosphorylation of 2′F-ara-FU than did 3′F-ara-FU. HCMV DNA polymerase was purified using affinity column chromatography, and the inhibitory effect of the 5′-triphosphate derivatives of 2′F-ara-FU (2′F-ara-FUTP) and 3′F-ara-FU (3′F-ara-FUTP) against viral and host DNA polymerase α was examined. No significant difference in the effectiveness of inhibition was observed between viral DNA polymerase and host polymerase α. However, viral polymerase incorporated 2′F-ara-FUTP into newly synthesized DNA, whereas polymerase α did not utilize 2′F-ara-FUTP as a substrate. Thus, viral polymerase differs from host polymerase α in its recognition and utilization of 2′F-ara-FUTP. This difference may be important to the design of selective antiviral agents for HCMV.

HCMV is a member of the herpesvirus groups and is an important human pathogen. Primary infection usually is asymptomatic; however, congenital cytomegalovirus and post-transfusion mononucleosis are two consequences of primary infection. Following primary infection, the virus is latent but reactivation may occur in immunosuppressed patients (1). HCMV is a major problem in organ transplant and acquired immunodeficient syndrome. Therefore, effective chemotherapy for HCMV is an important goal of research in antiviral chemotherapy. A few nucleoside analogues have been reported to be effective anti-HCMV compounds (2–4). The nucleoside analogues that have antiviral activity are initially phosphorylated in the infected cell. The phosphorylated nucleosides are then capable of inhibiting viral metabolic enzymes (5). However, it is difficult to develop selective antiviral chemotherapy for HCMV because HCMV does not produce viral specific TK (6, 7). Other human herpesviruses induce a viral specific TK which is the basis of the selective antiviral activity of many nucleoside analogues (8, 9).

It is known that pyrimidine nucleoside analogues that have a fluorine atom in the sugar moiety, such as FMAU, FIAU and FIAC, are highly effective against HCMV (2, 4). These compounds inhibited viral DNA and protein synthesis (4). Another nucleoside analogue, ara-FU, has been reported to be a possible bifunctional inhibitor of HCMV replication (10, 11). The mono-

This work was supported by Grants MT-5960 and MA-6425 from the Medical Research Council of Canada.

ABBREVIATIONS: HCMV, human cytomegalovirus; TK, thymidine kinase; FMAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil; FIAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil; FIAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine; ara-FU, 1-β-D-arabinofuranosyl-5-fluorouracil; ara-FUMP, 1-β-D-arabinofuranosyl-5-fluorouracil 5′-monophosphate; ara-FUTP, 1-β-D-arabinofuranosyl-5-fluorouracil 5′-triphosphate; 2′F-ara-U, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)uracil; 3′F-ara-U, 1-(3′-deoxy-3′-fluoro-β-D-arabinofuranosyl)uracil; 2′F-ara-FU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-fluorouracil; 3′F-ara-FU, 1-(3′-deoxy-3′-fluoro-β-D-arabinofuranosyl)-5-fluorouracil; 2′F-ara-FUMP, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-fluorouracil 5′-monophosphate; 3′F-ara-FUMP, 1-(3′-deoxy-3′-fluoro-β-D-arabinofuranosyl)-5-fluorouracil 5′-monophosphate; 2′F-ara-FUTP, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5′-fluorouracil 5′-triphosphate; 3′F-ara-FUTP, 1-(3′-deoxy-3′-fluoro-β-D-arabinofuranosyl)-5′-fluorouracil 5′-triphosphate; FMAUTP, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil 5′-triphosphate; ara-T, 1-β-D-arabinofuranosylthymine; ara-TTP, 1-β-D-arabinofuranosylthymine 5′-triphosphate; HPLC, high performance liquid chromatography; HFs, human fibroblasts; moi, multiplicity of infection; 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HSV, herpes simplex virus.

phosphate form of ara-FU (ara-FUMP) inhibits thymidylate synthetase, and the triphosphate derivative (ara-FUTP) inhibits viral DNA polymerase (10, 11).

However, there have been no reports describing the comparative effect of the thymidine analogues possessing a fluoro sugar moiety. We are interested in arabino nucleoside analogues which possess an electron-withdrawing substituent not only at the C-5 position of the uracil but also on sugar moiety as candidates for anti-HCMV activity. We now report the synthesis of 2′F-ara-FU, 3′F-ara-FU, and their 5′-triphosphates (2′F-ara-FUTP, 3′F-ara-FUTP). These nucleosides were studied to compare their antiviral activity in HCMV-infected cells. In this study, we also examined the ability of the TK to phosphorylate the nucleosides and the ability of 2′F-ara-FUTP and 3′F-ara-FUTP to inhibite the activity of viral and α DNA polymerases.

Materials and Methods

Compounds and chemicals. 2′F-ara-U and 3′F-ara-U were prepared using the procedures reported previously (12, 13). The 5-fluoro analogues of 2′F-ara-U and 3′F-ara-U were synthesized by the reaction of F_2 in acetic acid at room temperature which afforded 2′F-ara-FU and 3′F-ara-FU. These fluoro arabino nucleosides were phosphorylated by the procedure described in the literature (14, 15).

ara-T was purchased from Sigma Chemical Co. (St. Louis, MO). ara-TTP was kindly provided by Dr. Mineo Saneyoshi, Faculty of Pharmaceutical Sciences, Hokkaido University, Japan. The purity of all nucleosides and nucleotides was determined by HPLC analysis. The analytical HPLC system consisted of two HPLC pumps, model M-45 and M-510, a model 481 UV detector (267 nm), a model U6K universal injector, and an automatic gradient controller, model 680, from Waters Associates (Milford, MA). The separation was performed on a Waters M-9-ODS-3 stainless steel column (25 cm length × 9.4 mm, i.d.). A solvent program with appropriate solvent system using a flow rate of 4.00 ml/min was used for analysis. All of the analogues showed >99% purity. Unlabeled deoxyribonucleotides were purchased from Sigma. [^{3}H]dTTP (15.2 Ci/mmol), [^{3}H]dGTP (7.8 Ci/mmol), and [γ-^{32}P]ATP (3.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Activated DNA was used as a template primer for DNA polymerase assays. The salmon sperm DNA was activated as reported previously (16). Phosphocellulose (P-11), DEAE-cellulose (DE-22), and DEAE-cellulose paper (DE-81) were purchased from Whatman Inc. (Clifton, NJ). Single-stranded DNA cellulose was purchased from Pharmacia (Uppsala, Sweden). Proton magnetic resonance spectra (^{1}H-NMR) were recorded at 300 MHZ on a Bruker AM-300 spectrometer. Mass spectra were measured with a Hewlett-Packard 5995A gas-chromatograph mass spectrometer using the direct insertion probe method. UV absorbance was measured with a Beckman DU-7 spectrophotometer. All other reagents were analytical grade.

Synthesis of 2′F-ara-FU and 3′F-ara-FU. A freshly prepared solution of F_2 in glacial acetic acid (31.8 ml, 0.013 M, 1.10 mmol) was added to 92.0 mg (0.373 mmol) of 2′F-ara-U. The reaction mixture was stirred at room temperature for 15 min. The glacial acetic acid was removed on a rotary evaporator at 50°. The residue was redissolved twice in ethanol and the solvent was removed along with residual acetic acid to give a white foam in the flask. Treatment of this material with 0.5 ml of concentrated NH_4OH in 5 ml of methanol at room temperature for 18 hr afforded the product. The crude product was purified by column chromatography (2.5 × 15 cm, Merck Kieselgel 60, 70–230 mesh, methanol in chloroform) which gave a white foam (78.0 mg, 0.0295 mmol, 78.6%) of 2′F-ara-FU. The m.p., 166–167°, was similar to the reported m.p. of 167–168° (13). NMR (^{1}H, CD_3OD, TMS internal standard) δ (ppm) 8.1 (d, 1H, $J_{6,C\text{-}5F}$ = 6.8 Hz, H-6), 6.20 (m, 1H, $J_{1',F}$ = 16 Hz, $J_{1',2'}$ = 3.7 Hz, H-1′), 5.06 (m, 1H, $J_{2',F}$ = 52.24 Hz, $J_{1',2'}$ = 3.7 Hz, H-2′), 4.36 (m, 1H $J_{3',F}$ = 19.6 Hz, $J_{2',3'}$ = 4.7 Hz, H-3′), 3.94 (m, 1H, H-4′), 3.74 – 3.93 (complex m, 24, H-5′ and H-5″). Mass calculated for $C_9H_{10}N_2O_5F_2$: 264.05; found: 264.10 (M^+, 17.3% relative intensity), for $C_9H_9N_2O_5F$: 244.04; found 244.05 (M^+ – HF, 1.1% relative intensity).

Similarly, 3′F-ara-FU was prepared from 3′F-ara-U using 1.1 mol eq of F_2 in acetic acid solution, in 62.93% chemical yield as a viscous oil. NMR (^{1}H, CD_3OD, TMS internal standard) δ (ppm) (7.9 (d, 1H, $J_{6\text{-}C\text{-}5F}$ = 6.0 Hz, H-6), 6.04 (m, 1H, H-1′), 4.90 (m, 1H, $J_{3'F}$ = 51.85 Hz, $J_{2',3'} \simeq J_{3',4'} \simeq$ 2.5 Hz, H-3′) 4.36 (m, 1H, $J_{2',F'}$ = 13.9 Hz, $J_{2,3}$ = 2.5 Hz, $J_{1',2'}$ = 3.8 Hz, H-2′) 4.14 (m, 1H, $J_{3',4'}$ = 2.43, $J_{4',5'}$ = 4.9 Hz, H-4′), 3.74 – 3.96 (complex m, 2H, H-5′ and H-5″). Mass calculated for $C_9H_{10}N_2O_5F_2$: 264.05; found: 264.15 (M^+, 4.4% relative intensity), for $C_9H_9N_2O_5F$: 244.04; found: 244.05 (M^+ – HF, 5.0% relative intensity). HPLC analysis, using a C-18 reverse phase column with HPLC grade water:methanol (10:90, v/v) changing to water:methanol (50:50, v/v) during 10 min using the gradient program, indicated both products had a purity of greater than 99%.

General procedure for phosphorylation of 2′F-ara-FU and 3′F-ara-FU. 2′F-ara-FU or 3′F-ara-FU (0.079–0.082 mmol) in triethyl phosphate (0.8 ml) was phosphorylated with phosphorus oxychloride (20 μl, 0.22 mmol) under cooling at −20°. The reaction mixture was stirred for 18 hr at room temperature and then mixed with saturated aqueous sodium bicarbonate (2 ml) and chloroform (2 ml). The aqueous phase was washed with chloroform and then diluted with distilled water for a final volume of 30 ml. The solution was applied to a column of DEAE-cellulose (2.5 × 30 cm, bicarbonate form), which was eluted with a linear gradient from water (1 liter) to 0.25 M triethylammonium bicarbonate (pH 8.0, 1 liter). The desired 5′-monophosphate was eluted when the concentration of eluent was 0.15–0.18 M. The combined fractions were evaporated at 35° to dryness, followed by co-evaporation with 50% aqueous ethanol six times and with dry ethanol to remove residual triethylammonium bicarbonate and triethylamine. After measuring UV and homogeneity by thin layer chromatography, this 5′-monophosphate was converted to the 5′-triphosphate in the following manner. The 5′-monophosphate (0.029–0.060 mmol, as triethylammonium salt) in anhydrous dimethylformamide (2 ml) was added to *N,N′*-carbonyldiimidazol (0.27—0.142 mmol; 5 equimolar) and the mixture was stirred at room temperature for 2 hr. Methanol (4.5–8.7 μl; 4 equimolar to *N,N′*-carbonyldiimidazole) was added to the reaction mixture to decompose the excess reagent. The solution was stirred for 30 min at room temperature. A solution of tri-*N*-butylammonium pyrosphosphate (287–600 μl) in dry dimethylformamide was added to the reaction mixture and stirring continued for 14 hr at room temperature. Activated charcoal (>3 g) was added to the reaction mixture and was stirred for 1 hr. The mixture was diluted with distilled water and filtered over a Celite pad and washed well with water. The residue was eluted with 50% aqueous ethanol containing 1% ammonium hydroxide. The eluate was evaporated to dryness and residual nucleotide was further purified by DEAE-cellulose column chromatography. The nucleotide mixture in 10 ml of water was applied to a column of DEAE-cellulose (2.5 × 30 cm, bicarbonate form). Elution was performed with a linear gradient from water (1 liter) to 0.5 M triethylammonium bicarbonate (pH 8.0, 1 liter). The 5′-triphosphate was eluted when the concentration of eluent was 0.38—0.46 M. The fractions containing 5′-triphosphate were combined and evaporated to dryness under reduced pressure. These triphosphate derivatives of 2′F-ara-FU and 3′F-ara-FU were further analyzed by HPLC using a C-18 reverse phase ODS-Magnum-9 column with PIC-A in 75–80% methanol as solvent indicated both nucleotides had a purity of greater than 99%.

The structures of synthesized analogues are shown in Fig. 1. UV, R_f, and yields of the products are summarized in Table 1.

Cells and Viruses. The viruses used in the *in vitro* assay system were HCMV strains AD 169 and 3556 (a clinical isolate from the University of Alberta Hospital) passed in HFs two times. The cells were HFs, passage 15–30. Viral DNA polymerase was purified from HFs infected with HCMV AD 169.

Effect of the analogues on virus replication. Monolayers of HFs in 24-well plates (Falcon, Oxnard, CA) were infected with 40–70

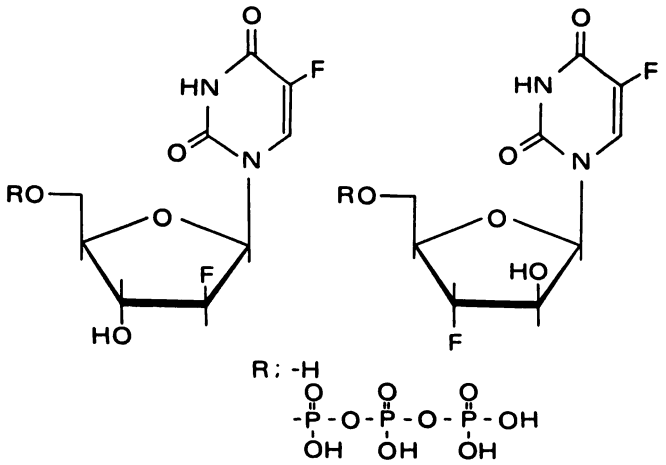

Fig. 1. Structures of 2′F-ara-FU, 3′F-ara-FU, and their 5′-triphosphates.

TABLE 1
Physical data of 2′F- and 3′F-ara-FUMP and -FUTP

Compound	UV λ_{max} in H_2O	UV λ_{min} in H_2O	R_f[a]	Yield from nucleoside
	nm	*nm*		%
2′F-ara-FUMP	266.5	233.0	0.22	72.7
2′F-ara-FUTP	266.5	235.0	0.06	34.8
3′F-ara-FUMP	268.0	249.5	0.25	36.1
3′F-ara-FUTP	268.5	239.5	0.06	22.8

[a] Thin layer chromatography was performed on a Whatman M6KF silica gel plate (1 × 3 inches) using solvent system *n*-butyl alcohol:acetic acid:H_2O, 4:1:1.

plaque-forming units per well. After a 1-hr adsorption period at 37°, media were removed and the cultures were overlaid with 1 ml of 0.5% agarose in media containing the inhibitors. After an incubation period of 8 days (AD 169) or 12 days (3556) at 37°, the plaques were counted microscopically.

Effect of the analogues on cell growth. Appropriate concentrations of drugs were added to the culture of HFs in six-well plates (Falcon) at 6 hr after seeding. After the incubation period of 72 hr at 37°, trypsinized cells were counted with a hemocytometer.

Phosphorylation of the analogues by TK. Crude enzyme extracts were prepared from HCMV AD 169-infected HFs (moi, 1.5) and from noninfected HFs. In the case of infected cells, monolayers of HFs were harvested at 48 hr after infection. HCMV-infected cells or noninfected cells (7.7×10^6 cells) were homogenized with a sonicator in 1 ml of 50 mM Tris-HCl (pH 7.8) containing 20% glycerol, 10 mM 2-ME, and 50 mM KCl. After centrifugation in an Eppendorf centrifuge for 10 min, the supernatant containing the enzyme was stored at −70° until use. ^{32}P transfer assays were performed as reported previously (17). A total volume of 100 μl of reaction mixture, which contained 63.5 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM NaF, 0.5 mM [γ-^{32}P]ATP (10 μCi/mmol), 60 μM substrate (nucleoside), and the crude enzyme (35 μg of protein), was incubated at 37°. After incubation, 100 μl of 2 N HCl were added to the reaction mixture which was then boiled for 12 min. This was followed by the addition of 800 μl of 1 mM K_2PO_4 and 400 μl of precipitation reagent (1 N perchloric acid:80 mM ammonium molybdate:0.2 M triethyl amine-HCl, pH 5.0, in a ratio of 4:2:1) to the reaction tubes. The mixture was centrifuged in an Eppendorf centrifuge for 10 min and the radioactivity present in a 700-μl aliquot of the supernatant was determined by liquid scintillation counting (LKB, 1215 Rackbeta).

DNA polymerase purification. HCMV DNA polymerase was purified from HFs infected with HCMV AD 169 at a moi of approximately 3. The virus-infected cells were harvested 72 hr after infection, when 90% of cells showed cytopathic effect. Cells (3.1 g) were homogenized as described previously (18). The resulting homogenates were centrifuged at 22,000 *xg* for 120 min. The 12 ml of supernatant were mixed with 12 g of phosphocellulose in buffer A (20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 10 mM 2-ME, 20% glycerol) containing 0.1 M KCl, and then eluted with a linear gradient from 0.1 M to 0.6 M KCl in buffer A. Viral polymerase activity was eluted at 0.34 M KCl, and cellular polymerase α, β, and γ activities were eluted at 0.26 M, 0.45 M, and 0.3 M, respectively. Viral polymerase fractions were combined and dialyzed against buffer A containing 0.1 M KCl and 0.5 mM PMSF, and then applied to a single-stranded DNA cellulose column (0.8 × 5 cm) to remove any γ polymerase contaminating the viral polymerase. After washing with buffer A containing 0.1 M KCl, the column was eluted with a KCl linear gradient from 0.1 M to 0.6 M in buffer A. The polymerase γ eluted at 0.28 M KCl whereas the viral polymerase eluted at 0.35 M KCl. No activities corresponding to α and β polymerase were detected. The combined fractions of viral polymerases had a specific activity of 10,600 units/mg of protein and were dialyzed against buffer B (100 mM Tris-HCl, pH 7.8, 4 mM $MgCl_2$, 10 mM 2-ME, 30% glycerol, and 0.5 mM PMSF) containing 50 mM KCl. The dialyzed sample was applied to 5′-AMP Sepharose (0.6 × 5.5 cm), which has been shown to function as an affinity column for 3′ to 5′ exonuclease (19). Since HCMV DNA polymerase has 3′ to 5′ exonuclease activity (20), this column was expected to bind the HCMV DNA polymerase. The column was washed with buffer B containing 50 mM KCl and then eluted with KCl linear gradient from 50 mM to 0.5 M in buffer B. Viral polymerase activity was eluted at 0.22 M KCl. The 3′ to 5′ exonuclease activity coeluted with the polymerase activity. Final fractions were dialyzed against buffer A containing 150 mM KCl and 60% glycerol, and were stored at −20°. This sample had a specific activity of >8,500 units/mg of protein, 450-fold greater than the crude extract. From 3.1 g of infected cells, 200 units of enzyme were recovered. Cellular DNA polymerase α was partially purified from noninfected HFs of 70–80% confluent log-phase culture using sequential column chromatography with phosphocellulose, DEAE-cellulose, and single-stranded DNA cellulose. The final sample had a specific activity of ≥4,340 units/mg of protein and did not contain other DNA polymerase activities.

Assay for DNA polymerase activity. The standard mixture for the assay of viral DNA polymerase contained 50 mM Tris-HCl, pH 7.8, 4 mM $MgCl_2$, 0.5 mM DTT, 100 mM ammonium sulfate, 100 μM dATP, dGTP, dCTP, and [^3H]dTTP (2.4 μCi/nmol), 2.5 μg of activated DNA, and the purified enzyme in 25 μl. Incubation was carried out at 37° for 20 min. The reaction mixture was chilled, and 20-μl samples were transferred to DE-81 paper disks. Disks were washed with 5% Na_2HPO_4 six times, washed twice with water, and finally washed with ethanol and dried. Radioactivity retained on the paper was measured. Assay mixtures for DNA polymerase α utilized the same reagents as described above for viral polymerase except that the 100 mM ammonium sulfate was omitted. Viral polymerase can be differentiated from host cell α polymerase by measuring the activity in the presence or absence of 100 mM ammonium sulfate in the assay mixture (20). Polymerase β and γ activities were measured utilizing an assay system described previously (21). One unit of activity was defined as the amount of enzyme that incorporated 1 nmol of deoxyribonucleoside monophosphates into DNA in 1 hr.

Results

Antiviral and anti-cellular activity. The nucleoside analogue concentrations which reduced plaque numbers to 50% (ED_{50}) and 90% (ED_{90}) for the viruses tested are shown in Table 2. 2′F-ara-FU was the most effective antiviral agent of the three compounds tested against HCMV. The clinical isolate (3556) was more susceptible than the AD 169 strain to the three compounds tested. As shown in Table 2, 2′F-ara-FU was more toxic to cell growth than the other compounds. That is, this compound inhibited cell growth by 50% (ID_{50}) at 50 μM. However, inhibition of cell growth was not observed with concentrations less than 20 μM for 2′F-ara-FU, a concentration which inhibited the virus plaque production by more than 50%.

Phosphorylation of the nucleoside analogues by cel-

TABLE 2

Anti-HCMV and anti-cell growth activity of the nucleoside analogues

Compound	Anti-HCMV Against: AD 169 ED_{50}[a]	AD 169 ED_{90}[a]	3556 ED_{50}[a]	3556 ED_{90}[a]	Anti-cell growth against HFs ID_{50}[b]
	μM		μM		μM
2′F-ara-FU	16	50	8	16	50
3′F-ara-FU	100	>100	65	>100	>100
ara-T	40	>100	22	>100	>100

[a] ED_{50} and ED_{90} represent the concentrations of compound that reduced the number of plaques by 50% and 90%, respectively, when compared with the virus control culture containing no compound. The data for AD169 and 3556 are the mean values of three experiments and two experiments, respectively.

[b] ID_{50} is the concentration of compound which inhibited the cell growth by 50% when compared with that of the culture containing no compound. The data are the mean values of two experiments.

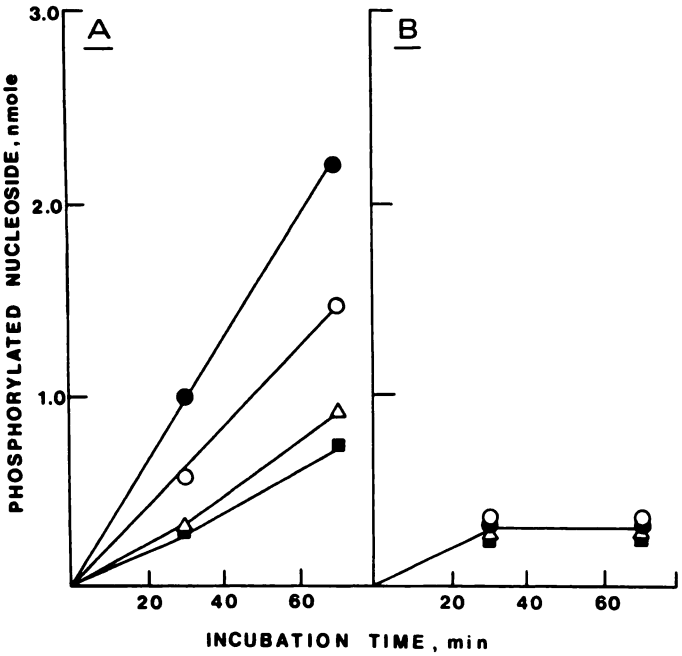

Fig. 2. Phosphorylation of thymidine (●), 2′F-ara-FU (○), 3′F-ara-FU (■), and ara-T (△) by TK of HCMV-infected cells (A) and noninfected cells (B).

lular TK. It is known that, although HCMV does not code for TK, it can increase cellular TK activity (6, 7). The phosphorylation of these nucleosides was examined and the data are shown in Fig. 2. TK activity in viral infected cells was increased severalfold in comparison with noninfected fibroblasts. 2′F-ara-FU was phosphorylated to 1.45 nmol during 70 min incubation by the TK from virus-infected cells, whereas 3′F-ara-FU and ara-T were phosphorylated to less than 1.0 nmol during the same incubation period (Fig. 2A). In contrast, noninfected confluent fibroblasts contained little TK activity, and the rate of phosphorylation was very low for all nucleosides (Fig. 2B).

Inhibition of DNA polymerases by 5′-triphosphate derivatives of the nucleoside analogues. The 5′-triphosphates of 2′F-ara-FU and 3′F-ara-FU were synthesized, and their inhibitory effects against HCMV and α polymerases were studied. Both polymerases were inhibited by ara-TTP and 2′F-ara-FUTP, but 3′F-ara-FUTP did not inhibit either polymerase. The inhibition effects were analyzed by using Lineweaver-Burk plots and the apparent inhibition constants (K_i) were calculated. Competitive inhibition of dTTP incorporation into DNA by the three analogues using HCMV polymerase is shown in Fig. 3. All compounds exhibited competitive inhibition with dTTP in both polymerases. The K_i values were determined by replotting the apparent K_m as shown in the *inset* of Fig. 3. The values obtained are summarized in Table 3. The K_m values for dTTP of viral and α polymerase were 3.9 μM and 1.74 μM, respectively. The 2′F-ara-FUTP had a K_i value of 3.00 μM for viral polymerase and 0.70 μM for polymerase α. The 3′F-ara-

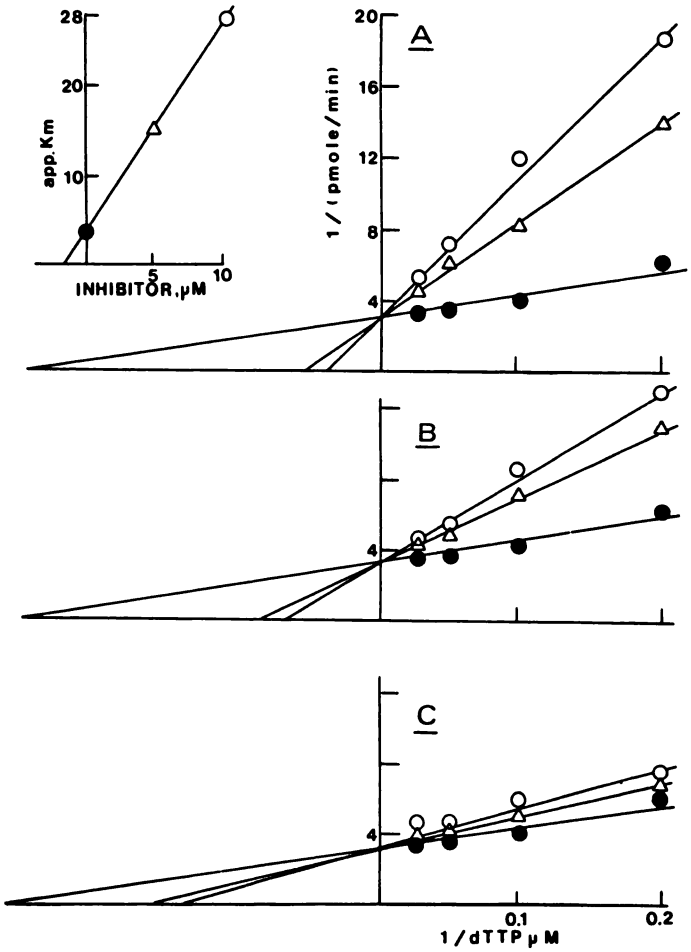

Fig. 3. Representative Lineweaver-Burk plots showing inhibition by ara-TTP (A), 2′F-ara-FUTP (B), and 3′F-ara-FUTP (C) in HCMV DNA polymerase. The reaction mixture (total 25 μl) contained 50 mM Tris-HCl, pH 7.8, 4 mM $MgCl_2$, 0.5 mM DTT, 100 mM ammonium sulfate (for viral polymerase), 2.5 μg of activated DNA, 100 μM each of dATP, dGTP, and dCTP and various concentrations of [^{3}H]dTTP (860–890 cpm/pmol), 0.36 unit of enzyme, and ara-TTP or 2′F-ara-FUTP at 0 μM (●), 5 μM (△), and 10 μM (○). In the case of 3′F-ara-FUTP, 0 μM (●), 10 μM (△), and 20 μM (○) concentrations were used. Incubation was for 20 min at 37°. The *inset* demonstrates the method for the determination of K_i.

TABLE 3

Kinetic constants for HCMV DNA polymerase and DNA polymerase α

Compound	HCMV DNA polymerase K_i	HCMV DNA polymerase K_i/K_m	DNA polymerase α K_i	DNA polymerase α K_i/K_m
	μM		μM	
dTTP	3.90 (K_m)		1.74 (K_m)	
2′F-ara-FUTP	3.00	0.77	2.00	1.15
3′F-ara-FUTP	18.00	4.62	24.00	13.79
ara-TTP	1.25	0.32	0.70	0.40

FUTP exhibited high K_i values for both polymerases, particularly for polymerase α. A comparison of K_i/K_m ratios for all three compounds demonstrated slightly lower values for viral polymerase than for polymerase α.

Utilization of the nucleoside 5′-triphosphates as substrates for DNA polymerases. The utilization of 2′F-ara-FUTP, 3′F-ara-FUTP, and ara-TTP as substrates for HCMV and α polymerases was studied. A dose response curve was determined for both polymerases using [^{3}H]dGTP incorporation into DNA as an indicator of DNA synthesis. Activated DNA was used as a template primer. Assays were performed at 37° for 20 min. Fig. 4 shows the effects of the compounds 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 substrate, the incorporation of the labeled precursor by both polymerases increased in a manner dependent on the concentration of dTTP. In subsequent experiments, 2′F-ara-FUTP, 3′F-ara-FUTP, or ara-TTP was substituted for dTTP. In experiments using HCMV polymerase, 2′F-ara-FUTP supported *in vitro* DNA synthesis, but 3′F-ara-FUTP and ara-TTP did not (Fig. 4A). The 2′F-ara-FUTP supported DNA synthesis at approximately 35–40% of the rate of the system using dTTP. In contrast, polymerase α did not utilize 2′F-ara-FUTP as a substrate in DNA synthesis. The DNA synthesis by polymerase α was decreased in the presence of any of the nucleotide analogues (Fig. 4B). The decrease in DNA synthesis using the polymerase α system may be due to suicidal inhibition of this enzyme by the analogues as reported previously in the HSV polymerase system (22).

Discussion

It has been reported that the thymidine analogue FMAU, which has a 2′-arabino fluorine atom in the sugar moiety, effectively inhibited HCMV replication (2, 4). More recently, Mar *et al.* (23) reported that the FMAUTP inhibited viral DNA polymerase and was incorporated into viral DNA but not cellular DNA. Halogenated pyrimidine nucleosides offer potential not only as antiviral compounds, but also as possible radiolabeled scanning agents to detect HSV-infected cells (24). It is important to know the precise relationship between the biological activity and the structure of the nucleoside analogues in order to design better antiviral compounds. Therefore, we synthesized the two difluoro-arabinofuranosyl-uracils 2′F-ara-FU and 3′F-ara-FU to investigate their biological activity in HCMV-infected cells.

Although the synthesis and determination of anti-HSV activity of 2′F-ara-FU has already been reported by Watanabe *et al.* (13), its anti-HCMV activity and the mechanism by which it inhibits viral replication were not investigated. We compared the anti-HCMV activity and phosphorylation in viral infected cells for these two di-fluoro analogues. We also studied the inhibition and utilization of the 5′-triphosphate of these nucleosides by viral DNA polymerase and cellular polymerase α. The results shown in Table 2 indicate that anti-HCMV activity and inhibition of cell growth by 2′F-ara-FU and 3′F-ara-FU are quite different. Cancer cells such as L5178Y and P815 have low ID_{50} values for 2′F-ara-FU (13) compared with the HFs used in our system. This may be based on the extent of nucleoside utilization in different cells. Since cellular TK activity is increased in rapidly proliferating cells (25), phosphorylation of the nucleoside analogues will increase significantly in these cells. TK is the key enzyme for activation of nucleoside analogues. The TK activity in HCMV-infected and noninfected cells have been compared. The cellular TK activity in HCMV-infected cells was increased severalfold compared to noninfected cells. HCMV-infected cells phosphorylated 2′F-ara-FU more readily than 3′F-ara-FU or ara-T (Fig. 2). The phosphorylated product, 2′F-ara-FUMP will inhibit the thymidylate synthetase (26). Since 2′F-ara-FUMP is converted to its 5′-triphosphate by cellular thymidylate kinases, the 5′-triphosphates of 2′F-ara-FU and 3′F-ara-FU were synthesized to investigate their effect on DNA polymerases. 2′F-ara-FUTP inhibited both viral DNA polymerase and host polymerase α. FMAUTP inhibits viral DNA polymerase more effectively than polymerase α (23). In this study with 2′F-ara-FUTP, the K_i/K_m ratios for HCMV polymerase were slightly lower than the K_i/K_m ratios for polymerase α, indicating that HCMV DNA polymerase inhibition is not very selective relative to polymer-

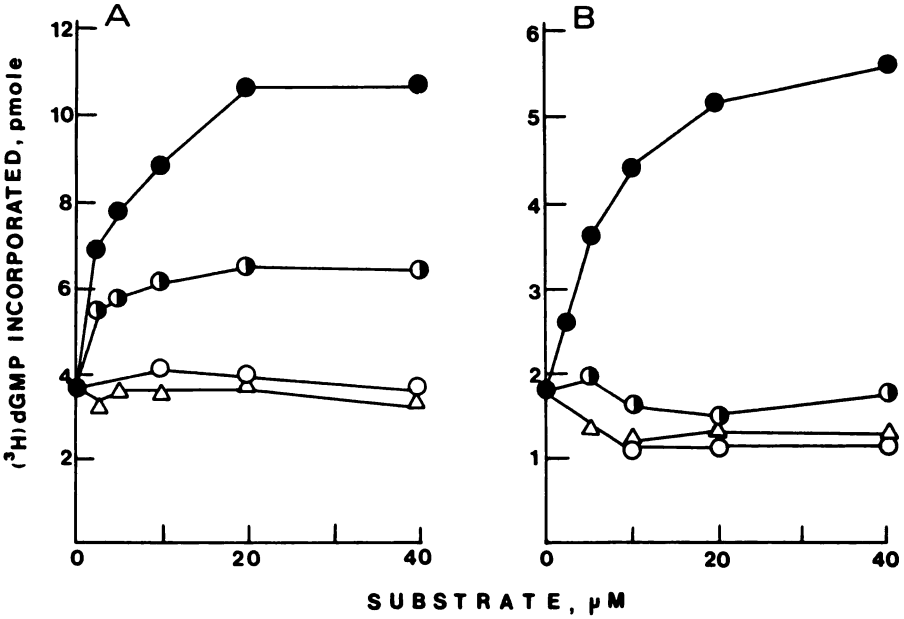

Fig. 4. Utilization of 2′F-ara-FUTP, 3′F-ara-FUTP, and ara-TTP by HCMV DNA polymerase (A) and DNA polymerase α (B). ●, dTTP; ◑, 2′F-ara-FUTP; ○, 3′F-ara-FUTP; △, ara-TTP. The reaction mixture contained 50 mM Tris-HCl, pH 7.8, 4 mM $MgCl_2$, 0.5 mM DTT, 100 mM ammonium sulfate (for viral polymerase), 2.5 μg of activated DNA, 100 μM each of dATP and dCTP, 10 μM [^{3}H]dGTP (240–330 cpm/pmol), and various concentrations of dTTP or nucleotide analogues. The enzymes used here were HCMV polymerase (0.36 unit) and polymerase α (0.18 unit). The incubation was for 20 min at 37°.

ase α (Table 3). On the basis of the results reported for FMAUTP (23), ara-FUTP (11), and the data for 2′F-ara-FUTP in this study, it is believed that the presence of a fluorine substituent at the C-5 position of the uracil base decreases affinity for viral DNA polymerase. This suggestion is supported by the inhibition data available for 5-halogenated (11) or 5-alkylated[1] ara-UTPs on HCMV polymerase.

A methyl substituent at the C-5 position of the uracil moiety seems to be a more effective inhibitor of HCMV polymerase than a C-5 fluorine substituent. Cellular TK recognizes the fluoro compounds having an arabino (2′-"up")-fluoro substituent as 2′-deoxy rather than 2′-hydroxy analogues, since cellular TK in HCMV-infected cells phosphorylates 2′F-ara-FU more readily than 3′F-ara-FU or ara-T. Viral DNA polymerase utilizes 2′F-ara-FUTP as an alternate substrate for DNA synthesis, although the utilization is not as efficient as the natural substrate. Previous studies have been shown that FIAC can be incorporated into cellular DNA (27, 28). However, purified DNA polymerase α does not use FIACTP as a substrate (23). Therefore, other DNA polymerases such as β and/or γ may be able to incorporate FIACTP into cellular DNA.

Watanabe *et al.* (13) reported that substitution of hydroxy by fluorine at the 2′-position resulted in selective antiviral activity. Our study indicated that viral DNA polymerase utilizes 2′F-ara-FUTP as a substrate, whereas cellular polymerase α does not. In addition, our study indicates the ED_{50} for HCMV is significantly lower than the ID_{50} for cell growth. Since the K_i/K_m ratios for HCMV polymerase and polymerase α are similar, one of the reasons for the selective antiviral effect should be based on the selective utilization of 2′F-ara-FUTP by HCMV DNA polymerase. This may be an important factor in designing antiviral nucleoside analogues for HCMV which have favorable therapeutic indices.

Finally, we should mention the recognition of 2′F-ara-FU by deoxypyrimidine nucleoside phosphorylase. Coderre *et al.* (26) have reported that 2′F-ara-FU was not degraded by deoxypyrimidine nucleoside phosphorylase. This suggests that this enzyme does not recognize 2′F-ara-FU as a 2′-deoxy species even though viral DNA polymerase or TK recognizes it as a 2′-deoxy compound. It is interesting that the metabolic and catabolic enzymes have different recognition mechanisms for the same substrate. These observations may be useful to future studies attempting to design nucleoside analogues which are stable to catabolic enzymes, but selectively utilized by HCMV metabolic enzymes.

Acknowledgments

We thank the Alberta Heritage Foundation for Medical Research for the award of postdoctoral fellowships to S. S. and H. K. M.

References

1. Meyers, J. D. Cytomegalovirus infection after organ allografting, in *The Herpesvirus* (B. Roizman and C. Lopez, eds.), Vol. 4. Plenum Press, New York, 201–227 (1985).
2. Calcino, J. M., and C. Lopez. Efficacy and selectivity of some nucleoside analogues as anti-human cytomegalovirus agent. *Antimicrob. Agents Chemother.* **24**:505–508 (1983).
3. Mar. E.-C., Y.-C. Cheng, and E.-S. Huang. Effect of 9-(1,3-dihydroxy-2-propoxymethyl) guanine on human cytomegalovirus replication *in vitro*. *Antimicrob. Agents Chemother.* **24**:518–521 (1983).
4. Mar. E.-C., P. C. Patel, Y.-C. Cheng, J. J. Fox, K. A. Watanabe, and E.-S. Huang. Effects of certain nucleoside analogues on human cytomegalovirus. *J. Gen. Virol.* **64**:47–53 (1984).
5. DeClerq, E. Biochemical aspects of the selective antiherpes activity of nucleoside analogues. *Biochem. Pharmacol.* **33**:2159–2169 (1984).
6. Estes, J., and E.-S. Huang. Stimulation of cellular thymidine kinase by human cytomegalovirus. *J. Virol.* **24**:13–21 (1977).
7. Zavada, V., V. Erban, D. Rezacova, and V. Vonka. Thymidine kinase in cytomegalovirus infected cells. *Arch. Virol.* **52**:333–339 (1976).
8. Miller, R. L., J. B. Ilitis, and F. Rapp. Differential effect of arabinofuranosylthymine on the replication of human herpesviruses. *J. Virol.* **23**:679–684 (1977).
9. Schaeffer, H. J., L. Beauchamp, P. DeMiranda, G. B. Elion, D. J. Bauer, and P. Collins. 9-(2-Hydroxethoxymethyl) guanine activity against viruses of the herpes group. *Nature (Lond.)* **272**:583–585 (1978).
10. Suzuki, S., M. Saneyoshi, C. Nakayama, and Y. Nishiyama. Selective inhibition of human cytomegalovirus replication by 1-β-D-arabinofuranosyl-5-fluorouracil: a possible bifunctional antiviral agent. in *Herpes Viruses and Virus Chemotherapy* (R. Kono, ed.). Elsevier Science Publishers, Amsterdam, 229–232 (1985).
11. Suzuki, S., M. Saneyoshi, C. Nakayama, Y. Nishiyama, and S. Yoshida. Mechanism of selective inhibition of human cytomegalovirus replication by 1-β-D-arabinofuranosyl-5-fluorouracil. *Antimicrob. Agents Chemother.* **28**:326–330 (1985).
12. Cushley, R. J., J. F. Codington, and J. J. Fox. Nuclear magnetic resonance studies of 2′- and 3′-halogeno nucleosides. The conformations of 2′-deoxy-2′-fluorouridine and 3′-deoxy-3′-fluoro-β-D-arabinofuranosyluracil. *Can. J. Chem.* **46**:1131–1140 (1968).
13. Watanabe, K. A., U. Reichman, K. Hirota, C. Lopez, and J. J. Fox. Nucleosides 110. Synthesis and antiherpes virus activity of some 2′-fluoro-2′-deoxyarabinofuranosylpyrimidine nucleosides. *J. Med. Chem.* **22**:21–24 (1978).
14. Maeda, M., A. D. Patel, and A. Hampton. Formation of ribonucleotide 2′,3′-cyclic carbonates during conversion of ribonucleoside 5′-phosphates to diphosphates and triphosphates by the phosphoroimidazolidate procedure. *Nucleic Acids Res.* **4**:2843–2853 (1977).
15. Yoshikawa, M., T. Kato, and T. Takenishi. A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett.* **50**:5065–5068 (1967).
16. Schlabach, A., B. Fridlender, A. Bolden, and A. Weissbach. DNA-dependent DNA polymerases from Hela cell nuclei. II. Template and substrate utilization. *Biochem. Biophys. Res. Commun.* **44**:879–885 (1971).
17. Dobersen, M. J., and S. Greer. An assay for pyrimidine deoxyribonucleoside kinase using γ-^{32}P-labeled ATP. *Anal. Biochem.* **67**:602–610 (1975).
18. Suzuki, S., T. Kimura, and M. Saneyoshi. Characterization of DNA polymerase induced by salmon herpesvirus, *Oncorhynchus masou* virus. *J. Gen. Virol.* **67**:405–408 (1986).
19. Lee, M. Y. W., and W. A. Whyte. Selective affinity chromatography of DNA polymerases with associated 3′ to 5′ exonuclease activities. *Anal. Biochem.* **138**:291–297 (1984).
20. Nishiyama, Y., K. Maeno, and S. Yoshida. Characterization of human cytomegalovirus-induced DNA polymerase and associated 3′ to 5′ exonuclease. *Virology* **124**:221–231 (1983).
21. Yamaguchi, M., A. Matsukage, and T. Takahashi. Chick embryo DNA polymerase γ. Purification and structural analysis of nearly homogeneous enzyme. *J. Biol. Chem.* **255**:7002–7009 (1980).
22. Furman, P. A., M. H. St. Clair, and T. Spector. Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. *J. Biol. Chem.* **259**:9575–9579 (1984).
23. Mar, E.-C., J.-F. Chiou, Y.-C. Cheng, and E.-S. Huang. Human cytomegalovirus-induced DNA polymerase and its interaction with the triphosphates of 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil, -5-iodocytosine and -5-methylcytosine. *J. Virol.* **56**:846–851 (1985).
24. Tovell, D. R., H. P. Yacyshyn, H. K. Misra, E. E. Knaus, L. I. Wiebe, J. Samuel, M. J. Gill, and D. L. J. Tyrrell. Effect of acyclovir on uptake of ^{131}I-labeled 1-(2′-fluoro-2′-deoxy-β-D arabinofuranosyl)-5-iodouracil in herpes infected cells. *J. Med. Virol.*, in press.
25. Bresnick, E., and S. S. Williams. Effects of 5-fluoromethyldeoxyuridine upon deoxythymidine kinase. *Biochem. Pharmacol.* **16**:503–507 (1967).
26. Coderre, J. A., D. V. Santi, A. Matsuda, K. A. Watanabe, and J. J. Fox. Mechanism of action of 2′-5-difluoro-1-arabinosyluracil. *J. Med. Chem.* **26**:1149–1152 (1983).
27. Grant A. J., A. Feinberg, T.-C. Chou, K. A. Watanabe, J. J. Fox, and F. S. Philips. Incorporation of metabolites of 2′-fluoro-5-iodo-1-β-D-arabinonucleic acid of neoplasmic and normal mammalian tissues. *Biochem. Pharmacol.* **31**:1103–1108 (1982).
28. Chou, T.-C., C. Lopez, J. M. Colacino, A. Feinberg, K. A. Watanabe, J. J. Fox, and F. S. Philips. Metabolic competition studies of 2′-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine in vero cells and herpes simplex type 1-infected cells. *Mol. Pharmacol.* **26**:587–593 (1984).

[1] S. Suzuki, S. Izuta, C. Nakayama, and M. Saneyoshi, submitted for publication.

Send reprint requests to: Dr. D. L. J. Tyrrell, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.