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2'-Fluoro-5-Iodo-Aracytosine, a Potent and Selective Anti-Herpesvirus Agent

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A newly synthesized pyrimidine analog, 2'-fluoro-5-iodo-aracytosine (FIAC), suppressed by 90% the replication of various strains of herpes simplex virus types 1 and 2 at concentrations of 0.0025 to 0.0126 μM. Cytotoxicity was minimal, as determined by trypan blue dye exclusion with normal Vero, WI-38, and NC-37 cell proliferation; the 50% inhibitory dose was 4 to 10 μM in a 4-day assay. When compared with other antiviral drugs, FIAC was active at much lower concentrations than arabinosylcytosine, iododeoxyuridine, and arabinosyladenine. It was slightly more active against herpes simplex virus type 1 than acycloquanosine and slightly more toxic to normal cells. FIAC was about 8,000 times more active against the replication of wild-type herpes simplex virus type 1 than against a mutant strain lacking the expression of virus-specified thymidine kinase. Since FIAC appears to be preferentially phosphorylated by the viral enzyme, this is probably responsible, at least in part, for the selectivity of its antiviral actions. Although FIAC appears to be an arabinosylcytosine analog, its antiviral activity was not reversed by deoxycytidine. The minimal cytotoxicity exhibited by FIAC for normal cells, however, was reversed by equimolar concentrations of deoxycytidine. Thymidine, which reversed the antiviral activity, was effective only when used in great excess.

Biochemical studies with the herpesviruses have revealed that infection of susceptible cells in a culture leads to the expression of at least one, and possibly two or more, enzymes coded for by the infecting virus (1, 5-7, 9-13). Infection with herpes simplex virus type 1 (HSV-1), HSV-2, herpes zoster virus (HZV), cytomegalovirus, or Epstein-Barr virus results in the expression of a new deoxyribonucleic acid polymerase that has properties different from those of the normal cellular enzymes (1, 5, 9, 11-13). In addition, HSV-1, HSV-2, and HZV also induce a virus-specific nucleotide kinase (4, 6, 7, 10), whereas cytomegalovirus induces increased expression of cellular thymidine kinase. Although these enzymes are thought to be coded for by the infecting virus, proof of this has been obtained only for HSV-1 (7, 13). These virus-specified enzymes appear to have substrate requirements which differentiate them from normal cellular enzymes and which, therefore, make them excellent targets for the development of selective antiviral chemotherapeutic drugs (2, 3).

Recently, Watanabe et al. (14) have synthesized a new series of nucleoside analogs, the 5-substituted 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-cytosines. One of this series of new compounds, 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (also called 2'-fluoro-5-iodo-aracytosine [FIAC]), was found in preliminary studies to be a potent inhibitor of HSV-1 replication in vitro, with minimal toxicity to mouse leukemic cell replication (14). In this report, we present further studies of the capacity of FIAC to suppress replication of HSV-1, HSV-2, and HZV and present evidence which indicates that the selectivity demonstrated by FIAC probably depends on the virus-specified nucleotide kinase.

MATERIALS AND METHODS

Virus strains. Most experiments with HSV-1 were carried out with strain 2931, which was isolated and characterized previously (8). Other HSV-1 strains included the Patton strain (obtained from M. Bernhardt, Sloan-Kettering Institute), the MacIntyre strain (obtained from the American Type Culture Collection), and the HEFM strain (obtained from Barry Bloom, Albert Einstein College of Medicine, Bronx, N.Y.). The G strain of HSV-2 was obtained from Bernard Roizman, University of Chicago, and strain 333 was obtained from Fred Rapp, Pennsylvania State University, Hershey, Pa. The Ellen strain of HZV was obtained from Richard Price, Sloan-Kettering Institute, and the thymidine kinase-deficient mutant of HSV-1 was a gift from Bernard Roizman.

Cell cultures. All HSV-1 and HSV-2 strains were propagated and quantitated on monolayers of Vero cells obtained from the American Type Culture Collection. Routine passage of virus was carried out at low multiplicities of infection to minimize defective particles. HZV was propagated and assayed on mono-

layers of WI-38 normal human fibroblasts, which were obtained from L. Hayflick, Stanford University, Stanford, Calif.

The Vero and FS-4 monolayers were propagated in Dulbecco modified Eagle medium containing 10% fetal calf serum and antibiotics (streptomycin and penicillin) (GIBCO Laboratories, Grand Island, N.Y.) and were maintained in the same medium containing 2% fetal calf serum. NC-37 cells were propagated and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (GIBCO).

Reagents. FIAC was synthesized and characterized as described by Watanabe et al. (14). 5-Iodo-2'-deoxyuridine and deoxycytidine were purchased from Calbiochem, La Jolla, Calif.; 9-β-D-arabinofuranosyladenine was obtained from Parke, Davis & Co., Detroit, Mich.; 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine) was a gift from James A. Fyfe, Burroughs Wellcome Co., Research Triangle Park, N.C.; 1-β-D-arabinofuranosylcytosine was obtained from Harry Wood, Drug Development Branch, National Cancer Institute; 1-β-D-arabinofuranosylthymine was obtained from I. Wempen, Sloan-Kettering Institute; and thymidine was obtained from P-L Biochemical, Inc., Milwaukee, Wis.

Inhibition of virus replication. Monolayers of Vero cells were established in wells of a Linbro FB-16-24-TC plate (Linbro Chemical Co., Inc., New Haven, Conn.) in 1 ml of medium. Monolayers were inoculated with virus at a multiplicity of infection of 1 plaque-forming unit per cell and after a 2-h absorption period were overlaid with maintenance medium containing varying concentrations of drugs or no drug (controls). After 18 to 20 h the supernatant fluids were removed, centrifuged to remove debris (10 min at 300 × *g*), and stored at −70°C until titrated. Virus was quantitated on Vero cell monolayers in microwells with flat bottoms (Cluster, Costar Co., Cambridge, Mass.), as described previously (7). Experiments were carried out at several drug concentrations (usually 100, 10, 1, 0.1, and 0.01 μM), and each drug was studied at least three times. Data from the experiments were used (after log/log transformation) for developing a linear regression and determining the concentration of drug required for a 90% reduction in virus yield (90% effective dose). In these experiments, the correlation coefficients were usually 0.95 or better, indicating a good fit of the data.

Inhibition of plaque formation. Monolayers of susceptible cells were established in Linbro plates and inoculated with approximately 10 to 20 plaque-forming units per well. After a 2-h absorption period, virus was washed off, and monolayers were covered with maintenance medium containing 1% methylcellulose. Drugs at the different concentrations were incorporated into the maintenance medium. Controls were overlaid with maintenance medium devoid of test drugs. When the plates were fully developed, the number of plaques was counted, and a linear regression was developed in order to calculate the concentration of drug required to reduce plaque formation by 50%. Each experiment was carried out in duplicate, and each experiment was repeated at least twice. Data from all experiments were pooled and used to develop the linear regression.

Cellular cytotoxicity. WI-38 and Vero (adherent) cells and a human lymphoblastoid (suspension) cell line, NC-37, were used for normal cytotoxicity studies. Adherent cells were plated onto Linbro plates at a concentration of 5×10^4 cells per ml per well, and the NC-37 cells were plated at 4×10^4 cells per ml per well. FIAC was added to individual wells to give a final concentration of 100, 10, 1, or 0.1 μM. The number of cells plated resulted in continuous replication through 4 days of culture. After 4 days of culture with the drug, monolayer cells were harvested with 0.025% trysin in phosphate-buffered saline by using a rubber policeman. Cell suspensions were triturated and mixed with an equal volume of 0.4% trypan blue dye (GIBCO), and viable cells were counted with a hemacytometer. Results with treated cells were compared with results with untreated cells to determine percent inhibition. The data were used to develop a linear regression, and from that regression the concentration of drug causing a 50% inhibition of replication (50% inhibitory dose) was determined.

RESULTS

Studies were performed with FIAC to determine whether four strains of HSV-1 and two strains of HSV-2 were susceptible to the antiviral activity of this drug. Table 1 shows that all four strains of HSV-1 were very susceptible to the antiviral activity of FIAC. The concentrations of FIAC which suppressed viral replication by 90% for the two strains of HSV-2 were 0.0044 and 0.0126 μM and thus were comparable to the activity of FIAC against the four strains of HSV-1 (Table 1).

We performed experiments in which the antiviral activity of FIAC was compared with the anti-herpesvirus activities of other antiviral compounds. Figure 1 shows the averaged results from two experiments. Of all drugs tested, FIAC was active at the lowest concentration; the 90% effective doses for acycloguanosine, arabinosylcytosine, 5-iododeoxyuridine, and arabinosyladenine were 0.6, 14.1, 421, and 5,263 times higher, respectively, than the 90% effective dose for FIAC. When compared with acycloguanosine at higher drug concentrations, FIAC had increased superiority. For example, at the 99.9% effective dose the difference between the activities of these compounds increased to 60-fold.

TABLE 1. *Capacity of FIAC to inhibit replication of various strains of HSV-1 and HSV-2 in Vero cell monolayers*

Strain	90% Effective dose (μM)
HSV-1	
Patton	0.0065
HFEM	0.0116
MacIntyre	0.0025
2931	0.009
HSV-2	
333	0.0126
G	0.0044

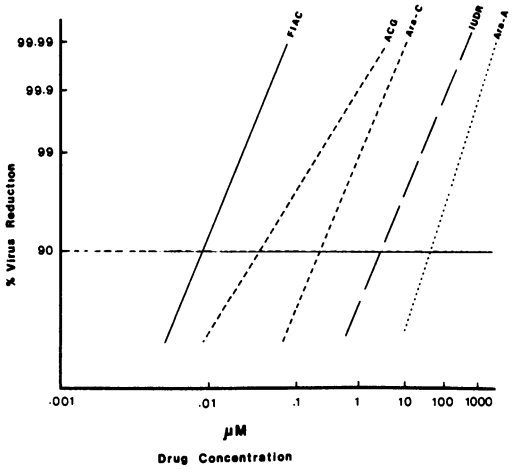

FIG. 1. *Comparison of anti-HSV-1 (strain 2931) activities of FIAC and other antiviral compounds. ACG, Acycloguanosine; Ara-C, 1-β-D-arabinofuranosylcytosine; IUDR, 5-iodo-2'-deoxyuridine; Ara-A, 9-β-D-arabinofuranosyladenine.*

The preliminary in vitro toxicity studies demonstrated minimal cytotoxicity (14). However, since these studies were carried out with mouse leukemic cells, further cytotoxicity studies were performed, in which we used Vero cells (which were used for the antiviral studies), as well as a strain of human fibroblasts (WI-38) and a human lymphoid cell line (NC-37). As Table 2 shows, the concentration of FIAC yielding a 50% reduction of cell proliferation over a 4-day period ranged from 4 to 10 μM. These toxicities were similar to those developed when mouse leukemic cells were used.

Studies were carried out with a thymidine kinase-negative mutant strain of HSV-1 to determine whether this virus-specified enzyme might be the target for the action of FIAC and thus might determine, at least in part, its specificity. The 90% effective dose with the HSV-1 thymidine kinase-negative strain was 78 μM; thus, compared with the wild-type strain of virus (thymidine kinase positive), the thymidine kinase-negative strain was about 8,000-fold less susceptible to FIAC.

Reversal studies were utilized to further evaluate the mechanism of FIAC specificity. The plaque reduction assay was performed with varying concentrations of FIAC (0.01 to 100 μM) either in the presence or absence of an equimolar concentration or a 10-fold excess of thymidine or deoxycytidine. At all concentrations of FIAC tested, neither an equimolar concentration nor a 10-fold excess of deoxycytidine reversed the anti-HSV-1 activity of the drug. Reversal of the antiviral activity of FIAC was found only with high concentrations of thymidine. A 10-fold excess of thymidine reversed by 33% the antiviral activity of 1 μM FIAC, and a 10-fold excess of thymidine reversed by 19% the antiviral activity of 10 μM FIAC (combined average of three experiments). By comparison, the antiviral activity of 1-β-D-arabinofuranosylthymine was readily reversed by equimolar concentrations of thymidine. In two experiments, 48% of the anti-HSV-1 activity of 10 μM 1-β-D-arabinofuranosylthymine was eliminated by the presence of an equimolar concentration of thymidine.

TABLE 2. *Cytotoxicity of FIAC with some uninfected cell lines*[a]

Cell line	50% Inhibitory dose (μM)
Vero	5
WI-38	4
NC-37	10

[a] Assay duration, 4 days.

Although deoxycytidine clearly had no inhibitory effect on the anti-HSV-1 activity of FIAC, this natural nucleoside was capable of completely blocking the minimal toxicity of FIAC on normal uninfected cells. For example, the toxicity of 100 μM FIAC on WI-38 cells was reduced from 98% inhibition of replication without deoxycytidine to 7% inhibition in the presence of an equimolar concentration of deoxycytidine.

Since HZV infection of cells has also been reported to be accompanied by the expression of a new, probably virus-specified, thymidine kinase (10), experiments were undertaken to determine whether FIAC was also effective against HZV. When the Ellen strain of HZV and WI-38 cells were used, 0.01 μM FIAC inhibited plaque formation by 50%.

DISCUSSION

Our studies indicate that FIAC is a very potent compound, which is equally active against various strains of HSV-1 and HSV-2, as well as HZV. When compared with other anti-herpesvirus drugs currently in clinical use or in experimental trials, FIAC was clearly active against HSV-1 at a lower concentration. The relative lack of cytotoxicity of FIAC with uninfected cells indicates selectivity of action, and the studies with the thymidine kinase-negative mutant indicate that the selectivity is probably determined, at least in part, by virus-specified pyrimidine nucleoside kinase. Thus, FIAC is probably phosphorylated much better by the viral enzyme than by the cellular enzyme, and this leads to activation of FIAC in infected cells but not in uninfected cells. In support of this possibility, preliminary data, which were derived with cytosols of HSV-1-infected and uninfected Vero cells, indicate that the virus-specified enzyme

phosphorylates FIAC much better than does the enzyme from uninfected cells (W. Kreis et al., manuscript in preparation).

As with the earlier studies with murine leukemic cells, our studies indicate minimal cytotoxicity of FIAC with uninfected Vero, WI-38, and NC-37 cells in culture. Since maximum selectivity would improve the therapeutic potential of any new antiviral drug, relatively low toxicity with normal cells is mandatory. Of special interest was the observation that this minimal toxicity could be almost completely obliterated by the presence in the culture medium of the naturally occurring nucleoside deoxycytidine. This observation suggests that, if necessary, in vivo toxicity of very large doses of FIAC might be reversed by the simultaneous infusion of deoxycytidine. Since this natural nucleoside had no adverse effects on the anti-HSV-1 activity of FIAC, such treatment would not compromise any potential antiviral effect. In addition, the observation that the low level of toxicity found with normal cells, but not the antiviral effect, could be reversed by deoxycytidine indicates that the former effect is probably mediated by a different mechanism than the latter effect. It is thus possible that the low level of cytotoxicity might depend on a cellular deoxycytidine kinase for phosphorylation of FIAC. Studies are in progress to evaluate this possibility further.

In summary, FIAC appears to be a highly potent, selective anti-herpesvirus drug equally effective against HSV-1, HSV-2, and HZV. Preliminary studies with mice inoculated with HSV-1 indicated a potent antiviral effect of FIAC against lethal infections (Lopez et al., manuscript in preparation). The drug is highly soluble (more than 150 mg/ml for the hydrochloride salt) and relatively nontoxic. Various studies, including in vivo toxicity and in vitro mutagenicity, must be carried out to determine whether FIAC might become a clinically useful drug.

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