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Metabolic Competition Studies of 2′-Fluoro-5-iodo-1-β-D-arabinofuranosylcytosine in Vero Cells and Herpes Simplex Type 1-Infected Vero Cells

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SUMMARY

2′-Fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC) is a potent antiviral agent with minimal cytotoxicity. In Vero cells, incorporation of labeled dCyd and dThd into the acid-insoluble DNA fraction was, respectively, competitively and noncompetitively inhibited by FIAC. In herpes simplex type 1 (HSV-1) infected Vero cells, these inhibition patterns became noncompetitive. The inhibition constants of FIAC on dThd and dCyd incorporation into the acid-insoluble fraction during a 15-min period were greater than 30 μM which were much higher than the antiviral concentration of FIAC (ED_{90} = 0.003–0.013 μM) for continuous exposure. Incorporation of dUrd into acid-insoluble DNA was inhibited by 10 μM FIAC in HSV-1-infected Vero cells, but not in uninfected cells. The radioactivity of [2-^{14}C]FIAC was incorporated into the acid-insoluble DNA fraction, and this incorporation in uninfected cells was strongly inhibited by 10 μM dCyd but not by dThd. By contrast, the incorporation in HSV-1-infected Vero cells was strongly inhibited by 10 μM dThd but not by dCyd. These data indicate that FIAC behaves metabolically like dThd, dUrd, or 5-iodo-dUrd in HSV-1-infected cells but like dCyd in noninfected cells. Thus, combined use of dCyd and FIAC may reduce cytotoxicity of FIAC or incorporation of FIAC into host cell DNA without affecting its antiviral activity. This finding is of significance since, for practical reasons, incorporation of FIAC into host cell DNA needs to be reduced as much as possible.

INTRODUCTION

2′-Fluoro-5-iodo-aracytosine (see Fig. 1) is a nucleoside with potent and broad spectrum antiviral activity (1–3). It suppressed by 90% the replication of HSV[5] types 1 and 2 at concentrations of 0.003–0.013 μM, whereas its cytotoxic effect on host cells as measured by inhibition of cell growth was minimal with a median-effect concentration of 4–10 μM (3). The selectivity of action of FIAC raised much interest in studying the biochemical basis for its antiviral effect. A report by Lopez *et al.* (2) showed that FIAC was about 3 log orders more active against the wild type HSV-1 than against a mutant strain lacking the expression of virus-specified thymidine kinase (HSV-1 TK$^-$). Recent reports showed that FIAC has higher affinity toward HSV-specified thymidine kinase than the mammalian enzyme (4, 5) and that the 5′-triphosphate of FIAC had higher affinity for the virus-specified DNA polymerase than the DNA polymerase of the host cells (6, 7). These results provide convincing supporting evidence for the selectivity of FIAC.

To explore further the biochemical effects of FIAC in cellular systems, we carried out metabolic competition studies analyzing the effects of FIAC on incorporation of labeled natural nucleosides into DNA in HSV-1-infected and uninfected Vero cells. Conversely, the effects of natural nucleosides on the incorporation of radioactivity of [2-^{14}C]FIAC into the acid-insoluble nucleic acid fraction were also analyzed. A preliminary report of this study has appeared (8).

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[5] The abbreviations used are: HSV, herpes simplex virus; FIAC, 2′-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine or 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine; FIAU, 2′-fluoro-5-iodo-1-β-D-arabinofuranosyluracil; ara-C, 1-β-D-arabinofuranosylcytosine; ara-U, 1-β-D-arabinofuranosyluracil; THU, 3,4,5,6-tetrahydrouridine; IdUrd, 5-iodo-2′-deoxyuridine; HPLC, high pressure liquid chromatography.

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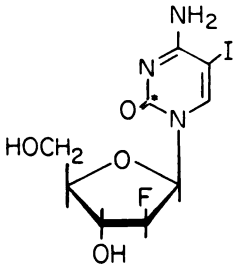

FIG. 1. *Chemical structure of 2'-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine*

The position of the ^{14}C-label is indicated by *.

MATERIALS AND METHODS

Cells and viruses. HSV-1 (strain 2931), which had been previously isolated and characterized, was used (9). The virus was propagated and quantitated on monolayers of Vero cells (African green monkey kidney cells) obtained from the American Type Culture Collection. Routine passage of virus was carried out at low multiplicities of infection to minimize the formation of defective particles. The Vero cell monolayers were propagated in Dulbecco's modified minimal essential medium containing 10% fetal calf serum and antibiotics (streptomycin and penicillin) (GIBCO, Grand Island, NY) and were maintained in the same medium containing 2% fetal calf serum. Vero cell monolayers were exposed to virus at multiplicities of infection of 1–1.5 plaque-forming units per cell in maintenance medium. The cultures were harvested at 12 hr postinfection and at a time when maximal viral and minimal cellular DNA replication could be found. In agreement with the earlier findings (10, 11), the host cell DNA synthesis was not detectable at 12 hr post-HSV-1 infection as shown by CsCl density equilibrium sedimentation analysis (12) using [^3H]dThd as a tracer. The cells were harvested from the monolayer with the use of a rubber scraper. The uninfected Vero cells were grown and harvested in the same manner except without virus infection. Cell numbers were determined with a hemocytometer.

Precursor incorporation. For studies of incorporation of the nucleoside into the perchloric acid-insoluble DNA fraction, incubation was carried out in Eagle's basal medium (GIBCO) containing 10% fetal calf serum and 2 mM freshly added L-glutamine and, when FIAC and/or dCyd were present in the reaction mixture, 0.1 mM of THU was added to prevent their deamination. Each ml of incubation mixture contained 0.5–3 × 10^6 uninfected or HSV-1-infected Vero cells and was gassed with a mixture of 95% oxygen and 5% CO_2. Incubations at 37° were 15 min for natural nucleosides and 45 min for [2-^{14}C]FIAC. Incorporation of nucleosides into acid-insoluble DNA fractions was analyzed by previously described procedures (15). The incorporation was linear for at least 30 min for natural nucleosides and 1 hr for labeled FIAC.

Deaminations. Deamination of cytosine nucleosides was determined as described (13) and was measured as acid-soluble extracts of cells, using an AG50-X8 cationic exchange column that separated cytosine nucleosides from uracil nucleosides. The deamination assay was also confirmed by descending paper chromatography using Whatman 3MM paper and the solvent system, isopropanol/concentrated HCl/H_2O, 68:17:15 (v/v). The R_F values of FIAC, FIAU, Cyd, Urd, dCyd, dUrd, ara-C and ara-U were 0.55, 0.82, 0.36, 0.52, 0.51, 0.70, 0.48, and 0.66, respectively.

Compounds. FIAC and [2-^{14}C]FIAC were synthesized by Fox and co-workers (1, 14). [6-^3H]dURD (15 Ci/mmol), [5-^3H]dCyd (5 Ci/mmol) and [2-^{14}C]IdUrd (48.5 mCi/mmol) were obtained from Schwarz-Mann, [5,6-^3H]ara-C (8.2 Ci/mmol) and [*methyl*-^3H]dThd (2.0 Ci/mmol) were from New England Nuclear, and [5-^3H]Cyd (28 Ci/mmol) was from ICN Pharmaceuticals. Labeled ara-C, dCyd, Cyd, dThd, and IdUrd, obtained from commercial sources, were purified by paper chromatography using the solvent system described above. The R_F values for dThd and IdUrd were 0.82 and 0.72, respectively. All nonlabeled natural nucleosides were obtained from Sigma Chemical Co., St. Louis, MO.

HPLC. 2-^{14}C-labeled FIAC, enzyme-degraded HSV-1 DNA was subjected to HPLC analysis using an SP 8000 HPLC with data system (Spectra-Physics, Piscataway, NJ) and SP 8300 UV detector. The Partisil ODS-3 (10 μM) column was purchased from Whatman, Inc. (Clifton, NJ). Methanol-potassium phosphate buffer, 0.02 M, pH 3.0, solvent system (12) was used for separating nucleosides.

RESULTS

Deamination of [2-^{14}C]FIAC. FIAC was deaminated by intact Vero cells at a rate equivalent to 19% of that for Cyd, or 20% of that for dCyd at an equimolar concentration (10 μM). The deamination of FIAC was increased 3.8-fold after HSV-1 infection (Table 1). Deamination of Cyd, dCyd, and ara-C by Vero cells was also increased by HSV-1 infection, but the increases were only 13, 10, and 22%, respectively. This metabolic change after HSV-1 infection is probably due to increased synthesis of pyrimidine nucleoside deaminase or to the release of the enzyme from the cellular compartment or both. This cellular deamination was inhibited more than 92 ± 7% by 0.1 mM THU, a pyrimidine nucleoside deaminase inhibitor. In order to maximize the complications in forming FIAU from FIAC and dUrd from dCyd, all studies involving these cytidine derivatives were carried out in the presence of 0.1 mM THU. Previously, we have shown that THU is nontoxic to animals (16) and to humans (17).

Effect of FIAC on natural nucleoside incorporation into DNA. When the concentration of labeled pyrimidine nucleosides, dCyd and dThd, were varied, their incorporation into DNA in Vero cells followed a hyperbola which yielded straight lines in a double-reciprocal plot and, thus conformed to simple Michaelis-Menten type kinetics (Fig. 2). The maximal rates of incorporation of dCyd and dThd into DNA were markedly increased after HSV-1 infection. These increases are mainly due to viral synthesis since, at 12 hr after infection, the host cell DNA synthesis was not detectable using CsCl density sedimentation (see Materials and Methods and refs. 10 and 11). In uninfected Vero cells, FIAC was a competitive inhibitor of dCyd, whereas in HSV-1-infected Vero cells,

TABLE 1

Relative rates of deamination of FIAC and other nucleosides by Vero cells and HSV-1-infected Vero cells

Incubation mixture in Eagle's basal medium with 10% fetal calf serum and 2 mM freshly added glutamine contained 3.2 × 10^6 Vero cells or 2.0 × 10^6 HSV-1-infected Vero cells and 10 μM labeled nucleosides. Incubation was carried out at 37° for 15 min. Deamination was terminated by adding 5% perchloric acid, and the deaminated metabolite was measured in acid-soluble extracts with AG50 cationic exchange column as described in Materials and Methods. Data given are mean ± deviation of two assays.

Substrate (10 μM)	Vero cells	HSV-1-infected Vero cells
	nmol/10^6 cells/15 min	
[5-^3H]Cyd	1.00 ± 0.01	1.13 ± 0.12
[5-^3H]dCyd	0.93 ± 0.02	1.08 ± 0.03
[2-^{14}C]FIAC	0.19 ± 0.01	0.72 ± 0.02
[5,6-^3H]ara-C	0.27 ± 0.03	0.35 ± 0.01

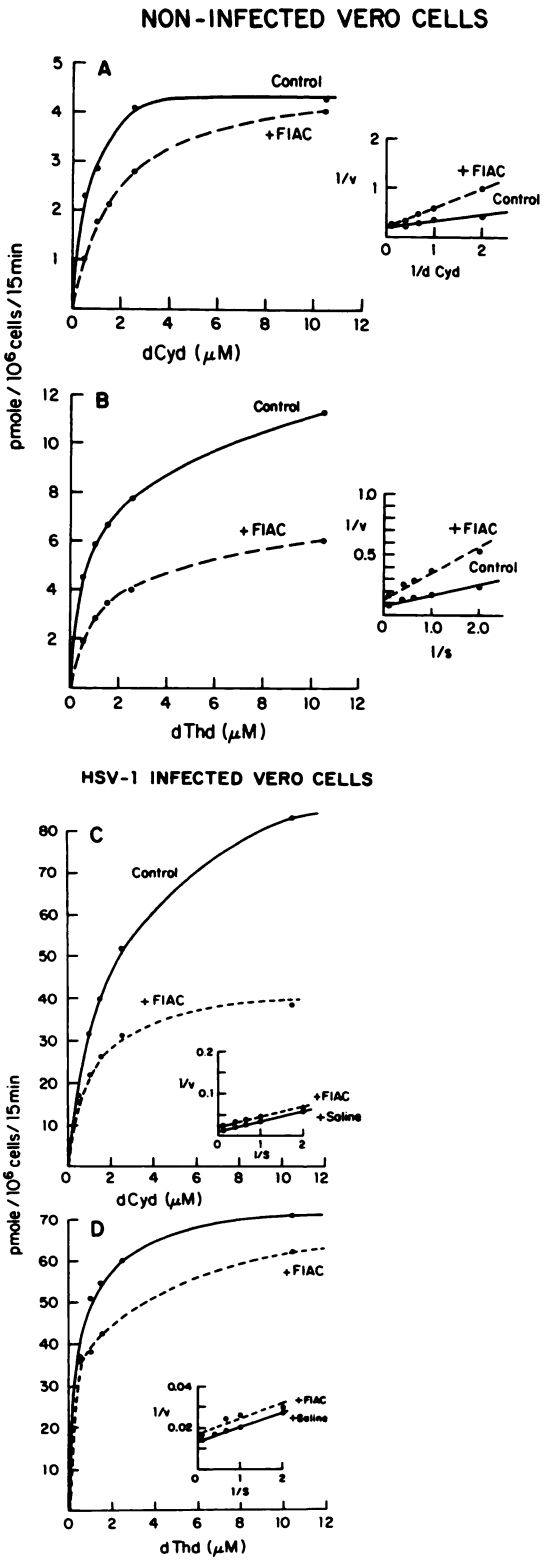

FIG. 2. *Effects of FIAC on incorporation of [5-³H]dCyd and [methyl-³H]dThd incorporation into acid-insoluble DNA*

Incubation was carried out at 37° for 15 min. Noninfected Vero cells with varying dCyd (A) and dThd (B) concentrations. HSV-1-infected Vero cells with varying dCyd (C) and dThd (D) concentrations. Control, ●; +FIAC (100 μM), O.

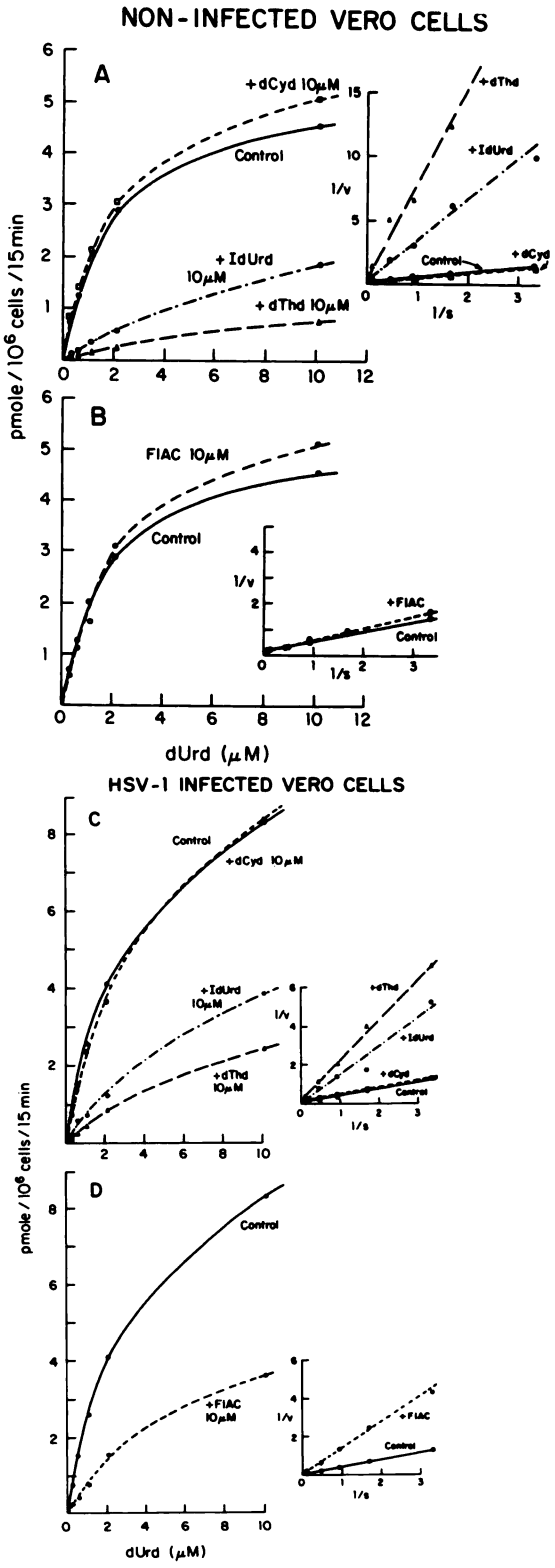

FIG. 3. *Effects of dCyd, IdUrd, and FIAC on incorporation of [6-³H]dUrd into acid-insoluble DNA in Vero cells (A) and (B) and in HSV-1-infected Vero cells (C) and (D).*

All inhibitors are 10 μM.

TABLE 2

Inhibition of incorporation of natural nucleosides into DNA by FIAC

The inhibiton constant, $K_i = [I]/[(\text{slope})_i/(\text{slope})_0 - 1]$ or $= [I]/[(\text{intercept})_i/(\text{intercept})_0 - 1]$, whichever is smaller, where $[I]$ is the concentration of inhibitor (FIAC), $(\text{slope})_i$ and $(\text{slope})_0$ are the slopes in the double reciprocal plot in the presence and absence of inhibitor, respectively, whereas $(\text{intercept})_i$ and $(\text{intercept})_0$ are the intercepts in the double reciprocal plot in the presence and absence of the inhibitor, respectively.

Substrate	K_i	
	Vero cells	HSV-1-infected Vero cells
	μM	
[*methyl*-^{3}H]dThd	93	178
[5-^{3}H]dCyd	38[a]	41
[6-^{3}H]dUrd	>1000	3.7

[a] Competitive inhibition. All others are noncompetitive inhibition.

FIAC inhibition of both dCyd and dThd gave a noncompetitive or uncompetitive type of inhibition.

In both uninfected and HSV-1-infected Vero cells, incorporation of [6-^{3}H]dUrd into DNA was competitively inhibited by dThd and IdUrd, but not by dCyd (Fig. 3). Interestingly, dUrd incorporation into DNA was not inhibited by FIAC in uninfected cells but was strongly inhibited in HSV-1-infected cells. These data indicate that FIAC behaves metabolically like dThd in HSV-1 infected cells but like dCyd in uninfected cells.

The cellular inhibition constants of FIAC obtained from Figs. 2 and 3 are summarized in Table 2. It is noted that FIAC with inhibition constants ranging from 38 to 178 μM with respect to dCyd and dThd is several log orders weaker than ara-C (18) in inhibiting natural nucleoside incorporation into DNA. Thus, FIAC (or its metabolites) does not seem to be a very potent inhibitor of cellular nucleoside kinases, nucleotide kinases, or DNA polymerases. FIAC inhibited dUrd incorporation more than it inhibited dThd incorporation, suggesting that thymidylate synthetase may be one of the possible targets of the chemotherapeutic effect of FIAC.

Rate of incorporation of [2-^{14}C]FIAC into acid-insoluble fractions. [2-^{14}C]FIAC radioactivity was incorporated into the acid-insoluble DNA fraction in Vero cells, but the maximal rate of incorporation was much slower than the rates for dThd, dUrd, and dCyd (Table 3). In addition, the half-saturation concentration ($S_{0.5}$) for FIAC was considerably higher than for natural nucleosides, suggesting that FIAC is less efficiently incorporated and may have less affinity for enzyme(s) in the anabolic pathway of uninfected cells. As in the natural nucleosides, dThd, dCyd, and dUrd, incorporation of [2-^{14}C]FIAC radioactivity into acid-insoluble viral DNA in Vero cells was greatly increased by HSV-1 infection (Table 3). The increase in the maximal rate of incorporation after HSV-1 infection was 4.5-fold for FIAC, 3.2-fold for dThd, 2.0-fold for dUrd, and 16.5-fold for dCyd. After HSV-1 infection, the half-saturation concentrations for dCyd, dUrd, and dThd were increased, whereas for FIAC it was slightly decreased, suggesting a higher affinity of anabolic enzyme(s) for FIAC.

As shown in Fig. 4, incorporation of radioactivity of [2-^{14}C]FIAC into the acid-insoluble fraction in noninfected Vero cells is strongly inhibited by dCyd, but not by dThd. By contrast, the incorporation into HSV-1-infected Vero cells is strongly inhibited by dThd, but not by dCyd. Since dUrd, after conversion via dThd kinase to the 5′-nucleotide, is the substrate for the *de novo* thymidylate synthesis, the results in Fig. 4 are consistent with the observation in Fig. 3 which showed that [^{3}H]dUrd incorporation into DNA is inhibited by FIAC in HSV-1-infected Vero cells, but not in noninfected Vero cells. Furthermore, IdUrd affected [2-^{14}C]FIAC incorporation in both infected and uninfected cells in the same manner as dThd. The relative potencies of inhibition of [2-^{14}C]FIAC and [6-^{3}H]dUrd incorporation by various nucleosides are summarized in Table 4. Inhibition constants for dThd, IdUrd, and dCyd on [2-^{14}C]FIAC incorporation showed marked changes after HSV-1 infection. The differences in inhibition constants were relatively small when the effects on [6-^{3}H]dUrd incorporation were studied. A notable exception was that [6-^{3}H]dUrd incorporation was unaffected by FIAC in uninfected Vero cells, but was inhibited by FIAC in HSV-1-infected Vero cells.

Incorporation of [2-^{14}C]FIAC radioactivity into acid-soluble DNA fractions after HSV-1 infection. The uninfected Vero cells and HSV-1-infected cells were exposed

TABLE 3

Rates of incorporation of [2-^{14}C]FIAC and other labeled nucleosides into acid-insoluble fraction

Incubation conditions were the same as those described in the legend of Table 1. The concentration of substrates was varied and the double-reciprocal plots were used for calculating the maximal rates of incorporation and the half-saturation concentrations of substrates. Data are given as mean ± standard error for three independent experiments, except for [6-^{3}H]dUrd where a single experiment was carried out.

Substrate	Maximal rate of incorporation		Half-saturation concentration ($S_{0.5}$)	
	Vero cells	HSV-1-infected Vero cells	Vero cells	HSV-1-infected Vero cells
	pmol/10^6 cells/15 min		*μM*	
[2-^{14}C]FIAC	0.77 ± 0.02	3.48 ± 1.18[a]	10.5 ± 3.0	7.7 ± 2.0
[5-*methyl*-^{3}H]dThd	12.7 ± 6.1	40.3 ± 18.7[a]	0.9 ± 0.2	1.6 ± 0.7
[5-^{3}H]dCyd	3.9 ± 1.8	64.3 ± 19.5[a]	0.7 ± 0.2	5.5 ± 2.3[a]
[6-^{3}H]dUrd	5.5	11.1	2.0	5.0

[a] $p < 0.05$ between infected and uninfected Vero cells.

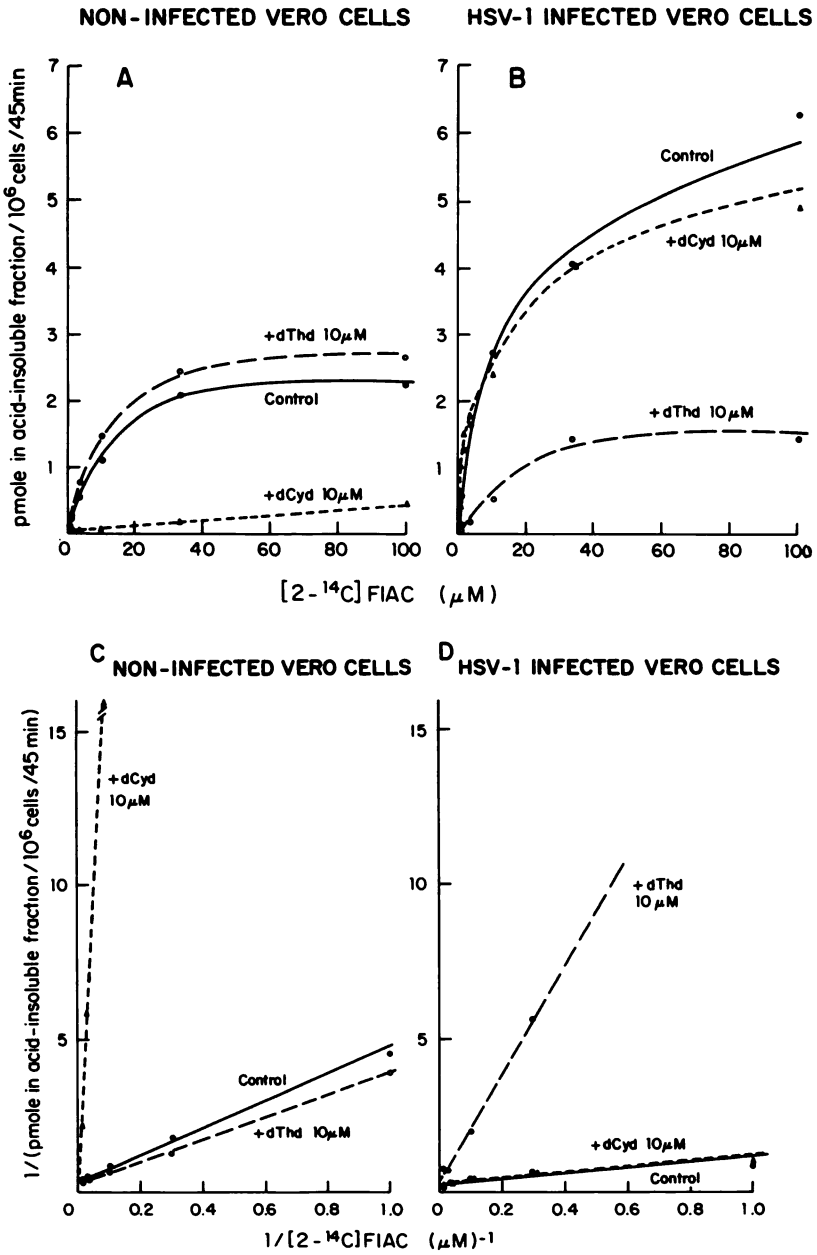

FIG. 4. *Effects of dThd and dCyd on incorporation of [2-^{14}C]FIAC into acid-insoluble DNA fraction*

A, noninfected Vero cells; B, HSV-1-infected Vero cells. Control, ●; +dCyd (10 μM), △; +dThd (10 μM), ○. Incubation was carried out at 37° for 45 min. The kinetic constants are given in Table 4.

to [2-^{14}C]FIAC (10 μM) for 45 min and then followed by extensive washing with medium. The perchloric acid precipitation or the phenol extraction procedure (12) was then used to measure radioactivity in these DNA fractions. In HSV-1-infected Vero cells, the perchloric acid procedure gave only 5.7 ± 1.6 pmol Eq of [2-^{14}C]FIAC/10^6 cells, whereas the phenol procedure gave 253 ± 84 pmol/10^6 cells. Interestingly, the perchloric acid and the phenol procedures gave nearly identical results for uninfected Vero cells (0.9–1.3 pmol Eq of [2-^{14}C]FIAC/10^6 cells). Thus, the phenol procedure revealed major biochemical changes in patterns of [2-^{14}C]FIAC radioactivity in HSV-1 infected cells. These findings have led to detailed further studies which will be described later.

HPLC analysis of DNA digests. HSV-1-infected cells were exposed to [2-^{14}C]FIAC, 5 μM, 0.7 μCi/ml in the presence and absence of 0.1 mM THU for 45 min. Labeled viral DNA was purified and digested with DNase, phosphodiesterase I, and bacterial alkaline phosphatase as described previously (12, 16). Reverse phase HPLC analysis using a Partisil 10, ODS-3 column indicated that the FIAC moiety in viral DNA was in fact FIAU (96%) and 2′-fluoro-1-β-D-arabinofuranosyluracil (2%). In the presence of 0.1 mM THU, no inhibition of the formation of the FIAU moiety in DNA was observed. There was no detectable FIAC moiety in viral DNA. [2-^{14}C]2′-fluoro-5-methyl-1-β-D-arabinofuranosyluracil, unlike [2-^{14}C]FIAC, has been shown recently to be metabolically stable in various systems (16, 21).

TABLE 4

Inhibition of incorporation of [2-^{14}C]FIAC and [6-^{3}H]dUrd into acid-insoluble DNA fractions by other nucleosides

Inhibition potency was described by inhibition constants (K_i), $K_i = [I]/[(\text{slope})_i/(\text{slope})_0 - 1]$, as described in the legend of Table 2. NA, not applicable.

Radioactive substrate	K_i potency of nucleoside							
	dThd		IdUrd		dCyd		FIAC	
	Vero cells	HSV-1 Vero cells	Vero cells	HSV-1 Vero cells	Vero cells	HSV-1 Vero cells	Vero cells	HSV-1 Vero Cells
	μM							
[2-^{14}C]FIAC	>100[a]	0.64[a]	>100	1.10	0.24[a]	100[a]	NA	NA
[6-^{3}H]dUrd	0.50[b]	2.08[b]	1.40[b]	3.28[b]	>100[b]	>100[b]	>100[b]	3.67[b]

[a] The K_i values are obtained from Fig. 4.

[b] The K_i values are obtained from Fig. 3.

DISCUSSION

The present studies carried out metabolic competition experiments on FIAC in HSV-1 infected and uninfected Vero cells. The results are in agreement with earlier findings that viral infection resulted in marked alterations in the metabolism of FIAC and in some other natural and synthetic nucleoside analogs (6, 8). The effects of FIAC, as shown in Figs. 2 and 3, were not due to FIAU since the incubation was carried out in the presence of 0.1 mM THU. However, the formation of FIAUMP from FIACMP via deoxycytidylate deaminase cannot be excluded. Thus, the deaminated product of FIAC (FIAUMP or FIAU) in virus-infected cells would competitively inhibit incorporation of dUrd into DNA (Fig. 3D) and act noncompetitively with dThd (Fig. 2D). In HSV-1-infected cells, FIAC behaves metabolically like dThd, dUrd, and IdUrd, whereas in uninfected cells, FIAC behaves like dCyd (Fig. 4 and Table 4) or like ara-C (18). Metabolic competition studies of FIAC in L1210 and P813 leukemic cells (18) showed patterns similar to uninfected Vero cells in the present studies. Since the selectivity of the antiviral activity of FIAC has been shown to be due, in part, to its high levels and high affinity of the virus-specified thymidine kinase (4, 5) and DNA polymerase (6, 7), our results suggest that the combined use of dCyd and FIAC may reduce the cytotoxicity of FIAC or its incorporation into host cell DNA without reducing its antiviral effects. FIAC has been subjected to clinical trials in immunosuppressed, virus-infected cancer patients and has been shown to have only minimal short-term host toxicity at therapeutic doses (19); in order for it to be a useful agent against herpes virus infection, the incorporation of FIAC (or its metabolites) into host cell DNA must be blocked or minimized to reduce the possible undesirable outcome of such incorporation.

The potency in inhibiting natural nucleoside incorporation into DNA in HSV-infected cells (Table 2) and the potency of the antiviral effect of FIAC are in poor correlation since they differ by several log orders of magnitude. Furthermore, FIAC serves as a poorer substrate for incorporation into the acid-insoluble DNA fraction than does dThd, dCyd, or dUrd in either HSV-1 infected or uninfected Vero cells (Table 3). The observation that FIAC behaves metabolically like dThd or dUrd in HSV-1-infected Vero cells may have more relevance to the selectivity of the antiviral effect of FIAC than to the mechanism of its antiviral effect. HPLC analysis of the labeled viral DNA digest indicates that the FIAC moiety incorporated into DNA of HSV-infected cells as FIAU was consistent with the competition studies showing that the incorporation of dUrd into DNA was inhibited by FIAC; this was probably due to its deaminated metabolites. A potent inhibitor of pyrimidine nucleoside deaminase, THU, at 0.1 mM was insufficient to block the formation of the FIAU moiety in DNA from the cells that were exposed to FIAC, suggesting that the deamination occurred at nucleotide level.

In HSV-1-infected Vero cells, the amount of radioactivity of [2-^{14}C]FIAC that is incorporated into the perchloric acid-insoluble DNA fraction is only 4.5-fold higher than when uninfected Vero cells are employed. Further studies indicated that the radioactive materials obtained from the phenol procedure in HSV-1-infected cells were nearly 200-fold higher than those in uninfected cells and that the former were largely perchloric acid-nonprecipitable and partially nondialyzable, suggesting the formation of small DNA pieces in HSV-1-infected Vero cells after exposure to FIAC. The locations of the chemical moieties of FIAC in DNA chain and the size of DNA fragments in HSV-1-infected cells after incubation with FIAC have been further studied (20).

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