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In Vitro Antiherpesviral Activity of 5-Alkyl Derivatives of 1-β-D-Arabinofuranosyluracil

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Several 5-alkyl derivatives of 1-β-D-arabinofuranosyluracil (araU) were tested for antiherpesviral activity and inhibitory action on cell growth in human embryonic lung fibroblasts. 1-β-D-Arabinofuranosylcytosine, 9-β-D-arabinofuranosyladenine, and 5-iododeoxyuridine (IUdR) were included as reference materials. Among the 5-alkyl derivatives of araU, arabinosylthymine was the most active, followed by 5-ethyl- and 5-propyl-araU. 5-Ethyl-araU was as active as IUdR and more active than 9-β-D-arabinofuranosyladenine against herpes simplex virus (HSV) type 1 and did not inhibit cell growth at a concentration as high as 1,000 μg/ml. 5-Butyl- and 5-methoxymethyl-araU, as well as araU, exhibited relatively low activity. The araU derivatives tested were as active against HSV WT-34, an isolate from a patient with keratitis, as against HSV type 1. Against an IUdR-resistant isolate, HSV WT-20, arabinosylthymine was less inhibitory than IUdR. Deoxyribonucleic acid synthesis in HSV type 1-infected cells was markedly inhibited by arabinosylthymine, IUdR, and 5-ethyl-araU, whereas cellular deoxyribonucleic acid synthesis in uninfected cells was significantly inhibited by IUdR but not by arabinosylthymine or 5-ethyl-araU.

Recently, several investigations concerned with antiviral compounds have focused on discovery and development of nontoxic antiviral substances. Selective inhibition of herpes simplex virus (HSV) by some thymidine analogs, such as 5-ethyl-deoxyuridine (8), 5-methoxymethyl-deoxyuridine (3, 14), 5'-amino-2',5'-dideoxy-5-iodouridine (6), 5-propyl-deoxyuridine, and 5-propynyloxy-deoxyuridine (7), has been reported. In addition, Gentry and Aswell (9) reported that 1-β-D-arabinofuranosylthymine (araT) completely inhibited HSV replication without effect on growth of BHK cells. Selective inhibition of HSV by 5-alkyl deoxyuridine analogs was also confirmed by Cheng et al. (4, 5). The selectivity appears to depend upon the fact that the above compounds can be phosphorylated by the virus-induced deoxypyrimidinenucleoside kinase but not by cellular thymidine kinase (1, 2, 4, 15).

We have synthesized 5-alkyl derivatives of 1-β-D-arabinofuranosyluracil (araU) and tested them for antiherpesviral activity (16). In this communication, inhibition of both HSV replication and cell growth by these derivatives was compared with that by several other nucleoside analogs possessing antiviral activity. It is of special interest that newly synthesized 5-ethyl-araU, as well as araT, effectively inhibited HSV type 1 (HSV-1) replication without any significant inhibition of the host cell growth and cellular deoxyribonucleic acid (DNA) synthesis.

MATERIALS AND METHODS

Cells and viruses. Human embryonic lung fibroblast (HEL-F) cells, kindly supplied by T. Kuwata, Chiba University, were used in this study. Eagle basal medium with Earle salts (GIBCO) supplemented with 10% fetal bovine serum and 0.08% $NaHCO_3$ was used as growth medium, and Eagle basal medium supplemented with 2.5% fetal bovine serum was used as maintenance medium. HSV-1 strain VR-3 and HSV type 2, strain MS (HSV-2), were kindly supplied by S. Yamazaki, National Institute of Health of Japan. Two isolates of HSV from patients with herpes keratitis (HSV WT-20 and HSV WT-34) were kindly supplied by T. Kurimura, Tottori University School of Medicine. These viruses were propagated in HEL-F cells. The culture fluids of HSV-infected HEL-F cells were titrated and stored at −80°C until use.

Antiherpesviral experiments. HEL-F cells grown in plastic microplates (Linbro Scientic Co. Inc., model FB 16-24 TC) were infected with 30 50% tissue culture infective doses of HSV. After 30 min, the viral inoculum was removed, and 0.5 ml of test compound prepared in maintenance medium was added to the appropriate well. Test compounds were diluted in 0.5 $\log_{10}$ dilutions, with 1,000 μg/ml being the maximal concentration used. The plates were incubated at 37°C. After 3 days, the virus-induced cytopathogenic

effect in each well was determined by microscope examination and compared with that of infected and uninfected control cells in the same plate. The effectiveness of each compound against a particular strain of HSV was evaluated numerically by using a modified virus rating procedure (18, 19). Antiviral activity was also expressed as the minimal discernible virus-inhibitory concentration of the compound at which cytopathogenic effect was depressed more than 50%.

Inhibition of cell growth. HEL-F cells were plated in a 35-mm plastic petri dish at 10^5 cells per dish. After 12 h, the cells were refed with fresh growth medium containing an appropriate amount of test compound. After an additional 4-day incubation, cells from duplicate plates were dispersed by trypsin, and the cell number was determined with a hemocytometer. Percent inhibition was calculated as follows: inhibition (percent) = [1 −(cell number increase in test culture/cell number increase in control culture)] × 100%.

DNA synthesis and cellular uptake of [^{3}H]thymidine. Confluent monolayers of HEL-F cells grown in a 35-mm plastic petri dish were infected with HSV-1 and supplemented with maintenance medium containing appropriate amounts of a test compound 1 h after infection. Fifty microliters of a 5-μCi/ml amount of [^{3}H]thymidine (41 Ci/mmol; The Radiochemical Centre) was added to each plate at 2 h postinfection, and after 10 h of incubation, the medium was removed. The cells (in duplicate) were washed three times with ice-cold phosphate-buffered saline and dissolved in 0.5 ml of 2.8% NH_4OH; total radioactivity incorporated into cells was determined by liquid scintillation counting. Cells from a duplicate were treated with 1 ml of 6% cold trichloroacetic acid, and trichloroacetic acid-insoluble radioactivity was counted. For examination of cellular DNA synthesis, exponentially growing cells from 2-day-old cultures were used, and 50 μl of a 20-μCi/ml amount of [^{3}H]thymidine was added 1 h after drug treatment. After 10 h of incubation, total radioactivity and trichloroacetic acid-insoluble radioactivity were measured. In another experiment, 10 μCi of [^{3}H]deoxyguanosine (2.3 Ci/mmol; The Radiochemical Centre) per ml was used instead of [^{3}H]thymidine. Trichloroacetic acid-insoluble material obtained by the above method was dissolved in 0.5 ml of 0.3 N KOH and allowed to stand for 18 h at 37°C. The alkaline solution was neutralized with 6 N HCl and treated with trichloroacetic acid. Radioactivity from the alkali-resistant and trichloroacetic acid-insoluble fraction (DNA fraction) was determined.

Compounds. 5-Ethyl-, 5-propyl-, and 5-butyl-araU were synthesized as described elsewhere (16). araT and 5-methoxymethyl-araU were prepared from ribosylthymine and 5-methoxymethyl-uridine, respectively, via 2,2′-anhydro derivatives by the method of Hampton and Nichol (10). 1-β-D-Arabinofuranosylcytosine (araC) and 5-iododeoxyuridine(IUdR) were commercial products of Yamasa Shoyu Co., Ltd. 9-β-D-Arabinofuranosyladenine (araA) was purchased from P-L Biochemicals, Inc.

RESULTS

Antiherpesviral activity in HEL-F cells. A typical experiment on the activities of 5-alkyl derivatives of araU against HSV-1 is shown in Table 1. Their activities against HSV-1 and HSV-2 were compared with those of several other known antiviral nucleoside derivatives (Table 2). The compounds listed in Table 2 did not cause visible cytotoxicity in HEL-F cells even at the highest concentration used (1,000 μg/ml, except that of araA was 100 μg/ml as a result of its relatively low solubility). Among 5-alkyl derivatives of araU, 5-methyl-araU (araT) was the most active against HSV-1, followed by 5-ethyl- and 5-propyl-araU. 5-Butyl- and 5-methoxymethyl-araU, as well as araU, exhibited relatively low activities.

Only 1 μg of araT per ml was required to prevent HSV-induced cytopathogenic effect distinctly, and at 3.2 μg/ml the compound completely inhibited development of cytopathogenic

TABLE 1. *Effect of 5-alkyl derivatives of araU on HSV-1 infection in HEL-F cells*

Concn of araU derivatives (μg/ml)	CPE[a]									
	araU		araT		5-Ethyl-araU		5-Propyl-araU		5-Butyl-araU	
1,000	0	0	0	0	0	0	0	0	0	0
320	0	0	0	0	0	0	0	0	1	1
10	2	2	0	0	0	0	0	0	2	2
32	3	3	0	0	0	0	1	1	3	3
10	4	4	0	0	1	1	3	3	4	4
3.2	4	4	0	0	2	2	4	4	4	4
1.0	4	4	1	1	4	4	4	4	4	4
0.32	4	4	3	3	4	4	4	4	4	4
0.10	4	4	4	4	4	4	4	4	4	4
VR[b]	1.1		2.8		2.1		1.6		1.0	
MIC (μg/ml)[b]	100		1.0		3.2		32		100	

[a] Graded from 0 (normal cells) to 4 (complete destruction of cells) 3 days after infection with HSV-1. Score of cytopathogenic effect (CPE) in virus control (cells with virus but without drug) was 4.

[b] VR, Virus rating; MIC, minimal inhibitory concentration; see text.

TABLE 2. *Comparison of the activities of 5-alkyl derivatives of araU with those of other antiviral agents against HSV-1 and HSV-2 in HEL-F cells*[a]

Compound	Anti-HSV-1		Anti-HSV-2	
	VR	MIC (μg/ml)	VR	MIC (μg/ml)
araC	3.2	0.32	3.0	0.32
araA	1.8	10	1.9	10
IUdR	2.4	3.2	2.2	3.2
5-Ethyl-deoxyuridine	2.1	3.2	1.9	10
araU	1.1	100	1.2	100
araT	2.8	1.0	2.6	1.0
5-Ethyl-araU	2.1	3.2	1.0	100
5-Propyl-araU	1.6	32	0.9	320
5-Butyl-araU	1.0	100	0.1	>1,000
5-Methoxy-methyl-araU	0.9	100	0.3	1,000

[a] VR, Virus rating; MIC, minimal inhibitory concentration.

effect induced by HSV-1 infection. The minimal inhibitory concentrations against HSV-1 and HSV-2 were only a little higher than that of araC. It should be noted that antiherpesviral activity of araT in HEL-F cells seems to be much lower than that in BHK cells reported by Gentry and Aswell (1, 9) and that araT was active against HSV-2 as well as against HSV-1 in HEL-F cells, although HSV-2 was reported not to respond as strikingly to araT in BHK cells as did HSV-1 (9). A newly synthesized compound, 5-ethyl-araU, was as active as 5-ethyldeoxyuridine and IUdR and more active than araA against HSV-1. However, 5-ethyl-araU had relatively low activity against HSV-2. In addition, 5-alkyl derivatives of both 1-β-D-xylofuranosyluracil and 2,2′-anhydro-uridine were synthesized and tested for antiherpesviral activity. These compounds were much less active than the corresponding arabinoside derivatives, and their virus ratings were less than 0.9.

The compounds active against HSV-1 were then tested for activity against two isolates of HSV, HSV WT-20 and HSV WT-34. As shown in Table 3, araC and araA were active against the isolates as well as against HSV-1 and HSV-2. On the other hand, araT and the other araU derivatives were less inhibitory than IUdR against HSV WT-20, which is resistant to IUdR (11), although the thymidine analogs were as active against HSV WT-34 as against HSV-1.

Inhibition of growth of HEL-F cells. As already mentioned, cytotoxicity of araT and the other compounds tested was not observed in HEL-F cells in stationary phase. Their inhibitory action on growth of HEL-F cells was then examined (Fig. 1). araC was the most growth inhibitory, and araA and IUdR also inhibited growth significantly. In marked contrast to these compounds, araT only slightly inhibited cell growth; at 50 μg of the compound per ml growth was inhibited only 10%. This observation agrees with the reports by Aswell et al. (1) and Miller et al. (15). araT did not completely inhibit cell growth at concentrations as high as 1,000 μg/ml. Other araU derivatives, 5-ethyl-, 5-propyl- and 5-methoxymethyl-araU, as well as araU, did not inhibit growth at all even at a concentration as high as 1,000 μg/ml.

For most of the compounds listed in Table 2, antiviral indexes were determined. The antiviral index was defined as 50% cell growth-inhibitory dose divided by the minimal inhibitory concentration against HSV-1 (see Table 4). An antiviral index < 1 was found for araC and araA, and both compounds are concluded to inhibit growth of HEL-F cells more effectively than replication

TABLE 3. *Effect of antiviral compounds on two isolates of HSV*[a]

Compound	Anti-HSV WT-20		Anti-HSV WT-34	
	VR	MIC (μg/ml)	VR	MIC (μg/ml)
araC	3.5	0.1	3.4	0.1
araA	2.1	10	1.9	10
IUdR	1.5	32	2.3	3.2
5-Ethyl-deoxyuridine	0.6	320	2.0	3.2
araT	0.8	100	2.7	1.0
5-Ethyl-araU	0	>1,000	2.2	3.2

[a] VR, Virus rating; MIC, minimal inhibitory concentration.

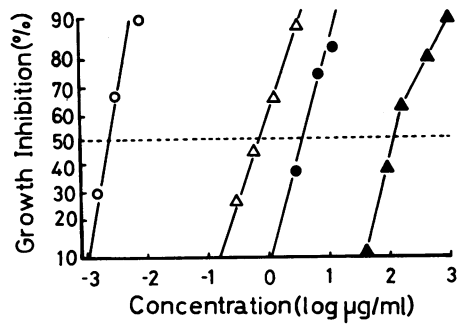

FIG. 1. *Inhibition of growth of HEL-F cells by araA, araC, IUdR, and araT. HEL-F cells were plated at 10^5 per dish. After 12 h of incubation, growth medium was replaced by fresh medium containing various concentrations of the following antiviral substances: araA (△); araC (○); IUdR (●); araT (▲). Cell numbers (in duplicate) were counted after an additional 4 days of incubation, and growth inhibition was calculated as described in the text.*

TABLE 4. *Comparison of the antiviral index of 5-alkyl-araU with those of other antiviral substances*

Compound	ID_{50} (μg/ml)[a]	Antiviral index[b]
araC	0.004	0.013
araA	1.2	0.12
IUdR	8.0	2.5
araT	150	150
5-Ethyl-araU	>1,000	>313
5-Propyl-araU	>1,000	>31.3
5-Butyl-araU	>1,000	>10
5-Methoxymethyl-araU	>1,000	>10

[a] ID_{50}, Concentration required to inhibit growth of HEL-F cells by 50% (see Fig. 1).

[b] Determined by dividing the ID_{50} by the minimal inhibitory concentration against HSV-1 (see Table 2).

of HSV-1. IUdR showed a modest antiviral index. A markedly higher antiviral index was obtained for araT. The highest safety margin was displayed by the new araU derivative, 5-ethyl-araU. This implies that 5-ethyl-araU, as well as araT, could inhibit viral replication without any actual inhibition of cell growth.

DNA synthesis and cellular uptake of [^{3}H]thymidine. HSV-1-infected HEL-F cells were treated with araT, 5-ethyl-araU, or IUdR, and the amounts of [^{3}H]thymidine incorporated into cells and trichloroacetic acid-insoluble materials were determined (Fig. 2A). Both uptake of [^{3}H]thymidine into cells and DNA synthesis were stimulated by infection with HSV-1 as much as 12-fold. Stimulation of DNA synthesis may be due to the formation of viral DNA (17). Uptake of [^{3}H]thymidine into HSV-1-infected cells was reduced by about 64% by 3.1 μg of araT per ml, whereas incorporation into DNA was almost completely inhibited (about 98%). 5-Ethyl-araU, as well as IUdR, also markedly inhibited DNA synthesis in the virus-infected cells. It is noteworthy that araT inhibited incorporation of [^{3}H]thymidine into DNA more effectively than IUdR, but the former inhibited the uptake of the precursor into infected cells less effectively than IUdR. The antiviral activity, therefore, correlates with the degree of inhibition of viral DNA synthesis rather than with the degree of inhibition of cellular uptake of [^{3}H]-thymidine.

araT might compete with [^{3}H]thymidine for the thymidine transport and phosphorylation systems of the infected cells (1); thus, it cannot be ruled out that the inhibition of [^{3}H]thymidine incorporation may be due to competition with added araT. [^{3}H]deoxyguanosine, therefore, was used as the DNA precursor, and its incorporation into alkali-resistant trichloroacetic acid-insoluble material (DNA fraction) was measured. All three antiviral substances inhibited incorporation of [^{3}H]deoxyguanosine into DNA in HSV-1-infected cells (Fig. 3). araT almost completely inhibited the incorporation, which confirms the previous finding with [^{3}H]-thymidine (Fig. 2A). The data presented here suggest that the antiviral actions of the three compounds are primarily due to inhibition of synthesis of viral DNA. Inhibition of cellular DNA synthesis by these compounds was also examined by using exponentially growing HEL-

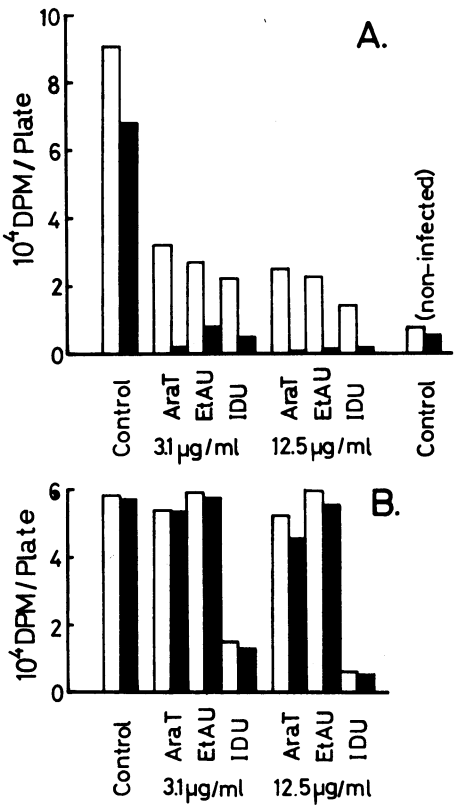

FIG. 2. *Influence of araT, 5-ethyl-araU, and IUdR (IDU) on uptake of [^{3}H]thymidine into cells and incorporation into DNA in HEL-F cells uninfected or infected with HSV-1. (A) Confluent monolayers of HEL-F cells infected with 3 × 10^6 50% tissue culture infective doses of HSV-1 per plate were supplemented with maintenance medium containing 3.1 or 12.5 μg of araT, IUdR, or 5-ethyl-araU (EtAU) per ml, or medium alone (control). At 2 h postinfection, 0.25 μCi of [^{3}H]thymidine per plate was added. At 12 h postinfection, radioactivity of whole cells (open bars) and trichloroacetic acid-insoluble radioactivity (closed bars) were determined. (B) HEL-F cells from 2-day-old cultures were refed with growth medium containing antiviral substances as described in (A). At 1 h after the treatment, 1 μCi of [^{3}H]thymidine per plate was added. After an additional 10 h of incubation, radioactivities were determined as in (A).*

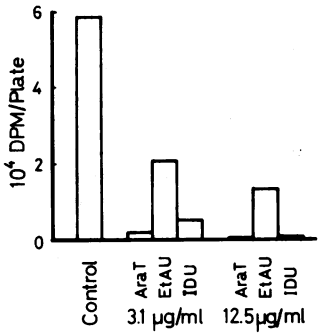

FIG. 3. *Influence of araT, 5-ethyl-araU (EtAU), and IUdR (IDU) on incorporation of ^{3}H-labeled 2'-deoxyguanosine into DNA in HEL-F cells infected with HSV-1. HEL-F cells were infected with HSV-1 and treated with drug-containing medium as in Fig. 2A. At 2 h postinfection, 0.5 μCi of [^{3}H]deoxyguanosine per plate was added. At 12 h postinfection, the alkali-resistant trichloroacetic acid-insoluble fraction (DNA fraction) was isolated and its radioactivity was determined.*

F cells (Fig. 2B). IUdR markedly inhibited cellular DNA synthesis, whereas 5-ethyl-araU did not. DNA synthesis was not affected by 3.1 μg of araT per ml and was only slightly inhibited by 12.5 μg/ml.

DISCUSSION

In the present paper, 5-alkyl derivatives of araU were tested for antiherpesviral activity. araT (5-methyl-araU) was confirmed to exhibit very high activity against HSV-1 and HSV-2 as reported by Gentry and Aswell (9) and Miller et al. (15). araT was the most effective among the 5-alkyl-araU derivatives tested. 5-Ethyl-araU was also active against HSV-1 to the same extent as IUdR and 5-ethyl-deoxyuridine. 5-Propyl-araU exhibited moderate activity, and 5-butyl- and 5-methoxymethyl-araU had relatively low activities. Thus, the antiviral activity of 5-alkyl derivatives of araU is concluded to decrease with increase in length of the alkyl group. In addition, 5-propyl-deoxyuridine (7) and 5-methoxymethyl-deoxyuridine (14) have been reported to be highly active against HSV, and it is considered that 5-alkyl derivatives of araU are generally less active than the corresponding derivatives of deoxyuridine.

Susceptibility of HSV to 5-alkyl-araU varied greatly from strain to strain. HSV-1 was susceptible to both araT and 5-ethyl-araU. One of the isolates, HSV WT-20, which is resistant to IUdR (11), was more resistant to araT than to IUdR and completely resistant to 5-ethyl-araU. In addition, 5-ethyl-araU was less effective against HSV-2 than IUdR or 5-ethyl-deoxyuridin. although the former was as effective as the latter against HSV-1. Deoxypyrimidinenucleoside kinase induced by HSV-1 or HSV WT-34 seems to act on a wider range of thymidine analogs than that induced by HSV-2 or HSV WT-20. Interestingly, pseudorabies virus- or vaccinia virus-induced deoxypyrimidinenucleoside kinase was reported to be able to phosphorylate thymidine but not deoxycytidine, whereas the kinase induced by HSV could phosphorylate both thymidine and deoxycytidine (12). In addition, the data presented here suggest that the degree of broadness in substrate specificity of HSV-induced deoxypyrimidine kinase varies from strain to strain of HSV.

araT was reported to have little effect on growth of BHK cells (9), LM cells (1), and human embryo fibroblasts (15). However, it should be noted that araT inhibited growth of CV-1 cells (15) and mouse leukemia cells, L5178Y (unpublished data), at 50 μg/ml. In our system, the growth of HEL-F cells was inhibited 10 and 63% by araT at 50 and 200 μg/ml, respectively. Surprisingly, 5-ethyl-araU did not influence the growth of HEL-F cells and L5178Y (unpublished data) at all even at a concentration as high as 1,000 μg/ml, whereas at a concentration as low as 3.2 μg/ml the compound reduced HSV-1-induced cytopathogenic effect by 50%. araC and araA inhibited the growth of HEL-F cells at a much lower concentration than araT, and antiviral indexes of the compounds in HEL-F cells were <1. Neither araC nor araA can be regarded as an antiviral substance with low toxicity. Antiviral indexes of araT and 5-ethyl-araU were 150 and >313, respectively. Therefore, 5-ethyl-araU, as well as araT, is concluded to be markedly selective in its anti-HSV-1 activity.

Antiviral activity of araT was proposed to be associated with an inhibition of viral DNA synthesis by phosphorylated araT, which was not formed in uninfected cells but was formed in HSV-infected cells (1, 15). It was shown by Matsukage et al. (13) that arabinosylthymine triphosphate markedly inhibited the action of DNA polymerase. Inhibition of DNA synthesis by 5-ethyl-araU selectively observed in HSV-infected cells (Fig. 2 and 3) and inhibition of DNA polymerase by 5-ethyl-arabinosyluracil triphosphate (K. Ono, personal communication) suggest that the mechanism operative with antiviral activity of 5-ethyl-araU is the same as that of araT. It is noteworthy that araT, IUdR, and 5-ethyl-araU inhibited not only incorporation of [^{3}H]thymidine into DNA of HSV-infected cells, but also that of [^{3}H]deoxyguanosine (Fig. 2A and 3). Incorporation of radioactive precursor into the DNA fraction in HSV-infected cells seems to be mainly due to synthesis of viral DNA, as DNA synthesis was markedly

stimulated by infection with HSV (Fig. 2A). Therefore, it may be concluded that viral DNA synthesis was markedly inhibited by araT, IUdR, and 5-ethyl-araU. However, at concentrations inhibiting viral DNA synthesis almost completely, cellular DNA synthesis in growing cells was not influenced by araT or 5-ethyl-araU, although it was markedly inhibited by IUdR (Fig. 2B). Thus, the significantly higher antiviral indexes of araT and 5-ethyl-araU may be due to their selective inhibition of viral DNA synthesis.

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