Effects of Certain Nucleoside Analogues on Human Cytomegalovirus Replication in vitro

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SUMMARY

Four nucleoside analogues, 1-(2'-deoxy-2'-fluoro-β-d-arabinofuranosyl)-5-methyluracil (FMAU), -5-iodouracil (FIAU), -5-methylcytosine (FMAC) and -5-iodocytosine (FIAC), were studied for their effect on human cytomegalovirus (HCMV) replication in vitro. FMAU, FIAU, FMAC and FIAC showed antiviral activities for four strains of HCMV (Major, Clegg, D550 and Towne) in a plaque reduction assay, with a dose required for 50% inhibition (ED50) in the range of 0.1 to 0.65 μM. At a concentration of 1 μM-FMAU or -FIAC, the synthesis of five virus-specific late polypeptides of molecular weights 150000, 120000, 67000, 54000 and 27000 was entirely blocked. Quantification of Towne viral DNA synthesis, using complementary RNA-DNA hybridization with a Towne-specific cRNA probe, demonstrated a complete inhibition of HCMV DNA replication at 1 μM of FMAU or FIAC. After the removal of the inhibitors, however, viral DNA synthesis resumed, and infectious virus reappeared, indicating that the inhibition of HCMV replication by these nucleoside analogues was of a virostatic reversible type.

INTRODUCTION

Human cytomegalovirus (HCMV) has become increasingly recognized as an important pathogen having various clinical manifestations ranging from asymptomatic infection to severe diseases such as congenital abnormality, mental retardation and interstitial pneumonia. Owing to the lack of effective treatment of HCMV infections, the search for effective antiviral drugs for chemotherapy of HCMV infection is pivotal.

Previously, it has been reported that phosphonoacetic acid (PAA) was effective against HCMV replication by virtue of interference with virus-induced DNA polymerase activity and therefore impeding viral DNA replication (Huang, 1975). However, PAA was found to have limited therapeutic value because of its potential bone deposition effect. Recently, we also examined the effect of a potent antipherpetic nucleoside analogue, namely acycloguanosine (ACV) (Elion et al., 1977), on HCMV replication. The results revealed that HCMV was somewhat resistant to ACV and viral DNA synthesis was not impaired by the drug even at the high concentration of 200 μM (Mar et al., 1982). With a continuing interest in the search for anti-HCMV drugs, and in view of a rapid development of drug-resistant mutants in herpes simplex systems, we have examined the antiviral activity of four new nucleoside derivatives of deoxyuridine and deoxycytidine on HCMV replication in vitro. These nucleoside analogues were potent anti-HCMV inhibitors, as compared to other existing antipherpetic compounds. Here, we report the study of the inhibitory effect of these nucleoside analogues on HCMV multiplication, at the replication and translation levels.
METHODS

Nucleoside analogues. 1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)-5-methyluracil (FMAU), 1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)-5-iodouracil (FIAU), 1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)-5-methylcytosine (FMAC) and 1-(2'-deoxy-2'-fluoro-beta-D-arabinosyl)-5-iodocytosine (FIAC) were synthesized in the laboratory of J. J. Fox; 9-(2-hydroxyethoxymethyl)guanine (ACV) was provided by G. B. Elion of the Burroughs Wellcome Co.

Virus and cells. Human fibroblastic cell lines (WI-38 and HEL; passages 21 to 26) and HCMV strains Towne (passage 42), Major (passage 29), Clegg (passage 4) and D550 (passage 26) were used throughout this study. The cell cultures were grown in minimum essential medium (MEM) supplemented with 8% foetal calf serum (FCS) and antibiotics (penicillin, 100 Units/ml; streptomycin, 100 µg/ml). After virus infection, the cells were maintained in the same medium containing 3% heat-inactivated FCS. The viruses were propagated by infection of WI-38 cultures at a multiplicity of infection (m.o.i.) of 1 to 2 p.f.u./cell and were harvested from the extracellular fluid, as described previously (Huang et al., 1973).

Antiviral activity. Antiviral activity was determined by a plaque reduction assay. Confluent monolayers in six-well plates (Costar no. 3524) were infected with appropriate dilutions of the virus suspension. After adsorption for 2 h, the virus inocula were removed, the infected cell monolayers overlayed with 1% agarose in medium with 3% FCS, and supplemented with or without antiviral compounds. A second overlay was applied on the 4th day. The plates were incubated at 37 °C for 5 to 7 more days until distinct plaque foci appeared in cultures infected with virus only. The plaques were washed once with phosphate-buffered saline (PBS, pH 7-2), fixed with a mixture of ethanol:acetic acid:formaldehyde (6:2:1) and stained with 0.1% crystal violet. The plaques were scored by using a dissecting microscope (Mar et al., 1982).

Estimation of viral DNA synthesis. The effect of the antivirals on HCMV DNA synthesis was monitored by using the cRNA–DNA membrane hybridization technique. Confluent WI-38 cells or HEL cells grown in 75 cm² flasks (Corning 25115) were virus-infected (Towne strain) or mock-infected at a m.o.i. of 1 to 2 p.f.u./cell. After adsorption for 2 h, virus was removed and medium with or without drug was added. At a designated time after infection the cells were lysed with a lysis buffer (0·01 M-Tris–HCl pH 8·0, 10 mM-EDTA, 1% SDS) and the lysates were stored at −20 °C. After all the samples had been collected, they were digested with Pronase (1 mg/ml) for 4 h at 37 °C, and ethanol-precipitated after phenol extraction. The precipitate containing DNA and RNA was dissolved in 2 × SSC (1 × SSC = 0·15 M-NaCl, 0·015 M-sodium citrate) and treated with pancreatic RNAse (40 µg/ml) for 3 h at 37 °C. The resulting mixture was subjected to one more cycle of phenol extraction and ethanol precipitation. The precipitate was dissolved in 0·1 × SSC. For cRNA–DNA hybridization, 30 µg DNA from each sample was immobilized on 25 mm nitrocellulose filters as described previously (Gillespie & Spiegelman, 1965; Huang, 1975). The filters were vacuum-dried at 80 °C for 2 h and prehybridized at 66 °C for 1 h in buffer A (0 × SSC, 0·1% SDS, 1 mg/ml yeast tRNA). Then HCMV 3H-cRNA having 200000 ct/min (sp. act. 1·2 × 10⁷ ct/min/µg) in buffer A was applied to each filter in glass scintillation vials. The hybridization was performed at 66 °C for 18 h. After thorough washing with 0·1 × SSC, the nitrocellulose filters were treated with RNase to remove the unhybridized RNA. After drying, the radioactivity on the filters was determined using a liquid scintillation counter.

Viral protein synthesis. WI-38 or HEL cell monolayers (90% confluent) grown in 25 cm² flasks were infected with HCMV Towne strain at a m.o.i. of 1 to 2 p.f.u./cell. The drugs were added to the infected cell cultures 2 h after virus adsorption and removed before labelling at the designated time. Prior to harvesting, the cultures were labelled for 2 h with [35S]methionine (5 µCi/ml, New England Nuclear) in MEM containing 3% heat-inactivated FCS and one-tenth the normal concentration of methionine. After labelling, cells were treated as follows. The monolayer was rinsed twice with TBS (50 mM-Tris–HCl pH 7·4, 150 mM-NaCl). The cells were scraped with a rubber policeman into a total volume of 5 ml TBS. The samples were centrifuged for 5 min at 2000 rev/min in a Sorvall (RC-5) centrifuge using the HB-4 rotor. The supernatants were discarded and pellets frozen at −70 °C. After all the samples were collected, 0·4 ml buffer (10 mM-Tris–HCl pH 8·0, 10 mM-EDTA, 10 mM-2-mercaptoethanol, 1 mM-p-mercaptoethylamine, 1 mM-pa-methylphenylsulphonyl fluoride) was added, and the resulting mixture was sonicated for 1 min on ice. Proteins were precipitated by trichloroacetic acid, washed with ethanol and dissolved in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) buffer (Laemmli, 1970). The [35S]methionine-labelled proteins were identified by SDS–PAGE through 9% polyacrylamide gels in a Tris buffer system as described by Laemmli (1970), and detailed previously (Mar et al., 1982). The gels were vacuum-dried onto Whatman 3 MM chromatography paper and exposed to X-ray film (Kodak RP1R2) for autoradiographs.

RESULTS

Antiviral and anticellular activities of FMAU, FIAU, FMAC and FIAC in vitro

The antiviral activities of FMAU, FIAU, FMAC and FIAC were determined by a plaque reduction assay and expressed in terms of ED₅₀. ED₅₀ values are defined as the concentrations of the
Anti-HCMV nucleoside analogues

Table 1. Anti-HCMV activity of FMAU, FIAU, FMAC and FIAC

<table>
<thead>
<tr>
<th>Strain</th>
<th>FMAU</th>
<th>FIAU</th>
<th>FMAC</th>
<th>FIAC</th>
<th>ACV†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Towne</td>
<td>0.10</td>
<td>0.36</td>
<td>0.26</td>
<td>0.30</td>
<td>96</td>
</tr>
<tr>
<td>Major</td>
<td>0.21</td>
<td>0.46</td>
<td>0.44</td>
<td>0.51</td>
<td>86</td>
</tr>
<tr>
<td>Clegg</td>
<td>0.18</td>
<td>0.41</td>
<td>0.34</td>
<td>0.48</td>
<td>100</td>
</tr>
<tr>
<td>Dsso</td>
<td>0.12</td>
<td>0.56</td>
<td>0.39</td>
<td>0.65</td>
<td>100</td>
</tr>
</tbody>
</table>

* Concentration required to inhibit 50% of plaque formation.
† The data obtained here are in agreement with our previous report (Mar et al., 1982) and used as a comparison.

Table 2. Effect of FMAU, FIAU, FMAC, and FIAC on cell growth*

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID₅₀, μM†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMAU</td>
<td>6</td>
</tr>
<tr>
<td>FIAU</td>
<td>10</td>
</tr>
<tr>
<td>FMAC</td>
<td>8</td>
</tr>
<tr>
<td>FIAC</td>
<td>16</td>
</tr>
<tr>
<td>ACV</td>
<td>250†</td>
</tr>
</tbody>
</table>

* The cytotoxicity of the inhibitors for WI-38 cells was monitored for 24 h (48 h after treatment) by measuring the inhibition of incorporation of [³H]thymidine into cellular DNA.
† ID₅₀ is defined as the concentration of a drug required to inhibit 50% incorporation of [³H]thymidine into DNA.
‡ The data obtained here are used as a comparison.

inhibitors at which viral replication was inhibited by 50%. These four nucleoside analogues exhibited anti-HCMV potency and had ED$_{50}$s ranging from 0-1 to 0-65 μM for the four HCMV isolates tested (Table 1).

The cytotoxicity of the inhibitors for WI-38 and HEL cells was monitored for 24 h (48 h after treatment) by measuring the inhibition of the incorporation of [³H]thymidine into cellular DNA. ID$_{50}$ represents the dose of a drug required to inhibit 50% incorporation of [³H]thymidine into WI-38 and HEL cell DNA. The ID$_{50}$s for FMAU, FIAU, FMAC and FIAC in WI-38 and HEL cells ranged from 6 to 16 μM (Table 2). There were no significant differences between WI-38 and HEL cell lines in their susceptibility to virus infection and sensitivity to the inhibitors.

Effect of FIAC and FMAU on viral DNA synthesis

The effect of the drugs on viral DNA replication in HCMV-infected WI-38 cells was determined by a nucleic acid membrane hybridization technique with HCMV-specific ³H-cRNA as a probe. Infection of WI-38 or HEL cells with HCMV Towne strain at a m.o.i. of 1 to 2 allowed the detection of viral DNA synthesis by cRNA–DNA hybridization at approximately 20 h after infection. Viral DNA continued to accumulate up to at least 96 h post-infection in this study, as shown in Fig. 1. On the other hand, we hardly detected the viral genomes in the drug-treated infected cells at a concentration of 1 μM-FIAC or -FMAU for up to 7 days after infection (Fig. 1). However, after the removal of drug at different times after infection the synthesis of viral DNA resumed.

Effect of FIAC and FMAU on HCMV-specific protein synthesis

In addition to the study of the effect of the drugs on viral DNA synthesis, we also investigated whether inhibition of viral DNA replication had any effect on virus-specific protein synthesis. At a concentration of 1 μM-FIAC or -FMAU we examined the effect of these two drugs on the kinetics of viral protein synthesis in Towne HCMV-infected WI-38 cells.
FIG. 1. Effect of FIAC and FMAU on HCMV Towne DNA replication in virus-infected WI-38 cells. Drug was added to the infected cultures at a final concentration of 1 μM immediately after virus adsorption. At various times after infection, the DNA was extracted from the infected cell culture for virus genome quantification by 3H-labelled cRNA-DNA membrane hybridization. Amounts of DNA and HCMV-specific 3H-cRNA applied to each filter were 30 μg and 4 × 10^{5} ct/min, respectively. The nucleic acid hybridization was performed as described previously (Gillespie & Spiegelman, 1965; Huang, 1975; Mar et al., 1982). ○, Viral DNA content of cultures without drug treatment; □—□, viral DNA content in the presence of 1 μM-FIAC and 1 μM-FMAU, respectively; △—△, recovery of DNA synthesis after the removal of 1 μM-FIAC and 1 μM-FMAU, respectively.

The results revealed that the inhibitory effect of FIAC and FMAU on the viral protein synthesis patterns were indistinguishable. Fig. 2 illustrates the autoradiographs of such a study using FMAU at a final concentration of 1 μM. The synthesis of five virus-specific polypeptides (VP150K, VP120K, VP67K, VP54K and VP27K) was completely blocked by 48 h and up to 96 h post-infection, as indicated by the arrows (Fig. 2). Because viral proteins appeared after viral DNA synthesis, they are virus-specific late antigens in nature.

**DISCUSSION**

A series of 2'-fluoro-5-substituted arabinofuranosylcytosines and -uracils has been synthesized (Watanabe et al., 1979; Fox et al., 1981). Four of these, FMAU, FIAU, FMAC and FIAC, were found to be very potent and highly selective against herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) at very low drug levels (Lopez et al., 1979). Furthermore, FIAC was reported to have anti-HCMV activity at a concentration as low as 0-1 μM (Lopez et al., 1979). In the present study, we have also demonstrated that FMAU, FIAU, FMAC and FIAC are potent anti-HCMV agents with viral ED<sub>50</sub> ranging from 0-1 to 0-65 μM, depending upon the virus strains used (Table 1). The ID<sub>50</sub> of these nucleosides for the cell growth were in the range of 6 to 16 μM (Table 2). Our data shown here are comparable to those reported by Lopez et al. (1979). In a direct comparison with an antitherpetic compound, ACV (Elion et al., 1977), having limited anti-HCMV activity (Crumpacker et al., 1979; Mar et al., 1982), FMAU, FIAU, FMAC and FIAC were found to be effective in vitro at concentrations 960 to 150 times lower and to be toxic at 1/40 to 1/15 the concentration of ACV, suggesting that FMAU, FIAU, FMAC and FIAC have higher therapeutic indices (ID<sub>50</sub>/ED<sub>50</sub>) than ACV.
Fig. 2. Time course of synthesis of virus-specific proteins in HCMV-infected WI-38 cells. The protein synthesis was studied by incorporation of [35S]methionine (5 μCi/ml) at the designated time (h) after infection. Autoradiography was done on radiolabelled polypeptides analysed by SDS-PAGE. Numbers at the right indicate mol. wt. × 10^{-3}; M, mock-infected cells. Arrows indicate that the synthesis of virus-specific proteins is inhibited by 1 μM-FMAU.

Quantification of viral DNA synthesis, using a cRNA–DNA hybridization technique, demonstrated a complete inhibition of HCMV DNA replication at 1 μM-FMAU or -FIAC (Fig. 1). This inhibition was confirmed by another experiment in which incorporation of [32P]orthophosphate into viral and cellular DNA was determined after separating DNA in CsCl gradients. At the drug concentration of 1 μM-FMAU, or -FIAC, no detectable viral DNA with a buoyant density of 1.7160 g/ml was observed. On the other hand, the cellular DNA (ρ = 1.7000 g/ml) was intact regardless of the presence of the inhibitor in cell cultures (data not shown). At 1 μM-FMAU, the synthesis of virus-specific late polypeptides (VP150, VP120, VP67, VP54 and VP27) was completely blocked for up to 96 h post-infection; in particular, the most abundant viral polypeptide (VP67) was strikingly inhibited (Fig. 2). This VP67 has been identified as the major phosphorylated virion protein (Mar et al., 1981, 1982). These results suggest that the mode
of action of these anti-HCMV nucleoside analogues might be inhibition of virus multiplication by impairing the viral DNA synthesis. Consequently, the synthesis of viral late proteins was impeded since their expression required viral DNA synthesis.

Upon removal of the inhibitor, the viral DNA synthesis resumed (Fig. 1), and virus obtained from the extracellular fluid became infectious, as compared with that from drug-treated infected cell cultures (data not shown). These results reflected others, that inhibition by this category of nucleoside analogues appears to be a virostatic reversible type of inhibition, as demonstrated previously for an anti-HCMV drug, PAA (Huang, 1975).

Table 1 shows that FIAU and FIAC exhibited an equipotency as judged by their ED50s against HCMV replication in a plaque reduction assay. Cytidine derivatives are prone to be deaminated by cytidine deaminase; Philips et al. (1980), using [2-14C]FIAC, demonstrated that FIAC and its deaminated metabolites (FIAU, or partly FMAU) are concomitantly incorporated in vivo into DNA in mice. Therefore the question is raised, of whether the antiviral activity of FIAC was in fact attributable to the formation of FIAU through a deamination pathway by cytidine deaminase in situ. Thus FIAC could be considered as a prodrug of FIAU. Accordingly, in the in vitro study, we manipulated the conditions by co-administration of FIAC plus tetrahydrouridine (THU), an inhibitor of cytidine deaminase (Camiener, 1968; Cohen & Wolfenden, 1971; Hanze, 1967; Neil et al., 1970). The results demonstrated that the antiviral potency was not lessened at all by the presence of THU in a molar ratio of 25:1 to FIAC (data not shown). Moreover, Chou et al. (1980) report that the deamination of FIAC in human leukaemic and monkey kidney (Vero) cells can be inhibited by THU. Taken together, these data suggest that the antiviral activity of FIAC was unlikely to be due to its deaminated product, FIAU.

The selectivity of FMAU, FIAU, FMAC and FIAC against HSV-1 and HSV-2 (Cheng et al., 1981; Lopez et al., 1980) was shown to depend on the virus-specific thymidine kinase (TK) in infected cell systems. This conclusion was based on the ineffectiveness of these nucleoside analogues against TK mutants of HSV-1 and HSV-2 (Cheng et al., 1981; Lopez et al., 1980).

No evidence was found which demonstrated that an HCMV-specific TK activity was present in infected cell systems (Estes & Huang, 1977; Zavada et al., 1976). However, these potent nucleoside analogues exhibited their anti-HCMV effect at ED50s ranging from 0.1 to 0.65 μM. This suggests that the mechanisms of anti-HCMV action of these derivatives might be different from those of their anti-HSV effect. Further examination of the mechanisms(s) underlying these nucleoside analogues' antiviral activity in HCMV-infected cells is deserved. Research in this field is continuing.

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REFERENCES


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