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Comparison of the in vitro and in vivo antiherpes virus activities of the acyclic nucleosides, acyclovir (Zovirax) and 9-[(2-hydroxy-1-hydroxymethyl-ethoxy)methyl]guanine (BWB759U)

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Summary

The antiherpes virus activities of acyclovir and its close analogue 9-[(2-hydroxy-1-hydroxymethylethoxy)methyl]guanine (BWB759U) were compared in vitro and in vivo. The activities of both compounds against herpes simplex virus and varicella-zoster virus were similar in the majority of cell lines. However, in mouse-derived and HeLa cells, BWB759U was more effective than acyclovir against herpes simplex virus. Mutants of herpes simplex virus deficient in thymidine kinase and resistant to acyclovir were found to vary in their sensitivity to BWB759U. In two mouse models of herpes simplex virus infection BWB759U was more effective than acyclovir.

antiviral; herpes viruses; acyclovir; BWB759U

Introduction

The action of acyclic nucleosides against the herpes group of viruses relies upon their specific phosphorylation by the virally coded thymidine kinase and subsequent inhibition of the viral DNA polymerase [1–3]. This is best exemplified by acyclovir (Zovirax™, ACV) which has been shown to be active against herpes simplex virus types 1 and 2 and varicella-zoster virus in cell culture [4–7], in animal models [7–9] and

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more recently in man [10]. This high level of antiviral activity is accompanied by low host cell toxicity permitting the compound to be used systemically.

9-[(2-Hydroxy-1-hydroxymethylethoxy)methyl]guanine (BWB759U), also referred to as DHPG, 2'NDG or BIOLF 62, is a close analogue of ACV which was prepared by chemists at Burroughs Wellcome Company, North Carolina, U.S.A. It has been shown to be active against herpes simplex virus types 1 and 2 [11–15], varicella-zoster virus [11,13], cytomegalovirus [13–16] (Dr. K. Biron, personal communication), Epstein–Barr virus [13,14] and also against the veterinary herpes viruses causing Aujeszky's disease and equine rhinopneumonitis [17]. In the present study we have compared the activities of ACV and BWB759U against herpes simplex virus (HSV) and varicella-zoster virus (VZV) in various cell lines and related the results to those obtained in animals.

Materials and Methods

Virus strains

The following strains of HSV were used: the laboratory strains ICI and MS, and the clinical isolates from immunocompetent patients, 9780, 10560, 2036 supplied by Dr. S. Sutherland, Middlesex Hospital, London; Evans and 5329 supplied by Ms. H. Dunne, Great Ormond Street Hospital, London; 19260* supplied by Dr. M. Ogilvie, Southampton General Hospital; 81.3973* supplied by Dr. I. Barton, Royal Hallamshire Hospital, Sheffield and 80.5550* and 79.5361* obtained from St. Bartholomew's Hospital, London. The strains 25784, 1760 and 5399 were isolates obtained from immunocompromised patients prior to acyclovir therapy. The strains 7294, 5771 and 5538 were the corresponding post therapy isolates. All were supplied by Mr. M. Ross, Royal Free Hospital, London. Virus stocks were stored at -70°C as 1 ml aliquots of crude infected cell extracts.

The Fortier strain of VZV was obtained as a clinical isolate from the Royal Marsden Hospital, London, and 6350 from the Great Ormond Street Hospital, London. Aliquots of cell associated virus were maintained in liquid nitrogen.

Cell cultures

Preliminary studies were carried out in the B_2 or H strains of Vero cells and subsequent comparative sensitivity studies were performed in HeLa, MRC5, RK13, BHK21, 3T3 and LM cells. The 3T3 cells were purchased from Flow Laboratories, Irvine, Scotland, and the LM cells were a gift from Dr. P. Furman, Burroughs Wellcome Company, U.S.A. The remaining cells were provided by the Cytology Department, Wellcome Research Laboratories, Beckenham, U.K.

All cells, with the exception of BHK21 and MRC5 were cultured and maintained in Eagle's minimal essential medium supplemented with foetal calf serum, sodium bicarbonate and penicillin-streptomycin. The MRC5 cells were grown in Eagle's basal

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* Type 2 strains

medium and the BHK21 cells in baby hamster kidney (BHK) medium with tryptose phosphate broth.

Animals

The CDI strain of albino mice weighing between 15 and 18 g, supplied by Charles River, Manston, Kent, were used in the herpes encephalitis model. The Balb/c strain of mice weighing between 15 and 18 g, supplied by Bantin and Kingman, were used in the generalised herpes model. All animals were housed in groups of 10–15 and were given food and water ad libitum.

Antiviral compounds

Acyclovir (ACV, Zovirax^{TM}, 9-(2-hydroxyethoxymethyl)guanine) and BWB759U (9-[2-hydroxy-1-hydroxymethoxyethoxy)methyl]guanine were supplied by Ms. L. Beauchamp, Burroughs Wellcome Company, U.S.A.

Plaque reduction assays

The in vitro potencies of the antiviral compounds were determined by the plaque reduction assay. Assays against HSV were performed in 60 mm tissue culture dishes (Corning Glass Works, Corning, NY, U.S.A.) under a solid agarose overlay [18] or in multiwell plates (Nunc U.K., Hounslow, Middlesex, U.K.) under a semi-liquid carboxymethyl cellulose overlay [7]. Against VZV, assays were performed under liquid overlay in 25 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY, U.S.A.). Doubling concentrations of test compound were incorporated into the overlay medium of cultures infected with sufficient virus to produce 100–200 plaques and incubated for 3 days at 37°C in an atmosphere of 5% CO_2 for HSV or for 7 days at 37°C for VZV. The resultant plaque counts were expressed as a percentage of the virus control and plotted against the $\log_{10}$ of the compound concentration, using a computer program. The concentration of compound required to prevent the formation of 50% of the plaques is termed the 50% inhibitory concentration (IC_{50}) from which all comparisons were made.

Animal models

1. *Herpes encephalitis in mice.* Ether anaesthetised CD1 mice were inoculated in the right cerebral hemisphere with 300 LD_{50} of the ICI strain of type 1 HSV contained in 25 µl of PBS'A'. The survival times of groups of 10 infected animals receiving 0.1 ml volumes of test compound intraperitoneally, subcutaneously or orally were compared with those of untreated animals with prolongation of survival time being indicative of an antiviral effect. Treatment was begun 2–3 h after infection and was continued twice daily (at 8.00 a.m. and 5.00 p.m.) for 5 days.

2. *Generalised herpes in mice.* Intraperitoneal inoculation of Balb/c mice with 5×10^5 p.f.u. of the MS strain of type 2 HSV contained in 0.1 ml of PBS'A' results in the death of the mice 6–10 days later. This model was used to compare subcutaneous and oral therapy as described for the encephalitis model.

Results

In vitro sensitivities

The high level of anti-HSV activity previously reported for BWB759U [11–15] was confirmed in plaque reduction assays using Vero H cells. BWB759U was 2–3-fold more active than acyclovir against 9 strains of HSV; however, against 4 strains of type 2 virus no differences in sensitivity were observed (Table 1). Assays in MRC5 cells showed that BWB759U and acyclovir had similar IC_{50} values against 2 strains of VZV (Table 1). The sensitivity of each strain was determined on at least 2 occasions with no more than a 2-fold variation in the individual IC_{50} values.

We received 3 strains of HSV type 1 from 3 patients who had been treated with intravenous acyclovir for chronic cutaneous herpes simplex whilst immunocompromised following bone marrow transplantation. Each of these strains was shown to be markedly less sensitive to acyclovir by plaque reduction in Vero H cells than their corresponding pre-treatment isolate (Table 2). Subsequent measurement of the thymidine kinase activities of these strains revealed that they each had less than 5% of the phosphorylating activity of their parent strain. Although these strains have yet to be further characterised they probably reflect mixed populations containing a very high proportion of thymidine kinase-deficient mutants. Whilst all parent strains were

TABLE 1

In vitro activities of BWB759U and acyclovir against strains of HSV types 1 and 2 and VZV

Virus	Mean IC_{50} values (μM)	
	BWB759U	ACV
HSV type 1	0.57 ± 0.30	1.34 ± 1.10 ($n = 9$)
HSV type 2	1.67 ± 0.43	1.78 ± 0.41 ($n = 4$)
VZV	3.71 ± 1.47	2.09 ± 0.18 ($n = 2$)

TABLE 2

Comparison of the response in Vero H cells of wild type and resistant strains of HSV to BWB759U and acyclovir

Virus strain (type 1)	IC_{50} (μM)	
	BWB759U	Acyclovir
25784 P	0.43, 0.48	0.36, 0.35
7294 R	56.42, 47.11	10.70, 10.15
1760 P	0.36, 0.24	1.03, 0.61
5771 R	1.69, 1.95	19.61, 25.09
5399 P	1.33, 0.82	0.92, 1.44
5538 R	3.04, 5.74	3.21, 6.31

Key: P, parent strain taken before acyclovir therapy; R, resistant strain post-therapy isolate.

highly sensitive to both acyclovir and BWB759U the post-treatment isolates were less sensitive to acyclovir and showed varying responses to BWB759U. Isolate 7294 appeared 5-fold more resistant to BWB759U, conversely 5771 was much more sensitive to BWB759U than to acyclovir although less sensitive than the parent strain, and 5538 was equally resistant to both compounds.

The activities of acyclovir and BWB759U against the ICI strain of HSV type 1 were compared by plaque reduction assay in different cell lines (Table 3). The virus was first titrated in each of the cell lines and the assay plates were then infected at similar multiplicities (150–200 p.f.u./cell culture). Each assay was repeated at least once and the individual IC_{50} values are presented in Table 3. In the majority of cells the activity of BWB759U was similar to or slightly greater than that of acyclovir. However, in HeLa and the mouse cell lines 3T3 and LM, BWB759U was between 10- and 20-fold more effective than acyclovir.

Animal models

1. *Herpes encephalitis in mice.* In a preliminary study BWB759U was found to be more effective than acyclovir ($P<0.01$) when administered orally at 100 mg/kg per dose twice a day to mice infected intracerebrally with HSV type 1 (Fig. 1). In this study acyclovir increased the mean survival time from 3.17 to 4.0 days ($P<0.05$) whereas BWB759U at the same dose increased the mean survival time to 6.1 days ($P<0.001$) with one mouse surviving. In a subsequent oral dose-ranging study treatment with doses of acyclovir up to 25 mg/kg (twice a day) afforded no significant protection and doses of 50 and 100 mg/kg only increased the mean survival time from 4.2 to 5.4 and 5.5 days, respectively (Table 4). Significant protection was achieved with all dose levels of BWB759U and this protection was greater than the corresponding acyclovir groups, attaining significance at 5, 25 and 100 mg/kg per dose.

To investigate whether the increased protection observed following oral administration resulted from improved bioavailability from the gut, subcutaneous and intraperitoneal routes of therapy were compared (Fig. 2). Following subcutaneous

TABLE 3

Comparison of the in vitro sensitivity of the ICI strain of HSV type 1 to BWB759U and acyclovir in different cell types

Cell type	IC_{50} (μM)	
	BWB759U	ACV
Vero B_2	0.05, 0.07	0.1, 0.14
Vero H	0.47, 0.5	0.77, 0.54
RK13	0.03, 0.09	0.07, 0.1
BHK21	0.3, 0.74	0.29, 0.44
HeLa	0.12, 0.08, 0.1	0.73, 1.3, 1.4
MRC5	1.04, 0.76	2.05, 0.77
3T3	0.004, 0.0014, 0.004	0.06, 0.06, 0.03
LM	0.001, 0.0005	0.01, 0.01

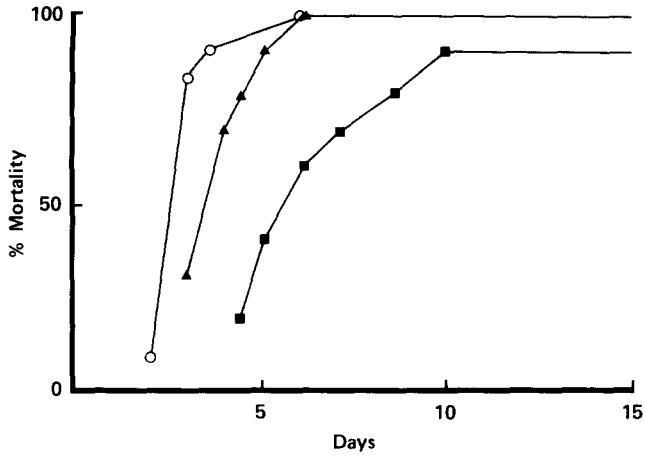

Fig. 1. Comparison of the efficacy of orally administered acyclovir and BWB759U in the fatal herpes encephalitis model in mice. Open symbols represent the cumulative mortalities of the untreated control mice; ▲——▲ acyclovir at 100 mg/kg per dose twice a day; ■——■ BWB759U at 100 mg/kg per dose twice a day.

administration at 100 mg/kg per dose twice a day mean survival times were increased from a control value of 3.09 to 6.1 days ($P<0.001$) for acyclovir and 12.1 days ($P<0.0001$) for BWB759U. The mean survival times after intraperitoneal administration at the same dose level were increased to 7 days ($P<0.001$) for acyclovir and to 12.9 days ($P<0.0001$) for BWB759U. By both routes BWB759U was significantly more

TABLE 4

Survival data comparing the activities of BWB759U and acyclovir in an oral dose-ranging study against HSV type 1 encephalitis in mice

Treatment group	Dose (mg/kg twice a day)	No. survivors/ total	Mean survival time (days ± S.E.)		
Virus control	0	0/15	4.2 ± 0.26		
Acyclovir	5	0/10	3.7 ± 0.34	NS[a]	
	10	0/10	5.1 ± 0.43	NS	
	25	0/10	4.1 ± 0.31	NS	
	50	0/10	5.4 ± 0.43	<0.05	
	100	0/10	5.5 ± 0.37	<0.05	
BWB759U	5	0/10	6.6 ± 0.56	<0.005	<0.001[b]
	10	0/10	6.1 ± 0.46	<0.005	NS
	25	2/10	8.5 ± 1.10	0.0001	<0.0005
	50	0/10	7.4 ± 0.91	<0.001	NS (0.06)
	100	2/10	9.2 ± 0.98	<0.0001	<0.005

[a] *P* values – Mantel-Cox and Breslow methods comparing with controls.
[b] Comparison of corresponding dose levels.

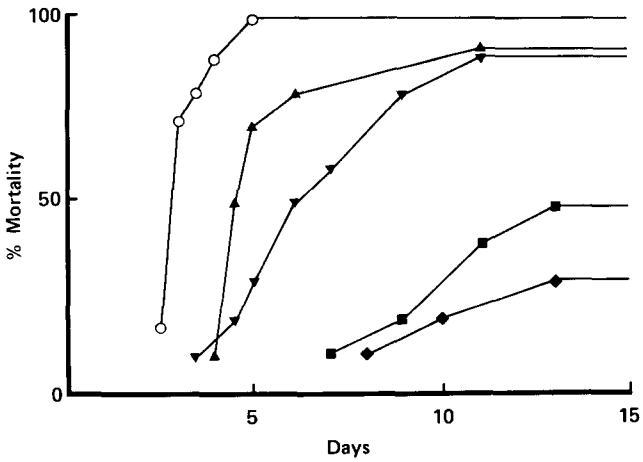

Fig. 2. Comparison of the efficacy of subcutaneously or intraperitoneally administered acyclovir and BWB759U in the fatal herpes encephalitis model in mice. Open symbols represent the cumulative mortalities of the untreated control mice; ▲——▲ subcutaneous acyclovir; ▼——▼ intraperitoneal acyclovir; ■——■ subcutaneous BWB759U; ◆——◆, intraperitoneal BWB759U. Both compounds were administered at 100 mg/kg per dose twice a day.

effective than acyclovir with P values of <0.005 for subcutaneous therapy and <0.001 for intraperitoneal therapy.

In a repeat experiment BWB759U was again significantly more effective than acyclovir by all 3 routes of administration, although the activities of both compounds were less than expected when given intraperitoneally, acyclovir failing to reach significance by this route (Table 5).

2. *Generalised herpes model.* Subcutaneous BWB759U at 100, 50 and 25 mg/kg per

TABLE 5

Survival data comparing the activities of BWB759U and acyclovir against HSV type 1 encephalitis in mice following subcutaneous (s.c.), intraperitoneal (i.p.) and oral therapy at 100 mg/kg per dose twice a day

Treatment group	Route	No. survivors/ total	Mean survival time (days ± S.E.)		
Virus control	–	0/11	2.91 ± 0.09		
Acyclovir	s.c.	1/9	6.22 ± 1.06	<0.0001[a]	<0.005[b]
BWB759U	s.c.	3/10	10.4 ± 1.06	<0.0001	
Virus control	–	0/10	3.5 ± 0.31		
Acyclovir	i.p.	0/10	4.3 ± 0.30	NS	<0.0001
BWB759U	i.p.	2/9	10.11 ± 1.16	<0.0001	
Virus control	–	0/11	2.91 ± 0.16		
Acyclovir	Oral	0/10	4.4 ± 0.40	<0.005	<0.005
BWB759U	Oral	0/10	7.3 ± 0.60	<0.0001	

[a] P values – Mantel-Cox and Breslow methods comparing with controls.

[b] P values comparing equivalent treatment groups.

TABLE 6

Survival data comparing the activities of BWB759U and acyclovir in a subcutaneous dose-ranging study against generalised HSV in mice

Treatment group	Dose (mg/kg twice a day)	No. survivors/ total	Mean survival time (days ± S.E.)		
Virus control	0	1/13	8.31 ± 1.09		
Acyclovir	0.5	0/10	7.80 ± 0.20	NS[a]	
	1.0	0/10	7.60 ± 0.34	NS	
	2.0	0/10	8.00 ± 0.37	NS	
	4.0	0/10	8.10 ± 0.23	NS	
	8.0	0/10	9.20 ± 0.65	NS	
	16.0	2/10	11.30 ± 1.66	<0.05	
	32.0	4/10	14.20 ± 2.00	<0.005	
	64.0	0/10	10.40 ± 1.08	0.05	
BWB759U	0.25	2/10	12.70 ± 1.55	<0.01	
	0.5	3/10	13.40 ± 1.91	<0.01	<0.01[b]
	1.0	3/10	14.90 ± 1.72	<0.001	0.0001
	2.0	4/10	16.90 ± 1.36	<0.001	<0.0001
	4.0	9/10	20.10 ± 0.85	<0.0001	<0.0001
	8.0	9/10	20.30 ± 0.66	<0.0001	<0.0001

[a] *P* values - Mantel-Cox and Breslow methods comparing with controls.
[b] Comparison of corresponding dose levels.

dose twice a day afforded complete protection to mice infected intraperitoneally with the MS strain of type 2 HSV. In contrast acyclovir therapy resulted in only 50% protection at doses in excess of 50 mg/kg per dose. In a subcutaneous dose-ranging comparison study, groups of 10 mice received BWB759U or acyclovir daily for 5 days (Table 6). Concentrations of acyclovir below 8 mg/kg per dose gave no significant protection. At doses above 16 mg/kg per dose significant protection was achieved with 2 and 4 survivors at 16 and 32 mg/kg per dose respectively and, although none survived at 64 mg/kg per dose, the mean survival time was increased from 8.31 to 10.4 days. BWB759U gave significant protection at all dose levels, with the lowest dose 0.25 mg/kg increasing the mean survival time to 12.7 days with 2 survivors. Doses of 4 and 8 mg/kg almost completely protected the mice with only one death in each group and mean survival times in excess of 20 days. At all corresponding dose levels between 0.5 and 8 mg/kg BWB759U was significantly more effective than acyclovir.

Discussion

Initial in vitro studies demonstrated that the acyclic nucleosides, acyclovir and BWB759U, were both highly active against members of the herpes virus group. In Vero cells BWB759U was approximately twice as active as acyclovir against HSV type 1,

substantiating previously reported work [11,15]. Others [13] reported no differences in activities determined in primary rabbit kidney cells, but this may reflect the test method employed or the small number of isolates examined. Type 2 strains were equally susceptible to both compounds but only 4 strains were evaluated. As expected VZV was less susceptible than HSV, but BWB759U was no more active against the virus than acyclovir. However, there are differences in the in vitro activities of these two compounds against other herpes viruses. Human cytomegalovirus is sensitive to BWB759U with IC_{50} values ranging from 0.4 to 7 μM compared with values from 10 to 140 μM for acyclovir [13–16] (Dr. K. Biron, personal communication). Significant activity has also been reported against Epstein–Barr virus [13,14,16]. Amongst the veterinary herpes viruses, BWB759U was 40-fold more active than acyclovir against equine rhinopneumonitis virus with IC_{50} values of 0.06 and 2.0 μM respectively and 2-fold more active against Aujeszky's disease virus with IC_{50} values of 19 and 34 μM respectively [17].

Studies with laboratory generated HSV mutants have revealed variable sensitivities to BWB759U, for example, DNA polymerase mutants resistant to acyclovir were sensitive to BWB759U [11,15], whereas some thymidine kinase-deficient mutants were resistant and others were sensitive. In our own studies, of three mutants which appeared to be thymidine kinase-deficient obtained from acyclovir clinical trials, 2 (7294 and 5538) were resistant to acyclovir and BWB759U, whereas one (5771) remained relatively sensitive to BWB759U, whilst resistant to acyclovir.

It was suggested [22,23] that the activities of antiherpes compounds could vary considerably in different cell lines. Therefore we studied the relative potencies of BWB759U and acyclovir in a number of cell types. In most the IC_{50} values obtained against a type 1 strain of HSV were similar. However, in 3 lines, HeLa and the mouse cells 3T3 and LM, BWB759U was 10–20-fold more effective than acyclovir.

The modes of action of BWB759U and acyclovir are similar. BWB759U, like acyclovir, has been shown to be preferentially phosphorylated to the monophosphate in herpes virus-infected cells by the herpes virus-coded thymidine kinase [13] (Dr. J. Fyfe, personal communication). Subsequent phosphorylation occurs under the influence of host cell enzymes. The BWB759U triphosphate successfully competes with dGTP for the viral DNA polymerase. However, the K_i for this reaction is 10-fold greater than that for acyclovir triphosphate [20]. Compensating for this deficiency and possibly explaining why the in vitro activities of the two compounds against HSV and VZV remain similar, 10-fold more BWB759U triphosphate than acyclovir triphosphate is produced in infected cells [20].

In mouse models of HSV infection, BWB759U was clearly more efficacious than acyclovir. In the mouse encephalitis model, where the virus is introduced directly into the brain, a significant increase in the mean survival time was achieved with orally administered BWB759U at 5 mg/kg per dose twice a day, whereas an increase in survival time was only obtained with doses of acyclovir of 50 mg/kg per dose or greater. BWB759U was also superior to acyclovir following subcutaneous or intraperitoneal administration substantiating previously reported work [21]. The least beneficial route of therapy was by mouth, reflecting the poor oral absorption of both compounds in the mouse (Dr. P. de Miranda, personal communication). In the

generalised HSV model BWB759U was up to 50-fold more effective after subcutaneous therapy, in agreement with others [12–14]. In a hamster model of equid herpes virus type 1, BWB759U was found to be considerably more effective than acyclovir [17].

Greater protection was achieved following treatment with BWB759U than acyclovir in both models, although complete protection was only observed when doses in excess of 25 mg/kg of BWB759U twice a day were employed in the generalised model. Increased protection following therapy with either compound may have been observed had continuous levels of drug been maintained as was shown by Field and De Clercq [24] by the incorporation of test compounds in the drinking water. However, the plasma half lives of both BWB759U and acyclovir are similar in the mouse, independent of the route of administration [25] (Dr. de Miranda, personal communication), which would justify a direct comparison irrespective of the frequency of therapy.

Investigations to date into the mode of action of BWB759U reveal no differences from that of acyclovir sufficient to explain the increase in vivo activity. Similarly in mice BWB759U is no better orally absorbed, a finding supported by the maintenance of its superiority following subcutaneous or intraperitoneal administration. BWB759U may be more readily absorbed into the central nervous system and so afford greater protection against encephalitis. However, in the generalised model where all major organs are involved (Dr. K. Biron, personal communication) BWB759U remains substantially more effective than acyclovir. Therefore the view that BWB759U has a greater predilection for nervous tissue [15] cannot be fully supported, although this could contribute to the observed increase in activity.

A more probable explanation arises from the observation of differential susceptibilities in various cell types; BWB759U was clearly more effective in mouse-derived cells as well as in HeLa cells. The reason for this is as yet unclear but may be accounted for by an increased production of BWB759U triphosphate. But this does not explain the high level of in vitro activity observed for BWB759U against human cytomegalovirus or the variable sensitivity observed with acyclovir resistant mutants of HSV unless another as yet undefined cellular mechanism of phosphorylation is invoked.

Advantages have been demonstrated for BWB759U over acyclovir in the management of herpes virus infections in cell cultures and in animal models but it is not clear whether these will be realised in man.

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