5-(2-BROMOETHYL)-2′-DEOXYURIDINE: A SELECTIVE INHIBITOR OF HERPES SIMPLEX VIRUSES IN VITRO

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Summary: A new pyrimidine analog, 5-(2-bromoethyl)-2′-deoxyuridine (BEUdR), was tested in vitro for antiviral activity on Herpes simplex virus types 1 and 2. As reference compounds, ACG, BVUdR and PAA were used. Compared to ACG and BVUdR, BEUdR resulted less potent on both HSV-1 and HSV-2. However, a 50% inhibition of the multiplication of uninfected cells could be obtained only at very high BEUdR concentration (ID_{50} = 8500 μM). This makes BEUdR the least toxic analog known and gives it a selective index comparable to, if not better, than of ACG and BVUdR.

INTRODUCTION

HSV-2 is the second leading cause of genital infections in man and its annual incidence is rising [1]. This virus has also been associated with cervical carcinoma but recent evidence indicates that it might be an initiating factor rather than the sole cause of this malignancy [2]. Antiviral chemotherapy studies have led to the synthesis of a number of antiherpes compounds. Among them, acyclovir has been licensed for the treatment of primary episodes of genital herpes as well as of recurring episodes [3, 4]. However, the lack of efficacy of acyclovir on latency, the rapid emergence of resistant mutants (especially in immunosuppressed patients) and its low but significant toxicity, stimulated the search for new antiviral agents.

Acyclovir derivatives have been synthesized with good antiherpes activity [5], but none has proved superior in treating HSV-2 infections. Among pyrimidine derivatives, 5-ethyl-2′-deoxyuridine has been shown to be a selective but not very potent inhibitor of both HSV-1 and HSV-2, while 5-vinyl-2′-deoxyuridine was found to be a potent but not very selective inhibitor of HSV-1 [6]. Interestingly, the substitution of the H atom on the C_2 of the vinyl group yielded a compound, 5-(2-bromovinyl)-2′-deoxyuridine, endowed with both potent and selective antiviral activity [7]; it was therefore interesting to check whether a similar substitution on the C_2 of the ethyl group could also result in a more selective compound. Thus, 5-(2-bromoethyl)-2′-deoxyuridine (BEUdR) was synthesized [8] and assayed for antiherpes activity and toxicity for uninfected cells. As reference compounds 5-(2-bromovinyl)2′-deoxyuridine (BVUdR), 5-iodo-2′-deoxyuridine(IUdR), 9-(2-hydroxy-ethoxymethyl)-guanine (ACG) and phosphonoacetic acid (PAA), were used.

MATERIALS AND METHODS

Antiviral compound

BEUdR was obtained as previously described [8]. ACG was kindly provided by Dr. Bernareggi, Valeas Spa, Milano. BVUdR was a gift from Dr. De Clercq, Katholieke Universiteit Leuven. IUdR and PAA were purchased from the Sigma Pharmaceutical Organisation.

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Table 1. – *Antiviral activity of BEUdR and other compounds on different herpes simplex virus strains.*

Virus strains	[a] ID_{50} (μM)				
	BEUdR	BVUdR	ACG	IUdR	PAA
HSV-2 ACC-VR-734	25	5.5	0.3	4	65
HSV-2 NM	21	7	0.3	3.9	65
HSV-1 ATCC-VR 733	16	0.05	0.25	3.25	60
HSV-1 CL	18	0.06	0.3	4.1	55
HSV-1 $IUdR^R$ N3	1500	7	30	200	14.5
HSV-1 PAA^R N14	15	0.05	4.95	3.5	670

[a] ID_{50}: dose capable of inhibiting plaque formation by 50% in PRT.
Number of plaques in untreated controls: 150 (HSV-1); 130 (HSV-2); 120 (HSV-1 N3); 135 (HSV-1 N14).

Cells and viruses

HEF, Hep-2, RK-13 and VERO cells were obtained from the Istituto Zooprofilattico Sperimentale, Brescia. Stock cultures were grown at 37° C in a humidified CO_2 (5%) atmosphere in Eagle's MEM supplemented with 10% new born calf serum (HEp-2, RK-13, VERO) or 10% foetal salf serum (HEF). Herpes simplex laboratory strains, (HSV-1, ATCC-VR-733 and HSV-2 ATCC-VR-734) and recent clinical isolates (HSV-1 CL and HSV-2 NM) were used throughout the experiments. The HSV-1 resistant strains N3-$IUdR^R$ and N14-PAA^R were isolated from plaques developed in the presence of 10 μg/ml IUdR and 100 μg/ml PAA, respectively. The mutants were then repeatedly grown in the presence of the selecting drug concentrations, plaque purified twice and propagated once in VERO cells in the absence of the drugs. Plaque assay and plaque and yield reduction tests were performed according to Collins and Bauer (9). The plaque counts and the virus titers obtained in treated cultures were expressed as percentages of untreated controls and plotted against the logarithm of drug concentrations. Dose-response lines, from which ID_{50}, ID_{90}, ID_{99}, etc., were calculated, were drawn by linear regression technique. Correlation coefficents were 0.96 or better, indicating a good fit of the data. For drug toxicity tests, cells were seeded at a concentration of 5×10^5 cells/flask (25 cm^2 Falcon) in growth medium and allowed to athere to the surface overnight at 37° C in a humidified, CO_2 incubator. New growth medium containing serial dilutions of the drugs was then added. After incubation for 3 days (HEp-2, RK-13 and VERO) or 5 days (HEF), the cultures were harvested, trypsinized, resuspended in maintenance medium mand counted in a hemocytometer. Variation between duplicate samples was less than 10%.

RESULTS

Effect of BEUdR on the multiplication of HSV-2 and HSV-1

The activity of BEUdR on Herpes simple virus strains has been evaluated in both plaque reduction tests (PRT) and yield reduction tests (YRT) and has been compared to that of BVUdR, IUdR, ACG and PAA.

As can be seen in tab. 1, the ability of the compounds to reduce HSV-2 plaque formation in VERO cells decreased in the following order: ACG > IUdR > BVUdR > BEUdR > PAA. On the contrary, in the case of HSV-1, BVUdR was the most active compound followed by ACG, IUdR, BEUdR and PAA, in decreasing order of activity. In any case, and for each compound, no significant variation in potency on laboratory strains and clinical isolates of both HSV-1 and HSV-2 was observed. When assayed on a HSV-1 mutant resistant to IUdR, BEUdR showed no significant antiviral activity behaving like all the other thymidine kinase-dependent analogs, which showed a 100-fold reduction of activity on this mutant. PAA was the only exception, being more active on the $IUdR^R$ mutant than on the wild type parental strain. On the contrary, when tested on a PAA-resistant mutant, BEUdR, as well as BVUdR and IUdR, maintained

Table 2. – *Effect of BEUdR and other compounds on the yields of different herpes simplex virus strains.*

Compound	Virus	μM		
		ID_{90}[a]	ID_{99}[a]	$ID_{99.9}$[a]
BEUdR	HSV-2 VR-734	13.5	56	315
	HSV-2 NM	9.5	49	286
	HSV-1 VR-733	7	25	115
BVUdR	HSV-2 VR-734	12	56	180
	HSV-2 NM	14.4	60	195
	HSV-1 VR-733	0.1	0.25	0.7
ACG	HSV-2 VR-734	2	5	10
	HSV-2 NM	2.4	5.9	12
	HSV-1 VR-733	1.7	2.6	4.5

[a] ID_{90}, ID_{99}, $ID_{99.9}$: dose capable of inhibiting virus yield by 90, 99, 99.9% in YRT.
Virus titers in untreated controls: $5\times10_7$ PFU/ml (HSV-2 VR-734); $3.9\times10_7$ PFU/ml (HSV-2 NM); $4.5\times10_8$ PFU/ml (HSV-1 VR-733).

its antiviral activity unchanged. It should be noted that this PAA^R mutant showed an increased resistance also to ACG.

The effects of BEUdR, BVUdR and ACG on HSV-2 and HSV-1 were compared also under single-cycle growth conditions (YRT). The results are shown in tab. 2. The most potent compound against HSV-1 was BVUdR, and the least BEUdR. On the other hand, while ACG was, as expected, the most active compound against HSV-2, BEUdR showed the same potency as BVUdR, irrespective of the degree of inhibition considered. It is worth noting that the ID_{90} of BEUdR on both HSV-2 and HSV-1 was one half of its ID_{50}, which implies a probable catabolization of the compound under multiple-cycle conditions (PRT).

Reversion of the antiviral activity of BEUdR

The ability of pyrimidine nucleosides to reverse the inhibition of BEUdR was determined in plaque reduction assays.

As shown in tab. 3, 10 μM thymidine fully reversed the anti-HSV action of BEUdR; at the same concentration d-Uridine was less effective, while d-Cytidine was completely ineffective at concentrations up to 250 μM.

Effect of BEUdR on the growth of uninfected cells

Toxicity of BEUdR, BVUdR and ACG was evaluated on various cell lines under conditions which allowed controls to grow for three cell cycles. The results (tab. 4) show that BEUdR was, in every cell liner tested, the least toxic compound. In fact, the ID_{50} (inhibitory dose necessary to reduce by 50% the number of cells) and the MNTD (maximum non toxic dose) of BEUdR were 10 and 15 times higher than those of ACG or thymidine, respectively. BVUdR, on the contrary, varied in toxicity according to the cell line used, being highly toxic on VERO and HEF cells.

Selectivity indices

Selectivity indices of BEUdR, BVUdR and ACG on HSV-1 and HSV-2 are reported in tab. 5. As could be foreseen

Table 3. – *Reversion of the anti-HSV action of BEUdR.*

Compounds	[a]Number of plaques: % of controls	
	HSV-1	HSV-2
Thymidine 10 μM	100	100
d-Uridine 10 μM	100	100
d-Cytidine 250 μM	100	100
BEUdR 50 μM	0	0
BEUdR 50 μM + Thymidine 10 μM	100	100
BEUdR 50 μM + d-Uridine 10 μM	70	75
BEUdR 50 μM + d-Cytidine 250 μM	0	0

[a] Numbers of plaques in untreated controls: 150 (HSV-1); 130 (HSV-2).

Table 4. – *Toxicity of BEUdR and other compounds on various cell lines.*

Compound	μM							
	VERO		HEp2		RK-13		HEF	
	[a]ID_{50}	MNTD[b]	[a]ID_{50}	MNTD[b]	[a]ID_{50}	MNTD[b]	[a]ID_{50}	MNTD[b]
BEUdR	8500	4600	8650	5200	8200	4700	8450	4600
BVUdR	0.6	0.15	200	40	250	14	10	0.4
ACG	1150	185	1100	100	1220	200	1200	190
TdR	1100	312	1150	300	1050	295	1350	320

[a]ID_{50}: dose capable of reducing by 50% the number of cells;
[b]MNTD: maximum non toxic dose.
Cell numbers determined after three cell-cycles of controls-cells.

from its high toxicity on VERO cells, BVUdR showed the lowest antiviral index. On the contrary, the extremely low toxicity of BEUdR resulted in antiviral indices comparable or superior to those calculated for ACG. Only when the ratio ID_{50} cells/ID_{50}HSV was considered, BEUdR showed a selectivity index 10 times lower than ACG.

DISCUSSION

BEUdR is a pyrimidine analog which shows a selective antiviral activity against both laboratory strains and recent clinical isolates of herpes simplex viruses in vitro. This drug has also been proved active on human cytomegalovirus ([8]).

Although the substitution of the H atom on the C_2 of the ethyl group by a bromine did not result in an increase of potency (as has been the case for the bromine addition to the vinyl group), this modification has led to a compound, BEUdR, which shows the lowest cytotoxicity among the nucleoside analogs so far synthesized. Surprisingly, even thymidine (TdR) is more toxic than BEUdR for uninfected cells. Consequently, BEUdR has antiviral indices equal or even superior not only to BVUdR but also to ACG.

Regarding its mode of action, our data suggest that BEUdR is among the inhibitors which owe their antiviral activity to a selective phosphorelation by the virus-induced thymidine kinase (TK). In fact, like ACG and BVUdR, it is inactive on a HSV-1 mutant resistant to IUdR (TK^- mutant) and, like BVUdR and IUdR, it is fully active on a HSV-1 mu-

Table 5. – *Antiviral indices of BEUdR, BVUdR and ACG.*

	[a]ID_{50} cell/ID_{50} HSV-2 (HSV-1)	[a]MNTD/ID_{90} HSV-2 (HSV-1)	[a]MNTD/ID_{99} HSV-2 (HSV-1)	[a]MNTD/$ID_{99.9}$ HSV-2 (HSV-1)
BEUdR	340 (530)	340 (655)	82 (185)	14.5 (40)
BVUdR	0.2 (12)	0.01 (1.5)	0.002 (1.6)	0.0008 (0.2)
ACG	3830 (5750)	92.5 (123)	37 (71)	18.5 (41)

[a]ID_{50} cell and MNTD values are from experiments on VERO cells.

tant with an altered DNA polymerase (PAA-resistant mutant). That BEUdR is a TK-dependent inhibitor is also supported by the fact that its anti-HSV activity can be reversed fully by thymidine, partially by deoxyuridine and not at all by deoxycytidine.

In conclusion, the extremely low toxicity of BEUdR, its very good antiviral indices against both HSV-1 and HSV-2, and its activity on herpes viruses resistant to ACG (PAA^R mutant in table 1), make this compound a very promising drug for the therapy of mucocutaneous and genital herpes.

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