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Preferential Inhibition of 5-Trifluoromethyl-2′-Deoxyuridine Phosphorylation by 5′-Amino-5′-Deoxythymidine in Uninfected versus Herpes Simplex Virus-Infected Cells

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SUMMARY

The cytotoxic effects of 5-trifluoromethyl-2′-deoxyuridine (CF_3dUrd) were effectively antagonized by 5′-amino-5′-deoxythymidine (5′-AdThd). The antiproliferative actions of CF_3dUrd were reduced in a dose-dependent manner by 5′-AdThd in both HeLa and Vero cells. In addition, the ability of CF_3dUrd to kill HeLa cells (95% at 1 μM and 99% at 3 μM), as measured by cloning efficiency, was ablated entirely by 5′-AdThd (300 μM). In contrast, the inhibition of herpes simplex virus Type 2 (HSV-2) replication in HeLa cells was not antagonized by 5′-AdThd. In Vero cells, the combination of CF_3dUrd and 5′-AdThd produced a greater antiviral effect than either agent alone. The reduction in CF_3dUrd cytotoxicity caused by 5′-AdThd in uninfected HeLa and Vero cells was associated with decreased intracellular levels of CF_3dUrd nucleotides. In contrast, in HSV-2-infected Vero cells the intracellular levels of CF_3dUrd nucleotides were slightly elevated by 5′-AdThd and, in virally infected HeLa cells, a 300-fold excess of 5′-AdThd reduced CF_3dUrd uptake only marginally. Since the relative abundance of these phosphorylated derivatives of CF_3dUrd was not markedly changed by 5′-AdThd, preferential inhibition of the mammalian thymidine kinase (EC 2.7.1.21) was suggested. Using CF_3dUrd as the substrate, the ability of 5′-AdThd to inhibit thymidine kinase activity in extracts prepared from parallel cultures of mock-infected or HSV-2 infected HeLa cells was compared. CF_3dUrd was phosphorylated to a lesser extent and the reaction was more potently inhibited by 5′-AdThd in the extracts prepared from the uninfected cells. The HeLa cell and HSV-2 thymidine kinases were purified by affinity column chromatography, and kinetic analyses were then done. Using CF_3dUrd as the variable substrate, the K_i values for 5′-AdThd were 2.2 μM for the HeLa enzyme and 36 μM for the viral enzyme. K_m values for CF_3dUrd were 2.6 μM and 4 μM for the viral and mammalian enzymes, respectively. These data account for the ability of 5′-AdThd to inhibit preferentially the phosphorylation of CF_3dUrd in uninfected host cells. The presence of 5′-AdThd substantially increased the therapeutic index of CF_3dUrd, indicating that this drug combination, an example of specific inhibition, warrants investigation *in vivo*.

INTRODUCTION

The replication of several DNA viruses, including herpes simplex virus and cytomegalovirus, is inhibited by CF_3dUrd[3] (1–4). Clinically, CF_3dUrd is an effective agent for the treatment of herpes keratitis (5). However, the compound is phosphorylated to active derivatives in uninfected tissues (6, 7), and cytotoxicity can result (3, 8, 9). Consequently, severe bone marrow depression can be produced when CF_3dUrd is administered systemically (10). Protection against the toxicity of CF_3dUrd without a parallel decrease in its antiviral activity would increase

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[3] The abbreviations used are: CF_3dUrd, 5′-trifluoromethyl-2′-deoxyuridine; CF_3dUMP, CF_3dUDP, CF_3dUTP, the 5′-mono-, di-, and triphosphates of CF_3dUrd; IdUrd, 5-iodo-2′-deoxyuridine; IdUTP, the 5′-triphosphate of IdUrd; 5′-AdThd, 5′-amino-5′-deoxythymidine; PBS, phosphate-buffered saline; HSV-2, herpes simplex virus Type 2; MOI, multiplicity of infection.

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the therapeutic safety of this drug. Recently, different approaches to this problem have been described and documented, including specific protection (11–13) and specific inhibition (11, 14).

The current study describes an attempt to reduce selectively the phosphorylation of CF_3dUrd in uninfected tissues with 5′-AdThd and, thereby, increase its therapeutic index. This strategy was based on the idea that 5′-AdThd, a potent inhibitor of the mammalian thymidine kinase (EC 2.7.1.21) (15, 16), should inhibit the phosphorylation of CF_3dUrd in uninfected cells. In addition, 5′-AdThd is a substrate for the virally encoded thymidine kinase (17), and it selectively inhibits herpes simplex virus replication *in vitro* and *in vivo* (18, 19). Thus, the intent of the drug combination was to reduce effectively the phosphorylation and the cytotoxicity of CF_3dUrd without compromising its antiviral activity. In a previous study we designed a similar drug combination, iododeoxyuridine plus 5′-AdThd, in which the target enzyme was also intended to be the host thymidine kinase (14). However, our data indicated that the critical protective interaction occurred with thymidylate kinase rather than with thymidine kinase (20). It was of interest, therefore, to examine the effects of 5′-AdThd on the metabolism of CF_3dUrd in both uninfected and virally infected cells and to elucidate the key site of interaction.

EXPERIMENTAL PROCEDURES

Materials. Moravek Biochemicals, Inc., supplied the [G-^{3}H] CF_3dUrd (specific activity 220 mCi/mmole) and the [2′-^{3}H]CF_3dUrd (specific activity 15 Ci/mmole). Unlabeled CF_3dUrd was obtained from Sigma Chemical Company (St. Louis, Mo.), and CF_3dUTP from P-L Biochemicals (Milwauee, Wisc.). The 5′-AdThd was kindly provided by Dr. T.-S. Lin, of the Yale University School of Medicine. The thin-layer chromatographic sheets, MN PEI Cel UV, were purchased from Brinkmann Instruments (Westbury, N. Y.).

Cells. The Vero and HeLa cells (Flow Laboratories, Rockville, Md.) were grown in Dulbecco's modified minimal essential medium (Flow Laboratories) supplemented with 10% newborn calf serum (K. C. Biologicals) at 37° in a humidified 5% CO_2 atmosphere. Porcine trypsin (0.1%) was used to remove the monolayers from the plastic cultureware, and the cells were passaged twice a week. The DNA staining technique was used to screen for mycoplasma, and all cultures were found to be negative (21).

Cytotoxicity. The cytotoxicity of CF_3dUrd was assessed by measuring the rate of cellular replication and the efficiency of colony formation. Cells were plated at 1.5×10^5 cells/dish and incubated for 24 hr prior to the addition of drugs. In the antiproliferative assays, the cells were exposed to the compounds for 72 hr, removed from the dishes with PBS (137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and 1.1 mM KH_2PO_4, pH 7.4) containing 2.5 mM EDTA and then counted with a model ZBI Coulter counter. The results are presented as the percentage of control cell number (mean ± standard error) for three different experiments.

HeLa cells were exposed to drugs for a 24-hr period in the viability experiments. The cells were washed with PBS and removed from the dishes with 0.1% trypsin. The cells were diluted with medium containing 10% newborn calf serum and then plated in 35-mm culture dishes. After 7–8 days of incubation the cells were stained with 1% methylene blue in methanol; colonies containing more than 50 cells were then counted.

Virus. HSV-2 (Strain G) was obtained from the American Type Culture Collection. The virus was propagated in confluent monolayers of Vero cells inoculated at an MOI of 0.01. The infected cells were harvested 48 hr after infection and concentrated by centrifugation. The virus stocks were stored at −70° and disrupted by three cycles of freezing and thawing prior to titration. Plaque assays, using confluent monolayers of Vero cells in medium containing 2% newborn calf serum and 0.05% human immune serum globulin (Travenol Laboratories, Glendale, Calif.), were carried out to determine the virus titers.

A yield reduction antiviral assay was used. Either HeLa or Vero cells growing in 25-cm flasks were infected with HSV-2 at a multiplicity of infection of 1 during an adsorption period of 1 hr. The virus inoculum was removed and relaced with medium and drugs or vehicle (PBS) in a total volume of 5 ml. The flasks were incubated at 37° for 24 hr and then frozen at −70°; after an additional cycle of freezing and thawing, virus titrations were done.

Thymidine kinase. Extracts of both uninfected and virally infected cells were prepared from washed, frozen (−70°) cell pellets. The cells were thawed and resuspended in an extraction buffer [5 mM Tris (pH 7.5), 10 mM KCl, 10% glycerol, 5 mM β-mercaptoethanol, 5 mM ATP, and 5 mM $MgCl_2$] for 15 min. The suspension was disrupted in a Dounce homogenizer (30 strokes) and centrifuged for 15 min at 12,000 rpm in a JA-20 rotor in a Beckman J21C centrifuge at 4°. The supernatant, used as the crude extract, could be stored at −70° without loss of dThd kinase activity. Prior to further purification with affinity chromatography, streptomycin sulfate precipitation (22) of the extracts was carried out. The affinity column was prepared by coupling *p*-aminophenylthymidine-3′-phosphate to CH-Sepharose 4B in the presence of 1-ethyl-3-(3′-dimethylaminopropyl)carboxamide (23). The HeLa cell enzyme was purified according to the procedures previously described (22, 24) except that the ammonium sulfate precipitation step was eliminated and 200 μM dThd was used to elute the enzyme from the column. HSV-2 dThd kinase was purified from virally infected Vero cells as described by Fyfe (25).

Thymidine kinase activity was assayed in a manner similar to procedures already reported (20). In enzyme preparations purified by affinity column chromatography, the reaction mixture contained 2.5 mM ATP, 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 50 mM Tris (pH 7.8), 1% bovine serum albumin, and the indicated amounts of tritiated CF_3dUrd or dThd in a volume of 80 μl. For experiments in which crude cellular extracts, prepared from either exponentially growing HeLa cells or from HeLa cells infected 24 hr previously with HSV-2 at a MOI = 1, were used, the reaction mixture included 4.5 mM phosphocreatine, creatine kinase (6 units/ml), and 20 mM NaF (16). For kinetic experiments the purified enzyme was passed over a G-50 column to remove the dThd. The reaction was carried out at 37°, and 30-μl samples were removed and spotted on Whatman DE81 paper discs after 15 and 30 min of incubation. The reaction velocities were linear with respect to time. The discs were washed in 95% ethanol, once in 1 mM ammonium formate, and thrice more in 95% ethanol. They were then dried and counted in HFP-20 liquid scintillation fluid (Research Products International) with a Tracor Mark III scintillation spectrometer.

Distribution of CF_3dUrd metabolites. The pattern of incorporation of [^{3}H]CF_3dUrd into CF_3dUMP, CF_3dUDP, and CF_3dUTP was determined using thin-layer chromatography. Exponentially growing cells, plated in 60-mm dishes, were used in these experiments. The cells were either mock-infected or infected with HSV-2 at a MOI = 1 in a volume of 0.9 ml/dish. After an absorption period of 1 hr, the inoculum was removed, the cells were washed with PBS, and 4.5 ml of fresh medium were added. Following 4.5 hr of incubation at 37°, the cultures were exposed for 1 hr to [^{3}H]CF_3dUrd (1 μM or 3 μM at 1 μCi/ml) in the presence or absence of various concentrations of 5′-AdThd. The cells were then washed with PBS and extracted with 60% methanol, as previously described (20). Portions of the concentrated supernatants (20) were applied to PEI Cel 300 strips and developed in 0.75 M LiCl. Under these conditions the R_F values were 0.86, 0.65, 0.27, and 0.11 for CF_3dUrd, CF_3dUMP, CF_3dUDP, and CF_3dUTP, respectively. These values were determined from authentic markers (CF_3dUrd and CF_3dUTP) and with [^{3}H]CF_3dUMP and [^{3}H]-CF_3dUDP enzymatically synthesized from [G-^{3}H]CF_3dUrd. A purified preparation of HeLa cell dThd kinase was used for the preparation of the monophosphate whereas a crude preparation, containing dTMP kinase, was used for the diphosphate. The reaction conditions were as described above. The chromatograms were cut into 0.75-cm strips, and the radioactivity was

eluted with 1 ml of 0.7 M $MgCl_2$/0.02 M Tris pH 7.5. The samples were counted in a Tracor Mark III scintillation spectrometer using ACS (Amersham).

RESULTS

Cytotoxicity. Using assays which measured either the rate of cellular replication or cloning efficiency, 5′-AdThd was found to diminish the cytotoxicity caused by CF_3dUrd. As shown in Fig. 1, the inhibition of HeLa cell growth induced by CF_3dUrd was antagonized in a dose-dependent manner by 5′-AdThd. At higher concentrations of CF_3dUrd, more 5′-AdThd was required to achieve the same degree of protection. Similarly, in Vero cells the growth-inhibitory effects of CF_3dUrd were counteracted by 5′-AdThd. In the presence of increasing concentrations of 5′-AdThd, the dose-response curve for growth inhibition by CF_3dUrd was shifted to the right (Fig. 2). The concentration of CF_3dUrd required to inhibit Vero cell replication by 50% was increased from 1 μM in the absence of 5′-AdThd to about 5 μM in the presence of 300 μM 5′-AdThd (data not shown). In addition, 5′-AdThd effectively antagonized the lethal actions of CF_3dUrd. Figure 2 illustrates the ability of 5′-AdThd to ablate entirely the reduction in cloning efficiency produced by CF_3dUrd in HeLa cells. Thus, 5′-AdThd antagonized both the inhibition of growth and the lethality produced by a toxic antiviral nucleoside.

Antiviral effects. In contrast to the effects on cytotoxicity, the antiviral activity of CF_3dUrd was not antagonized by 5′-AdThd. In Vero cells, the combination of the two drugs inhibited the replication of HSV-2 more effectively than either agent alone (Fig. 3A). More 5′-AdThd was required to inhibit HSV-2 replication in HeLa cells, but even 300 μM 5′-AdThd did not reduce the antiviral activity of CF_3dUrd (Fig. 3B). Therefore, the inclusion of 5′-AdThd dramatically lessened the cytotoxicity of CF_3dUrd without compromising inhibition of viral replication.

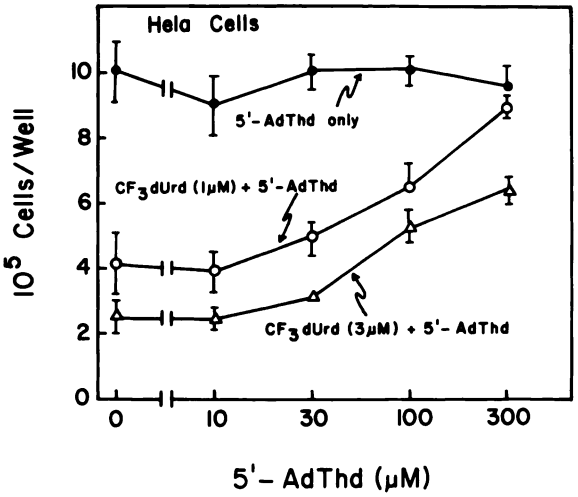

FIG. 1. *Effect of 5′-AdThd on the inhibition of HeLa cell growth caused by CF_3dUrd*

Exponentially growing HeLa cells were exposed for 72 hr to 5′-AdThd (●) or to 5′-AdThd and either 1 μM (○) or 3 μM (△) CF_3dUrd. The data are expressed as the mean ± standard error; $n = 3$.

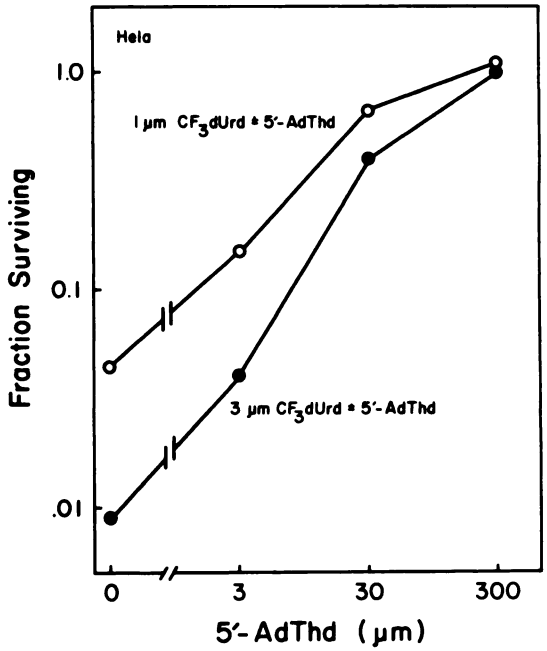

FIG. 2. *Effect of 5′-dThd on the reduction in HeLa cell survival produced by CF_3dUrd*

Exponentially growing HeLa cells were exposed for 24 hr to either 1 μM (○) or 3 μM (●) CF_3dUrd in the presence or absence of 5′-AdThd. The data are expressed as the fraction of cells surviving and are the average of two experiments. The plating efficiency for the control population was 50%.

Metabolism of CF_3dUrd. In an effort to explain the basis of these differential drug interactions, the effects on 5′-AdThd of the metabolism of CF_3dUrd in uninfected and HSV-2-infected cells were compared. In these experiments HeLa or Vero cells were either mock-infected or infected with HSV-2 at a MOI = 1. From 4.5 hr to 5.5 hr postinfection, the cells were exposed to [^{3}H]CF_3dUrd in

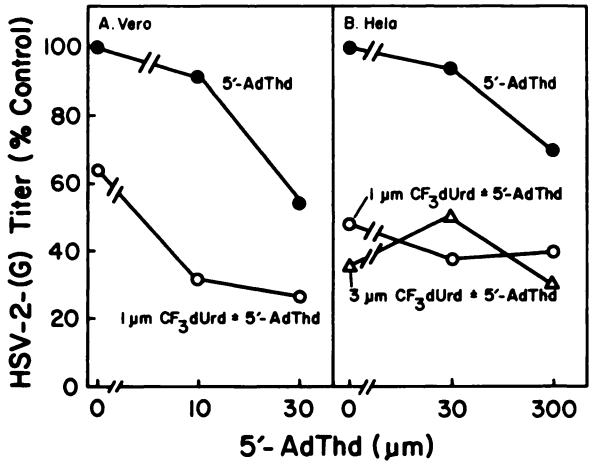

FIG. 3. *Effect of 5′-AdThd and CF_3dUrd on the replication of HSV-2*

Either Vero (A) or HeLa (B) cells were infected with HSV-2 (MOI = 1) and exposed to 5′-AdThd in the absence (●) or presence of 1 μM (○) or 3 μM (△) CF_3dUrd for 24 hr. A yield reduction assay (see Experimental Procedures) was used to assess viral replication. The untreated controls averaged 9.4×10^4 and 2.9×10^4 plaque-forming units/ml in A and B, respectively.

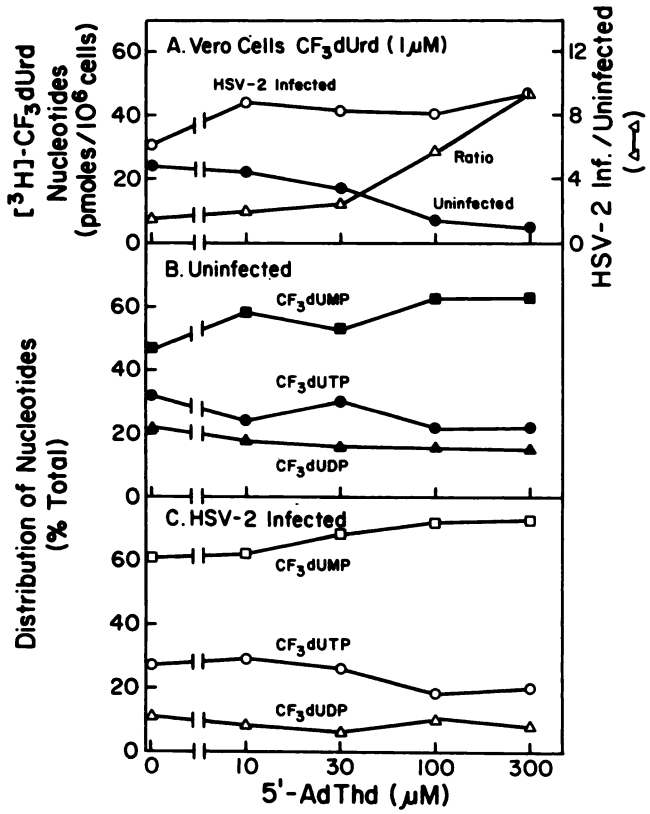

FIG. 4. *Effect of 5'-AdThd on the metabolism of CF_3dUrd*

Exponentially growing Vero cells were mock-infected or infected with HSV-2 at an MOI = 1. The cells were exposed to 1 μM [2'-^{3}H] CF_3dUrd (1 μCi/ml) and the indicated concentrations of 5'-AdThd from 4.5 to 5.5 hr postinfection. The cells were processed as described under Experimental Procedures. A, The accumulation of intracellular CF_3dUrd nucleotides in the HSV-2-infected (○) and uninfected cells (●). The ratio of accumulated nucleotides in the HSV-2-infected to the uninfected Vero cells is also shown (△). The effect of 5'-AdThd on the relative abundance of CF_3dUMP, CF_3dUDP, and CF_3dUTP in uninfected and HSV-2 infected Vero cells is shown in B and C, respectively.

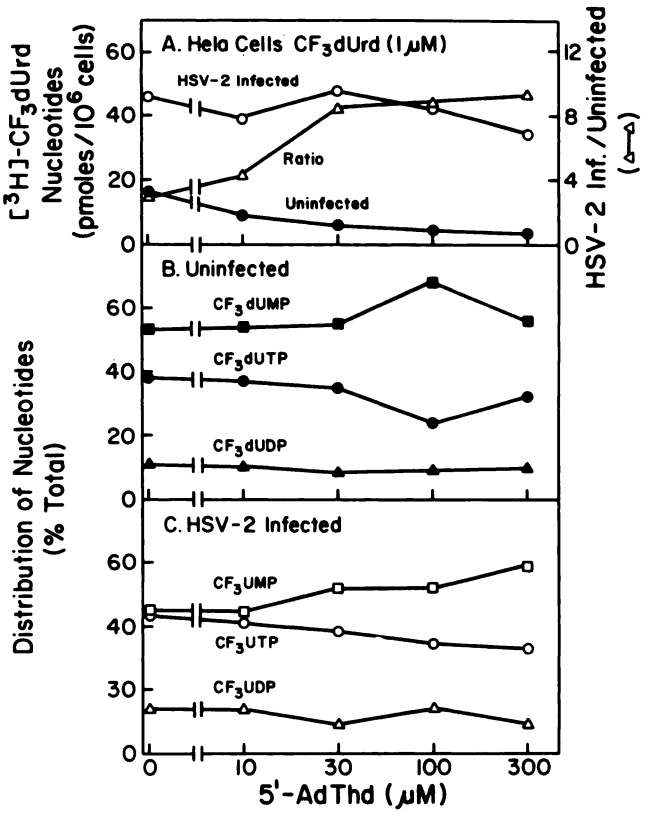

FIG. 5. *Effect of 5'-AdThd on the metabolism of CF_3dUrd*

Exponentially growing HeLa cells were mock-infected or infected with HSV-2 at an MOI = 1. The cells were exposed to 1 μM [G-^{3}H] CF_3dUrd (1 μCi/ml) and the indicated concentrations of 5'-AdThd from 4.5 to 5.5 hr postinfection. A, The accumulation of intracellular CF_3dUrd nucleotides in the HSV-2-infected (○) and uninfected HeLa cells (●). The ratio of accumulated CF_3dUrd nucleotides in HSV-2-infected to the uninfected HeLa cells is also shown (△). The effect of 5'-AdThd on the relative abundance of CF_3dUMP, CF_3dUDP, and CF_3dUTP in uninfected and HSF-2-infected HeLa cells is shown in B and C, respectively.

the presence or absence of 5'-AdThd. The relative distribution of the mono-, di-, and triphosphates of CF_3dUrd present in 60% methanol extracts was then determined. The data in Fig. 4 were obtained using Vero cells exposed to 1 μM CF_3dUrd. The presence of 5'-AdThd produced an increase in the intracellular CF_3dUrd nucleotides in the HSV-2 infected cells (Fig. 4A). In contrast, 5'-AdThd markedly depleted the levels of CF_3dUrd nucleotides in the uninfected cells. The ratio of the intracellular CF_3dUrd nucleotides in the virally infected to the uninfected cells increased from 1.4 in the absence of 5'-AdThd to 9.6 when 300 μM amino nucleoside was added (Fig. 4A). This favorable accumulation of CF_3dUrd nucleotides in the virally infected cells was consistent with the antiviral activity and the reduction in cytotoxicity produced by this combination in Vero cells. The distributions of the various nucleotides in the uninfected cells (Fig. 4B) and HSV-2 infected cells (Fig. 4C) are shown. A slight increase in the percentage of nucleotides present as CF_3dUMP was noted in both cases. In the uninfected HeLa cells exposed to either 1 μM or 3 μM CF_3dUrd, 5'-AdThd potently reduced the intracellular nucleotides derived from either 1 μM or 3 μM CF_3dUrd (Figs. 5A and 6A, respectively). In contrast to the Vero cells, 5'-AdThd did not produce increased intracellular levels of CF_3dUrd nucleotides in the HSV-2 infected HeLa cells. At 300 μM 5'-AdThd the uptake of CF_3dUrd was inhibited by about 25%. Nonetheless, the relative uptake of CF_3dUrd in the virally infected cells was increased 3-fold under these conditions (Figs. 5A and 6A). These data are consistent with the effects of 5'-AdThd on the cytotoxicity and antiviral activity of CF_3dUrd that were described earlier. No significant changes in the distribution of CF_3dUrd nucleotides were evident in the uninfected cells (Figs. 5B and 6B), but the relative abundance of CF_3dUMP was somewhat increased by 5'-AdThd in the HSV-2-infected cells (Figs. 5C and 6C).

Effects on thymidine kinase activity. Interactions between CF_3dUrd and 5'-AdThd with the viral and host dThd kinases were investigated, since 5'-AdThd did not produce major changes in the relative distribution of the CF_3dUrd nucleotides and it is known to be a good inhibitor of the mammalian enzyme. Enzyme activity was compared in extracts that were prepared from the HeLa

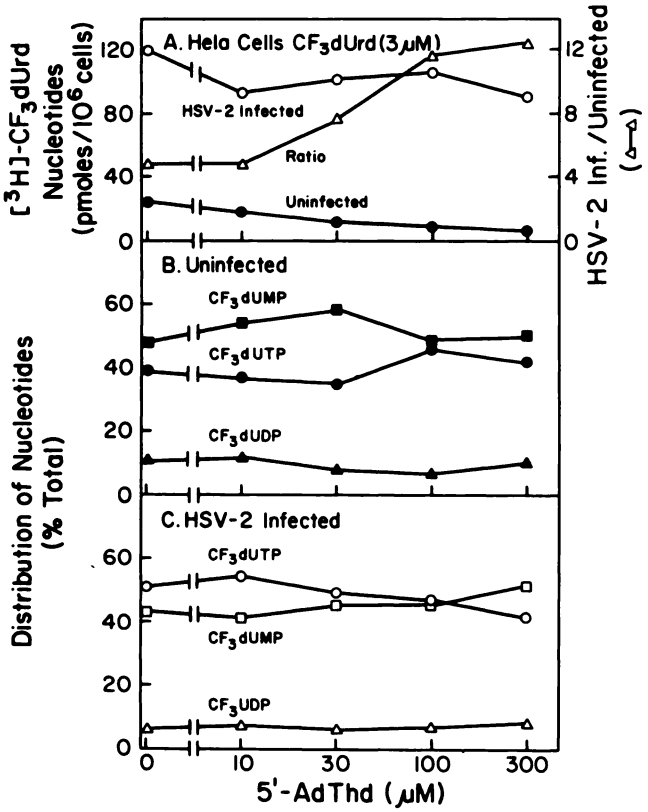

FIG. 6. *Effect of 5'-AdThd on the metabolism of CF_3dUrd*

Exponentially growing HeLa cells were mock-infected or infected with HSV-2 at an MOI = 1. The cells were exposed to 3 μM [G-^{3}H] CF_3dUrd (1 μCi/ml) and the indicated concentrations of 5'-AdThd from 4.5 to 5.5 hr postinfection. A, The accumulation of CF_3dUrd nucleotides intracellularly in the HSV-2-infected (○) and uninfected HeLa cells is also shown (△). The effect of 5'-AdThd on the relative distribution of CF_3dUMP, CF_3dUDP, and CF_3dUTP in uninfected and HSV-2-infected HeLa cells is shown in B and C, respectively.

cells that had been either mock-infected or infected with HSV-2 (MOI = 1) for 24 hr. Two major differences were seen. Because of the induction of the HSV-2 enzyme, considerably more phosphorylation of CF_3dUrd occurred in the extracts prepared from the virally infected cells. In order to ensure that the reaction kinetics were linear, extracts from the virally infected cells were diluted to a greater extent (100-fold) than for the uninfected cell extracts (25-fold). Different time points were taken for each condition to confirm linearity. Under these conditions, as shown in Fig. 7, 5'-AdThd inhibited the phosphorylation of CF_3dUrd much more effectively in the uninfected cells. These results, which were consistent with the metabolic, antiviral, and cytotoxicity studies, suggested that 5'-AdThd was a more effective inhibitor of the HeLa cell dThd kinase. In order to characterize these apparent differences, the HeLa cell dThd kinase and the HSV-2 enzyme were purified by affinity column chromatography. Kinetic analyses (Table 1) revealed marked differences in the K_i values for 5'-AdThd for the two enzymes using CF_3dUrd as the variable substrate. In both cases a competitive pattern of inhibition was seen. For the HeLa cell enzyme the K_i was 2.2 μM, whereas for the HSV-2 dThd kinase it was about 36 μM. The K_m values for CF_3dUrd were similar: 2.6 μM for the viral

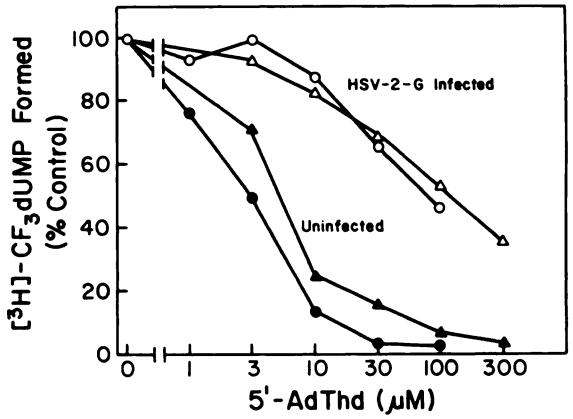

FIG. 7. *Inhibition of CF_3dUrd phosphorylation by 5'-AdThd*

The effect of 5'-AdThd on the phosphorylation of CF_3dUrd (1 μM, ○, ●; 3 μM, △, ▲) was compared in crude cellular extracts prepared from HSV-2-infected (○, △) and mock-infected HeLa cells (●, ▲). In the viral preparation, 19 and 54.4 pmoles of CF_3dUMP were formed in the control reaction at 1 and 3 μM CF_3dUrd, respectively. In the control reaction catalyzed by the uninfected cells, 2.6 and 6.7 pmoles of product were formed.

enzyme and 4 μM for the HeLa thymidine kinase. The much higher affinity of 5'-AdThd for the host enzyme appears to account for the preferential inhibition of CF_3dUrd phosphorylation in the uninfected cells.

DISCUSSION

The results of this study indicate that the selectivity of CF_3dUrd can be improved by the inclusion of a second drug. Utilizing 5'-AdThd, ablation of the cytotoxicity of CF_3dUrd was achieved without compromising its antiviral activity. Several lines of evidence indicate that selective inhibition of the host thymidine kinase with 5'-AdThd accounts for this improved therapeutic response. Data obtained using both cell-free extracts and purified enzyme preparations demonstrated that the phosphorylation of CF_3dUrd by the mammalian enzyme was 10–15 times more sensitive than the viral enzyme to inhibition by 5'-AdThd (Fig. 7; Table 1). With both enzymes the pattern of inhibition was competitive, and the K_m values for CF_3dUrd determined in this study are comparable to those reported by others (26, 27). However, these estimates were lower than those reported by Wigdahl and Parhurst (28), who used 100,000 × *g* supernatant rather than a purified enzyme preparation. The K_i value for 5'-AdThd obtained using the mammalian thymidine inase was similar to those previously reported (15, 16). Intra-

TABLE 1

K_m and K_i Values for the mammalian and viral dThd kinases

In these experiments ATP-MG^{2+} was held at 2.5 mM. The K_m values represent the averages of three separate determinations that were estimated graphically from 1/velocity versus 1/substate plots. The K_i values are also averages from three determinations which were estimated by replots of the slope versus the 5'-AdThd concentration.

Enzyme	K_m (CF_3dUrd)	K_i (5'-AdThd)
	μM	μM
HeLa dThd kinase	4	2.2
HSV-2 dThd kinase	2.6	36

cellularly, the consequence of these differences in enzyme binding was preferential inhibition of CF_3dUrd incorporation in the uninfected cells. The ratio of CF_3dUrd nucleotides which accumulated in HSV-2-infected cells as compared with uninfected cells was increased by 5′-AdThd in a dose-dependent manner (Figs. 4–6). This increase, reflecting enhanced selectivity toward the virus, was seen in both cell types. In HSV-2-infected Vero cells the accumulation of CF_3dUrd nucleotides was increased by 5′-AdThd, and, consequently, inhibition of viral replication was increased by the combination. In virally infected HeLa cells, however, more 5′-AdThd was required to achieve comparable antiviral activity. The accumulation of CF_3dUrd nucleotides was not increased, and additivity was not seen. Clearly, host cellular factors, as well as the presence of the virus, can influence the nature of this drug interaction.

Despite the structural similarities between CF_3dUrd and IdUrd, these two analogues interact quite differently with 5′-AdThd. At low concentrations, 5′-AdThd increased the phoshorylation and toxicity of IdUrd in uninfected HeLa and Vero cells (20). This enhancement of cytotoxicity was a consequence of the ability of 5′-AdThd to antagonize the feedback inhibition of dThd kinase that is normally produced by IdUTP and dTTP (20, 24). In contrast, as shown in this study, the cytotoxicity of CF_3dUrd was reduced by 5′-AdThd in a dose-dependent manner (Figs. 1–3). The absence of enhanced cytotoxicity in the case of CF_3dUrd may be explained by two findings. CF_3dUTP has been reported to inhibit dThd kinase 6- to 10-fold less effectively than dTTP (28), whereas IdUTP is a very good feedback inhibitor (29). Because of this, dThd kinase activity would be relatively less affected by the accumulation of CF_3dUTP, and any antagonism exerted by 5′-AdThd would be minimized. In addition, CF_3dUMP is a potent inhibitor of thymidylate synthetase (30), a critical enzyme in the *de novo* synthesis of dTTP. Consequently, dTTP pools would be depleted and the feedback inhibition of dThd kinase lessened. Under these conditions, stimulation (de-inhibition) of CF_3dUrd phosphorylation by 5′-AdThd would be minimal. It is likely that these effects are important, since we found that 5′-AdThd can, in the presence of dTTP, stimulate the phosphorylation of CF_3dUrd by a purified preparation of dThd kinase[4]. The relative influence of these interactions on the modulation of CF_3dUrd metabolism by 5′-AdThd in both HSV-2-infected and uninfected cells is under investigation.

5′-AdThd antagonizes the cytotoxicity of IdUrd by inhibiting the phosphorylation of IdUMP by TMP kinase (20). In contrast, the data in this study indicate that inhibition of dThd kinase accounts for the reduction in CF_3dUrd cytotoxicity produced by 5′-AdThd and that the phosphorylation of CF_3dUMP was not significantly reduced. The interactions of 5′-AdThd with CF_3dUrd at the level of the TMP kinase closely resemble those seen between 5′-AdThd and dThd (24). The differential interactions evident between these thymidine analogues emphasizes the uniqueness of each derivative.

[4] P. H. Fischer, D. G. Murphy, and R. Kawahara, unpublished results.

Different approaches can be taken to increase the selectivity of antiviral agents, including specific protection and specific inhibition. For example, dThd and dCyd have been used to protect selectively against the toxicity exerted by *E*-5-propenyl-2′-deoxyuridine (12) and 5-CIdCyd (13), respectively. In these cases preferential utilization of the normal nucleosides by the host enzymes bypassed the toxic effects of these analogues without reducing antiviral effectiveness. We previously demonstrated that high concentrations of 5′-AdThd could inhibit selectively the phosphorylation of IdUrd in uninfected cells and thereby antagonize cytotoxicity (14). However, in this example of specific protection, selectivity was not uniformly enhanced since low concentrations of 5′-AdThd increased the phosphorylation of IdUrd in the host cells (20). The present combination, also based on the concept of specific protection, has improved therapeutic potential since only reductions in the toxicity of CF_3dUrd were produced by 5′-AdThd. The potential usefulness of this combination warrants investigation *in vivo*, since the efficacy, as well as the toxicity, of CF_3dUrd are well documented (5, 31).

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