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Antiviral Action and Cellular Toxicity of Four Thymidine Analogues: 5-Ethyl-, 5-Vinyl-, 5-Propyl-, and 5-Allyl-2'-Deoxyuridine

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5-Ethyl-, 5-vinyl-, 5-propyl-, and 5-allyl-2'-deoxyuridine (dUrd) had antiviral activity against herpes simplex type 1 and type 2 grown in HeLa TK$^-$ cells, in the order 5-vinyl-dUrd, 5-ethyl-dUrd, 5-propyl-dUrd, 5-allyl-dUrd, but they were inactive against a TK$^-$ mutant of herpes simplex type 1. The antiviral activity of these compounds could be partially reversed by thymidine. Except for 5-vinyl-dUrd, they were not toxic to WI-38 and HeLa TK$^-$ cells at a concentration of 25 μM. All four analogues inhibited the growth of herpes simplex type 1-transformed HeLa TK$^-$ cells at a concentration of 1 μM.

Herpesviruses are responsible for many diseases in man, and recent evidence also suggests a strong correlation between herpes infections and the incidence of certain human malignancies (4). One characteristic of herpes simplex type I (HSV-1) and type II (HSV-2) is that they can induce virus-specific thymidine kinases (TK) in some human cells after infection. These viral TK's are different from the cytoplasmic and mitochondrial TK's of human cells not only with respect to their physical and chemical properties, such as electrophoretic mobility, activation energy, and salt sensitivity, but also in substrate specificity (Y. C. Cheng, submitted for publication; L. S. Lee and Y. C. Cheng, submitted for publication). This fact suggested to us that specific anti-HSV-1 and HSV-2 agents that specifically utilize viral TK might be developed.

The approach taken in this laboratory is to seek analogues of thymidine that could act as alternative substrates for viral TK but would not be substrates for normal mammalian cell TK's. Such analogues could be trapped inside the cells either as monophosphates or triphosphates to exert adverse effects on viral replication. Analogues of this nature would have no inhibitory effects on the growth of the uninfected cells.

Recently our laboratory purified TK's from HSV-1- and HSV-2-infected HeLa TK$^-$ cells (Y. C. Cheng and M. Ostrander, J. Biol. Chem., in press) as well as from the cytoplasmic and mitochondrial fractions of acute myelocytic leukemic blast cells (L. S. Lee and Y. C. Cheng, J. Biol. Chem., in press). A previous study on the binding specificity of some pyrimidine 2'-deoxyribonucleosides showed that 5-ethyl-, 5-propyl-, and 5-allyl-deoxyuridine (dUrd) had good binding affinity to HSV-1- and HSV-2-specific TK's (K_m of thymidine to K_i of analogue less than 0.2) but much poorer affinity for either human cytosol or mitochondrial TK's (K_m of thymidine to K_i of analogue larger than 0.005) and that 5-vinyl-dUrd had a high binding affinity to both viral and human mitochondrial TK's. For instance, the K_i of 5-ethyl-dUrd for human cytosol, human mitochondrial, HSV-1 (strain KOS), and HSV-2 (strain 333) TK's was 82, 305, 0.7, and 0.3 μM, respectively. The K_i of 5-vinyl-dUrd for human cytosol, human mitochondrial, HSV-1 (strain KOS), and HSV-2 (strain 333) TK's was 35, 1.7, 0.5, and 0.5 μM, respectively (Cheng and Lee, submitted for publication). This communication reports the results of a comparative study on the antiviral and cytotoxic effects of all four analogues and suggests that selective affinity to various TK's is responsible for their differential activity. Some studies on the biological activity of 5-ethyl-, 5-vinyl-, and 5-allyl-dUrd have been reported previously by other investigators (2, 7, 9-11).

MATERIALS AND METHODS

Cells and viruses. The stock viruses, HSV-1 (strains KOS and B2006) and HSV-2 (strain 333), were kindly given to us by W. Munyon of this institute (1, 3). Both the KOS and 333 strains of herpes simplex viruses but not the B2006 strain could induce TK in injected HeLa TK$^-$ cells. TK activities in those infected cells peak 12 h postinfection for strain KOS and 16 h postinfection for strain 333.

A viral suspension of low multiplicity (0.1 plaque-forming unit [PFU]/cell) was added to a 150-cm² confluent CV-I monolayer culture, and adsorption was allowed to proceed for 1 h. Forty milliliters of Eagle minimum essential medium containing 5% calf serum was then added, and the cells were incubated at 37 C for 24 h, followed by incubation at 34 C for 48 h (HSV-1) or 24 h (HSV-2). At the end of the incubation period, cells and medium were kept frozen at −70 C until titration. The yield of virus under these conditions was at least 5×10^9 PFU/ml for HSV-2.

HeLa TK⁻ (BU25), HSV-1 (strain KOS)-transformed HeLa TK⁻ cells, and CV-I cells were kindly given to us by W. Munyon and were grown as previously reported (1). WI-38 cells were obtained from Flow Laboratories (Rockville, Md.) and were free of mycoplasm.

Confluent HeLa TK⁻ cells (25 cm²) were used as host cells for virus infection. After a 1-h adsorption of virus with 10 PFU/cell, cell layers were rinsed twice with phosphate-buffered saline and 4 ml of Eagle minimum essential medium supplemented with 10% calf serum; various concentrations of drugs were then added, and the cells were incubated for 28 h at 37 C. At the end of the incubation they were stored frozen at −70 C until titration.

The various cell lines were grown at 37 C in different media (1) containing the analogues, and the cells were grown for three generations. The cytotoxic effect of the drugs is expressed as percentage of growth inhibition at the end of the third generation.

Materials. 5-Vinyl-2′-dUrd was synthesized as described by Sharma and Bobek (8). Briefly, catalytic hydrogenation of 1-(2-deoxy-3,5-di-*O*-*p*-toluoyl-β-D-erythropentofuranosyl)-5-vinyl uracil over 10% Pd-C in ethyl acetate, followed by removal of the protecting groups with sodium methoxide, yielded 5-ethyl-2′-dUrd. This material was identical in its melting point and ultraviolet spectrum to 5-ethyl-2′-dUrd prepared by Swierkowski and Shugar (11). 5-Allyl-2′-dUrd was kindly supplied by James F. Holland from Mount Sinai School of Medicine New York, N.Y. This material was purified by silica gel chromatography in ethyl acetate and finally recrystallized from ethyl acetate. 5-Propyl-2-dUrd was prepared by catalytic hydrogenation of 5-allyl-2′-dUrd on 5% Pd-C in methanol.

RESULTS

Antiviral activities. The effects of 5-ethyl-, 5-vinyl-, 5-propyl-, and 5-allyl-dUrd on the replication of HSV-1 (strain KOS) and HSV-2 (strain 333) in HeLa TK⁻ cells were compared with those of 5-iododeoxyuridine (Fig. 1). All analogues significantly inhibited the production of both types of virus. For HSV-1, 5-vinyl-dUrd was the most inhibitory, followed by 5-ethyl-, 5-propyl-, and 5-allyl-dUrd (Fig. 1A). For HSV-2, 5-ethyl- and 5-vinyl-dUrd were equally potent, followed by 5-propyl- and 5-allyl-dUrd (Fig. 1B). Preincubation of the two viral strains with 100 μM concentrations of any

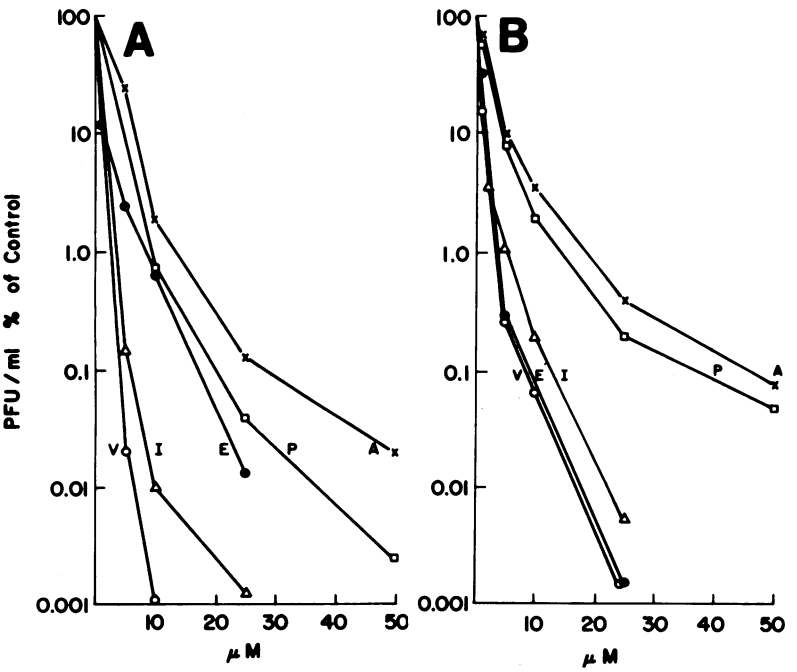

FIG. 1. *Effect of 5-ethyl-dUrd (E), 5-vinyl-dUrd (V), 5-propyl-dUrd (P), 5-allyl-dUrd (A), and 5-I-dUrd (I) on the replication of HSV-1 (A) and HSV-2 (B) in HeLa TK⁻ cells as a function of drug concentration. The experimental details are described in the text.*

of the four analogues for 1 h did not result in viral inactivation (data not shown).

Both HSV-1 (strain KOS) and HSV-2 (strain 333) induced TK in HeLa TK$^-$ cells after infection. A mutant of HSV-1, strain B2006, that could not induce TK activity in HeLa TK$^-$ cells was used for comparative tests of the antiviral activity of all four analogues. At 30 μM 5-ethyl- or 5-vinyl-dUrd and 100 μM 5-propyl- and 5-allyl-dUrd, no inhibition of B2006 growth in HeLa TK$^-$ was observed.

The anti-HSV-1 (strain KOS) and HSV-2 (strain 333) activities of these four analogues in HeLa TK$^-$ cells could be partially reversed by thymidine. The concentration of thymidine used, 25 μM, by itself had no activity against the growth of HSV-1 or HSV-2 in HeLa TK$^-$ cells (Table 1).

Cytotoxicity. Three human cell lines were chosen for examining the cytotoxicity of the analogues: (i) HeLa TK$^-$ cells, which are deficient in cytoplasmic TK but have mitochondrial TK (5); (ii) HSV-I (strain KOS)-transformed HeLa TK$^-$ cells, which possess HSV-1-specific TK (5); (ii) HSV-1 (strain KOS)-transformed (1); and (iii) WI-38 cells, which have both types of human TK. The results (Table 2) are expressed in three ways: no inhibition, less than 50% growth inhibition, or larger than 50% inhibition after three generations of cell growth. The cytotoxic effect of 25 μM 5-vinyl-dUrd against either WI-38 cells or HeLa TK$^-$ cells did not become apparent until the cells were incubated with this analogue for at least one generation. Under these conditions and for the same length of inhibition, 5-ethyl-, 5-propyl-, and 5-allyl-dUrd did not effect the growth of WI-38 and HeLa TK$^-$ cells. In contrast, all four analogues at a concentration of 1 μM inhibited the growth of HSV-1-transformed HeLa TK$^-$ cells even during the first generation of cell growth.

DISCUSSION

In summary, each of these four thymidine analogues, all of which had relatively good affinity for either HSV-1 (strain KOS)- or HSV-2 (strain 333)-induced TK (Cheng, submitted for publication), also showed significant antiviral activity against both types of viruses. An HSV-1 mutant (B2006) that lacked the ability to induce TK in infected cells was not inhibited by any of the analogues. The antiviral effects of these analogues could be partially reversed by thymidine. 5-Vinyl-dUrd, which has good affinity for human mitochondrial TK (Lee and Cheng, submitted for publication), inhibited

TABLE 1. *Reversal of antiviral activities of 5-ethyl-, 5-vinyl-, 5-propyl-, and 5-allyl-dUrd by thymidine*[a]

Compound	PFU/ml (% of control)	
	HSV-1 (KOS)	HSV-2 (333)
None	100	100
5-Ethyl-dUrd (10 μM)	0.7	0.08
5-Ethyl-dUrd (10 μM) Thymidine (25 μM)	5.5	9.7
5-Vinyl-dUrd (10 μM)	0.001	0.07
5-Vinyl-dUrd (10 μM) Thymidine (25 μM)	0.09	1.27
5-Propyl-dUrd (25 μM)	0.05	0.1
5-Propyl-dUrd (25 μM) Thymidine (25 μM)	1	1
5-Allyl-dUrd (25 μM)	0.1	0.5
5-Allyl-dUrd (25 μM) Thymidine)25 μM)	3	5
Thymidine (25 μM)	106	150

[a] Conditions of the experiments are described in the text.

TABLE 2. *Effects of 5-ethyl-, 5-vinyl-, 5-propyl-, and 5-allyl-dUrd on cell growth after three generations*[a]

Compound	Concn (μM)	Cell line	Growth inhibition		
			None	<50%	>50%
5-Ethyl-dUrd	25	HeLa TK$^-$	+		
5-Vinyl-dUrd	25	HeLa TK$^-$		+	
5-Propyl-Urd	25	HeLa TK$^-$	+		
5-Allyl-dUrd	25	HeLa TK$^-$	+		
5-Ethyl-dUrd	25	WI-38	+		
5-Vinyl-dUrd	25	WI-38		+	
5-Propyl-dUrd	25	WI-38	+		
5-Allyl-dUrd	25	WI-38	+		
5-Ethyl-dUrd	1	HSV-1-transformed HeLa TK$^-$		+	
5-Vinyl-dUrd	1	HSV-1-transformed HeLa TK$^-$			+
5-Propyl-dUrd	1	HSV-1-transformed HeLa TK$^-$		+	
5-Allyl-dUrd	1	HSV-1-transformed HeLa TK$^-$		+	

[a] Conditions of growth of the various cell lines are described in the text. Growth inhibition was calculated by the fold increase in number in the control cells minus that in the drug-treated cells divided by the fold increase in number in the control cells.

both HeLa TK$^-$ and WI-38 cell growth, but only after the first generation. There was no inhibition of cell growth in the first generation. The delay in 5-vinyl-dUrd toxicity could be due to a number of factors. For instance, this compound might specifically disturb mitochondrial deoxyribonucleic acid replication, and cells could therefore sustain their growth for one generation. While this manuscript was in preparation, the delayed type of toxicity exerted by 5-vinyl-dUrd against Ehrlich ascites carcinoma cells was reported (6). The selective biological effects of 5-ethyl-, 5-propyl-, and 5-allyl-dUrd on cells having viral TK seem to suggest that the action of the three analogues depends on the presence of the viral TK. Whether they act at the monophosphate or triphosphate level to exert their activities is still under investigation.

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