

Efficacy and Selectivity of Some Nucleoside Analogs as Anti-Human Cytomegalovirus Agents

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1-(2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC), 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-methyluridine (FMAU), 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouridine (FIAU), and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-ethyluridine (FEAU) were evaluated for antiviral activities against human cytomegalovirus (HCMV) and compared with 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir) and E-5-(2'-bromovinyl)-2'-deoxyuridine (BVDU). The relative anti-HCMV potencies of these compounds, as determined by calculating the dose of drug which inhibited 50% plaque formation, were in order of decreasing potency: FIAC > FIAU > FMAU > acyclovir > FEAU > BVDU. The antiviral activity of FIAC occurred at levels much lower than those that caused cytotoxic or cytostatic effects in uninfected fibroblasts. Neither thymidine nor deoxycytidine reversed the anti-HCMV activity of FIAC, indicating that this drug was not acting as an analog of the natural nucleosides. FIAC was not phosphorylated by cytosols of HCMV-infected cells to a greater extent than by those of uninfected cells, indicating that, unlike the antiviral activity against herpes simplex virus type 1, the selectivity of this drug is probably not based on a virus-specified pyrimidine kinase.

With the advent of aggressive immunosuppression for renal transplantation (1), bone marrow transplantation (19), and, more recently, heart transplantation (20) has come the recognition that human cytomegalovirus (HCMV) can cause life-threatening infections and is a major impediment to successful use of these treatments. In addition, HCMV has been associated with Kaposi's sarcoma, a malignancy which until recently was found only in older men in the United States (9), but which has been detected during the past 4 years in male homosexuals with acquired immunodeficiency syndrome (12). Recognition of HCMV as an important human pathogen has resulted in attempts to develop new chemotherapeutic agents for treatment of infections which it produces or with which it is associated. In this communication, we present evidence that one newly developed nucleoside analog has potent and selective activity against HCMV and that this activity is clearly different from that against herpes simplex virus type 1.

MATERIALS AND METHODS

Unlabeled and [2-¹⁴C]1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC), unlabeled and [2-¹⁴C]1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouridine (FIAU), 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-methyluridine (FMAU), and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-ethyluri-

dine (FEAU) were prepared with appropriate structural verification and supplied by J. Fox and K. Watanabe of this Institute (21, 23; K. A. Watanabe, T.-L. Su, U. Reichman, N. Greenberg, C. Lopez, and J. J. Fox, *J. Med. Chem.*, in press). 9-[(2-Hydroxyethoxy)methyl]guanine (ACV) was provided by G. B. Elion of Burroughs Wellcome Co., and E-5-(2'-bromovinyl)-2'-deoxyuridine (BVDU) was provided by R. T. Walker, University of Birmingham, Birmingham, England.

Cultures of primary human foreskin fibroblasts were started in this laboratory and used for no more than 10 passages. Cells were routinely checked for mycoplasma contamination (22). Monolayers of the fibroblasts were grown to about 80% confluency in 150-cm² flasks for routine subculture of HCMV. Strain AD169 was obtained from the American Type Culture Collection and was used for most of these studies. Some experiments were carried out with HCMV strain 303, isolated from the urine of a child with a perinatal infection (Lopez, unpublished data). Subculture of HCMV was carried out by using infected cells plus supernatant fluid.

The plaque reduction assay was performed with 24-well Linbro plates (Flow Laboratories, Inc., McLean, Va.). Monolayers of 80 to 90% confluent fibroblasts were inoculated with 10 to 15 PFU of HCMV. After a 2-h adsorption period at 37°C, unadsorbed virus was removed by a wash with phosphate-buffered saline, and the monolayers were covered with maintenance medium containing 0.5% methylcellulose (18). Drugs to be tested for antiviral activity were incorporated into the overlay at various concentrations. Monolay-

ers were monitored for viral cytopathic effect, and when the effect was well developed in the drug-free control (usually 10 to 12 days), the cells were washed from the overlay, fixed with methanol, and stained with Giemsa. Distinct viral plaques were counted, and the 50% effective dose was calculated by the method of Dougherty (5). Tests were carried out in duplicate, and each test was performed three times.

Cellular cytotoxicity was determined by using the same cells used in the plaque reduction assay described above. Briefly, human foreskin fibroblasts were plated into wells of the 24-well Linbro plates at a concentration of 50,000 cells per ml in growth medium. After 18 h of incubation at 37°C to allow attachment of cells to the plastic, the medium was replaced with growth medium containing drugs at a concentration of 100, 10, 1, 0.1, 0.01, or 0.001 μM . The number of cells initially plated allowed for their continuous replication for the 4 days of the experiment. The cells were then trypsinized from the monolayer, and viable cells were counted with the aid of trypan blue and a hemacytometer. The concentration of drug required to inhibit the replication of fibroblasts by 50% was determined by the method of Dougherty (5). Very few dead cells were found, indicating that the reduced cell number was the result of cytostasis rather than cytotoxicity.

Cell cytosols were prepared for evaluation of pyrimidine kinase activity by the procedure of Kit et al. (16). Briefly, uninfected fibroblasts were grown to ca. 80 to 90% confluency in 150-cm² flasks before being harvested for cytosol preparation. HCMV strain AD169-infected fibroblasts were prepared by inoculating 80 to 90% confluent monolayers with virus at a multiplicity of infection of about one. Typical cytopathic effect encompassed about 75% of the monolayer by about 7 days postinoculation. Cells were trypsinized to remove them from the monolayer and washed once with phosphate-buffered saline and once with reticulocyte swelling buffer (0.01 M KCl, 0.0015 M MgCl₂, 0.01 M Tris-hydrochloride, pH 7.4). The cells were then suspended in 4 volumes of reticulocyte swelling buffer containing 0.05 M ϵ -amino caproic acid, a protease inhibitor, and 0.2 mM thymidine. The cells were allowed to swell for 10 min at 4°C, after which time they were disrupted in a Dounce homogenizer. One-ninth volume of 1.5 M KCl-0.03 M 2-mercaptoethanol was then added to the homogenate. This was centrifuged at 9,500 $\times g$ for 10 min to remove cellular debris and at 105,000 $\times g$ for 1 h at 4°C. Nonidet P-40 was added to yield 0.5% (vol/vol), and the preparation was divided into aliquots and stored in liquid nitrogen until used.

Phosphorylation of FIAC and FIAU was evaluated as described earlier by Kreis et al. (17). Briefly, reaction mixtures contained 50 mM Tris-hydrochloride (pH 8.0), 10 mM ATP, 25 mM NaF, 0.3 mM creatine phosphate, 1 U of creatine phosphokinase per ml, 2.5 mg of dithiothreitol per ml, 16 mM MgCl₂, and 0.05 mg of bovine albumin per ml. The FIAC reaction mixture contained 10 μM [2-¹⁴C]-FIAC (1.3 $\mu\text{Ci/ml}$) and 0.2 mM tetrahydrouridine to prevent deamination. The FIAU reaction mixture contained 13.5 μM [2-¹⁴C] FIAU (5.2 $\mu\text{Ci/ml}$). Reactions were carried out at 37°C in a total volume of 0.250 ml (0.2 ml of the reaction mixture was added to 0.05 ml of enzyme source). Reactions were stopped by spotting 0.02-ml portions of the reaction mixture onto Whatman DE-81 filter

disks. The disks were washed twice with 15 ml of 1 mM ammonium formate, twice with 15 ml of distilled water, and once with 5 ml of 95% ethanol. The disks were then transferred to scintillation vials with 1 ml of 0.2 M KCl in 0.1 N HCl, and 10 ml of Biofluor (New England Nuclear Corp., Boston, Mass.) was added. The samples were refrigerated in the dark for 18 h before being counted.

RESULTS

Table 1 summarizes the results obtained in five experiments evaluating FIAC, FMAU, FIAU, ACV, and BVDU in the plaque reduction assay with HCMV and in a sensitive cytotoxicity assay with the same fibroblasts used in the plaque reduction assay. Only FIAC, FIAU, and FMAU consistently reduced plaque formation at drug concentrations below 1 μM . In most experiments, HCMV strain AD169 was used, but these drugs also were active with a recently isolated strain designated 303. In parallel experiments, however, two of these compounds FIAU and FMAU, demonstrated cytotoxicity with fibroblasts at levels equal to or below those showing antiviral effects. This cytotoxicity assay is very sensitive and yielded 50% inhibitory doses, which were lower than those published for ACV (6) and BVDU (4). Only FIAC demonstrated truly selective antiviral activity since it was active against HCMV at 1/13 of the concentration required to inhibit the cell replication. Addition of tetrahydrouridine, a deoxycytidine deaminase inhibitor, to either the cytotoxicity assay or to the plaque reduction assay failed to alter significantly these results, indicating that the active component in each assay was FIAC and not FIAU, its deamination product (data not shown).

We tried to determine whether FIAC was acting as an analog for one of the natural nucleosides by using them to block the antiviral activity of this compound. Addition of equimolar concentrations of thymidine failed to reverse the antiviral activity and had no significant effect on the cytotoxicity (data not shown). A 10-fold excess of the thymidine failed to reverse the antiviral activity of FIAC and at higher concen-

TABLE 1. Inhibitory action of nucleoside analogs

Drug	ED ₅₀ \pm SD ^a	ID ₅₀ \pm SD ^b
FIAU	0.2 \pm 0.14	0.13 \pm 0.025
FMAU	0.2 \pm 0.1	0.2 \pm 0.1
FIAC	0.6 \pm 0.13	7.6 \pm 3.3
ACV	11.0 \pm 7.0	14.0 \pm 9.0
FEAU	45.0 \pm 17	14.0 \pm 11
BVDU	115.0 \pm 26	20.0 \pm 4.0

^a ED₅₀, Concentration (μM) of drug required for 50% inhibition of plaque formation.

^b ID₅₀, Concentration (μM) of drug required to inhibit by 50% replication of human skin fibroblasts.

trations was toxic to the cells. In contrast, a 10-fold excess of deoxycytidine readily reversed the cellular cytotoxicity of FIAC but failed to inhibit plaque reduction.

Our earlier studies showed that herpes simplex virus type 1-induced pyrimidine nucleoside kinase was capable of phosphorylating FIAC far better than the cellular enzymes and that this substrate preference determined, at least in part, the selective antiviral activity of this drug (18). Studies were therefore undertaken to determine whether FIAC is preferentially phosphorylated by enzymes found in cytosols from HCMV-infected rather than uninfected cells. As others have noted (7, 14), we found that HCMV infection of fibroblasts resulted in a marked (up to 10-fold increase in thymidine kinase activity (data not shown). In addition, we found a slightly (twofold at most) increased deoxycytidine kinase activity in HCMV-infected cells (data not shown). Figure 1 presents the results of a typical experiment. Although both FIAC and FIAU were preferentially phosphorylated by enzymes found in herpes simplex virus type 1 cytosols, neither was preferentially activated by HCMV cytosols.

DISCUSSION

Infection of permissive cells by members of the herpesvirus group of viruses results in the expression in these cells of certain virally coded enzymes which have functions identical to those found in the uninfected cells but which differ in physical and chemical properties (3, 13, 15). Two of these enzymes, a pyrimidine nucleoside kinase and a DNA polymerase (3, 10), have been shown to have substrate specificities different from that of the normal cellular enzyme and have been employed as targets for the development of selective antiviral drugs. Studies with ACV, BVDU, and FIAC have strongly suggested that these compounds have selective activity against herpes simplex virus type 1 and herpes zoster virus because of the preferential phosphorylation of these compounds by the virally specified pyrimidine nucleoside kinase and, perhaps also because of a preferential interaction with the virally specified DNA polymerase (4, 8). Although HCMV is a member of the herpesvirus group of viruses, the studies of Estes and Huang (7) have shown that infection with this virus fails to induce a new pyrimidine nucleoside kinase. Others have shown that HCMV infection results in a virally specified DNA polymerase (10, 11).

The studies presented herein clearly demonstrate that FIAC, FMAU, and FIAU are potent inhibitors of HCMV replication in culture. Furthermore, FIAC activity appears to be selective in that it inhibited plaque formation at concen-

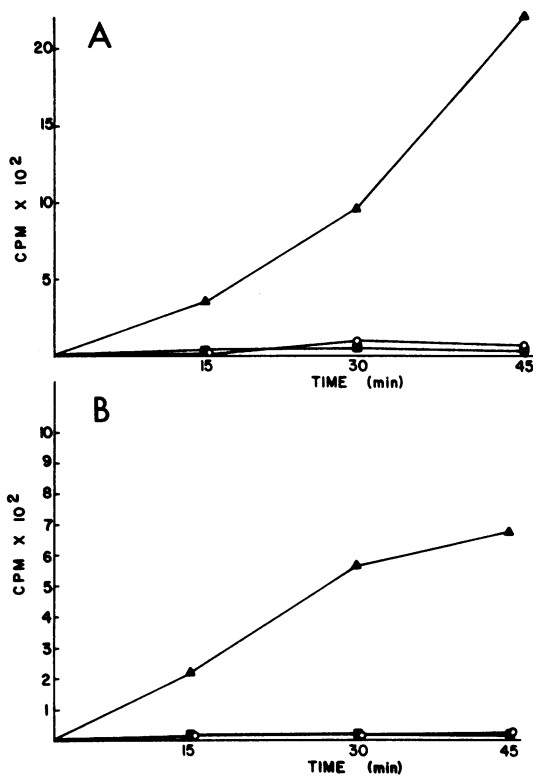


FIG. 1. (A) Phosphorylation of [¹⁴C]FIAC. (B) Phosphorylation of [¹⁴C]FIAU. Symbols: (■) uninfected skin fibroblast cytosol; (○) HCMV strain AD169-infected skin fibroblast cytosol; (▲) herpes simplex virus type 1 strain 2931-infected skin fibroblast cytosol. CPM, Counts per minute.

trations of drug which were not cytotoxic or cytostatic. The cytotoxicity assay used here is very sensitive but was used to determine whether an effect on the cellular metabolism might be responsible for the antiviral effects found. Since selectivity was only found with FIAC, the antiviral effects of the other compounds might be a result of an effect of the drug on a metabolic step in the cell which is essential for viral replication.

Our observation that FIAC selectively inhibits HCMV plaque formation suggests that it may act on a viral metabolic step either not shared by the uninfected cell or not as sensitive in it. Although FIAC is not selectively phosphorylated in HCMV-infected cells, enough of the drug may be converted to the triphosphate by cellular enzymes to selectively inhibit the viral DNA polymerase. Alternatively, FIAC may act by a mechanism which does not require that it be phosphorylated in HCMV-infected cells. In similar studies, Burns et al. (2) showed that ACV inhibits mouse cytomegalovirus replication without being phosphorylated selectively in infected cells. In preliminary studies, we found

that FIAC fails to be incorporated into HCMV DNA (Colacino and Lopez, unpublished data) again suggesting that its mechanism of action differs qualitatively from that found for herpes simplex virus type 1.

The selective activity of FIAC against HCMV suggests that it may be a candidate for clinical trials with this virus in humans. Since there appear to be no other clinically useful antiviral compounds which demonstrate selective activity against HCMV, an understanding of its mechanism of action will be important to our development of other drugs with perhaps even greater selective activity.

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LITERATURE CITED

1. Betts, R. F. 1982. Cytomegalovirus infection in transplant patients. *Prog. Med. Virol.* 28:44-64.
2. Burns, W., J. Wingard, W. Bender, and R. Saral. 1981. Thymidine kinase not required for antiviral activity of acyclovir against mouse cytomegalovirus. *J. Virol.* 39:889-893.
3. Cheng, Y.-C. 1977. A rational approach to the development of antiviral chemotherapy: alternative substrates of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) thymidine kinases. *Ann. N.Y. Acad. Sci.* 284:594-598.
4. Descamps, J., R. K. Sehgal, E. De Clercq, and H. S. Allaudeen. 1982. Inhibitory effect of *E*-5-(2-bromovinyl)-1- β -D-arabinofuranosyluracil on herpes simplex virus replication and DNA synthesis. *J. Virol.* 43:332-336.
5. Dougherty, R. M. 1964. Animal virus titration techniques, p. 169-223. *In* R. J. C. Harris (ed.), *Techniques in experimental virology*. Academic Press, Inc., New York.
6. Ellison, G. 1982. Mechanism of antiviral selectivity of acyclovir. *Am. J. Med.* 73:7-13.
7. Estes, J. E., and E.-S. Huang. 1977. Stimulation of cellular thymidine kinase by human cytomegalovirus. *J. Virol.* 24:13-21.
8. Furman, P. A., M. H. St. Clair, J. A. Fyfe, J. L. Rideout, P. M. Keller, and G. B. Ellison. 1979. Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)guanine and its triphosphate. *J. Virol.* 32:72-77.
9. Giraldo, G., E. Beth, W. Henle, G. Henle, V. Mike, B. Safai, J. M. Huraux, J. McHardy, and G. De-The. 1978. Antibody patterns to herpesviruses in Kaposi's sarcoma. II. Serological association of American Kaposi's sarcoma with cytomegalovirus. *Int. J. Cancer* 22:126-131.
10. Hirai, K., and Y. Watanabe. 1976. Induction of alpha-type DNA polymerase in human cytomegalovirus-infected WI-38 cells. *Biochim. Biophys. Acta* 447:238-239.
11. Huang, E.-S. 1975. Human cytomegalovirus, III. Virus-induced DNA polymerase. *J. Virol.* 16:298-310.
12. Hymes, K. B., T. Cheung, J. B. Greene, N. S. Prose, A. Marcus, H. Ballard, D. C. William, and L. Laubenstein. 1981. Kaposi's sarcoma in homosexual men—a report of eight cases. *Lancet* ii:598-600.
13. Jamieson, A. T., G. A. Gentry, and J. H. Subak-Sharpe. 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. *J. Gen. Virol.* 24:465-480.
14. Jerkovsky, M., M. Dobersen, and S. Greer. 1980. Inhibition of the replication of human cytomegalovirus by bromodeoxycytidine in the absence of detectable increases in bromodeoxycytidine kinase activity. *Intervirology* 14:233-238.
15. Kit, S. 1979. Viral-associated and induced enzymes. *Pharmacol. Ther.* 4:501-585.
16. Kit, S., W. C. Leung, D. Trkula, and G. Jorgensen. 1974. Gel electrophoresis and isoelectric focusing of mitochondrial and viral induced thymidine kinases. *Int. J. Cancer* 13:203-218.
17. Kreis, W., L. Damin, J. Colacino, and C. Lopez. 1982. *In vitro* metabolism of 1- β -D-arabinofuranosylcytosine and 1- β -2'-fluoroarabino-5-iodocytosine in normal and herpes simplex type 1 virus-infected cells. *Biochem. Pharmacol.* 31:767-773.
18. Lopez, C., K. A. Watanabe, and J. J. Fox. 1980. 2'-Fluoro-5-iodoarabicytosine, a potent and selective anti-herpesvirus agent. *Antimicrob. Agents Chemother.* 17:803-806.
19. Meyers, J. D., H. C. Spencer, J. C. Watts, M. B. Gregg, J. A. Stewart, R. H. Troupin, and E. D. Thomas. 1975. Cytomegalovirus pneumonia after human marrow transplantation. *Ann. Intern. Med.* 82:181-188.
20. Pollard, P. B., K. H. Rand, A. M. Arvin, and T. C. Merigan. 1978. Cell-mediated immunity to cytomegalovirus infection in normal subjects and cardiac transplant patients. *J. Infect. Dis.* 137:541-549.
21. Reichman, U., K. A. Watanabe, and J. J. Fox. 1975. A practical synthesis of 2-deoxy-2-fluoro-D-arabinofuranose derivatives. *Carbohydr. Res.* 42:233-240.
22. Schneider, E. L., E. J. Stanbridge, and C. J. Epstein. 1974. Incorporation of ^3H -uridine and ^3H -uracil into RNA. A simple technique for the detection of mycoplasma contamination of cultured cells. *Exp. Cell Res.* 84:311-318.
23. Watanabe, K. A., U. Reichman, K. Hirota, C. Lopez, and J. J. Fox. 1979. Nucleosides 110. Synthesis and anti-herpes virus activity of some 2'-fluoro-2'-deoxyarabinofuranosyl-pyrimidine nucleosides. *J. Med. Chem.* 22:21-24.