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Comparison of Two Bromovinyl Nucleoside Analogs, 1-β-D-Arabinofuranosyl-*E*-5-(2-Bromovinyl)Uracil and *E*-5-(2-Bromovinyl)-2′-Deoxyuridine, with Acyclovir in Inhibition of Epstein-Barr Virus Replication

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The effect of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU), a new antiviral drug, on Epstein-Barr virus (EBV) was studied and compared with those of *E*-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) and acyclovir (ACV). BV-araU effectively inhibited EBV replication both in superinfected Raji cells and in virus producer P3HR-1(LS) cells, as determined by density gradient centrifugation, in situ cytohybridization with an EBV DNA probe, and cRNA-DNA hybridization. The 50% effective doses for viral DNA replication were 0.26, 0.06, and 0.3 μM for BV-araU, BVdU, and ACV, respectively. The relative efficacy on the basis of the in vitro therapeutic index was BVdU (6,500) > BV-araU (1,500) > ACV (850). Synthesis of EBV-induced polypeptides with molecular weights of 145,000 and 140,000 was inhibited by these drugs. Kinetic analysis of reversibility of inhibition of EBV DNA replication after removal of the drugs indicated that BV-araU, like BVdU, has a more prolonged inhibitory effect than ACV. These results indicate that the 2′ OH group in the arabinosyl configuration of BV-araU results in marked reduction in anti-EBV activity while slightly diminishing cytotoxicity.

1-β-D-Arabinofuranosyl-*E*-5(2-bromovinyl)uracil (BV-araU), a congener of *E*-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) (2), has been shown to inhibit herpes simplex virus type 1 and varicella-zoster virus (17–19). The only difference between BV-araU and BVdU is that BV-araU has a 2′ OH group in the arabinosyl configuration (Fig. 1). Although all herpesviruses encode functional viral DNA polymerases, the responses of the different herpesviruses to various nucleoside analogs are unpredictable. As part of our attempts to evaluate structure-activity relations (1, 6), we decided to test and compare the relative efficacies of BV-araU and BVdU in parallel with acyclovir (ACV). The results clearly indicate that BV-araU, although less active against Epstein-Barr virus (EBV) than BVdU, is as potent as ACV and has a comparable in vitro therapeutic index and a more prolonged inhibitory effect than ACV.

MATERIALS AND METHODS

Cell cultures. A highly productive virus-producer cell line, P3HR-1(LS), derived by low-serum cloning (7) and nonproducer (Raji) cells were propagated in RPMI 1640 medium as described previously (10). Cells were maintained in exponential growth (13) by seeding at a density of 4×10^5 to 6×10^5 cells per ml.

Treatment of cells with drugs. Exponentially growing cells were spun down and suspended for 14 days in fresh medium containing different concentrations of drugs (15). At the end of drug treatment, cells were harvested and the number of EBV genome copies per cell was determined. In some experiments, the drug-treated cells were released into drug-free medium and incubated in medium containing 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for an appropriate time, as indicated.

Determination of EBV genome copies per cell. EBV DNA was purified from virus isolated from the supernatant fluids of TPA-induced P3HR-1(LS) cells (10). In vitro synthesis of cRNA and cRNA-DNA hybridization on nitrocellulose filters were carried out as described elsewhere (14).

Determination of ED_{50} for virus replication and ID_{50} for cell growth. Cells were treated with various drug concentrations. During drug treatment, cells were counted daily. The number of cells after 4 days of growth was plotted against drug concentration, and the cell 50% inhibitory dose (ID_{50}) was determined from the plot (15). The number of EBV genome copies per cell after 14 days of drug treatment was plotted against drug concentration, and the virus 50% effective dose (ED_{50}) was determined as detailed previously (15).

Equilibrium density centrifugation. Both superinfected and mock-infected Raji cells were pelleted, and DNA was isolated and processed for analysis in cesium chloride density gradients (13).

In situ cytohybridization with a recombinant EBV DNA probe. Cloned *Bam*HI fragment V DNA, which contains the large internal repeated sequence (IR1) within the EBV genome, was labeled with biotin (Enzo Biochem) by nick translation, according to the protocol of the manufacturer and as described elsewhere (5).

In situ cytohybridization was performed in cell smears on slides as detailed previously (9).

Polyacrylamide gel electrophoresis of EBV-induced polypeptides. Superinfected Raji cells were labeled with [^{35}S]methionine in the presence and absence of drugs (15, 16), and the proteins were analyzed on 7.5% polyacrylamide gels (4).

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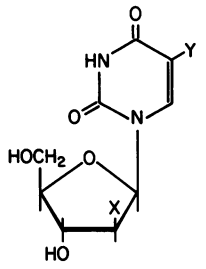

Nucleoside	X	Y
BV-araU	OH	CH=CHBr
BVdU	H	CH=CHBr
BUdR	H	Br

FIG. 1. Structures of BV-araU, BVdU, and BUdR.

RESULTS

Dose-dependent inhibition. Figure 2 shows the dose-dependent effects of BV-araU, BVdU, and ACV on viral genomes in P3HR-1(LS) cells cultured for 14 days in the presence of drugs. EBV genome copies per cell decreased with increasing drug concentrations in all cases but at different rates. Assuming that the residual genome level (30 copies per cell) achieved by an effective drug concentration (100 μM) is zero and that the viral genome level before drug treatment is 100, from the plot we determined that the ED_{50}s for viral inhibition were 0.26, 0.055, and 0.3 μM for BV-araU, BVdU, and ACV, respectively. The same graphical method was used to determine the ID_{50}s for cell growth. On the basis of these data, we calculated the therapeutic index (ID_{50}/ED_{50}). The

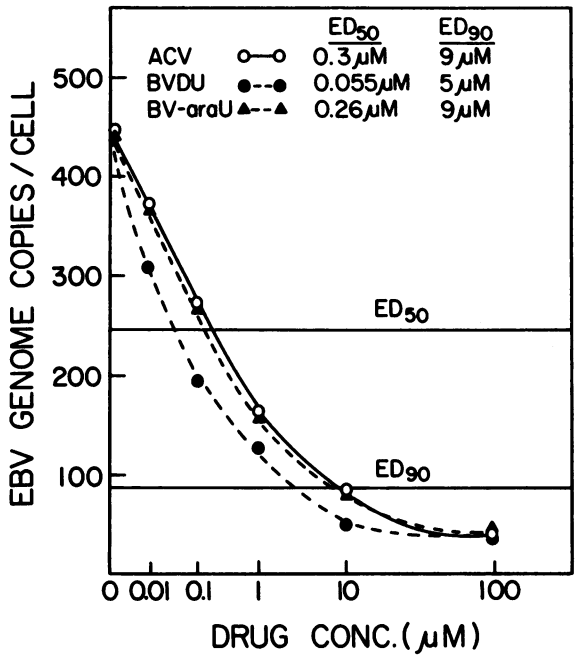

FIG. 2. Dose-dependent inhibition of EBV DNA replication. Exponentially growing P3HR-1(LS) cells were seeded at a density of 10^6 per ml and incubated in various concentrations of drugs for 14 days. EBV genome copy numbers per cell determined at each drug concentration were the average of two determinations.

TABLE 1. Inhibitory action of BV-araU, BVdU, ACV

Drug[a]	ED_{50} (μM)	ID_{50} (μM)	Therapeutic index (ID_{50}/ED_{50})
BV-araU	0.26	390	1,500
BVdU	0.055	360	6,500
ACV	0.3	255	850

[a] The results obtained for BVdU and ACV were not significantly different from those reported previously (15, 16).

results are shown in Table 1. The relative efficacy on the basis of the therapeutic index was BVdU (6,500) > BV-araU (1,500) > ACV (850).

Effects of BV-araU on superinfected Raji cells. To establish that the inhibitory effect of BV-araU on EBV DNA replication is a general phenomenon, we tested the drug in an infection system instead of the virus-producing cell line. Superinfection of Raji cells with P3HR-1(LS) virus results in the shutdown of host functions and stimulation of viral DNA synthesis of both the exogenous virus and the endogenous EBV genomes (11, 15). A large quantity of EBV DNA was synthesized after superinfection of Raji cells (Fig. 3B) compared with the control (Fig. 3A), as measured by the radioactivity incorporated into DNA banding at 1.718 g/cm^3, the density of EBV DNA. In contrast, viral DNA replication was greatly reduced in the presence of 90 μM BV-araU (Fig. 3C). As observed in other studies, cell DNA synthesis is suppressed in superinfected Raji cells even in presence of an antiviral drug (11, 15). This effect is attributed to superinfection, not to an effect of the drug on cell DNA synthesis (6).

Inhibition of EBV DNA replication detected by in situ cytohybridization. Since there is residual viral DNA in the presence of an effective concentration of BV-araU, we performed viral DNA hybridization in situ in both cell systems, i.e., superinfected Raji and P3HR-1(LS) cells. Figure 4 clearly indicates that EBV DNA synthesis was detected in approximately 40% of P3HR-1(LS) cells (Fig. 4A) and in 80% of superinfected Raji cells (Fig. 4D). The sensitivity of hybridization was calibrated so that endogenous EBV episomes in P3HR-1(LS) and Raji cells were below the level of detection. However, viral DNA synthesis was completely inhibited in both cell systems in the presence of BV-araU (Fig. 4B and C), indicating that the residual viral DNA (see Table 2) was not due to a few cells continuing to synthesize new linear EBV DNA.

Persistent effect of BV-araU. We previously reported that the inhibitory action exerted by ACV was completely reversed by 11 days after removal of the drug (15). The same reversibility experiment was performed for BV-araU. Figure 5 indicates that 14 days of drug treatment were needed for BV-araU to reduce the viral genome numbers to the residual level (30 copies per cell). Upon removal of BV-araU, the viral genome copies per cell remained at low levels (30% of the control level) for about 13 days, becoming gradually restored to control levels of untreated cells after 35 days.

To determine whether TPA can override the inhibitory effect of the drug and speed up the reversibility of viral replication after drug removal, we applied TPA to P3HR-1(LS) cells after exposure for 14 days to BV-araU, BVdU, or ACV. Table 2 shows that without antiviral drug treatment, TPA produced a fivefold increase in EBV genome copies (440 to 2,395). In ACV-treated cells, EBV genome numbers increased to 98% of the control levels within 7 days, whereas it took significantly longer to reach the control level in cells treated with either BVdU or BV-araU. The most effective

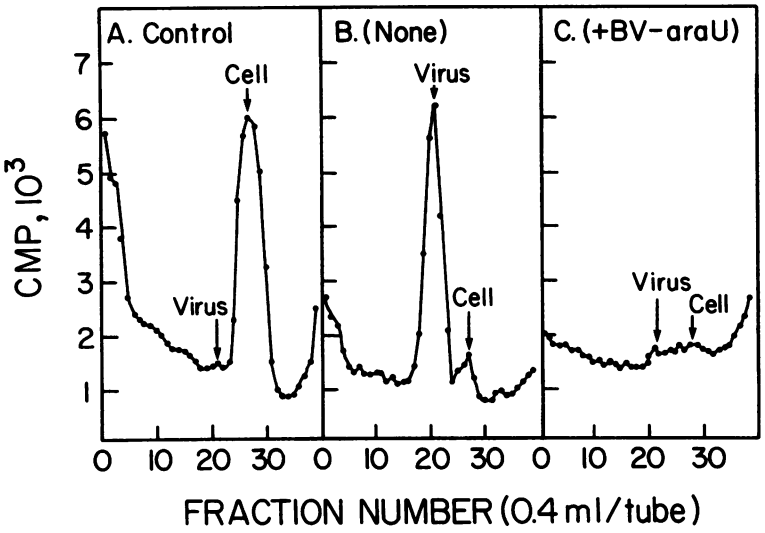

FIG. 3. Inhibition of EBV DNA synthesis by BV-araU in superinfected Raji cells. EBV DNA synthesis was determined by measuring the incorporation of ^{32}P into viral DNA and analyzing the DNA by cesium chloride density gradient centrifugation.

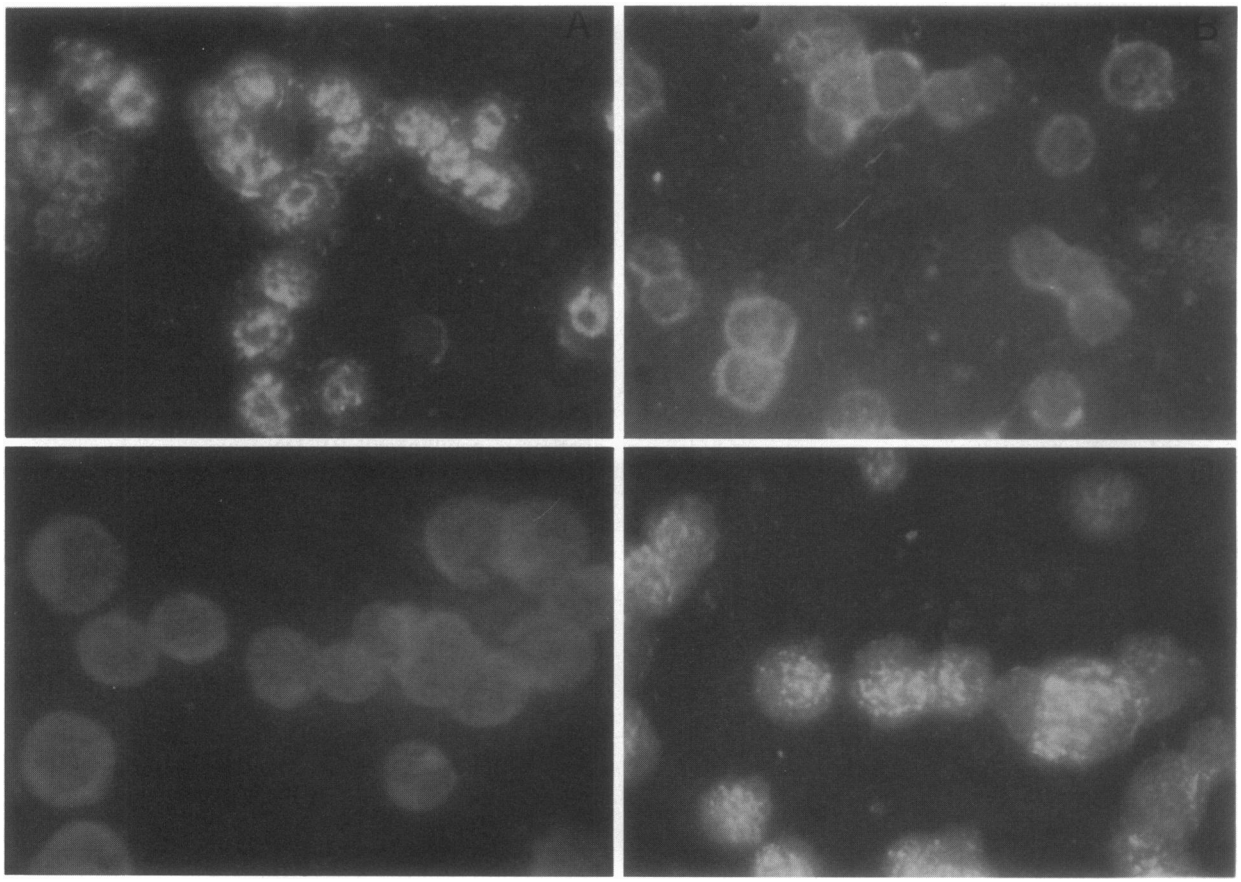

FIG. 4. Inhibition of EBV DNA synthesis detected by in situ cytohybridization. The probe used was cloned *Bam*HI fragment V DNA, which contains the large internal repeated (IR1) sequence. The probe was labeled with biotin by nick translation. The technique clearly distinguishes those cells that are synthesizing viral DNA at levels above the endogenous episomal content. (A) P3HR-1(LS); (B) P3HR-1(LS) in the presence of BV-araU (90 μM); (C) superinfected Raji cells in the presence of BV-araU (90 μM); (D) superinfected Raji cells.

TABLE 2. Effect of TPA on the replication of residual EBV genomes in P3HR-1(LS) cells after treatment with BV-araU, BVdU, and ACV

Drug (concn)	No. of EBV genome copies/cell		% Control
	Drug treatment[a]	TPA induction[b] (day)	
ACV (90 μM)	30	1,452 (4)	60
		2,347 (7)	98
BVdU (50 μM)	30	98 (4)	4
		220 (7)	9
		487 (10)	20
		1,896 (14)	79
BV-araU (90 μM)	30	395 (4)	16
		950 (7)	40
		2,270 (10)	95
		2,289 (14)	96
None	440	2,395 (4)	100

[a] A 10-fold ED_{90} was used for 14 days.
[b] After 14 days of drug treatment, cells were spun down, washed, resuspended in TPA medium, and incubated for various times as specified.

antiviral drug, BVdU, had the most prolonged inhibitory effect even in the presence of TPA.

Inhibition of EBV-associated polypeptides. Figure 6 shows the results of an electropherogram of ^{35}S-labeled polypeptides synthesized in superinfected Raji cells in the presence and absence of drugs. Superinfected Raji cells (lane S), compared with mock-infected cells (lane M), resulted in the synthesis of at least seven new polypeptides with molecular weights of 145,000 (145K), 140K, 135K, 110K, 85K, 55K, and 32K, detected 24 h postinfection in a continuous labeling experiment. In the presence of 90 μM BV-araU (lane B), synthesis of 145K, 140K, 110K, and 85K polypeptides was significantly inhibited. For comparison, the effects of 50 μM BVdU (lane C) and 90 μM ACV (lane A) on polypeptide synthesis are shown in the adjacent lanes. The 135K protein also appears relatively reduced by BV-araU, as do the 145K and 140K proteins with BVdU and ACV. The three drugs had similar effects on EBV-induced polypeptide synthesis, except that the 85K polypeptide was markedly reduced by BV-araU.

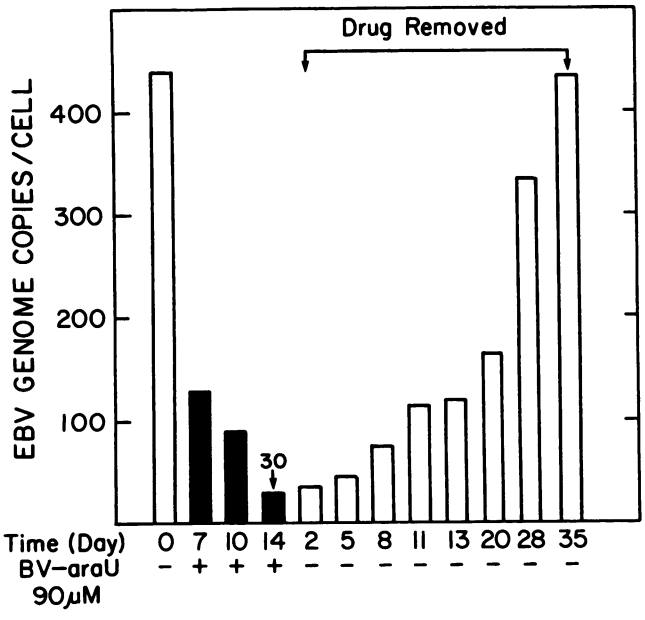

FIG. 5. Kinetics of inhibition and reversibility of EBV DNA replication in P3HR-1(LS) cells treated with BV-araU (90 μM).

DISCUSSION

The present results show that the anti-EBV activity of BV-araU decreases approximately fivefold (in terms of ED_{50}s) upon substitution of 2′ H with an OH group in the arabinosyl configuration (Fig. 1).

It should be noted that the dose-response curve (see Fig. 2) near the ED_{50} is more linear than that near the 90% effective dose. Thus, for comparison of the potencies of different drugs, ED_{50}s are more precise and useful than 90% effective doses.

Despite the loss of activity, the data clearly indicate that BV-araU is more potent than ACV in inhibition of EBV in vitro, as evidenced by the lower ED_{50} and higher therapeutic index. It should be noted that both 5-iodo-2′-deoxyuridine (IUdR) and 5-bromo-2′-deoxyuridine (BUdR), which are structurely related to BVdU and BV-araU (Fig. 1), are

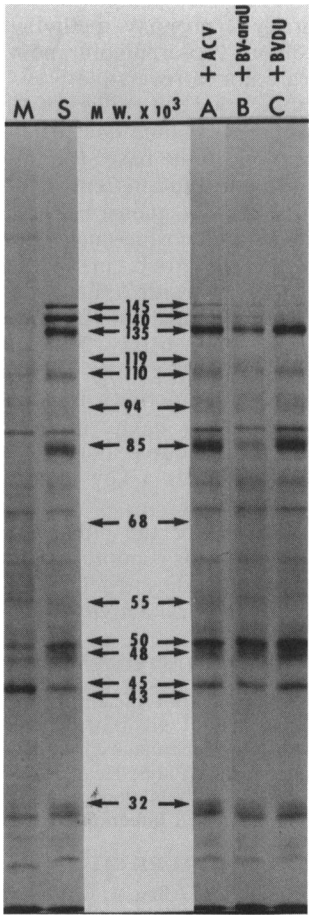

FIG. 6. Differential effects of BV-araU, BVdU, and ACV on the synthesis of EBV-associated polypeptides. Lanes: S, superinfected Raji cells; M, mock-infected Raji cells; A, B, and C, superinfected Raji cells in the presence of ACV, BV-araU, and BVdU, respectively.

potent inducers rather than inhibitors of EBV replication (our unpublished data); treatment of D98/HR-1 cells with IUdR stimulates EBV replication (8).

The anti-EBV activity of BV-araU appeared to be selective in that it inhibited virus replication at drug concentrations which were not cytotoxic or cytostatic in vitro. The effect of BV-araU on EBV-associated polypeptides is consistent with its antiviral activity. As is the case for most antiviral drugs, the effects of these compounds on viral polypeptide synthesis, although a secondary effect of the drugs, could play a role in the inhibition of virus replication (15).

DNA polymerases induced by both types 1 and 2 of herpes simplex virus have integrally associated exonuclease activity (20). Although not yet confirmed by others, there is some indication that EBV-induced DNA polymerase also has exonuclease activity (1a). Exonuclease may play a role in affecting drug action through excision of the terminally incorporated drug moieties, allowing at least temporary recovery of primer-template capability. Under these circumstances, the antiviral activity of a nucleoside analog with a potential of chain termination due to lack of the 3′ OH group, as in the case of ACV (3), would be expected to be more reversible than an analog that incorporates (12) but does not possess the capability of termination, such as BVdU and BV-araU. In fact, we have observed that ACV inhibition of EBV is readily reversible, whereas BVdU and BV-araU, like 9-(1,3-dihydroxy-2-propoxy-methyl)guanine (DHPG) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) (11), are not as reversible.

We have recently carried out studies of structure-activity relations with acyclic pyrimidine nucleosides (1). We have found that modifications in the base structure reduce antiviral activity, whereas substitutions on or in the pyrimidine ring exhibit selective activity against EBV (1). Furthermore, in studies with acyclic adenosine analogs, substitutions of the 2,3-dihydroxypropyl group in (S)-9-(2,3-dihydroxypropyl)adenine [(S)-DHPA] with 2-phosphonylmethyl derivatives such as in 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and in (S)-9-(3-hydroxy- 2-phosphonylmethoxypropyl)adenine [(S)-HPMPA] greatly enhance antiviral activity (6). Similar structure-activity relation studies on 5-substituted deoxyuridines have been reported (21). Conjugation of the C-C double bond of the olefinic 5-substituent was suspected as a key molecular feature affecting antiviral activity (21).

The biochemical basis for the differences in biological activities of these compounds is poorly understood. There is clearly a need for further studies to define fully the molecular features that promote antiviral activity.

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