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Treatment of experimental herpes simplex virus type 1 encephalitis in mice with (*E*)-5-(2-bromovinyl)- and 5-vinyl-1-β-D-arabinofuranosyluracil: comparison with bromovinyldeoxyuridine and acyclovir

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Summary

The efficiency of (*E*)-5-(2-bromovinyl)- and 5-vinyl-1-β-D-arabinofuranosyluracil (BrVaraU, VaraU) as inhibitors of three herpes simplex virus type 1 (HSV-1) strains was assessed in comparison to (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BrVUdR), 9-(2-hydroxyethoxymethyl)guanine (ACV), and trisodium phosphonoformate (Na_3PFA) using a plaque assay in human embryonic lung fibroblast (HELF) cell cultures. The following order of decreasing activity was found: BrVaraU>VaraU>BrVUdR>ACV>>Na_3PFA. In HELF cell cultures, the selectivity indexes of VaraU and BrVaraU were 10 times higher than those of BrVUdR and ACV. Protection of mice from encephalitis and death due to intracerebral (i.c.) infection with a clinical HSV-1 isolate was nearly complete if mice were treated intraperitoneally (i.p.) with two daily doses of VaraU and BrVaraU (100 or 200 mg/kg per day) over a period of 5 or 10 days. The efficacy was similar to ACV, but, using a treatment schedule of three daily i.p. doses over 10 days, with equimolar amounts of the nucleoside analogs, VaraU and BrVaraU (140 and 180 mg/kg per day) were superior to ACV (130 mg/kg per day) ($P<0.05$).

(*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil; 5-vinyl-1-β-D-arabinofuranosyluracil; bromovinyldeoxyuridine; acyclovir; herpes simplex virus strains; HSV-1 encephalitis in mice

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Introduction

The discovery of the selective antiviral action of 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV, Zovirax®) [14,37] and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (bromovinyldeoxyuridine, BrVUdR) [9,31] against herpes simplex virus (HSV) and varicella-zoster virus (VZV) hallmarked the beginning of a new era in anti-herpes chemotherapy, the opportunity of systemic treatment of life-threatening herpesvirus infections. The demonstration of in vivo activity of ACV [28] and BrVUdR [13,30,32] against experimental HSV type 1 (HSV-1) encephalitis in mice has been followed by initial clinical trials of BrVUdR for local and generalized severe VZV infections of tumor patients ([7,10] and P. Wutzler et al., unpublished data), and in the mean time ACV has been licensed for clinical use for topical and systemic treatment of HSV infections.

While several acyclic BrVUdR analogs [36] as well as 5-(2-X-vinyl)-UdR derivatives [3,11,19,34,35] did not have improved properties compared with BrVUdR, the search for selective antiherpetics among the 5-substituted 1-β-D-arabinofuranosyluracil (araU) nucleoside analogs was successful, yielding two highly active compounds: (*E*)-5-(2-bromovinyl)-araU (BrVaraU) [2,12,23,33] and 5-vinyl-araU (VaraU) [23,33]. Compared with BrVUdR, both BrVaraU and VaraU are at least as specific as inhibitors of HSV-1 in human diploid fibroblasts, BrVaraU having a stronger effect against VZV [24,38] while VaraU is more active against HSV-2 [23,33]. It should also be recognized that the antiviral activity of BrVaraU is strongly cell-dependent, with virtually no activity expressed in simian cell lines [5,33].

We have now examined the effects of BrVaraU and VaraU, in comparison with ACV, BrVUdR (Fig. 1) and trisodium phosphonoformate (Na_3PFA), on plaque formation of 3 HSV-1 strains in human embryonic lung fibroblast (HELF) cultures and on the proliferation of HELF cells. Furthermore, the in vivo activity of these compounds (except Na_3PFA) against experimental HSV-1 encephalitis in mice was determined. Some results on BrVaraU in a similar animal model were published by Machida et al. [26] and later compared to BrVUdR [27], but no attempts were made to define the optimal treatment regimen(s).

Materials and Methods

Compounds

BrVaraU, BrVUdR, and VaraU were prepared as described previously [31,33]. ACV was a gift from Burroughs Wellcome Company (Research Triangle Park, NC, U.S.A.) and Na_3PFA was kindly supplied by Dr. Bertram, VEB Arzneimittelwerk (Leipzig, G.D.R.). For plaque assays and cell growth inhibition studies 20 mM stocks and dilutions were prepared in phosphate-buffered saline (PBS) and stored at -20°C. For animal experiments fresh solutions of all compounds (at 5 mg/ml, 10 mg/ml (not for ACV) or 0.02 M) in Hank's balanced salt solution were made separately for each experiment.

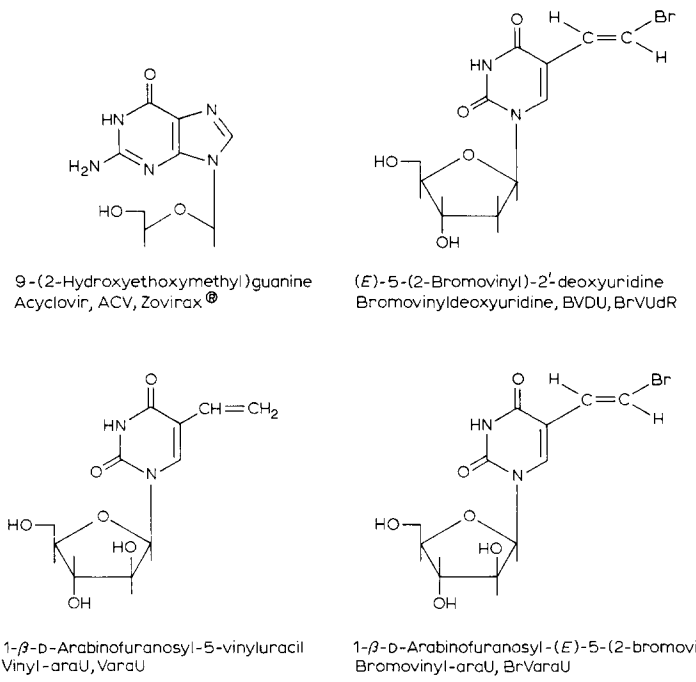

Fig. 1. Structural formulae of anti-herpes nucleoside analogs.

Viruses

The two recent clinical isolates HSV-1 strain 64 (source: muco-cutaneous herpes) and HSV-1 strain 511 (source: herpetic encephalitis) as well as the laboratory strain HSV-1-Kupka (source: herpetic keratitis) were differentiated according to their origin, by quantitative microneutralization with type-specific antiherpes serum from rabbits, and by plaque-forming ability in primary chick embryo cell cultures. They were further characterized biologically by demonstrating high neurovirulence and liver necrosis in mice after intracerebral (i.c.) or intravenous (i.v.) infection, respectively. After isolation the viruses were propagated in HELF or in primary rabbit testes (PRT) cells yielding titers of 2–5 $\times$ 10^7 pfu/ml. Aliquots of virus batches were stored in liquid nitrogen until use for cell culture or animal experiments.

Cytotoxicity assay

Human embryonic lung fibroblast (HELF) cell cultures prepared from 3–4-month-old embryos were cultured at 37°C in monolayers with Eagle's minimal essential medium (EMEM; Institut für Immunpräparate und Nährmedien, Berlin, G.D.R.) and Earle's salts supplemented with 10% fetal calf serum (Gibco Bio-Cult Laboratory, Glasgow, Scotland) (EMEM/10). Neomycin (50 µg/ml) (Spofa, Prague, Czechoslovakia) was used as an antibiotic. For growth inhibition studies, 4–6 $\times$ 10^5 cells in 5 ml EMEM/10 were seeded into 50-ml culture bottles, and allowed to grow in the presence

of varying concentrations of the test compounds. After 72 h the medium was removed, cell sheets were treated with trypsin/versene and the cell number in the collected suspensions was determined in a Fuchs-Rosenthal chamber using the trypan blue-exclusion method. The inhibition data were plotted as dose-effect curves (not shown) from which the 50% inhibitory doses (ID_{50}) were obtained. The ID_{50} values are the means of three cytotoxicity assays with three concentrations within the inhibitory range of the compounds (duplicate cultures and duplicate cell counts). The cytostatic effect of a drug (ID_{50}) is expressed as the concentration of a compound that reduced the cell number in a treated culture by 50% as compared to the number of cells in an untreated control culture.

Plaque inhibition assay

Nearly confluent HELF monolayers in 50-ml culture bottles were used 48–72 h after seeding. Cells were infected with 0.2 ml of a virus suspension yielding 50–100 plaques per bottle. After a 1-h adsorption period 3.6 ml of a methylcellulose (0.5% w/v) overlay medium containing 5% calf serum and 0.2 ml of a solution containing varying amounts of the test compounds were added to each culture. After a 72-h incubation period at 34°C the cell cultures were stained with crystal violet dye for 1 h at 34°C, fixed with methanal/methanol after removing the overlay medium, and plaques were counted microscopically. The resulting plaque counts were expressed as percentages of the counts obtained for untreated virus control cultures and the data were plotted on a decimal scale against the logarithm of the concentration. The concentrations of the compounds inhibiting plaque formation by 50% (ID_{50}) compared to untreated virus control cultures were obtained from the dose–response curves. The ID_{50} values are the means of three plaque inhibition assays performed with three concentrations each within the inhibitory range of the compounds and with triplicate cultures.

Experimental herpes encephalitis in mice

Groups of SPF-F1 grey female hybrids (ABD2), weighing 20–23 g (Central Institute of Microbiology and Experimental Therapy, Jena, G.D.R.), were infected i.c. with 0.9×10^4 pfu of HSV-1 strain 64 in 0.1 ml of PBS (Exp. 1 and 2) or with 1.8×10^4 pfu of HSV-1 strain Kupka (Exp. 3). For i.c. infection mice were anaesthesized with diethyl ether and disinfected at the site of injection, which was the center of an imaginative line connecting the anterior parts of the ears. Beginning 2 h post-infection groups of 10 mice were treated intraperitoneally (i.p.) twice (at 8 a.m. and 4 p.m.; Exp. 1 and 2) or three times per day (every 8 h; Exp. 3) with 0.2 ml of the drug solutions for 5 days (Exp. 1 and 2) or for 10 days (Exp. 3). Because of the limited supply of ACV, this compound was excluded from Exp. 2. Control mice were treated with 0.2 ml of Hank's salt solution. The concentrations of the compounds were 5 or 10 mg per ml corresponding to 100 or 200 mg/kg per day in experiments 1 and 2, respectively. In the third experiment equimolar amounts (0.02 M) of BrVaraU, VaraU, BrVUdR, and ACV were used, yielding 180, 140, 170, and 130 mg/kg per day, corresponding to daily doses of 4.2, 3.2, 4.0, and 3.0 mg per mouse, respectively. The animals were examined twice daily and typical signs of encephalitis were taken to be: rotation phenomenon, tremor, apathy, and pareses of the extremities. The time of death post-infection (p.i.) was

recorded in comparison with non-treated control groups. The mean survival times of the mice that died were estimated. Animals were considered to be cured if they remained without discernible symptoms up to 28 days p.i.

Demonstration of antibody

From all surviving infected mice blood was collected by heart puncture after 28 days p.i. for antibody assay by the fluorescence antibody technique (FAT) [45]. HSV-1-infected human struma cells fixed on slides for 1 h at -20°C served as antigen. They were incubated with the 1 : 10 diluted sera for 1 h at room temperature, rinsed twice with PBS (pH 7.3) for 5 min and visualized with fluoresceinisothiocyanate (FITC)-labeled goat anti-mouse globulin (Staatliches Institut für Immunpräparate und Nährmedien, Berlin, G.D.R.) diluted 1 : 6 in PBS containing 2% Tween 80 and 0.1 g/l Evans blue. Following a 1-h incubation period at room temperature the slides were again rinsed twice with PBS and inspected under the fluorescence microscope using a HBO 200 bulb with the filter combination BG 12 and OG 1. Mice sera with known antibody titers were run as positive, and sera of uninfected animals as negative controls. Typical nuclear or cytoplasmic fluorescence was judged as positive.

Statistical analyses

Differences in the efficacy of the nucleoside analogs were evaluated with the Gehan test on the basis of differences in survival times of mice between the various treated groups, and between treated groups and the control group. All mice which survived by the end of the test (day 28) were assigned a survival time of 29 days. Statistical evaluation of differences between the mean survival times of the mice that died in the treated and control groups was done by Student's *t*-test.

Results

Anti-HSV-1 and anti-HELF cell growth activity, selectivity index

The antiviral activity of BrVaraU and VaraU against the laboratory strain HSV-1-K and the two recent clinical isolates HSV-1 strain 64 and HSV-1 strain 511 was evaluated in comparison to BrVUdR, ACV, and Na_3PFA in a plaque inhibition assay in HELF cell cultures. The following order of decreasing potency (on a molar basis) was observed: BrVaraU > VaraU > BrVUdR > ACV >> Na_3PFA, with only one exception: HSV-1 strain 511 was nearly equally sensitive towards VaraU and BrVUdR (Table 1). The new analogs BrVaraU and VaraU were 2–9 times more potent than ACV and up to 5000 times more potent than Na_3PFA. An inhibitory effect on HELF cell proliferation (ID_{50}) was achieved by the four nucleoside analogs only at concentrations 10^3–10^4 times higher than those required for inhibition of virus plaque formation. The order of increasing cytostatic activity was: VaraU = BrVaraU < ACV < Na_3-PFA < BrVUdR (Table 2). The arabinosyl nucleosides were less cytostatic than the known antiherpes compounds, resulting in a 10 times higher selectivity of antiviral action than noted for ACV and BrVUdR (Table 2). A very low index of selectivity (ID_{50} for HELF cells divided by ID_{50} for HSV-1) was found for Na_3PFA ($I = 3$), whereas 10^4 times higher indexes were estimated for BrVaraU and VaraU.

TABLE 1

Inhibition of HSV-1 plaque formation in HELF cells by different anti-herpes compounds

Compound	ID_{50} (μM)[a]			Mean ID_{50} ± S.D. for all HSV-1 strains
	HSV-1-64	HSV-1-511	HSV-1-K	
BrVaraU	0.020	0.024	0.024	0.023 ± 0.002
VaraU	0.042	0.064	0.061	0.056 ± 0.012
BrVUdR	0.082	0.069	0.090	0.080 ± 0.011
ACV	0.187	0.120	0.139	0.149 ± 0.035
Na_3PFA	98	78	103	93 ± 13

[a] ID_{50} = concentration required to inhibit plaque formation by 50%.

TABLE 2

Inhibition of HELF cell proliferation by different anti-herpes compounds

Compound	ID_{50} (μM)[a] (HELF cells)	Selectivity index (I) = $\frac{ID_{50}\text{ (HELF cells)}}{ID_{50}\text{ (HSV-1)}^b}$
BrVaraU	>1000	>43478
VaraU	>1000	>17857
BrVUdR	170	2125
ACV	360	2416
Na_3PFA	280	3

[a] ID_{50} = concentration required to reduce cell proliferation by 50%.
[b] Data from Table 1.

Therapeutic efficacy of different nucleoside analogs on HSV-1 encephalitis in mice

Initially, the efficacy of BrVaraU, VaraU, BrVUdR and ACV at a dose of 100 mg per kg per day against experimental encephalitis induced by i.c. infection with HSV-1 strain 64 was evaluated. As shown in Table 3, two daily i.p. doses of 50 mg per kg of either compound continuing for 5 days were effective in increasing the final survival rates of the infected mice compared with the untreated control group where all mice died between the 3rd and the 8th day (mean: 5.4 days). Whereas the survival rates were 80, 70, 60, and 50% for ACV, VaraU, BrVaraU, and BrVUdR, respectively, prolonged mean survival times of the mice that died were found only for BrVaraU ($P>0.05$) and VaraU ($P<0.05$), but not for BrVUdR and ACV. Using a higher dose of 200 mg per kg per day in a second experiment the final survival rates and the mean survival times were not considerably increased, as compared with the dose of 100 mg/kg per day (Table 3).

Because of the differences in the molecular weights of the four investigated nucleoside analogs the real potentials of the compounds in treating herpes encephalitis in mice were compared in a final experiment in which equimolar amounts of the drugs were administered (Table 4). Whereas all untreated control mice infected with a high

TABLE 3

Treatment of experimental HSV-1 encephalitis in mice with different nucleoside analogs

Treatment group/ dose (mg/kg per day)	Survivors/total	Mean survival time of mice that died (days ± S.D.)	Time interval of death (days)
Hank's/Control	0/15	5.4 ± 1.68	3–8
BrVaraU/100	6/10	7.3 ± 1.53	6–9
200	7/10	8.5 ± 1.32[a]	7.5–10
VaraU/100	7/10	8.7 ± 1.53[a]	7–10
200	7/10	8.5 ± 0.71[a]	8–9
BrVUdR/100	5/10	6.0 ± 1.00	5–7
200	6/10	7.3 ± 1.32	6–9
ACV/100	8/10	4.0 ± 2.83	2–6

Mice were infected i.c. with 0.9×10^4 pfu of HSV-1 strain 64 and treated i.p. twice daily for 5 days.

[a] Significantly different from the Hank's-treated mice (with $P<0.01$ and <0.05 for BrVaraU and VaraU, respectively; Student's t-test).

TABLE 4

Treatment of experimental HSV-1 encephalitis in mice with different nucleoside analogs in equimolar amounts

Treatment group/ dose (mg/kg per day)	Survivors/total	Mean survival time of mice that died (days ± S.D.)	Time interval of death (days)
Hank's/Control	0/15	4.0 ± 1.38	2.5–6.5
BrVaraU/180	9/10	18.0	-
VaraU/140	9/10	9.0	-
BrVUdR/170	7/10	5.5 ± 2.60	2.5–7
ACV/130	5/10	8.5 ± 1.70[a]	6–10.5

Mice were infected i.c. with 1.8×10^4 pfu of HSV-1 strain Kupka and treated i.p. three times per day for 10 days.

[a] Significantly different from the Hank's-treated mice (with $P<0.001$; Student's t-test).

dose of HSV-1 strain Kupka died between the 3rd and the 7th day (mean: 4.0 days), death was prevented in 90% of the mice after treatment with three daily doses (every 8 h) of BrVaraU and VaraU for 10 days. When evaluated under the same conditions, BrVUdR and ACV showed final survival rates of 70 and 50%, respectively. The specific antibody response against HSV measured in surviving infected mice by means of FAT proved positive in all treated animals (data not shown).

Discussion

The chemotherapy of life-threatening human herpes simplex encephalitis and of neonatal herpes infections, which is usually also associated with encephalitis, presents an unsolved problem. Although the infusion of 9-β-D-arabinofuranosyladenine (vidarabine, adenine arabinoside, Ara-A) or its 5'-monophosphate [41,42,44] has proven beneficial, the disadvantages of low solubility (high infusion volume), low antiviral activity and low selectivity cannot be surmounted. The therapeutic results obtained with 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) [39,43] appear more promising than those noted for vidarabine. Trials of several other antiherpetics [20], which are far superior to Ara-A in vitro, i.e. 5-ethyl-2'-deoxyuridine (EUdR [4]), 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine and -thymine (FIAC and FMAU [18]), ACV [21,28], 1-β-D-arabinofuranosylthymine (Ara-T [22,25]), (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BrVUdR [13,30,32]), 5-(2-chloroethyl)-2'-deoxyuridine (ClEtUdR [8]), and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, BIOLF-62, 2'-NDG [1]), against experimentally induced encephalitis in mice yielded results indicative for their possible efficacy in humans. When injected i.p. twice daily, (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BrVaraU) at doses of 100 or 200 mg/kg per day for 5 days, successfully prevented encephalitis in mice inoculated i.c. with HSV-1 ($P<<0.01$) (ca. 70% survival rates). These results are different from those of Machida et al. [26], who, by applying a similar treatment schedule, did not obtain any survivors in the treated groups. But recently, they reported successful oral treatment with BrVaraU of an infection initiated by a lower virus dose [27]. In addition, we showed that 5-vinyl-araU (VaraU) [23,33] which is highly selective in its antiherpes effect in vitro (Table 2) also protected mice against HSV-1 encephalitis ($P<<0.01$) (Table 3). Three daily i.p. injections (8-h intervals) over 10 days led to nearly complete protection of the mice with both arabinosyl nucleosides, whereas, in the untreated group, all animals died between the 3rd and 7th day (Table 4). It remains to be investigated whether the equimolar doses of BrVaraU and VaraU applied in this schedule can be reduced without affecting the nearly complete protection of the mice. BrVaraU was twice as effective as VaraU and both arabinosyl analogs were clearly more effective (2–9 times) than ACV in vitro (Table 1), but not in vivo (Table 3). At equimolar doses, however, BrVaraU and VaraU were superior to ACV in vivo ($P<0.05$; Table 4). This was confirmed by the results of a study where mice inoculated i.c. with HSV-1 were treated by the oral route with compounds dissolved in the drinking water (J. Reefschläger, P. Wutzler, K.-D. Thiel and G. Herrmann, unpublished data). It seems that relatively constant drug levels such as those attained by oral treatment via the drinking water are more effective than high concentrations spread over too long intervals. Our data (Tables 3 and 4) indicate that BrVaraU and VaraU are at least as effective, if not more effective than BrVUdR, whereas ACV and BrVUdR showed comparable results as in the treatment of orofacial HSV-1 infections of hairless mice [29]. However, BrVUdR was inferior to ACV in the oral treatment, via the drinking water, of an acute ear infection with HSV-1 in mice [15]. In the latter study, the molar concentration of BrVUdR was 2/3 that used for ACV. Recently, Field et al. [17] quantified the inhibition by nucleosides of HSV-1 replication in different

parts of the mouse brain and demonstrated the superiority of DHPG over ACV and BrVUdR, again, however, not at an equimolar base. The presence of specific antibodies against HSV in all sera of surviving mice can be regarded as an indicator for a normal humoral immune reaction of the mice under treatment with the different nucleoside analogs.

Compared with BrVUdR which is weakly active against HSV-2 in vitro and in vivo [6,11,32], BrVaraU is even markedly less active [33], while VaraU not only has an appreciable inhibitory effect on HSV-2 in vitro [23,33] but also in vivo against HSV-2 encephalitis in mice (J. Reefschläger, P. Wutzler, K.-D. Thiel, G. Herrmann, manuscript in preparation). Thus, by virtue of their high anti-herpes activity and selectivity in vitro and their therapeutic efficacy against HSV-1 encephalitis in mice, BrVaraU and VaraU are promising candidates for the treatment of HSV-1 encephalitis in humans. Because BrVUdR-resistant HSV-1 mutants may still be sensitive to ACV [16] and ACV-resistant strains can be inhibited by BIOLF-62 [40], it may be useful to consider drug combinations as a means to suppress the emergence of resistant HSV-1 mutants. BrVaraU and VaraU may be part of such drug combinations.

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