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EFFICACY OF (*E*)-5-(2-BROMOVINYL)-2′-DEOXYURIDINE AGAINST DIFFERENT HERPES SIMPLEX VIRUS STRAINS IN CELL CULTURE AND AGAINST EXPERIMENTAL HERPES ENCEPHALITIS IN MICE*

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(*E*)-5-(2-Bromovinyl-2′-deoxyuridine (BrVUdR) showed strong antiviral activity against different laboratory strains and clinical isolates of herpes simplex virus type 1 (HSV-1) on primary rabbit testes (PRT) cells with a 50% inhibition of plaque formation (ID_{50}) at 0.01–0.02 μM. One laboratory strain (HSV-1-S), however, was completely refractory even at concentrations as high as 100 μM. In contrast, the ID_{50}s for all herpes simplex virus type 2 (HSV-2) strains were about 10^2–10^3 times higher (8–25 μM) than for the HSV-1 strains. No toxicity in mice treated with 140 mg BrVUdR/kg/day for 14 days was observed, and successful treatments of herpes encephalitis in mice induced experimentally by intracerebral infection with one laboratory strain (HSV-1-Kupka) and one clinical isolate (HSV-1-64) were achieved. Treatment of encephalitis in mice induced by the strain HSV-1-S insensitive to BrVUdR in cell culture failed to be effective. Similar antibody titers against HSV-1 were found in surviving mice of the control and of the BrVUdR-treated groups.

(*E*)-5-(2-bromovinyl)-2′-deoxyuridine herpes simplex virus strains primary rabbit testes cells plaque inhibiton assay herpes encephalitis in mice

INTRODUCTION

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BrVUdR) has proved to be a very potent inhibitor of herpes simplex virus type 1 (HSV-1) replication in various mammalian cell lines [7,31] (for a review, see refs. 10, 13). It is equally effective in vitro against clinical isolates of varicella-zoster virus (VZV) at concentrations of 0.003–0.03 μM [14] or 0.03–0.075 μM [2] as well as pseudorabies virus at 0.02–0.2 μM [31], while an inhibition of herpes simplex virus type 2 (HSV-2) is only achieved at more than 100-fold higher concentrations [7,9,31]. The antiherpetic action of BrVUdR is extraordinarily selective, since the proliferation of different cell lines, e.g. primary rabbit kidney cells [7] or

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synchronized baby hamster kidney (BHK 21/C 13/2P) suspension cells [31], is only affected at about 1000-times the antiviral concentration. The slight inhibition of the growth of BHK 21/C 13/2P cells at high BrVUdR concentrations (10–50 μM) is totally reversible after removal of the substance [31]. BrVUdR is also effective in vivo, e.g. in the treatment of herpes virus infections of the rabbit eye [26] and the skin of guinea pigs and athymic nude mice [7,15,17] as well as in simian varicella virus infection of African green monkeys [34]. It is well absorbed when administered orally [8].

We have now examined the in vitro sensitivity of a series of laboratory strains and clinical isolates of HSV-1 and HSV-2 towards BrVUdR in the plaque inhibition assay on primary rabbit testes (PRT) cells and compared these results with the in vivo influence of BrVUdR on herpes encephalitis of the mouse induced by intracerebral infection with three of the HSV-1 strains evaluated in vitro.

MATERIALS AND METHODS

Preparation of primary rabbit testes (PRT) cells

Rabbit testes were removed sterilely and halved after separating the tunica vaginalis and the epididymis. The parenchyma was detached from the inner side of the tunica albuginea and prepared mechanically and enzymatically as described [38]. Following two subcultivations the uniformly fibroblastic PRT cells were seeded into 50 ml rectangular flasks at 2×10^5 cells/ml and, having formed confluent monolayers, used for plaquing. Details of cultivation and media have been previously published [37,41].

Viruses

The origin, typing and some biological properties of the investigated HSV-1 and HSV-2 laboratory strains and clinical isolates are summarized in Table 1. The strains HSV-1-S and HSV-2-S were received from Professor Krech (Bakteriologisches Untersuchungsamt, St. Gallen, Switzerland).

Compound

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BrVUdR) was synthesized as described [31] and dissolved in saline at appropriate concentrations.

Plaque inhibition assay

The modified plaque technique with methylcellulose (0.5% w/v) in the overlay used for testing antiviral activity [35,36] and for differentiation of virus strains has been described [42]. BrVUdR was added to the methylcellulose overlay after a 1 h virus adsorption period.

Mice

Four to five week old SPF-F1 grey female hybrids (ABD2) with an average weight of

13–16 g per animal breeded in the Central Institute of Microbiology and Experimental Therapy (Jena, G.D.R.) were used. Groups of 10 mice per cage received standardized pellet food and water ad libitum.

Experimentally induced encephalitis

Twenty animals were each infected intracerebrally (i.c.) or intraperitoneally (i.p.) with the HSV-1 strains listed in Table 2 at doses of 10^2 and 10^4 $TCID_{50}$ in 0.1 ml. For i.c. infection the mice were narcotized with ether and disinfected at the site of injection, which was the center of an assumed connecting line between the anterior parts of the ears. I.p. application was into the left lower abdomen, intramuscular (i.m.) infection into the left hind leg.

Beginning at the day of infection until day 8 or 11, 10 animals each received 140 mg/kg BrVUdR daily in two single doses of 0.2 ml (2×1 mg/mouse/day) injected i.m. The control group of 10 infected animals was treated with two single doses of 0.2 ml saline daily; the other, non-infected control group of 10 mice was treated analogously with BrVUdR over a period of 14 days (the first dose was injected i.c.; all further treatments were i.m.). The animals were examined twice daily at the time of treatment. 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 the non-treated control group. Animals were considered to be cured if they remained without discernible symptoms up to 28 days p.i. and produced specific antibodies against HSV-1.

Demonstration of antibody

From all surviving infected mice heart blood was withdrawn for antibody assay by the fluorescence antibody technique (FAT) on the 28th day p.i. [43]. HSV-1-infected human struma cells fixed on slides for 1 h at -20°C served as antigen. They were incubated with the geometrically diluted sera for 1 h at room temperature, rinsed twice with phosphate-buffered saline (PBS) (pH 7.3) for 5 min and visualized with FITC-labeled anti-mouse globulin of the goat (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, sera of uninfected animals as negative controls. The typical nuclear or cytoplasmic fluorescence was judged as positive.

TABLE 1

Characterization of herpes simplex virus strains and their sensitivity to BrVUdR

Virus strain	Origin	Differentiation[a]	Biological characteristics (mouse)[b]	No. of passages in vitro[c]	ID_{50} (μM)[d]
HSV-1-Kupka[e]	Keratitis herpetica	NT PF (neg.)	High N., i.c. appl. L neg.	16 (L)	0.01–0.02
HSV-1-Klone 70[e]	Herpes tracheitis	NT PF (neg.)	Low N, i.c. L neg.	9 (L)	0.01–0.02
HSV-1-S[e]	Unknown	NT PF (pos./neg.)	High N, i.c., i.m., i.p. L. pos.	>100 (L)	>100
HSV-1-77[f]	Herpes labialis	PF (neg.)	High N, i.c. L pos.	7 (I)	0.01
HSV-1-64[f]	Herpes integumentalis	PF (neg.)	High N, i.c. L. pos.	5 (I)	0.01–0.02
HSV-2-US[e]	Herpes dermatose	NT PF (pos.)	Low N, i.c., i.m., i.p. L. neg.	14 (L)	8
HSV-2-S[e]	Unknown	NT PF (pos.)	High N, i.c. L not done	>100 (L)	20
HSV-2-74/66[f]	Herpes genitalis	PF (pos.)	High N, i.c., i.m. L pos.	4 (I)	25
HSV-2-42/78[f]	Herpes progenitalis	PF (pos.)	N not done L neg.	2 (I)	25
HSV-2-82[f]	Herpes integumentalis	NT PF (pos./neg.)	Low N, i.c. L pos.	7 (I)	25

[a] Quantitative microneutralization (NT); plaque formation (PF) on chicken embryo fibroblast cells.

[b] Neurovirulence (N); liver necrosis (L).

[c] After isolation (I) or after arrival at our laboratory (L).

[d] Concentration of BrVUdR which inhibits plaque formation on PRT cells by 50% compared with an untreated infected control culture.

[e] Laboratory strain.

[f] Clinical isolate.

RESULTS

Plaque inhibition assay

The ID_{50} values (Table 1) for the HSV-1 laboratory strains as well as the clinical isolates are in the range of 0.01–0.02 μM BrVUdR. One HSV-1 strain, however, the laboratory strain HSV-1-S, was insensitive even at 100 μM. The tested HSV-2 strains were all considerably less sensitive to BrVUdR, requiring 8–25 μM for 50% reduction of plaque counts.

Experimental herpes encephalitis in mice

The results of treating experimentally induced herpes encephalitis in mice caused by different herpes virus strains are shown in Table 2.

After i.c. infection with the clinical isolate HSV-1-64 the mice of the untreated control group died between the 3rd and the 10th day (mean: 5.6 d); one mouse survived (10%). In the treated group (140 mg/kg/day) only six mice died between the 5th and 10th day (mean: 7.3 d); four mice were still alive after 28 days (40%). After i.c. injection of the laboratory strain HSV-1-Kupka, obviously highly virulent in mice, all members of the untreated control group died between days 3 and 5 (mean: 4.2 d). In the group receiving therapy nine mice died between days 3 and 9 (mean: 6.2 d) and two mice survived (18%). The encephalitis induced by i.c. or i.p. infection with the laboratory strain HSV-1-S could not, in any case, be influenced by treatment with 1 or 2 mg BrVUdR/day/mouse; there were neither survivors in the treated groups nor differences between the mean survival times of the groups. This virus strain had proved resistant to the compound in plaque inhibition assays on PRT (Table 1) and Vero cells (not shown).

Two groups of non-infected mice treated with 140 mg/kg daily (first injection i.c. or i.m., all following treatments i.m.) over a period of 14 days were free of any toxic symptoms even after 6 weeks.

Assays for specific HSV-1 antibody in the experimentally infected mice with and without BrVUdR treatment showed that the antibody titers were as high in the surviving untreated as in the treated animals (1 : 10–1 : 80).

DISCUSSION

BrVUdR [7,31], along with 1-β-D-arabinofuranosylthymine (ara-T) [19], 9-(2-hydroxyethoxymethyl)guanine (ACG, acyclovir) [18], 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) [39], and 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil (FMAU) [39] as well as the recently developed 1-β-D-arabinofuranosyl-5-vinyluracil (VaraU) [23,32,33], 1-β-D-arabinofuranosyl-5-(2-bromovinyl)uracil (BrVaraU) [12,32,33], and 5-(2-bromovinyl)-3′-amino-2′,3′-dideoxyuridine (3′-NH_2-BVDU) [12] belongs to the nucleoside analogs which have become known as potent and selective anti-

TABLE 2

Treatment of experimental herpes encephalitis in mice with (*E*)-5-(2-bromovinyl)-2′-deoxyuridine

Virus strain	Infection dose ($TCID_{50}$)	Group	Survival time (days)										Mean survival time of mice that died (days)
HSV-1-64	i.c.10^4	C[a]	3.5	4	4	4.5	4.5	5.5	5.5	8.5	10.5	S[f]	5.6 ± 2.36
(Clin. isol.)		T[b]	5.5	6.5	6.5	6.5	8.5	10.5	S	S	S	S	7.3 ± 1.84[d]
HSV-1-Kupka	i.c.10^4	C	3	3.5	3.5	3.5	4	4.5	4.5	5	5	5.5	4.2 ± 0.82
(Lab. strain)		T[b,g]	3.5	4.5	4.5	5.5	6.5	6.5	7.5	8	9.5	S	6.2 ± 1.92[e]
HSV-1-S	i.c.10^2	C	4.5	5	6.5	7.5	7.5	8.5	8.5	9	9.5	12.5	7.9 ± 2.31
(Resist. strain)		T_1[c]	4.5	6	7	7.5	9	9.5	10.5	11.5	11.5	11.5	8.9 ± 2.51
		T[c]	4.5	6.5	6.5	7	7.5	7.5	8	8.5	8.5	9.5	7.4 ± 1.39
HSV-1-S	i.p.10^4	C	7.5	7.5	8	8.5	8.5	9.5	12.5	12.5	13.5	S	9.8 ± 2.39
		T[c]	7.5	8.5	8.5	9	9.5	9.5	10.5	10.5	10.5	11.5	9.6 ± 1.21

[a] Control.
[b] Treated for 11 days with 2 mg/day/mouse.
[c] Treated for 8 days with 2 mg/day/mouse; T_1 = 1 mg/day/mouse.
[d,e] Significance ($P > 0.1$ and $P < 0.01$, respectively), different from the saline-treated mice (Student's *t*-test).
[f] S = Survived at 28 days.
[g] Eleven animals were treated in this group.

herpes compounds during the past years. They have been tested against HSV-1 and HSV-2 strains [5,9,12,21,23,24,29] and found effective against most of these viruses; BrVUdR, VaraU, BrVaraU and 3'-NH_2-BVDU are exceptional in preferentially inhibiting HSV-1 strains.

Other members of the herpetoviridae family are also susceptible, such as varicella-zoster virus towards ara-T, ACG, FIAC, and BrVUdR [2,5,21,29], cytomegalovirus to ACG and FIAC [5,20] and Epstein–Barr virus to ACG [4]. BrVUdR also inhibits herpes viruses pathogenic to animals, such as pseudorabies virus [31], bovid herpes virus type 1 [40] and at concentrations of nearly 1 μM BrVUdR the formation of plaques by Marek's disease virus in duck fibroblast cultures and in the chicken embryo chorioalantoic membrane (G. Heider and J. Reefschläger, unpublished data).

In earlier studies of the inhibition of different HSV-1 strains by BrVUdR we have used a plaque inhibition assay on Vero cells and found ID_{50}s in the range of 0.06–0.23 μM [31]; however, most HSV-1 laboratory strains and clinical isolates were significantly more sensitive in PRT host cells (Table 1) (ID_{50}=0.01–0.02 μM). These data confirm the results of De Clercq and coworkers [9] who found an ID_{50} of about 0.02 μM for 11 tested HSV-1 strains. In agreement with the results presented by De Clercq et al. [9] we also identified a resistant HSV-1 laboratory strain (HSV-1-S; $ID_{50} > 100$ μM) which is presumably a mutant strain altered in its ability to metabolize BrVUdR or in its sensitivity to BrVUTP. Deoxypyrimidine nucleoside kinase and DNA polymerase of HSV-1 have been recognized as the key enzymes for the selective antiviral activity of BrVUdR [1,3]. We could not find any difference between the sensitivity of the HSV-1 laboratory strains and clinical isolates towards BrVUdR, but noticed a marked difference between these and HSV-2 strains which only responded to doses of 8–25 μM (ID_{50}). Therefore, the drug may not be suitable for systemic treatment of human HSV-2 infections, as also suggested by other studies [16].

The advantages of an experimental herpes encephalitis induced intracerebrally in the mouse as an animal model system have been exploited to demonstrate the activity of some of the above-mentioned nucleoside analogs, for example ara-T [22], 5-ethyl-UdR [6], ACG [30] and FIAC [20]. We found that an encephalitis of mice induced by intracerebral inoculation of a laboratory strain (HSV-1-Kupka) or a clinical isolate (HSV-1-64) could be treated successfully with BrVUdR administered intramuscularly (140 mg/kg/day) over a period of 11 days. The survival times and rates were significantly enhanced in comparison with the untreated control groups (Table 2). De Clercq et al. have reported that a herpes encephalitis of the mouse which eventually develops after intracutaneous virus application can be treated successfully with BrVUdR [7,15,17]. Our results prove that even the immediate and devastating threat presented to the brain by intracerebrally injected virus, which might bear more likeness to the state of clinical manifest human encephalitis, can be influenced by BrVUdR. The fact that an encephalitis induced by i.c. or i.p. injection of the strain HSV-1-S insensitive to BrVUdR in tissue culture did not respond to BrVUdR by changes in either survival times or rates (Table 2) emphasizes the significance of the in vitro results.

A significant increase in the survival rate of mice infected i.c. with HSV-1 has also been obtained upon oral and subcutaneous administration of BrVUdR, provided that the treatment was initiated shortly after virus infection (i.e. day 0 or 2, or day 4, if BrVUdR was administered subcutaneously) at a dosage of 80 mg/kg/day or higher (E. De Clercq, Z.-X. Zhang and I.S. Sim, submitted for publication, 1982).

Assays for specific HSV-1 antibody in the experimentally infected mice with and without BrVUdR treatment showed that the antibody titers were as high in the surviving untreated as in the treated animals (1 : 10–1 : 80), indicating a normal immune reaction under BrVUdR treatment. Since antibody is produced despite immediate therapy, it is clear that the establishment of infection is not prevented.

It has recently been concluded from results in different in vitro assays that BrVUdR does not exert toxic effects on the immune system [25]. One of the properties required for an antiviral agent is absence of immunosuppression. After removal of the compound the immune system becomes responsible for the course of the disease. This might be of importance in the case of BrVUdR, because its antiviral activity against VZV was shown to be reversible after removal of the compound [2].

The potent antiviral effect of BrVUdR on HSV-1 and VZV [2,14] in vitro in the mouse encephalitis model described here and in the previously published animal models [7,17,26,34], the lack of an immunosuppressive activity, and the recently reported successful treatments of human herpes simplex keratitis by Maudgal et al. [27] and by Töpke et al. (Augenklinik der Medizinischen Akademie, Erfurt, G.D.R., in preparation) and of severe herpes zoster cases [11] as well as ophthalmic zoster [28] give us hope that BrVUdR will prove effective for the therapy of generalized human HSV-1 and VZV infections and herpes encephalitis.

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