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# Anti-Herpes Simplex Virus and Anti-Human Cell Growth Activity of *E*-5-Propenyl-2'-Deoxyuridine and the Concept of Selective Protection in Antivirus Chemotherapy

YUNG-CHI CHENG,[1]* SUSAN GRILL,[1] JERRY RUTH,[1] AND DONALD E. BERGSTROM[2]†

*Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27514[1], and Department of Chemistry, University of California at Davis, Davis, California 95616[2]*

*E*-5-Propenyl-2'-deoxyuridine (*E*-5-propenyl-dUrd) inhibited the growth of herpes simplex virus (HSV) types 1 (HSV-1) and 2 in culture. The concentration of drug required to give a 2-log reduction in virus titer was 5 μM for HSV-1 and 23 μM for HSV-2. The anti-HSV-1 activity of this agent was more potent than 5-propyl-dUrd, equivalent to *E*-5-(3,3,3-trifluoropropenyl)-dUrd, and less potent than *E*-5-bromovinyl-dUrd. The HSV-1 mutant (B2006) lacking the ability to induce virus-specific thymidine kinase could not be inhibited by *E*-5-propenyl-dUrd. The binding constants of *E*-5-propenyl-dUrd to HSV-1, HSV-2, varicella-zoster virus, and human mitochondrial thymidine kinases were established to be 0.2, 6.2, 0.3, and 0.8 μM, respectively. Thymidine phosphorylation catalyzed by human cytosol thymidine kinase could not be inhibited by *E*-5-propenyl-dUrd at a concentration 10-fold higher than the thymidine in the assay. When thymidine and *E*-5-propenyl-dUrd were added concomitantly at equal concentrations to virus-infected cells, the antiviral activity was not reversed in HSV-1 and only partially reversed in HSV-2. *E*-5-Propenyl-dUrd also inhibited the growth of human cells in culture with 50% inhibitory dose of 50 μM. Since this inhibition could be readily reversed by a lower concentration of thymidine, the idea of selective protection is proposed. This approach could avoid the cytotoxic effect of an antiviral agent with properties similar to *E*-5-propenyl-dUrd without sacrificing antiviral activity.

Since we first postulated the concept of selective alternative substrate approach for the rational development of selective anti-herpes simplex virus (HSV) agents (2), a large number of thymidine (dThd) or deoxycytidine analogs with a modification at the 5 position were synthesized and examined for their antiviral activity (9, 18). Some of these analogs have shown more potent anti-HSV effects than 5-propyl- or 5-ethyl-2'-deoxyuridine (5-ethyl-dUrd), which were used as the examples for demonstrating specific alternative substrate approach (4). Unfortunately, all of these analogs have some degree of toxicity against cell growth in culture which could limit their potential use and safety in the clinic. Instead of trying to further modify these compounds, ideally the uninfected cells could be protected against the cytotoxicity of these nucleoside analogs without sacrificing their antiviral effects. This is why two approaches were postulated by which cytotoxicity could be avoided. The first, the selective inhibitor approach (3), was independently proven to be effective by Fisher et al. (11) with the combination of iodo-dUrd and 5'-aminothymidine. The second, the selective protection approach, is described in this report with a newly synthesized drug, *E*-5-propenyl-dUrd. Although this paper deals with the cytotoxicity and antiviral activity of this compound, this selective protection approach could be applied to other agents of similar properties.

† Present address: Department of Chemistry, University of North Dakota, Grand Forks, ND 58201.

## MATERIALS AND METHODS

**Compounds.** *E*-5-Propenyl-dUrd, *E*-5-trifluoropropenyl-dUrd, and 5-propyl-dUrd were synthesized by the procedure described previously (17, 19). *E*-5-Bromovinyl-dUrd was kindly provided by D. Walker of the University of Birmingham, England, and E. DeClercq of the Rega Institute for Medical Research, Belgium. dThd was purchased from P-L Biochemicals, Inc.

**Enzymes.** Human cytosol and mitochondrial thymidine kinases were extracted from peripheral blasts of acute myelocytic leukemia patients. HSV type 1 (HSV-1) and HSV-2 thymidine kinases were purified from virus-infected HeLa BU (cytosol thymidine kinase deficient) cells 16 h postinfection. The procedures for purifying different types of thymidine kinase by affinity column chromatography and the enzyme assay were described previously (5, 14). Enzyme amounts of

from $5 \times 10^{-2}$ to $1.0 \times 10^{-2}$ U were used for determining the $K_i$ values of different analogs.

**Cells.** The cells were grown at 37°C in RPMI 1640 medium containing 100 μg of kanamycin per ml and supplemented with 5% calf serum for CV-1 and HeLa BU cells and 5% fetal calf serum for HeLa and KB cells. All cultures were found to be mycoplasma free when tested by the 4,6-diamidino-2-phenylindole fluorescence technique (16).

**Virus stocks.** HSV-1 (strains KOS and B2006) or HSV-2 (strain 333) was added at a low multiplicity of infection (0.1 plaque-forming unit [PFU] per cell) to 150-$cm^2$ flasks of confluent CV-1 monolayers. After a 1-h adsorption period, 40 ml of RPMI 1640 supplemented with 5% calf serum was added. The cells were then incubated at 37°C for 24 h, followed by incubation at 34°C for 48 (HSV-1) or 24 h (HSV-2). At the end of this incubation period, the cells were suspended and aliquoted into sterile plastic tubes (12 by 75 mm) and kept frozen until titration. The yield of virus under these conditions was at least $5 \times 10^8$ PFU/ml.

Thymidine kinase was induced in infected HeLa BU cells by strains KOS and 333 but not by B2006.

**Cell and virus growth inhibition.** The cells were seeded in 25-$cm^2$ flasks in their respective growth media. They were then incubated for 24 h before adding the drug to allow the cells time to attach to the flask and to ensure that they were in log phase. The cell number was determined at the time of drug addition and 24, 48, and 72 h later by hemacytometer counts. All cell numbers were determined by an average of at least two flasks for each condition.

Confluent HeLa BU cells in 25-$cm^2$ flasks were used as host cells for virus infection. After a 1-h adsorption period of virus at 5 to 10 PFU per cell, the monolayers were rinsed twice with phosphate-buffered saline followed by addition of 5 ml of growth medium containing various concentrations of drug. The cells were incubated at 37°C for 28 h, then stored frozen at −70°C until titration. A suspension of CV-1 cells containing 0.1% methylcellulose (15 cps) was added to sterile 24-well plates and incubated at 37°C for 24 h. A serial dilution was prepared with complete growth medium from the virus samples. The medium was removed from the CV-1 monolayers, and 100 μl of the virus dilutions was added to each well. The plates were incubated for 1 h at 37°C. This was followed by adding to each well 0.5 ml of a mixture containing equal parts of 2× growth medium and 2% methylcellulose (4,000 cps). After a 2-day incubation, the medium-methylcellulose overlay was aspirated off and replaced with 0.25 ml of a 0.8% crystal violet solution in 50% ethanol. After 10 min the stain was removed, and the plates were rinsed with water. They were then allowed to air dry, and the plaques were counted by the use of a light box. Two duplicate trials were carried out to ensure reproducibility.

## RESULTS

**Affinity of *E*-5-propenyl-dUrd to human- and virus-induced thymidine kinases.** When *E*-5-propenyl-dUrd was examined against various types of thymidine kinase, the drug could not inactivate any of these enzymes. This was demonstrated by preincubating thymidine kinase with 0.5 mM of *E*-5-propenyl-dUrd in the presence of 2 mM adenosine triphosphate for 30 min at 37°C, where no enzyme activity was lost during the incubation (data not shown). Yet this drug acted as a competitive inhibitor against [$^{14}$C]dThd phosphorylation catalyzed by human mitochondria, HSV-1, HSV-2, and varicella-zoster virus thymidine kinases. The $K_i$ values are reported in Table 1. *E*-5-Propenyl-dUrd could not inhibit [$^{14}$C]thymidine phosphorylation catalyzed by human cytosol enzyme at a concentration ten times higher than the concentration of dThd (100 μM) in the assay.

**Effects of *E*-5-propenyl-dUrd and related analogs on HSV growth.** The antiviral effects of *E*-5-propenyl-dUrd and related analogs on the growth of HSV-1 (KOS), HSV-2 (333), and HSV-1 thymidine kinase-deficient ($TK^-$) mutant (B2006) in HeLa BU cells were compared (Table 2). *E*-5-Bromovinyl-dUrd, which was shown previously to have potent anti-HSV-1 effects (8),

TABLE 1. *Inhibition constant of E-5-propenyl-dUrd for thymidine kinase derived from various sources*[a]

| Enzyme source | $K_i$ (μM) |
|---|---|
| Human cytosol | >400[b] |
| Human mitochondria | 0.8 |
| HSV-1 | 0.2 |
| HSV-2 | 6.2 |
| Varicella-zoster virus | 0.3[c] |

[a] Thymidine kinase from various sources was purified with the procedures as described previously (1, 5). $K_i$ values were obtained with the same method as described (8). Competitive inhibition of [$^{14}$C]dThd phosphorylation by *E*-5-propenyl-dUrd catalyzed by various isozymes was observed.

[b] Values obtained based on the sensitivity of our assay.

[c] This $K_i$ value, published previously (7), is included for comparison.

TABLE 2. *Effects of E-5-propenyl-dUrd and related analogs on virus growth*[a]

| Additives (10 μM) | Control PFU/ml (%) | | |
|---|---|---|---|
| | HSV-1 (KOS) | HSV-2 (333) | HSV-1 ($TK^-$) (B2006) |
| None | 100 | 100 | 100 |
| 5-Propyl-dUrd | 7 | 9 | 107 |
| *E*-5-Propenyl-dUrd | 0.1 | 4 | 100 |
| *E*-5-Trifluoro-propenyl-dUrd | 0.1 | 19 | |
| *E*-5-Bromovinyl-dUrd | <0.1 | 2 | |

[a] Drugs were added at 0 h postinfection, and viruses were harvested at 28 h postinfection and titrated as described in the text. The number of PFU per milliliter in control assays was $5.1 \times 10^8$ for HSV-1, $2.5 \times 10^7$ for HSV-2, and $2.4 \times 10^8$ for HSV-1 $TK^-$.

was more potent than *E*-5-propenyl-dUrd, *E*-5-(3,3,3-trifluoropropenyl)-dUrd, or 5-propyl-dUrd against HSV-1 growth; all four compounds exhibited comparable activity (within an order of magnitude) against HSV-2 growth in HeLa BU cells. The HSV-1 $TK^-$ mutant, which lacked the ability to induce virus-specific thymidine kinase, could not be inhibited by *E*-5-propenyl-dUrd. The concentration-dependent inhibition of virus growth by *E*-5-propenyl-dUrd is shown in Fig. 1. The difference in the potency of *E*-5-propenyl-dUrd against HSV-1, HSV-2, or HSV-1 $TK^-$ growth in HeLa BU is clearly demonstrated.

**Effects of *E*-5-propenyl-dUrd and related analogs on human cell growth.** KB cells were grown in culture as described, and the effects of *E*-5-propenyl-dUrd and related analogs on the growth of KB cells were examined (Tables 3 and 4). At 50 μM, 5-propyl-dUrd could not inhibit KB cell growth, whereas *E*-5-propenyl-dUrd, *E*-5-(3,3,3-trifluoropropenyl)-dUrd, and *E*-5-bromovinyl-dUrd could inhibit the cell growth about 50%. The anti-cell growth potency was the same in HeLa, HeLa BU, and KB cells. The growth inhibitory activity of *E*-5-propenyl-dUrd on KB cells could be reversed by using one-fifth the concentration of dThd (Table 3).

**Effects of dThd on the antiviral activity of *E*-5-propenyl-dUrd.** dThd was added to virus-infected cell cultures which contained 10 and 25 μM *E*-5-propenyl-dUrd for HSV-1 and HSV-2, respectively. Its effect on the inhibitory activity of *E*-5-propenyl-dUrd on virus growth was evaluated (Table 5). Whereas dThd at equimolar to four times the concentration of *E*-5-propenyl-dUrd could only partially reverse the antiviral effect of the drug in HSV-2, 10 times more dThd than drug could not reverse the action on HSV-1 growth. This may be related to the fact that dThd could prevent the phosphorylation of *E*-5-propenyl-dUrd by HSV-2 thymidine kinase more efficiently than by HSV-1 thymidine kinase (Table 1).

## DISCUSSION

The antiviral activity of *E*-5-propenyl-dUrd is not dependent on the activity of cytosol thymidine kinase since similar antiviral activity was observed when HeLa or HeLa BU cells were used as the host cells for HSV-1 and HSV-2

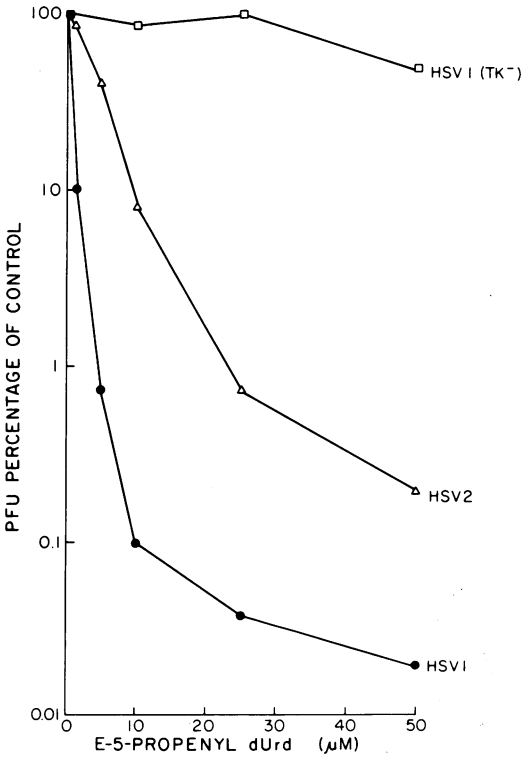


FIG. 1. *E-5-Propenyl-dUrd was added to HeLa BU cells at 0 h postinfection. Viruses were harvested and titrated 28 h postinfection as described in the text.*

TABLE 3. *Effects of 5-propenyl-dUrd and related analogs on human KB cell growth*[a]

| Additive | Concn (μM) | Cell growth (%) |
|---|---|---|
| None | | 100.0 ± 0 |
| dThd | 10 | 96.3 ± 5 |
| 5-Propyl-dUrd | 50 | 99.8 ± 1 |
| *E*-5-Propenyl-dUrd | 50 | 43.8 ± 8 |
| *E*-5-Trifluoro-propenyl-dUrd | 50 | 54.2 ± 3 |
| *E*-5-Bromovinyl-dUrd | 50 | 48.1 ± 3 |
| *E*-5-Propenyl-dUrd + dThd | 50 + 10 | 96.3 ± 5 |

[a] KB cells were grown under the conditions as described in the text for 48 h. The percentages of cell growth were compared with control, which increased cell number 3.2-fold in comparison with the initial number.

TABLE 4. *Inhibitory effect of E-5-propenyl-dUrd on the growth of human KB cells*[a]

| Concn (μM) | Cell no. (×$10^5$) per flask at time postinoculation (h): | | | |
|---|---|---|---|---|
| | 0 | 24 | 48 | 72 |
| None | 0.6 | 1.3 | 2.8 | 5.2 |
| 25 | 0.6 | 0.9 | 2.1 | 3.4 |
| 50 | 0.6 | 0.8 | 1.5 | 2.9 |

[a] Cells were grown under the conditions described in the text.

TABLE 5. *Effects of dThd on antiviral activity of E-5-propenyl-dUrd*[a]

| E-5-Propenyl-dUrd (μM) | dThd (μM) | Control PFU/ml (%) | |
|---|---|---|---|
| | | HSV-1 | HSV-2 |
| None | None | 100 | 100 |
| None | 100 | 100 | 100 |
| 10 | None | 0.10 | |
| 10 | 10 | 0.15 | |
| 10 | 25 | 0.15 | |
| 10 | 50 | 0.10 | |
| 10 | 100 | 0.10 | |
| 25 | None | | 0.40 |
| 25 | 10 | | 0.75 |
| 25 | 25 | | 1.50 |
| 25 | 50 | | 3.50 |
| 25 | 100 | | 10.0 |

[a] *E*-5-Propenyl-dUrd or dThd or both were added at 0 h postinfection. Viruses were harvested 28 h postinfection and titrated as described in the text. The PFU per milliliter for control assays was $3.8 \times 10^8$ for HSV-1 and $1.9 \times 10^7$ for HSV-2.

infection (data not shown). This is consistent with the observation that the cytosol thymidine kinase had very poor binding affinity to *E*-5-propenyl-dUrd (Table 1). The antiviral activity of this drug seems to be related to its binding affinity to viral thymidine kinase, based on the following observations: *E*-5-propenyl-dUrd has more affinity to HSV-1 thymidine kinase than to HSV-2 thymidine kinase, and the antiviral activity of *E*-5-propenyl-dUrd was more potent against HSV-1 than HSV-2, whereas 5-propyl-dUrd had similar binding affinity to HSV-1 and HSV-2 thymidine kinases and similar antiviral activity against HSV-1 and HSV-2 growth in culture (Tables 1 and 2). The HSV-1 $TK^-$ mutant lacking the ability to induce viral thymidine kinase upon its infection was insensitive to either 5-propenyl-dUrd or 5-propyl-dUrd. It should be noted that the specificity of thymidine kinase is only a first-line determining factor for dThd analogs; their potency against virus growth will also depend on the behavior of their phosphorylated derivatives on the metabolic targets. In general, the substrate specificity of HSV-1 or varicella-zoster virus thymidine kinase is less than HSV-2 thymidine kinase, as was suggested previously (1, 6, 15). This is also consistent with the observation in Table 1. Therefore, a dThd analog which was shown to have activity against HSV-1 would not always have equal activity against HSV-2. The detailed interactions of *E*-5-propenyl-dUrd will be examined when the radioactive compound becomes available.

The inhibitory effects of *E*-5-propenyl-dUrd on the growth of human cells in culture may be related to its strong binding affinity to human mitochondrial thymidine kinase. HeLa BU cells, which are deficient in cytosol thymidine kinase, have a sensitivity to *E*-5-propenyl-dUrd similar to that of HeLa or KB cells. Thus, the mitochondrial thymidine kinase is most likely responsible for the cytotoxicity of this agent by converting it to a nucleotide. 5-Vinyl-dUrd and *E*-5-propenyl-dUrd behave similarly toward human cytosol and mitochondrial thymidine kinases. On the basis of the structural relationship, it is conceivable that *E*-5-trifluoropropenyl-dUrd and *E*-5-bromovinyl-dUrd may also have a strong affinity to mitochondrial thymidine kinase but not to the cytosol enzyme. Unpublished results from this laboratory in collaboration with R. T. Walker and E. DeClercq support this notion. It should be noted that although the cytosol thymidine kinase is not involved in the cytotoxicity of *E*-5-propenyl-dUrd, it may play an important role in the reversal process of the cytotoxicity of this agent by dThd. Its role is under current investigation.

The in vitro results presented here indicate that the inclusion of a one-fifth equivalent of dThd reverses the growth inhibitory activity of *E*-5-propenyl-dUrd without decreasing its antiviral effects; even much higher levels of dThd leave the virucidal activity unaffected. Since 5-alkyl-dUrd analogs are generally substrates for human dThd phosphorylase (unpublished data), the combination of dThd with these analogs could offer a second advantage, with dThd serving as a strong competitive substrate for the phosphorylase and as a result prolonging the action of the analogs. Although as yet untested in vivo, this type of selective protection approach appears to have good potential in a variety of applications for the treatment of HSV-induced disease. For example, both *E*-5-bromovinyl-dUrd and acyclovir (acycloguanosine) have potent antiviral activity (8, 10, 12, 13), but inhibit cell growth at higher concentrations. The cytotoxic effects of *E*-5-bromovinyl-dUrd (and other 5-substituted dUrd analogs) may be decreased or eliminated by the inclusion of dThd (or other agents) without sacrificing the antiviral activity. The mechanism may also be applied to agents acting in related processes, such as acyclovir. Although the cytotoxic mechanism of acyclovir has not been established, if it is mediated through the phosphorylation process which normally utilizes guanosine or deoxyguanosine, the inclusion of one of these with acyclovir may minimize the cytotoxicity, leaving its antiviral activity unaffected (since acyclovir behaves as a dThd analog during its activation in HSV-infected cells) (13). Further experiments are needed to prove these points.

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