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Structure-activity relationship between (*E*)-5-(2-bromovinyl)- and 5-vinyl-1-β-D-arabinofuranosyluracil (BV-araU, V-araU) in inhibition of Epstein-Barr virus replication

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Summary

The structure-activity relationship between (*E*)-5-(2-bromovinyl)- and 5-vinyl-1-β-D-arabinofuranosyluracil (BV-araU and V-araU) in inhibition of Epstein-Barr virus (EBV) was evaluated. Both V-araU and BV-araU effectively inhibited EBV replication in virus-producer P3HR-1(LS) cells, as determined by DNA-DNA hybridization. The 50% effective doses (ED_{50}) for viral DNA replication were 0.005 and 0.3 μM for V-araU and BV-araU, respectively. The in vitro therapeutic index was 4000 for V-araU and 1300 for BV-araU. Synthesis of EBV-induced polypeptides with molecular weights of 145 000 (145, 140, 130, and 110 kDa) was significantly inhibited by both drugs. Only V-araU inhibited the synthesis of 85-, 55-, and 32-kDa polypeptides by approx. 50%. Kinetic analysis of inhibition and reversibility of EBV DNA replication after removal of the drugs indicated that BV-araU has a more prolonged inhibitory effect than V-araU. These results indicate that the substitution of H by Br in the 5-vinyl group results in marked reduction in anti-EBV activity while prolonging the drug effect and diminishing cytotoxicity.

Epstein-Barr virus; EBV; BV-araU; V-araU

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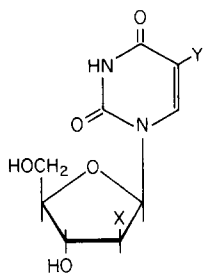
Introduction

(*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BV-araU), a congener of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) (De Clercq et al., 1979), has been shown to inhibit replication of herpes simplex virus type 1 and varicella-zoster virus (VZV) (Machida, 1986; Machida et al., 1981). Recently we have reported comparative studies on the in vitro efficacy of BV-araU and BVdU in inhibition of Epstein-Barr virus (EBV) replication (Lin and Machida, 1988). The only difference between BV-araU and BVdU is that BV-araU has a 2′OH group in the arabinosyl configuration (Fig. 1). This structural change of BV-araU resulted in marked reduction in anti-EBV activity while slightly diminishing cytotoxicity (Lin and Machida, 1988). Recently, we have synthesized several new 5′-substituted araU analogs (Reefschläger et al., 1983); their activities were comparable to that of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) (Watanabe et al., 1979). Although FIAC and FMAU were also potent inhibitors of EBV replication, their low therapeutic indices due to cytotoxicity have hindered their clinical applications for treatment of EBV infection (Lin et al., 1983b). As part of our attempts to evaluate structure-activity relations (Beauchamp et al., 1988; Lin et al., 1987; Lin and Machida, 1988), we decided to test and compare the relative efficacies of BV-araU and V-araU on EBV replication. The results indicate that V-araU, although more cytotoxic than BV-araU, is approx. 60-fold more active against EBV than BV-araU and has a higher therapeutic index, but a less prolonged inhibitory action.



Nucleoside	X	Y
BV-araU	OH	CH=CHBr
BVdU	H	CH=CHBr
V-araU	OH	$CH=CH_2$

Fig. 1. Structure of BV-araU, V-araU and BVdU.

Materials and Methods

Cell cultures

Raji cells (nonvirus-producer) and a highly productive virus-producer cell line, P3HR-1(LS), derived by low-serum cloning (Lin et al., 1986), were propagated in RPMI 1640 medium as described previously (Lin et al., 1983a). Cells were maintained in exponential growth (Lin et al., 1982) 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 (Lin et al., 1984). 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 for different time periods as indicated.

Determination of EBV genome copies per cell

Cellular DNA was isolated and purified from P3HR-1(LS) cells. Cloned *Bam*HI W DNA, which contains the large internal repeated sequence within the EBV genome, was labeled with ^{32}P by nick translation. DNA-DNA hybridization on nitrocellulose filters was carried out (Lin and Raab-Traub, 1987) and EBV genome copy numbers were determined as described (Lin et al., 1984).

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 50% cellular inhibitory dose (ID_{50}) was determined from the plot (Lin et al., 1984). During the four-day assay the untreated control cultures were growing exponentially. 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 described previously (Lin et al., 1984).

Polyacrylamide gel electrophoresis of EBV-induced polypeptides

Superinfected Raji cells were labeled with [^{35}S]methionine in the presence and absence of drugs (Lin et al., 1984) and the proteins were analyzed on 7.5% polyacrylamide gels (Laemmli, 1970).

Results

Dose-dependent inhibition

Fig. 2 shows the dose-dependent effects of BV-araU and V-araU on viral genomes in P3HR-1(LS) cells cultured for 14 days in the presence of the drugs. EBV genome copies per cell decreased with increasing drug concentrations in both cases but at different rates. Assuming that the residual genome level (30 copies per cell) achieved by an effective drug concentration (5 μM for V-araU and 50 μM for BV-araU) is the zero point and that the viral genome level before drug treatment (460 copies/cell) is 100 (Lin et al., 1984), ED_{50} represents the mid-point between 460 and 30. From the plot we determined that the ED_{50}s for viral inhibition were 0.005 and 0.3 μM for V-araU and BV-araU, respectively; the ED_{90}s were 0.45 μM for V-araU and 8 μM for BV-araU. 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 results are shown in Table 1. The relative efficacy on the basis of the therapeutic index was V-araU (4000) > BV-araU (1300). Although V-araU is much more toxic to the cells (ID_{50}: 20 μM) due to its low ED_{50} (0.005 μM) it achieves a therapeutic index comparable to that of BVdU (Lin and Machida, 1988).

The same experiments were performed with Raji cells and similar ID_{50} values as for P3HR-1(LS) cells were observed (data not shown). Concentrations of the drugs effective against viral genome copy numbers in P3HR-1(LS) cells did not affect the EBV DNA content of nonproducer Raji cells (data not shown).

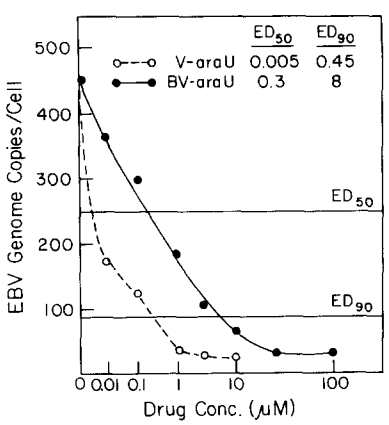


Fig. 2. Dose-dependent inhibition of EBV DNA replication. Exponentially growing P3HR-1(LS) cells were seeded at a density of 10^6 per ml and incubated in various concentrations of drugs for 14 days. EBV genome copy numbers per cell determined at each drug concentration were the average of two determinations.

TABLE 1

Inhibitory action of V-araU, BV-araU and BVdU

Drug	ED_{50} (μM)	ID_{50} (μM)	Therapeutic index (ID_{50}/ED_{50})
BV-araU[a]	0.3	400	1300
V-araU	0.005	20	4000
BVdU[b]	0.055	360	6500

[a]The results obtained for BV-araU were not significantly different from those reported previously (Lin and Machida, 1988).
[b]The data reported previously (Lin and Machida, 1988) are included.

Reversibility of drug effect

We previously reported that the inhibitory action of ACV is completely reversed by 11 days after removal of the drug (Lin et al., 1984). The same reversibility experiments were performed for BV-araU and V-araU. Fig. 3 indicates that 14 days of drug treatment were needed for both BV-araU and V-araU to reduce the viral genome numbers to the residual level [EBV episomes (30 copies per cell)]. Upon removal of BV-araU, the viral genome copies per cell remained at low levels (27% of the control level) for 12 days, becoming gradually restored to 67% of the control level by 26 days. In contrast, the V-araU-treated cells exhibited faster kinetics of reversibility than BV-araU-treated cells. The viral genome numbers were restored to 38% and 61% by 12 and 19 days, respectively. After 26 days of drug removal, the viral genome copies per cell were completely restored to the control level.

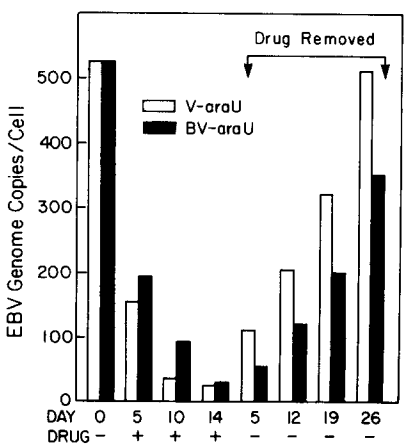


Fig. 3. Kinetics of inhibition and reversibility of EBV DNA replication in P3HR-1(LS) cells treated with 10 times the ED_{90} of BV-araU (80 μM) and V-araU (5 μM).

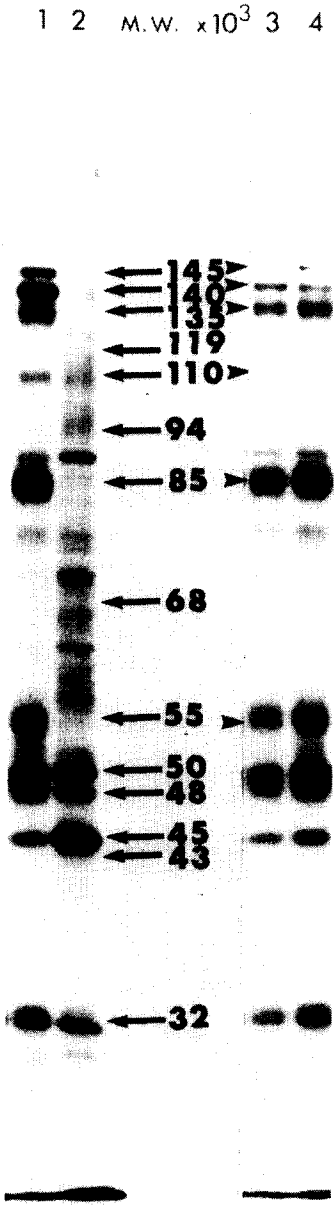


Fig. 4. Differential effects of BV-araU and V-araU on the synthesis of EBV-induced polypeptides. Lane 1, superinfected Raji cells; lane 2, mock-infected Raji cells; lane 3, superinfected Raji cells in the presence of 10 times the ED_{90} of V-araU (5 μM); lane 4, superinfected Raji cells in the presence of 10 times the ED_{90} of BV-araU (80 μM).

Inhibition of EBV-associated polypeptides

Fig. 4 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 1), compared with mock-infected cells (lane 2), resulted in the synthesis of at least seven new polypeptides with relative molecular masses of 145, 140, 135, 110, 83, 55 and 32 kDa, detected 24 h postinfection in a continuous labeling experiment. In the presence of 5 μM V-araU (lane 3) and 80 μM V-araU (lane 4), synthesis of 145-, 140-, 135- and 110-kDa polypeptides was significantly inhibited by both drugs. Furthermore, V-araU reduced the synthesis of 85-, 55- and 32-kDa polypeptides by approx. 50%. It should be noted that the 110-kDa polypeptide has been recently identified as EBV DNA polymerase (Lin et al., 1991).

Discussion

In this study we demonstrated that the anti-EBV activity of BV-araU decreased by approx. 60-fold (in terms of ED_{50}) upon substitution of H with a Br atom in the vinyl group (Fig. 1). BV-araU is much less toxic to the cells than V-araU, as evidenced by the higher ID_{50} value (Table 1). Despite the cytotoxicity of V-araU, the extraordinarily low ED_{50} value, and thus high therapeutic index, makes this drug potentially more useful (Table 1).

The present results are somewhat different from a previous report in which a less sensitive assay (viral antigens detected by immunofluorescence) was used to assess the antiviral activity of BV-araU and V-araU (Färber et al., 1987). Several reasons could account for these differences. First, the P3HR-1 cells used in those experiments had a low level of spontaneous virus production (less than 7% of cells); we used a highly productive cloned cell line P3HR-1(LS) (Lin et al., 1986) in which more than 40% of cells were producing virus. Thus, the decrease in percentage of VCA-positive cells resulting from drug treatment was more difficult and less accurately evaluated in the previous study. In addition, the difference in cytotoxicity results may simply reflect the difference in virus-producing state; the higher the virus-producing rate, the lower the cell viability. Secondly, EBV-positive human serum contains a mixture of antibodies against EA and VCA, although a serum with a high titer of VCA was selected for the other study. It is known that the synthesis of EA is not affected by nucleoside analogues. In the present report we employed a more specific and sensitive nucleic acid hybridization method to quantify precisely even small changes in viral genome copy numbers.

The anti-EBV activity of V-araU and BV-araU appeared to be selective in that they inhibited virus replication at drug concentrations which were not cytotoxic or cytostatic in vitro. It should be noted that both 5-iodo-2'-deoxyuridine (IUdR) and 5-bromo-2'-deoxyuridine (BUdR), which are structurally related to BVdU, BV-araU and V-araU, are potent inducers rather than inhibitors of EBV replication (Lin and Pagano, 1987).

The inhibitory effect of BV-araU and V-araU on the synthesis of the EBV-associated polypeptides which could play a role in virus replication (Lin et al., 1984) is consistent with their antiviral activities. Recently we have identified the 110-kDa polypeptide appearing in superinfected Raji cells as being EBV DNA polymerase (Lin et al., 1991). Fig. 4 clearly shows that synthesis of the 110-kDa polypeptide is more affected by V-araU than by BV-araU. This finding may account for the lower ED_{50} of V-araU since DNA polymerase is essential for viral replication.

Exonuclease activity is associated with DNA polymerases induced by herpesviruses (Clough, 1979; Ostrander and Cheng, 1980). Excision of the terminally incorporated drug moieties by exonuclease may affect drug potency. In this connection, the antiviral activity of a nucleoside analogue with a potential for chain termination due to a lack of the 3′OH group, as in the case of ACV (Elion, 1983), would be expected to be more reversible than an analogue that incorporates (Lin et al., 1985b) but does not possess the capability of termination, such as BVdU. In fact, we have observed that ACV inhibition of EBV is readily reversible (Lin et al., 1984), whereas the inhibitory effects of BVdU and BV-araU, like that of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), FIAC and FMAU (Lin et al., 1983b), are not as reversible. In the present study we observed that the inhibitory effect of BV-araU on EBV replication is less reversible than that of V-araU. The molecular basis for this difference is not known. It is speculated that the Br atom in BV-araU, once incorporated into the growing DNA chain, may render the drug moiety less accessible to exonuclease due to steric hindrance. This may account for its more prolonged inhibitory action as compared to V-araU.

Recently we have focused our studies on the structure-activity relationship of acyclic pyrimidine nucleosides (Beauchamp et al., 1988; Lin and Machida, 1988) and nucleosides with either modifications or substitutions in the base (Lin and Machida, 1988). We have found that modifications in the base structure reduce antiviral activity, whereas substitutions in the pyrimidine ring allow selective activity against EBV (Beauchamp et al., 1988). Furthermore, introduction of a phosphonylmethyl group into the 2,3-dihydroxypropyl side chain of (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], thus leading to the formation of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], greatly enhance antiviral activity (Lin et al., 1987). Similar structure-activity relationship studies with 5-substituted deoxyuridines have been reported (Lin and Machida, 1988; Sim et al., 1983).

Conjugation of the C-C double bond of the olefinic 5-substituent was suspected as being a key molecular feature affecting antiviral activity (Lin and Machida, 1988; Sim et al., 1983). The biochemical basis for the differences in biological activities of these compounds is poorly understood. There clearly is a need for further studies to fully determine the molecular features that promote antiviral activity in general and anti-EBV activity in particular.

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