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EFFICACY OF (*E*)-5-(2-BROMOVINYL)- AND 5-VINYL-1-β-D-ARABINOFURANOSYLURACIL AGAINST ACUTE HERPES SIMPLEX VIRUS KERATITIS AND THE ESTABLISHMENT OF LATENCY: COMPARISON WITH ACYCLOVIR AND BROMOVINYLDEOXYURIDINE

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Summary. — Four nucleoside analogues — acyclovir [9-(2-hydroxyethoxymethyl)guanine], bromovinyldeoxyuridine [(*E*)-5-(2-bromovinyl)-2-deoxyuridine], vinylarauracil 5-vinyl-1-β-D-arabinofuranosyluracil and bromovinylarauracil [(*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil] — were compared in the therapy of acute keratitis induced in the rabbit cornea by inoculation of the KUPKA strain of herpes simplex virus type 1 (HSV-1). In comparison to placebo-treated animals, the drugs reduced the mean plaque counts in conjunctival swabs as follows: acyclovir to 0.16—1.73 %, bromovinyldeoxyuridine to 0.02—0.25 %, vinylarauracil to 0.55—5.96 % and bromovinylarauracil to 0.12—3.39 % of control values. Latency was established to a most limited extent in 1 or 2 out of 5 rabbits treated with vinylarauracil or bromovinylarauracil, respectively. One or 6 out of 84 or 98 explanted ganglion fragments (1.3 or 6 %) were positive for HSV-1 as compared to 72 fragments out of 173 (43%) from placebo-treated rabbits. Acyclovir and bromovinyldeoxyuridine completely prevented latency.

Key words: Vinylarauracil; bromovinylarauracil; herpes simplex virus keratitis; nucleoside analogues; latency; rabbits

Introduction

Bromovinyldeoxyuridine [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine, BrVUdR, BVDU] — a synthetic thymidine analogue (Langen and Bärwolff, 1975; Bärwolff, 1978) — is a potent and selective inhibitor of herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV) and Epstein-Barr virus (EBV) (De Clercq *et al.*, 1979; Shigeta *et al.*, 1983; Reefschläger *et al.*, 1982*a*; 1987*b*; Lin *et al.*, 1985) in cell culture. Whereas these herpesviruses were

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inhibited at BrVUdR concentrations from 0.006 to 0.2 μmol/l, depending on the choice of cell line, herpes simplex virus type 2 (HSV-2) is quite insensitive (Reefschläger *et al.*, 1982*b*). Normal cell DNA metabolism and the growth of different mammalian cell lines were affected only at 1 000 to 10 000-fold higher drug concentrations (De Clercq *et al.*, 1979; Reefschläger *et al.*, 1982*a*; 1986, 1987*b*; Reefschläger, 1986). The selectivity of bromovinyldeoxyuridine as an antiherpes agent can be attributed to the specific phosphorylation by the HSV-1 and VZV encoded thymidine kinases to the mono- and diphosphate levels (Allaudeen *et al.*, 1981; Cheng *et al.*, 1981; Descamps and De Clercq, 1981; Ayisi *et al.*, 1984). The 5′-triphosphate of BrVUdR inhibits the HSV-1 DNA polymerase to a greater extent than cellular DNA polymerases (Allaudeen *et al.*, 1981; Ruth and Cheng, 1981), thereby serving as an alternative substrate of the DNA polymerase which incorporates BrVdUMP into herpesviral DNA (Allaudeen *et al.*, 1982; Mancini *et al.*, 1983).

BrVUdR proved highly effective in both topical and systemic treatments of HSV-1 and VZV animal model infections, i.e. orofacial and cutaneous HSV-1 infection in mice (Park *et al.*, 1982; De Clercq, 1984), HSV-1 encephalitis in mice (Reefschläger *et al.*, 1982*b*; 1986, 1987*b*), varicella virus infection of monkeys (Soike *et al.*, 1981) and different herpetic eye infections in rabbits, including epithelial and stromal keratitis as well as iritis and keratouveitis (Maudgal *et al.*, 1982; 1984*a*; Töpke *et al.*, 1984). Following topical application of 0.5% eyedrops of ^{125}I/IVDU, the label appeared in the aqueous humor as well as in the anterior chamber fluid at drug levels surpassing the minimal antiviral concentration (Maudgal *et al.*, 1985*a*).

Topical treatment of patients with herpetic keratitis (dendritic and geographic corneal ulcers or deep stromal keratitis) using 0.1% BrVUdR eyedrops and combined oral and topical application for herpes zoster ophthalmicus (Maudgal *et al.*, 1984*a*; 1985*b*, *c*; Töpke *et al.*, 1984) and the promising results in the oral BrVUdR therapy of severe HSV and VZV infections in immunosuppressed patients, including varicella and zoster infections in children with cancer, point to a significant advance in both topical and systemic treatment of human HSV-1 and VZV infect ons (Wildiers and De Clercq, 1984; Benoit *et al.*, 1985; Tricot *et al.*, 1986).

9-(2-Hydroxyethoxymethyl(guanine)acyclovir, (ACV) is a potent and selective inhibitor of HSV-1 and HSV-2 replication but it is less effective againts VZV, CMV and EBV (Elion *et al.*, 1977; Schaeffer *et al.*, 1978). The drug is converted by the HSV-coded thymidine kinase to the monophosphate and further by cellular kinases to the active triphosphate which, in addition to its affinity to the HSV-polymerases, functions as a substrate causing chain termination of the template (Elion, 1983). ACV was successfully used for topical as well as oral treatment of experimental herpes keratitis (Trousdale and Nesburn, 1982; Kaufmann *et al.*, 1983) and for the management of human herpes simplex virus infections (Lau *et al.*, 1982; Falcon, 1983; Sundmacher, 1983). The 2′-0-glycylacyclovir, a water-soluble ester of acyclovir, has been synthesized to overcome the poor solubility of AVC. This derivative was

ound effective as 1% eyedrops in the topical treatment of experimental herpes simplex keratouveitis (Colla *et al.*, 1983; Maudgal *et al.*, 1984*b*).

Of a series of 5-substituted 1-β-D-arabinofuranosyluracil (araU) analogues (Sakata *et al.*, 1980; Busson *et al.*, 1981; Reefschläger *et al.*, 1983), (*E*)-5--(2-bromovinyl)-araU (BrVaraU) and 5-vinyl-araU (VaraU) emerged as potent and selective inhibitors of some members of the herpesvirus group. BrVaraU is one of the most effective inhibitors of HSV-1 and VZV in vitro (Machida *et al.*, 1980, 1981, 1982; Reefschläger *et al.*, 1983). In vivo it was successful against HSV-1 encephalitis in mice (Machida and Sakata, 1984; Reefschläger *et al.*, 1986) and against cutaneous herpes elicited with HSV-1, (De Clercq, 1984), and effective against simian varicella virus infection of monkeys (Soike *et al.*, 1984). BrVaraU is practically inactive against HSV-2 (Reefschläger *et al.*, 1983) and also not active against human cytomegalovirus replication in culture (Reefschläger, unpublished data), although strongly decreases the expression of EBV capsid antigens in human lymphoblastoid P3HR-1 cells (Färber *et al.*, 1987). To exert its antiviral activity, the nucleoside analogue BrVaraU needs to be phosphorylated by the virus--induced deoxypyrimidine kinase (Descamps *et al.*, 1982). The triphosphate (BrVaraUTP) inhibits the herpesviral DNA polymerases competitively to dTTP, as reflected by low inhibition constants, but does not act as an alternative substrate in the polymerase reaction and, therefore, it is not incorporated into the replicating DNA (Ruth and Cheng, 1981).

In contrast to BrVaraU, VaraU is not only a strong and selective inhibitor of HSV-1, VZV and EBV in vitro (Machida *et al.*, 1980; Machida, 1986*a*,*b*; Reefschläger *et al.*, 1983, 1986, 1987*a*; Färber *et al.*, 1987) and effective against experimental HSV-1 encephalitis in mice (Reefschläger *et al.*, 1986), but also selectively inhibits HSV-2 replication of different clinical isolates at a concentration similar to that of acyclovir. The latter drug showed therapeutic efficacy against experimental HSV-2 encephalitis after oral or i.p. treatment in mice (Reefschläger *et al.*, 1983, 1987*a*). No activity of VaraU against CMV replication in human embryonic lung fibroblast cell cultures was demonstrated (Reefschläger *et al.*, 1987*a*).

In the present study we report on topical treatment of experimental herpes keratitis in rabbits with ACV, BrVUdR, VaraU and BrVaraU and describe their effect on the establishment of latency.

Materials and Methods

Drugs. BrVUdR, BrVaraU and VaraU were synthesized as described previously (Reefschläger *et al.*, 1982*a*; 1983). ACV was a gift from Burroughs Wellcome Comp. (Research Triangle Park, N.C., U.S.A.). The compounds (Fig. 1) were dissolved as 0.1 % drops (w/v) in phosphate buffered saline (PBS) pH 7.2. Saline alone (containing no drug) was used as placebo eyedrops.

Infection and treatment of animals. Adult albino rabbits (33 animals) weighing 3 000 g were inoculated with 10^6 PFU/50 µl of HSV-1 (strain KUPKA) into the right scarified cornea; among these, there were 13 placebo-treated controls. Twenty animals were divided into 4 groups (5 animals each) and treated for consecutive days with 0.1 % drug solution in phosphate buffered saline (PBS, pH 7.2), starting 2 hr post-infection p.i. (day 0) 4 hourly (50 µl each) and then on day 5 and 6 with 0.2 % solution, 8 hourly. From 24 hr p.i. on, swabs were taken before dropping the drug solution at daily intervals. The swabs were immediately immersed into 1 ml BEM

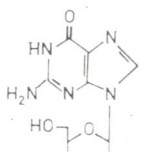
containing 2 % inactivated calf serum (ICS) and antibiotics for 1 hr. The eluted fluids were titrated by plaque assay under 0.9 % methylcellulose overlay on Vero cells, and the number of plaques was calculated per the total 1 ml volume of the tested fluids.

Testing and quantitation of latency. To detect latency, both trigeminal ganglia were removed at day 59 to 70 p.i. The right ganglion samples were minced, and each fragment was cultured separately in Leighton tubes in medium CMRL-1415 supplemented with 10 % newborn calf serum. The presence of virus in the culture fluid was checked on explantation days 4, 7 and 10. This procedure allowed us to quantify the relative incidence of virus-yielding fragments per ganglion and to compare the extent of latency in the ganglia of treated versus untreated animals, provided that the number of fragments from each ganglion was relatively constant (16—20 per ganglion). The left ganglia were cultured by usual procedures.

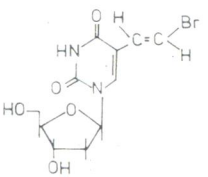
Virus neutralization tests were performed with preinfection serum samples and with the sera of animals sacrificed at ganglion removal. Assays were done on Vero cells versus 100 PFU of HSV-1 (KOS) in the presence and absence of complement (6 units per 0.1 ml of fresh guinea pig serum).

Results

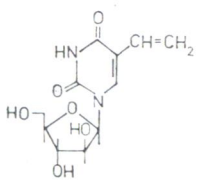
In the course of ACV treatment the mean plaque counts ranged from 10—44 PFU, i.e. from 0.16 to 1.73% fo the untreated controls (Table 1). None of the trigeminal ganglia removed 63 days p.i. yielded latent virus when cultured for 11 days. No neutralizing antibodies were found in any of the ACV-treated animals. After cessation of treatment (day 6, 140 hr p.i.), a relative increase of plaque counts was noted in 2 animals by 7 and 8 days p.i., indicating a "secondary increase" of virus replication at the time of spontaneous decline of virus titre in the conjunctival swabs of placebo-treated (positive control) rabbits. The results of BrVUdR treatment were similar.



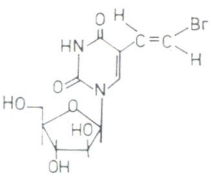
9-(2-Hydroxyethoxymethyl) guanine
Acyclovir, ACV, Zovirax®



(E)-5-(2-Bromovinyl)-2'-deoxyuridine
Bromovinyldeoxyuridine, BVDU, BrVUdR



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

Fig. 1

Table 1. Mean plaque counts in the conju swabs from treated and untreated rabbits during herpes conjunctivitis and the establishment of latency

Days p.i.[a]	Rabbit number	Drug: None	ACV	BrVUdR	VaraU	BrVaraU	Comment
		13	5	5	5	5	
1		8020*	44 (0.54%)	19 (0.24%)	478 (5.96%)	98 (1.22%)	0.1%/4 hr
2		8832	14 (0.16%)	5 (0.06%)	411 (4.65%)	300 (3.39%)	
3		4320	13 (0.3%)	0	202 (4.67%)	52 (1.2%)	
4		5770	16 (0.27%)	1 (0.02%)	32 (0.55%)	7 (0.12%)	
5		576	10 (1.73%)	0	0 [c]	0	0.2%/8 hr
6		787	55 (6.99%)	2 (0.25%)	38 (4.82%)	63 (8%)	
7		449	49 (10.91%)	11 (2.45%)	320 (71.2%)	246 (55%)	No treatment
8		210	83 (39.5%)	2 (0.95%)	208 (99%)	6 (29 %)	
9		152	1 (0.65%)	3 (1.97%)	3 (1.97%)	0	
10		97	3 (3.09%)	68 (70.1%)	80 (82%)	2 (2.06%)	
59 to 70[b]	Rabbit no.	9/9	0/5	0/5	1/5	2/5	Right
	Fragment no.	72/173**	0/100	0/100	1/84	6/98	Gasserian
	Positive rate	(43%)	0	0	(1.3%)	(6%)	ganglion

[a] Treatment from day 0, 2 hr p.i.; animals infected on day 0 with 10^6 PFU/50 µl HSV-1 (KUPKA) inoculum into the right scarified cornea

[b] At given intervals both trigeminal ganglia were removed, minced and each fragment from the right trigeminal ganglion was cultured separately for 11 days (separately tested for virus presence)

[c] Four of 5 rabbits had no virus; in 1 animal the titres ranged from 50 to 1500 throughout (this particular rabbit developed neutralizing antibodies)

* average absolute number of plaques per ml of the conjunctival swab (per cent of controls in brackets).

** positive fragments out total (latency developed in each nontreated rabbit)

The plaque counts in the course of treatment were 0.02—0.25% of the controls, zero values being noted on days 3 and 5 p.i. Despite of the "secondary peak" evident in 2 of 5 rabbits especially on day 10 p.i., no latency was detected. No neutralizing antibodies were found in BrVUdR-treated animals.

Reduction of plaque counts was less clear-cut in rabbits treated with the 5-substituted arauracil derivatives. Reduction of plaque counts during VaraU treatment reached 0.55—5.96% of controls and in 4 of 5 animals no virus was found on day 5 p.i. Relatively severe conjunctivitis with titres ranging from 50—1500 PFU/ml was found in one animal throughout the observation period. Virus-neutralizing antibodies developed in this particular rabbit and latency was detected in its Gasserian ganglion (1 positive fragment out of 16).

In BrVaraU-treated rabbits virus titres decreased to zero on day 5 p.i.; the reduction of plaque counts during the acute keratitis was a little more evident than in the VaraU-treated group (0.12—3.39%). The "secondary replication peak" was clearly expressed in 1 VaraU- and in 2 BrVaraU-treated animals. Latency has been found unrelated to the above-mentioned "secondary peak" in Gasserian ganglia of 2 BrVaraU-treated rabbits (6 of 98 fragments were positive, Table 1). Virus neutralizing antibodies were detected at a threshold titre of 4 (no complement) and 16 (in the presence of complement) in the latter animals. For comparison, the geometric mean titres in nontreated rabbits 70 days p.i. were 76 and 151, respectively (based on examination of 40 serum pairs).

Discussion

As expected, BrVUdR was the most effective drug, causing a 10^3—10^4-fold titre reduction within 48 hr. Interestingly enough, a second replication peak was observed even in 2 of these animals as soon as 24 hr after treatment termination. ACV treatment was also highly efficient, however, a little less effective than BrVUdR. The lowest plaque reduction rate (in the range of 10—100 fold) was observed in rabbits treated with the araU derivatives. This is in accord with the previous finding that BrVaraU as either 0.1% or 0.5% eyedrops promoted a significant healing of epithelial keratitis in rabbits in comparison with placebo eyedrops, but the reduction in keratitis score achieved by BrVUdR 0.1% eye drops was significantly greater (Maudgal and De Clercq, 1985).

Of special interest is the reduction or absence of latency in 2 groups of drug-treated HSV-infected rabbits. It seems that neural spread and latency may be prevented when topical and/or systemic treatment starts no later than within 24 hr p.i., more often when treatment starts 3—6 hr p.i. (Pavan-Langston *et al.*, 1979; Klein *et al.*, 1984, 1985). When treatment started later than 48 hr p.i., it could not prevent the establishment of latency (Trousdale *et al.*, 1980). Pavan-Langston *et al.* (1981) reported that if ACV treatment started 24 hr p.i. and lasted for 15 days the detection of latency depended on the time elapsed between finishing treatment and ganglion removal. When ganglia were cultured beginning on day 2 after treatment termination,

the detectable latency rate was low, while when ganglia were explanted 16 days after finishing treatment, latency was frequently detected. However, when treatment started 3 hr p.i., the latency rate was low even when a longer time had elapsed between cessation of treatment and explantation.

Once established, latency cannot be eradicated from the ganglion tissue (Blyth *et al.*, 1980; Soennerholm *et al.*, 1981; Field and De Clercq, 1981; Kaufman *et al.*, 1983; Mayo *et al.*, 1983). Even when antiherpetic drugs do not eradicate the latent virus due to nonproductive persistence of the genome, topical treatment may lessen additional centripetal spread of virus during recurrencies (Nesburn *et al.*, 1983). In the light of the "round trip" hypothesis, reinfection of the ganglion which contributes to the maintenance of latency (Klein, 9182), would be prevented by treatment of recurrent lesions. In addition, it is known that thymidine kinase-deficient HSV strains do not invade the nervous system (Gordon *et al.*, 1982). One could speculate that emergence of such drug-resistant strains in outcome of antiviral chemotherapy of human herpetic infections would be beneficial in fighting latency.

In our case ACV and BrVUdR treatment beginning from 2 hr p.i. completely prevented latency, while the uracil derivatives reduced the number of animals and the number of ganglion cells which became virus carriers. Our results showed that 10^6 PFU in 50 µl spread over the scarified cornea may not be enough to achieve neural uptake from the inoculum. It had been demonstrated that a small inoculum of pseudorabies virus deposited into the ear skin can spread immediately to the ganglion (Field *et al.*, 1975). Nevertheless, after gentle surface scarification of the cornea the inoculum became adsorbed to epithelium cells rather than to nerve endings, perhaps because the free nerve endings ramify mainly in the deeper epithelium layer. Thus, the crucial event for limited development of latency was the extent of early virus replication in epithelium cells during the first 24—72 hr p.i.; titres in the conjunctival swabs were in the range of 100—1000 PFU when neural uptake and axonal spread took place. Interestingly enough, limited latency was not observed in any of the 7 rabbits which, after cessation of therapy, revealed the "secondary replication peak". Secondary shedding of the virus into the preocular tear film after cessation of ACV therapy has been described by others (Green *et al.*, 1981). Such rebound shedding, possibly due to reversible inhibition of the virus replication, may occur also in association with other antiherpetic drugs. In all these cases neural uptake was prevented, indicating that the virus reservoir had been restricted to a limited number of surface epithelium cells. In addition, our results showed that the secondary shedding after cessation of therapy could not be caused by reversed transfer of the reactivated virus from the ganglion, because it occured in rabbits whose ganglia were free of the latent genome.

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