JOURNAL OF VIROLOGY, July 1982, p. 332-336
0022-538X/82/070332-05$02.00/0

Vol. 43, No. 1

Inhibitory Effect of *E*-5-(2-Bromovinyl)-1-β-D-Arabinofuranosyluracil on Herpes Simplex Virus Replication and DNA Synthesis

J. DESCAMPS,[1] R. K. SEHGAL,[1] E. DE CLERCQ,[2] AND H. S. ALLAUDEEN[1]*

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510,[1] and Rega Institute, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium[2]

Received 23 October 1981/Accepted 2 March 1982

The effect of *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BVaraU) on herpes simplex virus (HSV) replication was examined and compared with that of *E*-5-(2-bromovinyl)-2'-deoxyuridine (BVdUrd). The 50% inhibitory dose against HSV type 1 (HSV-1) was 0.1 μg/ml compared with 0.008 μg/ml for BVdUrd; the antimetabolic 50% inhibitory dose of BVaraU ranged from 20 to 95 μg/ml. The addition of 50 μg of BVaraU per ml to HSV-1-infected Vero cells decreased the synthesis of viral and cellular DNA by 37 and 28%, respectively. The 5'-triphosphate (BVaraUTP) competed with dTTP in DNA synthesis by the herpesviral and cellular DNA polymerases; the apparent K_i values of HSV-1 DNA polymerase, DNA polymerase α, and DNA polymerase β were 0.14, 0.32, and 5 μM, respectively. Thus, BVaraU was a less effective antiherpesvirus agent than BVdUrd; unlike BVdUrd, it did not appear to be internally incorporated into replicating DNA in virus-infected cells.

Among the newly developed analogs, *E*-5-(2-bromovinyl)-2'-deoxyuridine (BVdUrd) emerged as one of the most promising agents against herpes simplex virus type 1 (HSV-1) replication both in cell culture and in experimental animals (5, 6). More recently, an arabinose analog of BVdUrd, *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BVaraU), was synthesized by Sakata et al. (12) and at the Rega Institute, Leuven, Belgium. In its initial in vitro trials, it showed promising selective antiviral action. (E. De Clercq, R. Busson, L. Colla, J. Descamps, J. Balzarini, and H. Vanderhaeghe, Program Abstr. Int. Congr. Chemother., 12th, Florence, Italy, abstr. no. 316, 1981). To elucidate the mode of selective antiherpesviral action of BVaraU, we investigated its antiviral and antimetabolic properties and examined its effect on DNA replication in virus-infected cells. The effect of chemically synthesized 5'-triphosphate on the activities of purified viral and cellular DNA polymerases is also described in this communication.

The antiviral assays were carried out with primary rabbit kidney (PRK) cell cultures infected with HSV (4). The results are expressed as ID_{50} (50% inhibitory dose), the concentration of compound required to reduce viral cytopathogenic effect (CPE) by 50% when virus-infected control PRK cells reached complete CPE. The data presented are an average of at least three separate experiments. The ID_{50} value for BVaraU against three strains of HSV-1 was 0.1 μg/ml compared with 0.007 to 0.009 μg/ml for BVdUrd (Table 1). BVaraU was less effective against HSV-2 replication; the ID_{50} ranged from 15 to 70 μg/ml compared with 1 to 1.5 μg/ml for BVdUrd. Neither BVaraU nor BVdUrd was active against an HSV-1 TK^- (B2006) mutant, indicating that both nucleoside analogs need to be phosphorylated by the virus-induced thymidine kinase to exert their antiviral activity. BVaraU was not active against vaccinia virus or vesicular stomatitis virus. The antimetabolic activity of BVaraU was monitored by the inhibition of dThd or dUrd incorporation into DNA of PRK cells (4); [1',2'-^{3}H]2'-deoxyuridine (specific activity, 42 Ci/mmol), and [*methyl*-^{3}H]thymidine (specific activity 12 Ci/mmol) were used as the labeled DNA precursors. BVaraU inhibited cellular DNA synthesis only at doses much higher than that required to inhibit the viral-induced CPE (Table 1).

To confirm that the inhibitory effect of BVaraU on CPE is due to its ability to inhibit the virus multiplication, we tested its effect on the replication of HSV-1 (KOS) in PRK cells. Both BVaraU and BVdUrd inhibited the viral multiplication, albeit to a different extent. For example, at 100 μg/ml after 24 h, BVaraU reduced the virus replication by 3.9 $\log_{10}$, whereas BVdUrd reduced it by 5.9 $\log_{10}$; after 48 h, BVaraU at 100 μg/ml reduced virus replication by 3.4 $\log_{10}$, whereas BVdUrd reduced it by 5.4 $\log_{10}$.

TABLE 1. Antiviral and antimetabolic activities of BVaraU and BVdUrd in PRK cell cultures[a]

Antiviral or -metabolic activity	ID_{50} (μg/ml)[b]	
	BVaraU	BVdUrd
Virus		
HSV-1 (KOS)	0.1	0.007
HSV-1 (F)	0.1	0.007
HSV-1 (Mac Intyre)	0.1	0.009
HSV-1 TK⁻ (B2006)	>200	100
HSV-2 (G)	15	1
HSV-2 (Lyons)	20	1
HSV-2 (196)	70	1.5
Vaccinia	>200	7
Vesicular stomatitis virus	>200	>200
Antimetabolic activity[c]		
[*methyl*-^{3}H]dThd incorporation	20	70
[1′,2′-^{3}H]dUrd incorporation	95	20

[a] PRK cells were inoculated with 100 cell culture infecting doses of virus for 1 h at 37°C and exposed to various concentrations of the test compound (in Eagle minimal essential medium plus 3% fetal bovine serum). CPE was recorded daily. In virus-infected but untreated cell culture controls, the viral CPE was generally complete after 3 days following viral inoculation.

[b] Results are expressed as ID_{50}, i.e., the concentration required to inhibit viral CPE or incorporation of labeled precursors by 50% compared with untreated controls; 0.1 μg/ml is equivalent to 0.29 and 0.3 μM of BVaraU and BVdUrd, respectively.

[c] [1′2′-^{3}H]2′-deoxyuridine (specific activity, 42 Ci/mmol) and [*methyl*-^{3}H]thymidine (specific activity, 12 Ci/mmol) were used as the labeled DNA precursors in experiments to determine the antimetabolic activity.

The effect of the 5′-triphosphate (BVaraUTP) on the utilization of dTTP by viral and cellular DNA polymerases was determined. The BVaraU was converted to its 5′-triphosphate by procedures described previously (3). The DNA polymerases used in these experiments were purified by successive chromatography, using phosphocellulose and DNA-cellulose columns. These enzymes were free of cross-contamination; for example, HSV-1 DNA polymerase was free of DNA polymerase α and vice versa. In their properties such as elution positions in ion-exchange columns, primer-template preference, effects of mono- and divalent cations, sensitivity to *N*-ethylmaleimide and aphidicolin, these enzymes resembled the respective viral and cellular DNA polymerases. The polymerase assays were performed under conditions optimum for the individual enzymes with the concentration of [^{3}H]dTTP maintained at two to three times the K_m value for the respective polymerase (1 μM for HSV-1 DNA polymerase, 10 μM for DNA polymerase α, and 30 μM for DNA polymerase β and Epstein-Barr virus [EBV] DNA polymerase). HSV-1 DNA polymerase was the most sensitive among the polymerases tested; DNA polymerase α was the next sensitive enzyme. However, DNA polymerase β and EBV DNA polymerase were relatively insensitive. At 1 μM, BVaraUTP inhibited the activities of HSV-1 DNA polymerase, DNA polymerase α, DNA polymerase β, and EBV DNA polymerase by 60, 48, 15, and 10%, respectively; at 10 μM, it inhibited their activities by 90, 82, 34, and 23%, respectively.

To determine the nature of BVaraUTP inhibition, further experiments were performed with increasing concentrations of dTTP. Results show that the inhibition was competitive with dTTP (8); the mode of inhibition appears to be the same for all of the enzymes examined (Fig. 1). The K_m values for dTTP and the K_i value for BVaraUTP are depicted in Table 2. Although the K_i values for HSV-1 DNA polymerase (0.14 μM BVaraUTP) and DNA polymerase α (0.32

TABLE 2. Kinetic analysis of BVaraUTP inhibition[a]

Enzyme	K_m (dTTP) (μM)	K_i (BVaraUTP) (μM)	K_m/K_i
HSV-1 DNA polymerase	0.35	0.14	2.5
Human DNA polymerase α	5.16	0.32	16.12
Human DNA polymerase β	14.8	5	2.96

[a] DNA polymerase α activity was assayed in a 50-μl reaction mixture consisting of 50 mM Tris-hydrochloride (pH 8.0), 2 mM dithiothreitol, 8 mM $MgCl_2$, 100 μM each of dATP, dCTP, and dGTP, and various concentrations of [^{3}H]dTTP (530 cpm/pmol), 10 μg of activated calf thymus DNA, 10 to 20 μg of bovine serum albumin, 5 to 10% glycerol, and enzyme. Incubation was at 37°C for 30 min. Acid-insoluble radioactivity was collected on a nitrocellulose filter (Gelman or Millipore, 0.45 μm) washed several times with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate, washed once with 70% ethanol, dried, and measured in a liquid scintillation counter. DNA polymerase β activity was assayed under similar conditions except that a pH 9.0 Tris-hydrochloride buffer, 50 μM of nonradioactive triphosphates and 40 mM KCl were used. The reaction mixture for assaying HSV-1 DNA polymerase activity contained 50 mM Tris-hydrochloride (pH 8.3), 2 mM dithiothreitol, 4 mM $MgCl_2$, 10 μM each of dATP, dCTP, and dGTP, and various concentrations of [^{3}H]dTTP (4,300 cpm/pmol), 5 μg of activated calf thymus DNA, 50 mM ammonium sulfate, 10 μg of bovine serum albumin, 5 to 10% glycerol, and enzyme. Other conditions were similar to those described above for measuring DNA polymerase α activity. EBV DNA polymerase activity was assayed under similar conditions for DNA polymerase α except that 4 mM $MgCl_2$ and 100 mM KCl were used.

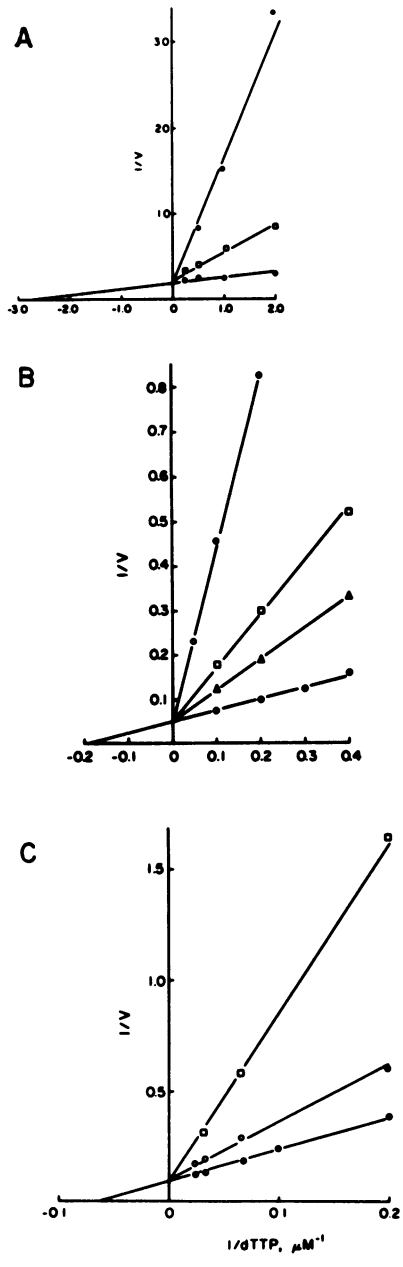

FIG. 1. DNA polymerases α and β were purified from leukocytes of a patient with acute myelogenous leukemia. EBV DNA polymerase was purified from a virus-producing cell line, P3HR-1, originally derived from a patient with Burkitt's lymphoma. The isolation procedures were described previously (1). The HSV-1 DNA polymerase was purified from KB cells infected with HSV-1 strain HF. Growth conditions and isolation procedures of HSV-1-infected KB cells were described by Shipman et al. (13). (A) Effect of BVaraUTP on HSV-1 DNA polymerase reaction in the presence of different concentrations of [^{3}H]dTTP with activated DNA template. Symbols: ○, no inhibitor, □, 1 μM BVaraUTP; and ●, 4 μM BVaraUTP. (B) Effect of BVaraUTP on DNA polymerase α reaction in the presence of different concentrations of [^{3}H]dTTP with activated DNA template. Other assay conditions were optimal for the DNA polymerase α activity. Symbols: ●, no inhibitor; ▲, 0.5 μM BVaraUTP; □, 1 μM BVaraUTP; and ○, 4 μM BVaraUTP. (C) Effect of BVaraUTP on DNA polymerase β reaction in the presence of different concentrations of [^{3}H]dTTP with activated DNA template. Other assay conditions were optimal for the DNA polymerase β activity. Symbols: ●, no inhibitor; ○, 4 μM BVaraUTP; and □, 20 μM BVaraUTP.

μM BVaraUTP) are almost similar, the K_m/K_i ratio for DNA polymerase α was six times more than that for the HSV-1 DNA polymerase. This implies that BVaraUTP is less selective than BVdUTP. Since the submission of this manuscript, Ruth and Cheng reported a K_i value of 0.013 μM for BVaraUTP of HSV-1 DNA polymerase (11).

DNA polymerases require all four natural deoxyribonucleoside triphosphates for optimum activity. However, a limited DNA synthesis occurs when one or two triphosphate substrates are omitted from the reaction mixture. Using this phenomenon, we tested whether BVaraUTP can substitute dTTP in a DNA polymerization reaction. BVaraUTP was added to the HSV-1 DNA polymerase reaction mixture containing all triphosphates except dTTP, and the reaction was followed for 180 min (Fig. 2). The addition of BVaraUTP instead of dTTP did not enhance the polymerization reaction. There was no increase in DNA synthesis even when excess dTTP was added at the end of 120 min. However, the addition of dTTP to the reaction mixture containing only the three nucleotides at 120 min increased DNA synthesis as expected. The addition of new enzyme and dTTP to the reaction mixture containing the analog did not enhance DNA synthesis; the addition of excess template and dTTP increased DNA synthesis only slightly. It appears that BVaraUTP was not an alternate substrate for DNA polymerase and that it did not get internally incorporated into replicating DNA (Fig. 2). A similar pattern was obtained for DNA polymerase α. Preincubation of the enzyme in the presence of different concentrations of BVaraUTP for 18 h at 4°C did not alter the enzyme reaction (data not shown). These results are consistent with the view that BVaraUTP may be a chain terminator for the polymerization reaction.

To study the effect of BVaraU on DNA synthesis in virus-infected cells, Vero cells were infected with HSV-1 and grown in the presence of BVaraU; the viral and cellular DNAs were separated by isopycnic CsCl centrifugation (2).

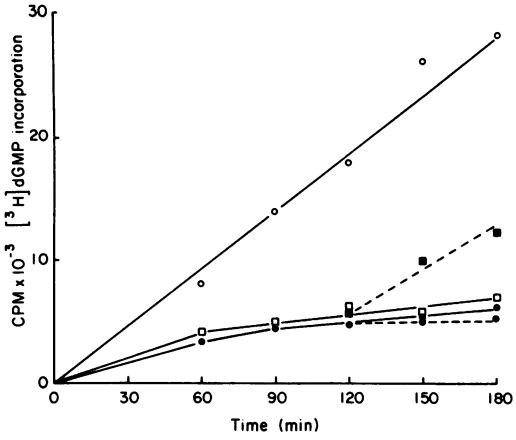

FIG. 2. Time course of BVaraUTP inhibition of HSV-1 DNA polymerase reaction. Activated DNA was used as template and the conditions were optimal for HSV-1 DNA polymerase. Symbols: ○, all four nucleotides present; □, dATP, dCTP, and [³H]dGTP present; ●, dATP, dCTP, and [³H]dGTP, plus 40 μM BVaraUTP present. At 120 min, 40 μM dTTP was added to the reaction mixture containing either three nucleotides (■) or three nucleotides plus 40 μM BVaraUTP (◑) and the reaction was stopped at 30 and 60 min after the addition.

BVaraU inhibited the synthesis of both viral and cellular DNAs (Fig. 3). For example, the addition of 50 μg of BVaraU per ml resulted in a decrease in synthesis of both viral and cellular DNA by 37 and 28%, respectively. The inhibition was proportional to the concentration of the nucleoside analog added. The effect of the addition of 50 μg of BVdUrd per ml is shown in Fig. 3D. We have also examined whether there was any qualitative change in the viral and cellular DNAs synthesized in the presence of BVaraU. Previously, it has been shown that growing virus-infected cells in the presence of either 5-iodo-2'-deoxyuridine (IdUrd) or 5'-amino-5-iodo-2'-deoxyuridine (AIdUrd) resulted in the incorporation of these analogs into DNA, which was measured by an increase in densities of the DNAs (7). Substitution of 2% or more of thymidine by BVaraU into DNA would have resulted in an increase in densities of viral and cellular DNAs. There was no difference in densities of either viral or cellular DNA isolated from the virus-infected cells grown in presence of BVaraU (Fig. 3). This indicated that the analog was either not internally incorporated or insufficiently incorporated into replicating DNAs in virus-infected cells, which is consistent with results shown in Fig. 2. However, virus-infected cells grown in the presence of 50 μg of BVdUrd per ml showed an increase in density of the viral DNA (from 1.725 to 1.74 g/ml) but not in cellular DNA (2). Recently, Pelling et al. (10) demonstrated that another arabinose analog, 9-β-D-arabinofuranosyladenine (araA), is not an absolute chain terminator and is internally incorporated into viral and cellular DNAs.

The results presented in this communication demonstrate that BVaraU is approximately 10 times less active than BVdUrd against HSV-1 replication in PRK cells. However, recently Machida et al., using different HSV-1 strains and human embryonic lung fibroblast cells, observed no difference between BVaraU and BVdUrd in their abilities to inhibit replication of HSV-1 (0.032 μg/ml) and HSV-2 (100 μg/ml) (9). The reason for differences in values of the anti-herpesvirus activity between the two labora-

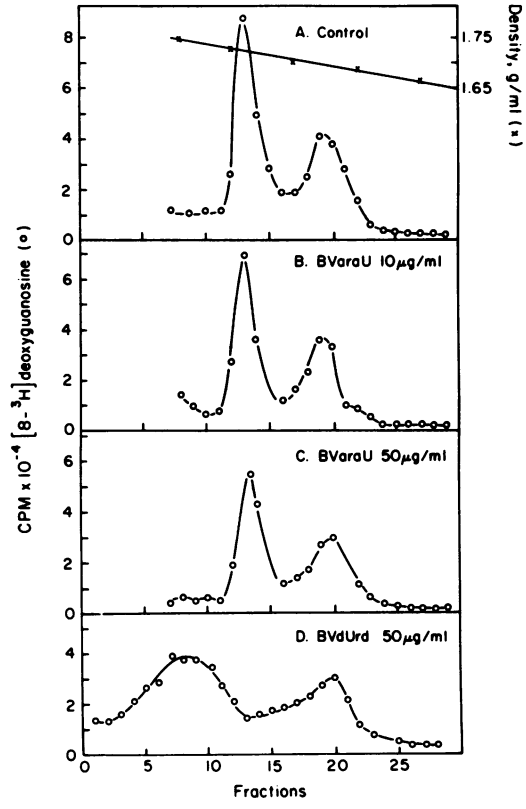

FIG. 3. CsCl density gradient analysis of cellular and viral DNA in HSV-1 (Cl 101)-infected Vero cells at a multiplicity of infection of four. After 1 h of contact at 37°C, residual virus was removed, and 4 ml of growth medium containing either no nucleoside or BVaraU was added. To each petri dish, 20 μCi of [³H]deoxyguanosine (specific activity, 14.5 Ci/mmol) was added and incubated for 24 h. (A) Cellular DNA density, 1.695 g/ml; HSV-1 DNA density, 1.725 g/ml in control; (B) BVaraU, 10 μg/ml or 29 μM; (C) BVaraU 50 μg/ml or 144 μM; and (D) BVdUrd 50 μg/ml or 150 μM.

tories is not yet known. HSV-1 DNA polymerase and cellular polymerase α are the most sensitive to the BVaraUTP inhibition among the enzymes tested. The K_i values for BVaraUTP of DNA polymerase specific to two strains of HSV-1, i.e., strain Cl 101 propagated in Vero cells and strain HF propagated in human KB cells are similar (0.14 to 0.2 μM). The analog does not appear to get internally incorporated into replicating DNA. Our results show that BVaraU was approximately 100 times less effective in inhibiting the replication of HSV-2 than HSV-1. Since HSV-2 markedly differs from HSV-1 in its susceptibility to BVaraU, the differential sensitivity can be exploited in distinguishing the two types of HSV.

This research was supported by Public Health Service grant CA 28852-01 and American Cancer Society grant CH47-C.

LITERATURE CITED

1. **Allaudeen, H. S., and J. R. Bertino.** 1978. Isolation of herpesvirus-specific DNA polymerase from tissues of an American patient with Burkitt's lymphoma. Proc. Natl. Acad. Sci. U.S.A. **75**:4504–4508.
2. **Allaudeen, H. S., M. S. Chen, J. J. Lee, E. De Clercq, and W. H. Prusoff.** 1982. Incorporation of E-5-(2-halovinyl)-2'-deoxyuridines into deoxyribonucleic acids of herpes simplex virus type-1 infected cells. J. Biol. Chem. **257**:603–606.
3. **Allaudeen, H. S., J. W. Kozarich, J. R. Bertino, and E. De Clercq.** 1981. On the mechanism of selective inhibition of herpesvirus replication by E-5-(2-bromovinyl)-2' deoxyuridine. Proc. Natl. Acad. Sci. U.S.A. **78**:2698–2702.
4. **De Clercq, E., J. Descamps, G. F. Huang, and P. F. Torrence.** 1978. 5-Nitro-2'-deoxyuridine and 5-nitro-2'-deoxyuridine 5'-monophosphate: antiviral activity and inhibition of thymidylate synthetase in vivo. Mol. Pharmacol. **14**:422–430.
5. **De Clercq, E., J. Descamps, P. De Somer, P. J. Barr, A. S. Jones, and R. T. Walker.** 1979. (E)-5-(2-bromovinyl)-2'-deoxyuridine: a potent and selective antiherpes agent. Proc. Natl. Acad. Sci. U.S.A. **76**:2947–2951.
6. **De Clercq, E., J. Descamps, G. Verhelst, R. T. Walker, A. S. James, P. F. Torrence, and D. Shugar.** 1980. Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. J. Infect. Dis. **14**:563–574.
7. **Fisher, P. H., M. S. Chen, and W. H. Prusoff.** 1980. The incorporation of 5-iodo-5'-amino-2',5'-dideoxyuridine and 5-iodo-2'-deoxyuridine into herpes simplex virus DNA. Biochim. Biophys. Acta **606**:236–246.
8. **Lineweaver, H., and O. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
9. **Machida, H., S. Sakata, A. Kuninaka, and H. Yoshino.** 1981. Antiherpesviral and anticellular effects of 1-β-D-arabinofuranosyl-*E*-5-(2-halogenovinyl)uracils. Antimicrob. Agents Chemother. **20**:47–52.
10. **Pelling, J. C., J. C. Drach, and C. Shipman, Jr.** 1981. Internucleotide incorporation of arabinosyladenine into herpes simplex virus and mammalian cell DNA. Virology **109**:323–335.
11. **Ruth, J. L., and Y. C. Cheng.** 1981. Nucleoside analogs with clinical potential in antivirus chemotherapy. The effect of several thymidine and 2'-deoxycytidine analog 5'-triphosphates on purified human (α,β) and herpes simplex virus (types 1, 2) DNA polymerases. Mol. Pharmacol. **20**:415–422.
12. **Sakata, S., S. Shibuya, H. Machida, H. Yoshino, K. Mirota, K. S. Senda, K. Ikeda, and Y. Mizuno.** 1980. Synthesis and antiherpesviral activity of 5-C-substituted uracil nucleosides. Nucleic Acids Res. Symp. Ser. **8**:539–542.
13. **Shipman, C., Jr., S. M. Smith, R. M. Carlson, and J. C. Drach.** 1976. Antiviral activity of arabinosyladenine and arabinosylhypoxanthine in herpes simplex virus-infected KB cells: selective inhibition of viral deoxyribonucleic acid synthesis in synchronized suspension cultures. Antimicrob. Agents Chemother. **9**:120–127.