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EFFECT OF (*E*)-5-(2-BROMOVINYL)- AND 5-VINYL-1-β-D--ARABINOFURANOSYLURACIL ON EPSTEIN-BARR VIRUS ANTIGEN EXPRESSION IN P3HR-1 CELLS: COMPARISON WITH ACYCLOVIR

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Summary. — The effect of (*E*)-5-(2-bromovinyl)- and 5-vinyl-1--β-D-arabinofuranosyluracil (BrVaraU, VaraU) in comparison to 9-(2-hydroxyethoxymethyl)guanine (ACV) on the proliferation of human lymphoblastoid P3HR-1 cells in culture and on the expression of Epstein-Barr virus capsid antigen (VCA) in the same cells was evaluated. After 7 days of cell growth, at 100 μmol/l the total number of new generations in drug-treated cultures was similar or 5 and 10 % below that in drug-free control cultures, for VaraU, ACV, and BrVaraU, respectively. During the same time the percentage of VCA-expressing cells decreased from 6.3 % in drug-free cultures to 1.3, 1.5, and 2.0 % in cultures treated with VaraU, ACV and BrVaraU, respectively. In VaraU-treated cultures a further decrease in the percentage of VCA-positive cells down to 0.5 % was revealed 7 days after drug removal. VaraU was also effective in reducing the proportion of VCA-expressing cells at 10 and 1 μmol/l. At 14 days after drug removal, the inhibitory effect of ACV was nearly reversed, whereas BrVaraU showed a prolonged VCA- suppressing effect.

Key words: Epstein-Barr virus antigen expression; lymphoblastoid P3HR-1 cells; (E)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil; 5-vinyl-1-β-D-arabinofuranosyluracil; anti-herpesvirus nucleoside analogues

Introduction

During the last years considerable efforts have been made in the search for drugs which selectively inhibit the replication of Epstein-Barr virus (EBV). Acyclovir (ACV, 9-(2-hydroxyethoxymethyl)guanine) is one of a

group of nucleoside analogues recently shown to be effective in inhibiting EBV replication at drug dosage levels that are not toxic to host cells (Colby *et al.*, 1980). In contrast to the ready reversibility of EBV inhibition by ACV, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BrVUdR), 1-(2-deoxy-2-fluoro--β-D-arabinofuranosyl)-5-iodocytosine (FIAC), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU), and 9-(1, 3-dihydroxy-2-propoxymethyl)guanine (DHPG) were reported to have a more prolonged inhibitory effect on EBV genome replication in virus-producing cells upon removal of drugs (Lin *et al.*, 1983, 1984). However, all these compounds were without influence on the replication of viral DNA in latently infected nonproducer cells (Colby *et al.*, 1980; Lin *et al.*, 1983, 1984, 1985).

The two new nucleoside analogues, (*E*)-5-(2-bromovinyl)- and 5-vinyl-1--β-D-arabinofuranosyluracil (BrVaraU, VaraU), were proved to be potent and selective inhibitors of some members of the herpesvirus group. BrVaraU inhibits herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) in vitro (Machida *et al.*, 1981, 1982; Reefschläger *et al.*, 1983) and in vivo (Machida and Sakata, 1984; Reefschläger *et al.*, 1986*a*; Soike *et al.*, 1984), whereas VaraU was shown to be effective in vitro against HSV-1, HSV type 2 (HSV-2), and VZV (Machida *et al.*, 1981, 1982; Reefschläger *et al.*, 1983) as well as in vivo against HSV-1 and HSV-2 (Reefschläger *et al.*, 1986, *a*, *b*). This prompted us to test whether these compounds are also active against EBV. In this study we report the effect of BrVaraU and Varall in comparison to ACV on the proliferation of the lymphoblastoid P3HR-1 cells in culture and on the expression of EB viral capsid antigen (VCA) in these spontaneously EBV-producing cells.

Materials and Methods

For testing the drug effects on EBV, P3HR-1 cells cultivated until they reached a density of $1-2\times 10^6$ cells per ml were seeded at a density of 2×10^5 cells per ml in tissue culture flasks in Eagle's MEM supplemented with 20 % newborn calf serum. ACV and BrVaraU were added at concentrations of 100 μmol/l. VaraU was tested at 100, 10, and 1 μmol/l, respectively. The cultures were incubated at 37 °C. During drug treatment, cells were counted on day 2, 3, 4, and 6. On day 7, 14, and 21, drug-treated and control cells were counted, collected by centrifugation and resuspended in the same volume of drug-free medium. Cultures treated with VaraU were seeded into drug-free medium on day 28 and incubated for an additional 7 days. Cell viability was determined by the trypan blue exclusion method.

For detection of viral antigens, samples were taken every 7th day and prepared as follows: The cells were spun down and washed with buffered saline. The pellets were resuspended at a concentration of 3×10^6 cells per ml in saline. Drops of cell suspension were placed on slides, dried and fixed in acetone at −20 °C for 30 min.

VCA was detected by indirect immunofluorescence using human sera with high titres to VCA ($\geqq 1:2500$) and negative for antibodies to early antigens. The immunofluorescence test was carried out according to the method of Henle and Henle (1973). The nuclear antigen of EBV (EBNA) was detected by the method of Reedman and Klein (1966) using human anti--EBNA-positive and -negative sera. The number of antigen-expressing cells was evaluated by counting the cells with bright fluorescence out of a total of 2000 cells under the fluorescence microscope (FLUOVAL, VEB Carl Zeiss Jena).

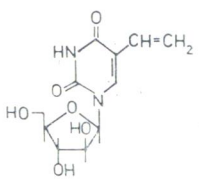
BrVaraU and VaraU (Fig. 1) were synthesized as previously described (Reefschläger *et al.*, 1983). ACV was kindly provided by Burroughs Wellcome Co. (Research Triangle Park, NC, U.S.A.).

Results

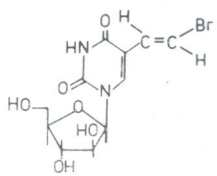
After 7 days of cell growth at 100 μmol/l drug concentrations VaraU had no inhibitory effect on P3HR-1 cell multiplication, whereas the total cell counts of ACV- and BrVaraU-treated cultures were about 5 and 10 %, respectively, below those in drug-free control cultures (data not shown). During the 7-day period the viability of cells was maintained between 90 and 93 % in all drug-treated and control cultures.

The effect of ACV, BrVaraU, and VaraU on VCA expression is shown in Table 1. As revealed by immunofluorescence, in contrast to the 6.0 to 6.7 % VCA-positive cells in drug-free control cultures, the percentage of VCA-expressing cells was reduced under drug treatment. After 7 days, the cultures treated with ACV or BrVaraU exhibited a decrease of the percentage of VCA-expressing cells to 1.5 and 2 %, respectively. By 14 days after drug removal, the inhibitory effect of ACV was nearly reversed. In BrVaraU-treated cultures drug removal resulted in an increase of VCA-expressing cells up to 3.0 %. In cultures treated with 100, 10, and 1 μmol/l VaraU for 7 days, the percentages of VCA-positive cells were 1.3, 1.7, and 3.7 %, respectively (Table 1). 7 days after VaraU removal a further decrease of VCA-expressing cells was revealed. Extending the incubation period in drug-free medium for 28—35 days, the percentages of VCA-positive cells reached approximately the value of the control cultures.

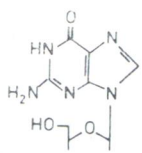
In drug-treated cultures there was no difference in the expression of EBNA in P3HR-1 cells in comparison to untreated controls.



1-ß-D-Arabinofuranosyl-5-vinyluracil
Vinyl-ara U, Vara U



1-ß-D-Arabinofuranosyl-(E)-5-(2-bromovinyl)uracil
Bromovinyl-ara U, Br Vara U



9-(2-Hydroxyethoxymethyl) guanine
Acyclovir, ACV, Zovirax®

Fig. 1
Structural formulae of anti-herpesviral nucleoside analogues.

Table 1. Inhibitory effects of ACV, BrVaraU, and VaraU on the expression of Epstein-Barr virus capsid antigen (VCA) in P3HR-1 cells

Drug	Concentration (μmol/l)	VCA — expressing cells (%) Under drug treatment at day 7	After drug removal at day 7	14	21	28	35
ACV	100	1.5	3.8	6.4	n.d.	n.d	n.d.
BrVaraU	100	2.0	2.3	3.0	n.d.	n.d.	n.d.
VaraU	100	1.3	0.5	1.0	2.9	4.4	5.8
	10	1.7	1.0	1.8	3.5	4.1	5.9
	1	3.7	3.4	3.8	5.3	5.9	5.9
Control		6.3	6.1	6.7	6.5	6.3	6.0

The percentages are mean values from three separate determinations
n.d. — not done

Discussion

Since VCA are late antigens and, therefore, their transcription and translation depends on viral DNA synthesis, the influence of antiviral drugs on EBV replication can be evaluated by determination of VCA-positive cells in EBV-producing cell lines. This simple method is useful for obtaining first information on the efficacy of new drugs.

In our experiments, VaraU was superior to the other drugs tested in suppressing VCA expression by EBV-producing cells. In contrast to the readily reversed inhibitory action of ACV after drug removal, the prolonged suppression of VCA formation by VaraU and BrVaraU appears to be comparable with the anti-EB viral activity of DHPG, BrVUdR, FIAC, and FMAU (Lin *et al.*, 1983, 1984).

Just like these drugs, ACV, BrVaraU, and VaraU are without effect on EBNA expression in the fraction of nonpermissively infected P3HR-1 cells, which is a function of the plasmid form of the EBV genome. Approximately 30 % of this covalentely closed circular EBV DNA in P3HR-1 cells is resistant to inhibitors of the viral DNA polymerase activity (Shaw, 1985) and presumably replicated by cellular DNA polymerases like the EBV DNA in nonproducer cell lines (Henry *et al.*, 1983). These cellular enzymes are much less sensitive to inhibition by the triphosphates of several pyrimidine nucleoside analogues including BrVaraU (Ruth and Cheng, 1981). Therefore, the inhibition of VCA expression in the human lymphoblastoid cell line P3HR-1 by BrVaraU and VaraU (shown in Table 1) appears not to be a consequence of inhibition of host cell functions. Furthermore, the proliferation of P3HR-1 cells is nearly unaffected by 100 μmol/l of the analogues, and concentrations as high as 1 mmol/l do not inhibit the growth of human embryonic lung fibroblasts by 50 % (Reefschläger *et al.*, 1986*a*).

In order to get more precise information on the activity of VaraU and BrVaraU against EBV, further investigations including the determination of the EBV genome copy numbers in permissively infected human lymphoblastoid cell lines are indispensable.

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