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5,6-Dihydro-5-Azathymidine: In Vitro Antiviral Properties Against Human Herpesviruses

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5,6-Dihydro-5-azathymidine (DHAdT), a novel water-soluble nucleoside antibiotic, inhibits herpes simplex virus type 1 (HSV-1) in appropriately infected cell cultures to a greater extent than herpes simplex virus type 2 (HSV-2). Vaccinia virus was less susceptible than HSV-2, and pseudorabies virus yields were not reduced at the concentrations studied. Plaque formation by varicella-zoster virus was suppressed by DHAdT. DHAdT was slightly toxic to cells at concentrations that were inhibitory for HSV-1 and varicella-zoster virus. Thymidine and deoxyuridine completely reversed the anti-HSV-1 activity of DHAdT, whereas deoxycytidine was partially effective. Compared with other nucleoside analogs with activity for HSV-1, DHAdT was less active than 5-iodo-2'-deoxyuridine or 1-β-D-arabinofuranosylcytosine and nearly equipotent with 9-β-D-arabinofuranosyladenine.

Nucleoside analogs have received considerable attention in the past because of their potential utility as antiviral agents. The impetus for this interest began with the discovery that 5-iodo-2'-deoxy-uridine (IUdR) was inhibitory for herpes simplex virus (HSV) in cell culture (6) and the subsequent demonstration of antiviral activity in rabbits with experimental herpes keratitis (7) and in clinical disease in humans (8). Since then, many nucleoside analogs have been prepared and evaluated for their potential antiherpes activity.

Recently, a novel nucleoside antibiotic was discovered in the fermentation broths of *Streptomyces platensis* var. *clarensis* (C. DeBoer, B. Bannister, A. Dietz, C. Lewis, and J. E. Gray, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, O29, p. 185). The structure of the nucleoside (Fig. 1) was shown to be 5,6-dihydro-5-azathymidine (DHAdT) (DeBoer et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, O29, p. 185). DHAdT has been shown to be effective against cutaneous HSV infection in hairless mice (14). This report describes the in vitro antiviral properties of this nucleoside.

MATERIALS AND METHODS

Cells. Primary rabbit kidney (PRK) monolayers were prepared from trypsin-treated kidneys from 100- to 150-g rabbits. The cell count was 5×10^5 cells per ml; 5 ml of cell suspension was used to seed 60-mm plastic petri dishes (Falcon Plastics, Oxnard, Calif). The cultures were incubated at 37°C in a gassed incubator and were used 5 to 7 days after planting.

Cultures of RK13, BHK-21, and Vero cells were purchased from Flow Laboratories, Rockville, Md. Human fibroblasts, derived from foreskin, were provided by R. D. Hamilton, of these laboratories. The cells were serially propagated in Eagle minimal essential medium (EMEM) containing 10% fetal bovine serum with antibiotics.

Viruses. HSV types 1 and 2 (HSV-1 and HSV-2, respectively, strains 42D and 35D) were provided by L. Chien, University of Tennessee. Vaccinia virus (vaccine strain) was originally obtained from the Michigan Department of Health and has undergone numerous passages in PRK. Pseudorabies virus (Aujeszky strain) was originally obtained from the American Type Culture Collection. Virus stocks were prepared by inoculating PRK monolayers and incubating at 37°C. Twenty-four to 48 h after infection, the cultures were frozen and thawed. The virus stocks were maintained at −70°C (11, 13).

The varicella-zoster virus (VZV) isolates were provided by Sandor Feldman, St. Jude's Children's Hospital, Memphis, Tenn., as frozen, infected cell suspensions. The strains used in these studies were VZV-195, P2 Collins and VZV-189, P2 Bennett. The virus-infected cell suspensions were prepared with human foreskin fibroblast monolayers. When >50% of the cells were rounded, they were dislodged by scraping, and the cells were suspended in 1 to 2 ml of EMEM containing 10% fetal bovine serum and 10% glycerol. The virus-infected cell suspensions were stored at −70°C.

Drugs. DHAdT and 1-β-D-arabinofuranosylcytosine (ara-C; cytarabine, Cytosar) were provided by B. Bannister and B. D. Aspergrin, respectively, of these laboratories. 9-β-D-Arabinofuranosyladenine (ara-A) was purchased from Pfanstiehl Laboratories, Waukegan, Ill. This preparation was not micronized and was used in the absence of inhibitors of adenosine deaminase. IUdR, deoxycytidine hydrochloride, deoxyuridine, and thymidine (dThd) were purchased from Calbiochem, Los Angeles, Calif. All drugs were prepared in Hanks solution.

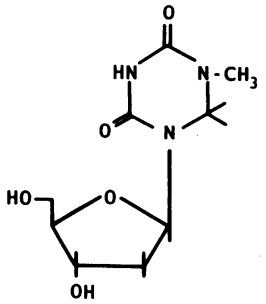

FIG. 1. *Structure of DHAdT.*

Antiviral studies. With HSV-1, HSV-2, pseudorabies, and vaccinia viruses, contiguous monolayers of cells were infected by adding 0.5 ml of the appropriate virus at a multiplicity of about 1. After 60 min at 37°C, the excess virus was removed, and the monolayers were washed twice with 2 ml of Hanks solution. EMEM containing 3% fetal bovine serum and antibiotics was added (4.5 ml), and 0.5 ml of the drug solution was appropriately diluted to contain the indicated final concentrations of drug. The cultures were incubated at 37°C until 50 to 75% of the cells in the control cultures showed evidence of viral infection. The cultures were frozen and thawed, and the virus titers were determined by the plaque method on PRK monolayers (11).

The assay of the antiviral properties of DHAdT for VZV was slightly modified from that described by Rapp (10). Human foreskin fibroblast monolayers, 24 h after refeeding with EMEM containing 4% fetal bovine serum, were inoculated by adding 0.5 ml of VZV-infected cell suspension. EMEM containing 4% fetal calf serum with the indicated concentrations of DHAdT was added to each of triplicate plates. The cultures were refed with drug-containing medium 7 days after infection, and the plaques were visualized by staining 10 to 14 days after infection.

Toxicity studies. Cells, 24 to 48 h after planting in plastic petri dishes, were refed with EMEM containing 10% fetal bovine serum and different concentrations of DHAdT. At the time when the control (non-drug-treated) cultures were nearly contiguous, the cells were removed by trypsin treatment and counted with the aid of a hemocytometer. Three plates were included for each drug concentration, and six were included for the controls. The mean (and standard error of the mean) cell counts were determined.

RESULTS

The yields of HSV-1, HSV-2, vaccinia, and pseudorabies viruses from infected PRK monolayers treated with different concentrations of DHAdT are shown in Fig. 2. HSV-1 replication was the most susceptible to inhibition by DHAdT, whereas HSV-2 appears to be two- to fourfold less susceptible than HSV-1. Vaccinia virus yields were less affected by DHAdT than either HSV-1 or HSV-2. Pseudorabies virus yields were not reduced by DHAdT at the concentrations included in this study.

The effect of DHAdT on plaque formation by VZV is shown in Table 1. Plaque formation was completely inhibited by DHAdT at 100 and 400 μM. When the DHAdT concentration was 25 μM, the plaque number and the plaque size were only slightly reduced. Under these conditions, no evidence of drug toxicity was observed by microscopic examination at the time of staining.

Table 2 compares the virus (HSV-1, HSV-2, and vaccinia) yields from RK13, BHK-21, and Vero cells treated with different concentrations of DHAdT. The greatest reductions in virus yields were observed with HSV-1 infection, regardless of the host cell. HSV-2 infection of RH13 cells appeared to be more susceptible to DHAdT than was HSV-2 infection of BHK-21 or Vero cells; e.g., DHAdT at concentrations >25 μM reduced the HSV-2 yields from RK13 cells by 2 logs, whereas at much higher DHAdT concentrations, the HSV-2 yield from BHK-21 and Vero cells was reduced by <1 log. Similarly, vaccinia virus infection was more susceptible to DHAdT when the virus yields from RK13 cells

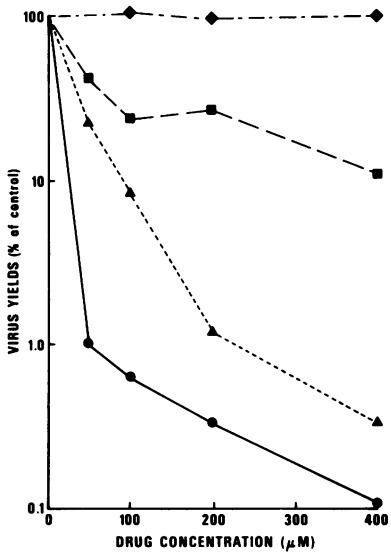

FIG. 2. *Effect of DHAdT on yields of HSV-1 (●), HSV-2 (▲), vaccinia (■), and pseudorabies (◆) viruses from PRK.*

TABLE 1. *Effect of DHAdT on plaque formation by VZV in human foreskin fibroblasts*

DHAdT concn (μM)	VZV plaques/plate	
	Strain 189	Strain 195
0	131[a]	134
25	117	122
100	0	0
400	0	0

[a] Average plaque count from three plates.

TABLE 2. *HSV-1, HSV-2, and vaccinia virus yields from RK-13, BHK-21, and Vero cell monolayers treated with different concentrations of DHAdT*

DHAdT (μM)	Plaque-forming units/0.5 ml								
	RK-13			BHK-21			Vero		
	HSV-1	HSV-2	Vaccinia	HSV-1	HSV-2	Vaccinia	HSV-1	HSV-2	Vaccinia
400	1.1×10^4	1.9×10^2	3.5×10^5	3.4×10^4	8.1×10^4	4.5×10^6	4.0×10^5	1.5×10^5	2.0×10^6
200	1.7×10^4	1.2×10^2	7.7×10^5	2.9×10^4	6.4×10^4	4.6×10^6	4.0×10^5	1.4×10^5	1.9×10^6
100	1.8×10^4	1.4×10^2	8.4×10^5	3.2×10^4	6.2×10^4	3.5×10^6	5.0×10^5	1.4×10^5	1.2×10^6
50	2.2×10^4	2.4×10^2	9.8×10^5	3.6×10^4	4.3×10^4	4.2×10^6	4.8×10^6	2.7×10^5	1.8×10^6
25	3.8×10^4	8.2×10^2	1.1×10^6	2.4×10^5	8.9×10^4	3.7×10^6	1.4×10^7	6.5×10^5	2.1×10^6
0	3.7×10^6	4.0×10^4	6.5×10^6	4.5×10^6	4.4×10^5	5.2×10^6	2.9×10^7	4.5×10^5	2.9×10^6

were compared to those from BHK-21 or Vero cells. However, even at 400 μM DHAdT, the vaccinia virus yields from RK13 were reduced by about 1.3 logs compared to >2-log reductions of HSV-1 yields from the same cells at the same drug concentration.

Table 3 summarizes the toxicity studies with DHAdT for PRK and BHK-21 cells. At a drug concentration of 250 μM, the cell count was approximately 50% of the non-drug-treated controls. Lesser drug allowed for greater increases in cell numbers; with BHK-21 cells, nearly the same yields of cells were obtained with concentrations of 62 μM or less as was obtained from the controls. At 1,000 μM, cell division was completely arrested, as indicated by comparing the cell counts resulting from this drug concentration to the initial (T_0) cell counts.

The antiviral (HSV-1) activity for DHAdT was compared to that of other nucleoside antiherpes agents (Fig. 3). Ara-C and IUdR were much more effective in reducing the virus yields from PRK than were either ara-A or DHAdT. At drug concentrations below 100 μM, ara-A and DHAdT appeared to be equivalent. Because of problems associated with the solubility of the nonmicronized ara-A, it was not evaluated at higher concentrations.

The effect of DHAdT on HSV-1 yields from PRK in medium containing different deoxynucleosides is shown in Table 4. DHAdT (200 μM) treatment reduced the virus titer about 1.5 logs when compared to the nontreated controls. In medium containing deoxycytidine hydrochloride, the virus titers from the DHAdT-treated cultures were about 0.5 log less than those from deoxycytidine hydrochloride-treated controls. Deoxyuridine and dThd both completely prevented the reduction of HSV-1 yields due to DHAdT. In this study the reversal of antiviral activity of ara-C and IUdR by deoxycytidine hydrochloride and dThd, respectively, are included as controls (11).

Figure 4 shows the HSV-1 titers from PRK in the presence of different concentrations of DHAdT at different times after inoculation. DHAdT reduced the yield of HSV-1, as well as the rate at which the maximum virus titer was reached at all times studied beyond 4 h. Of interest is the observation that, between 24 and 48 h, the virus titers in the drug-treated group remained essentially unchanged.

DISCUSSION

Several laboratories have reported that infection of cells with HSV results in the induction of several thymidine-metabolizing enzymes, which are different from those of the host cells. Considerable effort has been devoted to the devel-

TABLE 3. *Toxicity of DHAdT for PRK and BHK-21 cells*[a]

DHAdT (μM)	PRK (144 h)			BHK-21 (72 h)		
	Mean	SEM	% Control	Mean	SEM	% Control
T_0	55	5.3		65.3	2.0	
0	160	7.8	100	374	7.5	100
31	144	23	90	409	7.5	109
62	123	11	77	395	19.6	105
125	106	11	67	320	13.7	86
250	75	1.7	47	238	18.5	64
500	48	5.5	30	118	9.4	32
1,000	40	3.8	25	65	6.9	17

[a] Cell counts after trypsin treatment were determined at the indicated times and expressed as cells × 10^4 per petri dish. SEM, Standard error of the mean.

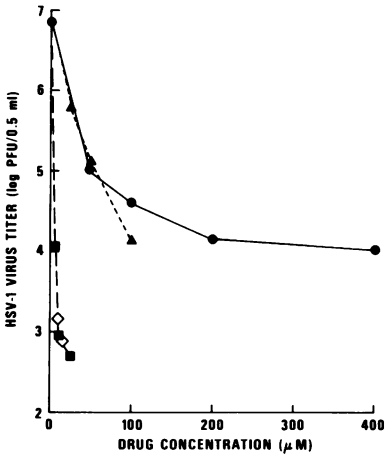

FIG. 3. *HSV-1 yields from PRK treated with different concentrations of DHAdT (●), ara-A (▲), ara-C (■), and IUdR (◇).*

opment of thymidine analogs with enhanced activity toward these virus-induced enzymes in anticipation of more effective antiviral nucleosides (2, 9). As a result, nucleoside analogs that have improved therapeutic indexes in virus-infected cell culture systems have been reported. These analogs have included 5-iodo-5'-amino-2',5'-dideoxyuridine (3) and 1-β-D-arabinofuranosylthymine (1, 5). DHAdT, a novel nucleoside analog with gram-negative antibacterial activity (De Boer et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, O29, p. 185), has been shown in the present study to inhibit certain deoxyribonucleic acid (DNA) viruses in vitro. For example, DHAdT was found to inhibit HSV-1 to a greater extent than HSV-2 in cell cultures and to suppress plaque formation by VZV. DHAdT was only slightly inhibitory for vaccinia virus and was noninhibitory for pseudorabies virus. DHAdT did not inactivate HSV-1 on prolonged incubation, indicating that DHAdT was not virucidal for this virus (data not shown). The antiviral activity was observed in virus-infected cell cultures derived from several animal species, including those from humans, rabbits, hamsters, and monkeys. It has not been possible to demonstrate antiviral activity with any of several different ribonucleic acid viruses (coxsackie A-21, influenza A/PR/8/34, parainfluenza-3, and vesicular stomatitis virus; data not shown).

In addition to the rather selective inhibition of HSV-1, DHAdT was relatively nontoxic for PRK and BHK-21 cells and in these respects resembles 5-iodo-5'-amino-2',5'-dideoxyuridine (9) and 1-β-D-arabinofuranosylthymine (1, 5). For HSV-1, DHAdT was less active than ara-C or IUdR, and at the concentrations studied it appeared nearly as active as ara-A. The relative antiviral activity of ara-C, IUdR, and ara-A as determined by measuring HSV-1 yields from PRK is nearly the same as that reported for HSV-2 in the same cell (12).

It is known that ara-C is toxic to cells in addition to reducing DNA virus yields (11). Drach and Shipman (4) have recently shown that certain antiviral agents such as ara-A are more selective inhibitors of viral DNA synthesis than of cellular DNA synthesis. In heavily infected cells, HSV DNA synthesis was reduced by ara-A concentrations that did not reduce cellular DNA synthesis. However, in these same studies ara-C appeared to be a more effective inhibitor of cellular DNA synthesis in growing cells than of viral DNA. The molecular basis for the antiviral activity of DHAdT is not known, and it remains to be established whether DHAdT has selectivity for viral (HSV-1) DNA synthesis.

It is not known whether DHAdT must be phosphorylated to the nucleotide in order to inhibit viral replication. The reversal of the antiviral activity by dThd and deoxyuridine suggests that the action of DHAdT (or its nucleotide) may be associated with inhibition of dThd (or dThd nucleotide) phosphorylation in the infected cell and thereby reduces the availability

TABLE 4. *Reversal of antiviral activity of DHAdT by deoxynucleosides*[a]

Inhibitor	μM	HSV-1 yield (PFU/0.5 ml)			
		None	dCyd · HCl	dUrd	dThd
None		1.6×10^7	1.2×10^7	1.2×10^7	1.1×10^7
DHAdT	200	6.3×10^5	6.5×10^6	1.1×10^7	1.4×10^7
Ara-C	20	6.0×10^4	4.0×10^6		
IUdR	30	8.0×10^4			1.3×10^6

[a] Medium was supplemented with 100 μg of deoxycytidine · HCl (dCyd · HCl), deoxyuridine (dUrd), or thymidine (dThd per ml). PFU, Plaque-forming units.

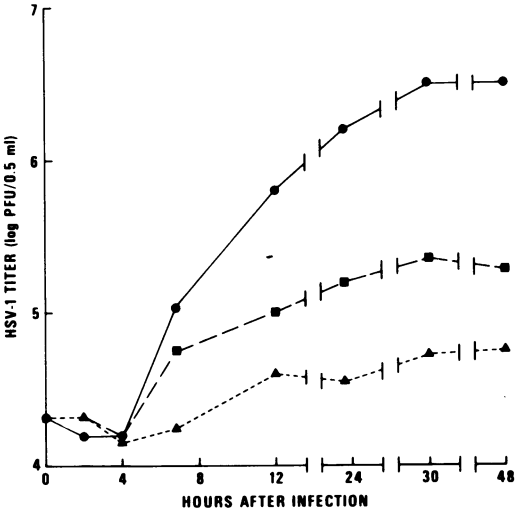

FIG. 4. *HSV-1 titers from PRK in the presence of DHAdT at 0 (●), 25 (■), and 100 (▲) μM at different times after inoculation.*

of pool dThd nucleotide (triphosphate) for subsequent DNA synthesis. Deoxyuridine, after phosphorylation and methylation, may circumvent this block by supplying dThd (as the nucleotide) via thymidylate synthetase. The partial reversal of the antiviral activity of DHAdT by deoxycytidine may reflect the ability of this nucleoside to provide deoxyuridine (or the phosphate) after deamination by cellular cytidine deaminase.

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