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Mode of Action, Toxicity, Pharmacokinetics, and Efficacy of Some New Antiherpesvirus Guanosine Analogs Related to Buciclovir

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9-[4-Hydroxy-3-(hydroxymethyl)butyl]guanine (3HM-HBG), (*RS*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine ([±]2HM-HBG), and *cis*-9-(4-hydroxy-2-butenyl)guanine (2EN-HBG), new acyclic guanosine analogs structurally related to buciclovir (BCV [(*R*)-9-(3,4-dihydroxybutyl)guanine]), were evaluated in parallel with buciclovir as anti-herpes simplex virus (HSV) agents. In cell cultures, replication of different strains of HSV type 1 (HSV-1) and HSV-2 was inhibited at nontoxic drug concentrations. The concentrations giving 50% inhibition of plaque formation were, however, dependent on virus strain and cell type. In most cell types, the order of activity against HSV-1 strains was 3HM-HBG > (±)2HM-HBG > BCV > 2EN-HBG, whereas the drugs showed an approximately equivalent activity against HSV-2 strains in different cells. The cytotoxic effects of the drugs were also cell type dependent, the order of activity being BCV > 3HM-HBG = (±)2HM-HBG > 2EN-HBG. At growth-inhibitory concentrations, the guanosine analogs BCV, 3HM-HBG, and (±)2HM-HBG showed clastogenic effects in human lymphocytes, mainly because of the induction of chromatid breaks. When evaluated for their anti-HSV effects in systemic HSV-1 infections in mice, the order of activity was BCV = 3HM-HBG > (±)2HM-HBG > 2EN-HBG, and in mice infected systemically with HSV-2, only BCV and 3HM-HBG showed efficacy. The differences between efficacy in vitro and in vivo could be explained in part by differences in kinetics of the drugs in mouse plasma, as the more efficacious drugs, BCV and 3HM-HBG, showed lower clearances and longer half-lives than the less efficacious ones, (±)2HM-HBG and 2EN-HBG. When used topically against a cutaneous HSV-1 infection in guinea pigs, 3HM-HBG showed an effect equivalent to that of BCV, whereas (±)2HM-HBG and 2EN-HBG were inactive. Mechanistically, the guanosine analogs were characterized by a high affinity for the viral thymidine kinase and a low affinity for a cellular thymidine kinase and by their inhibition of viral DNA synthesis in infected cells.

Some acyclic guanosine analogs were recently reported to be selective antiherpesvirus agents (for reviews, see references 9, 25, 28). We reported that the acyclic guanosine analogs 9-(4-hydroxybutyl)guanine (HBG) and (*R*)-9(3,4-dihydroxybutyl)guanine (buciclovir [BCV]) were inhibitors of herpes simplex virus (HSV) replication in cell cultures (10, 21, 22). In addition, BCV showed efficacy in some experimental HSV infections in mice and guinea pigs (10, 22). The specificities of HBG and BCV are determined by their selective phosphorylation to monophosphates by viral thymidine kinases and a preferential inhibition of viral DNA synthesis by their triphosphates (21–23, 33). For example, in HSV type 1 (HSV-1)-infected cells, BCV is rapidly phosphorylated to BCV triphosphate, a specific inhibitor of the viral DNA polymerases (33).

A number of new acyclic guanosine analogs, structurally related to BCV and HBG, were synthesized, and a few of these were inhibitors of HSV-1 replication in cell culture (19). The properties of the active new guanosine analogs, the structures of which are shown in Table 1, were further studied. We showed that the antiviral activity of these new compounds is dependent on the virus-induced thymidine kinase for phosphorylation and on inhibition of viral DNA synthesis. However, despite the similarities in their modes of action, the individual compounds differ from each other in several respects, as shown below.

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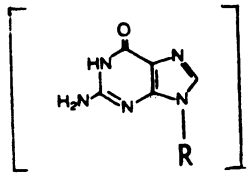
MATERIALS AND METHODS

Materials. The acyclic guanosine analogs BCV, 9-[4-hydroxy-3-(hydroxymethyl)butyl]guanine (3HM-HBG), (*RS*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine ([±]2HM-HBG), and *cis*-9-(4-hydroxy-2-butenyl)guanine (2EN-HBG) were synthesized at Astra Alab AB, Södertälje, Sweden (manuscript in preparation). The monophosphates of the guanosine analogs were prepared enzymatically by using wheat shoot extracts (18). [*methyl*-^{3}H]thymidine (77.2 Ci/mmol) and [α-^{32}P]ATP (>5,000 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass.). Other nucleosides and nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. Hog brain guanylate kinase was from Boehringer GmbH, Mannheim, Federal Republic of Germany.

Cells and viruses. Baby hamster kidney (BHK) cells, African green monkey kidney (Vero) cells, rabbit cornea (SIRC) cells, human embryo lung (HEL) cells, and the guinea pig cell line 1405 (GP 1405) were cultivated to confluent monolayers as described earlier (10) and were used for plaque reduction tests (29). Primary mouse embryo cells and primary guinea pig embryo cells were obtained from the National Bacteriological Laboratory, Stockholm, Sweden. The cells were grown in Eagle minimal essential medium containing Earle salts and 10% fetal calf serum and supplemented with nonessential amino acids. For plaque reduction tests, confluent monolayers of the third or fourth passage were used as described previously (29).

TABLE 1. Chemical structures of the guanosine analogs investigated[a]



R	Systemic name	Abbreviation
$-CH_2-CH_2-CH_2-CH_2OH$	9-(4-hydroxybutyl)guanine	HBG
$-CH_2-CH_2-\underset{\displaystyle OH}{\overset{\displaystyle H}{\underset{|}{\overset{|}{C}}}}-CH_2OH$	Buciclovir (R)-9-(3,4-dihydroxybutyl)guanine	BCV (R-DHBG)
$-CH_2-CH_2-CH\begin{matrix}\diagup CH_2OH\\ \diagdown CH_2OH\end{matrix}$	9-[4-hydroxy-3-(hydroxymethyl)-butyl]guanine	3HM-HBG
$-CH_2-\underset{\displaystyle CH_2OH}{\underset{|}{CH}}-CH_2-CH_2OH$	(RS)-9-[4-hydroxy-2-(hydroxy-methyl)butyl]guanine	(±)2HM-HBG
$-CH_2-CH=CH-CH_2OH$ (cis)	cis 9-(4-hydroxy-2-butenyl)guanine	2EN-HBG

[a] The guanosine ring was substituted in the 9 position with various side chains (R) as indicated.

HSV-1 C-42, 7935-72, and KJ502 and HSV-2 91075, 72, and B4327 were clinical isolates and were previously described (10). HSV-1 strains C1(101) and Sc16 were kindly provided by H. J. Field, Cambridge, United Kingdom. The acyclovir-resistant strains were plaque purified, and infected thymidine-kinase-negative cells produced ≤5% of the thymidine kinase activity of cells infected with the parental strain (10).

Effects on cell proliferation were studied as described previously (32), test compounds being added to actively growing cells and the effects being measured after 48 h. Cell numbers were determined by a cell counter (Analysinstrument AB, Stockholm, Sweden).

Enzyme assays and DNA synthesis. Purified viral thymidine kinase from HSV-1 C-42-infected Vero cells and purified cellular cytosol thymidine kinase from Vero cells, obtained as described previously (22), were used in enzyme kinetic experiments. The K_m and K_i values were determined by linear regression analysis of data in double reciprocal plots (22). Phosphorylation rates were determined by the procedure of Dobersen and Greer (8) with [α-^{32}P]ATP as a substrate as described previously (21–23). The kinetics of phosphorylation of monophosphates of guanosine analogs by guanylate kinase were determined spectrophotometrically by coupling the phosphorylation to the pyruvate kinase-lactate dehydrogenase system and measuring the oxidation of NADH at 340 nm (33).

The inhibition of viral (HSV-1 C-42) DNA synthesis in infected Vero cells was determined by a nucleic acid spot hybridization technique as previously described (16).

Measurement of genotoxicity. Clastogenicity tests were done in vitro with Ficoll-Isopaque (Pharmacia, Uppsala,

Sweden)-separated human peripheral lymphocytes mainly as described by Evans and O'Riordan (11). The cells were grown in RPMI 1640 (Dutch modification) medium containing 20% fetal calf serum (both ingredients from Flow Laboratories, Ayshire, Scotland) at a density of 10^6 cells per ml.

The test compounds were added from stock solutions in dimethyl sulfoxide (DMSO) 22 h after initiation of cultivation (1% DMSO [vol/vol] final concentration) and were present during the remaining incubation period (control cultures also received 1% DMSO). The highest concentrations of test compounds permitting analyses were 1 mM BCV, 2 mM 3HM-HBG, and 2 mM (±)2HM-HBG. The lymphocytes were incubated for 67 h and not, as is usual, for approximately 48 h, as the test compounds induced cell cycle delay.

From each concentration and test compound, 100 Giemsa-stained metaphases were investigated in experiment B (see Table 4). However, in experiment A, the intention was to analyze 200 metaphases, but due to a low mitotic index, only 67 to 191 metaphases could be analyzed. Gaps were not scored as aberrations.

Plasma pharmacokinetics. Male NMRI mice (Anticimex, Sollentuna, Sweden), acclimatized for 3 days and weighing 20 to 22 g, were used in this study. Food was removed 2 h before drug administration. The guanosine analogs were dissolved in saline (0.2 ml) for the intraperitoneal (i.p.) (abdominal cavity) and intravenous (i.v.) (tail vein) administrations of 62.8 μmol of each analog per kg of body weight. Blood samples were taken, deproteinated, and prepared for analysis as previously described (10). The determination of nucleoside analog concentrations in plasma was by high-pressure liquid chromatography on a C18 microsphere column (100 by 46 mm; 3-μm pore size) at a flow rate of 1 ml/min with 10% methanol (vol/vol) in phosphate buffer (pH 6.5; ionic strength, 0.1) as an eluant. Detection was by UV light at 280 nm, the detection limit being 0.3 μM. The retention times for BCV, 3HM-HBG, (±)2HM-HBG, and 2EN-HBG were 2.66, 3.55, 4.08, and 6.01 min, respectively. The data for drug concentrations in plasma were analyzed for model-dependent pharmacokinetic parameters as described before (26). The data were also analyzed by a model-independent method, and parameters such as clearance, area under the concentration-time curve (AUC), and bioavailability were calculated (10).

Animals and virus inoculation. For cutaneous infections, guinea pigs were inoculated as described previously (1) with ca. 10^5 PFU of HSV-1 applied to each of four sites on the back. Unless stated otherwise, HSV-1 C-42 was used. Animals were treated with a 5% suspension (wt/wt) of the guanosine analogs in oil-in-water creams (10) or with 25 μl of a 5% (wt/vol) solution in DMSO. The creams were applied on the back to cover the infected area (1). Treatment was carried out three times daily at 7 a.m., 12 noon, and 5 p.m. for 4 days and was started 24 h after infection. Scores were determined each day for 10 days.

For systemic HSV infections, female NMRI mice (Anticimex) weighing 14 to 15 g were inoculated with HSV-2 or HSV-1 i.p. to produce 80 to 100% mortality in nontreated animals (10, 20). To this end, mice were inoculated with 10^4 PFU of HSV-2 91075 or 10^5 PFU of HSV-1 C-42. For intranasal infections (3), mice were infected with 10^4 PFU of HSV-2 91075. Deaths were recorded for 2 weeks (intranasal infection) or 3 weeks (i.p. infection). i.p. treatment, twice daily at 7 a.m. and 4 p.m., was started 1 h after inoculation (unless indicated otherwise) and continued for 5 days. Drugs were dissolved in phosphate-buffered saline for i.p. treatment or in tap water when given orally via drinking water. Treatment via drinking water was for 10 days starting immediately after infection.

TABLE 2. Inhibition of HSV plaque formation in Vero cells by acyclic guanosine analogs

Virus strain[a]	Mean IC_{50} (μM) ± SD of[b]:			
	BCV	3HM-HBG	(±)2HM-HBG	2EN-HBG
HSV-1 C-42	2.1 ± 1.0	0.3	0.5 ± 0.3	0.8 ± 0.2
C-42 Acv^r	230.0	55.0	60.0	150.0
7935-72	1.2 ± 1.0	0.2 ± 0.1	0.15 ± 0.08	
KJ502	7.0 ± 1.0	0.5 ± 0.2	0.3 ± 0.2	1.0
C1(101)	6.8 ± 2.1	1.5 ± 1.0	1.7 ± 0.7	
Sc16	5.0 ± 2.0	1.9 ± 0.6	1.4 ± 0.3	8.6 ± 1.3
HSV-2 91075	4.0 ± 1.8	7.0 ± 1.0	5.0	
91075 Acv^r	≥250	230	≥250	≥250
72	4.0 ± 3.0	3.6 ± 1.2	10.0 ± 1.0	3.2
B4327	11.0 ± 8.3	7.0 ± 0.9	14.0 ± 3.4	3.7

[a] Acyclovir-resistant strains were obtained from the parenteral strains (see Materials and Methods).

[b] The IC_{50} values were derived from dose-response studies with six different concentrations of each drug.

Statistical analysis. Student's t test was used for evaluation of the mean number of days to death in infected animals; the χ^2 test was used for evaluation of differences in mortality in the mice and for differences in the frequencies of aberrant metaphases. The cutaneous infections were compared by the Mann-Whitney U test. Differences of $P < 0.05$ for two-tailed tests were considered significant.

RESULTS

Antiviral effects in cell cultures. The concentrations of BCV, 3HM-HBG, (±)2HM-HBG, and 2EN-HBG required to inhibit plaque formation in Vero cells by 50% (IC_{50}) by different strains of HSV-1 and HSV-2 are shown in Table 2. In this cell culture system, BCV, 3HM-HBG, and (±)2HM-HBG were less active against the HSV-2 strains than against the HSV-1 strains. 3HM-HBG and (±)2HM-HBG were somewhat more active than BCV and 2EN-HBG against the HSV-1 strains, whereas the anti-HSV-2 activities of these analogs were similar. HSV strains selected for resistance to acyclovir and shown to be defective in thymidine kinase activity (10) were also resistant to BCV, 3HM-HBG, (±)2HM-HBG, and 2EN-HBG (Table 2), showing the role for viral thymidine kinase in the antiherpesvirus activity of these compounds (see also below).

The anti-herpesvirus activity of these guanosine analogs was dependent on the type of host cell (Table 3). Thus, in Vero cells and HEL cells, BCV, 3-HM-HBG, and (±)2HM-HBG were somewhat more active against HSV-1 C-42 than against HSV-2 91075, whereas in SIRC and BHK cells, similar activities for the four guanosine analogs were observed. Inhibition of virus replication in guinea pig cells is peculiar, as high IC_{50} values were observed for the antiviral guanosine analogs (Table 3). This could also be shown with another guinea pig cell line and primary guinea pig embryo cells (data not shown).

Toxic effects in vitro. The toxic effects of the nucleoside analogs were determined by studying the inhibition of cell growth after 48 h of treatment. The IC_{50}s are shown in Table 3. The toxicities were dependent on cell type for all compounds tested. The toxicity of (±)2HM-HBG in the guinea pig cell line GP 1405 was higher than in the other cell lines (Table 3). 2EN-HBG showed low toxicity in all cell lines,

TABLE 3. Inhibition of HSV-1 C-42 and HSV-2 91075 plaque formation and cell growth by the guanosine analogs in different cell lines

Cell type	Mean plaque formation IC_{50} (μM) ± SD in strain with compound[a]								Mean cell growth IC_{50} (μM) ± SD with compound[a]			
	HSV-1 C-42				HSV-2 91075							
	BCV	3HM-HBG	(±)2HM-HBG	2EN-HBG	BCV	3HM-HBG	(±)2HM-HBG	2EN-HBG	BCV	3HM-HBG	(±)2HM-HBG	2EN-HBG
Vero	2.1 ± 1.0	0.3	0.5 ± 0.3	0.8 ± 0.2	4.0 ± 1.8	7.0 ± 1.9	5.0		2,300 ± 300	2,200 ± 210	930 ± 250	≥2,500
SIRC	2.2 ± 0.4	1.3 ± 0.3	1.4		0.9 ± 0.2	1.4 ± 0.5	2.7 ± 1.1	3.6 ± 1.6	800	1,700	≥2,000	1,500
HEL	1.0 ± 0.3	0.3 ± 0.1	0.2 ± 0.1	1.6 ± 0.6	5.9 ± 1.9	5.1 ± 1.9	23 ± 2	16 ± 7	170 ± 11	500 ± 70	870 ± 35	1,790 ± 53
BHK	23 ± 7	3.2 ± 0.8	1.4 ± 0.1	25 ± 3	11	3.0 ± 1.1	1.1 ± 0.1	26 ± 5	130 ± 38	260 ± 7	230 ± 92	≥2,000
GP 1405	68 ± 8	14 ± 3	19 ± 11	100	170	35	11	100	200 ± 70	460 ± 53	95 ± 12	1,310 ± 330
Primary mouse	0.2	0.1	0.1	0.2								

[a] The IC_{50}s were derived from dose-response studies with six (plaque formation) or eight (cell growth) different concentrations of each drug.

whereas BCV, 3HM-HBG, and (±)2HM-HBG were 2 to 20 times more toxic in BHK, HEL, and GP 1405 cells than in SIRC cells. The toxicities of 3HM-HBG, (±)2HM-HBG, and 2EN-HBG in SIRC, BHK, and HEL cells were considerably lower than the antiviral activities of the drugs in these cells (Table 3).

The guanosine analogs 3HM-HBG, (±)2HM-HBG, and BCV were tested for their ability to induce chromosome aberrations in cultures of human lymphocytes. At concentrations of 100 μM or less, the percentage of abnormal metaphases was not higher than in control cultures. However, at concentrations of 1 mM or more, a significant increase in abnormal metaphases was observed (Table 4). At a concentration of 1 mM, BCV caused a statistically significantly greater proportion of abnormal metaphases than 3HM-HBG or (±)2HM-HBG ($P < 0.01$), whereas the difference between (±)2HM-HBG and 3HM-HBG was not significant. At a concentration of 2 mM, (±)2HM-HBG was more genotoxic than 3HM-HBG ($P < 0.001$). The type of aberration induced by these compounds was almost exclusively chromatid breaks.

Effect of treatment with guanosine analogs on mortality of mice infected with HSV. In i.p. infected mice, virus replicates initially in visceral organs and then spreads to the central nervous system and causes death (20). The results on mortality of treatment with BCV, 3HM-HBG, (±)2HM-HBG, and 2EN-HBG in HSV-1 C-42-infected animals are shown in Table 5. When drugs were given by the i.p. route, 20 mg of 3HM-HBG or BCV per kg per day gave complete protection. In this experiment, (±)2HM-HBG or 2EN-HBG even at higher doses was less efficacious; high mortality still resulted. Oral treatment by supplying the drugs in the drinking water (1 mg/ml) gave good therapeutic effects (<10% mortality) with BCV, 3HM-HBG, and (±)2HM-HBG (not shown). In HSV-2 91075, infected animals i.p. treated with 20 mg of 3HM-HBG per kg per day showed decreased mortality (94 to 20%), whereas (±)2HM-HBG and 2EN-HBG given i.p. did not protect the animals even at the highest dose tested (Table 6). By supplying the drugs in the drinking water, good protection was obtained with 3HM-HBG but not with (±)2HM-HBG, despite similar oral bioavailabilities (see below).

After intranasal inoculation of mice with HSV-2, virus is transmitted by neural routes to the olfactory lobe, cerebellum, and brain stem (3, 20) and the mice die 6 days postinfection. BCV, 3HM-HBG, and (±)2HM-HBG supplied in the drinking water as above did not reduce the mortality nor the mean day to death of mice infected intranasally with strain 91075 of HSV-2 (data not shown; 2EN-HBG was not tested).

Topical treatment with guanosine analogs of cutaneous HSV infections in guinea pigs. In cutaneous HSV-1 infections in guinea pigs, viral replication occurs in the skin 1 to 7 days postinfection; lesions are formed, which crust over and heal during 6 to 12 days postinfection (1). In HSV-1 C42-infected animals, topical treatment with 50 mM of 3HM-HBG or BCV in DMSO gave therapeutic effects with reductions in the cumulative score of 36 or 27%, respectively. No effects

TABLE 4. Effect of acyclic guanosine analogs on the frequency of chromosome abnormalities in human lymphocytes in vitro

Drug	Concn (μM)	Mitoses/1,000 cells		% Abnormal metaphases		No. of chromatid breaks/100 metaphases	
		Expt A	Expt B	Expt A	Expt B	Expt A	Expt B
None		11	24	3.7	9.0[a]	3	7
BCV	500	12	14	11.5[b]	12.0	10	10
	1,000	4	9	30.8[c]	44.0[c]	32	59
3HM-HBG	500	10	14	3.1	11.0	2	9
	1,000	4	11	13.7[d]	18.0	10	17
	2,000	5	NT[e]	20.3[c]	NT	18	NT
(±)2HM-HBG	500	11	13	7.5	8.0	6	7
	1,000	8	16	14.1[d]	12.0	11	10
	2,000	6	NT	51.3[c]	NT	105	NT

[a] A 9% aberration frequency in the untreated control is exceptionally high. Usually, this value is 2 to 5%.
[b] $P < 0.05$ (χ^2 test) relative to untreated controls.
[c] $P < 0.001$ (χ^2 test) relative to untreated controls.
[d] $P < 0.01$ (χ^2 test) relative to untreated controls.
[e] NT, Not tested.

were seen with (±)2HM-HBG or 2EN-HBG (100 mM in DMSO). Topical treatment with 5% 3HM-HBG (200 mM) in a cream decreased, compared with placebo cream, the cumulative score of infection caused by HSV-1 C-42, 7935-72, and KJ 502 by 34, 33, and 48%, respectively, whereas an infection caused by a thymidine kinase-deficient strain of HSV-1 (see above, C915 TK^-) was refractive to treatment with 3HM-HBG.

Pharmacokinetic studies. The concentration-time curves in plasma of the three BCV analogs administered as a single i.v. bolus or i.p. injection are shown in Fig. 1. The relevant pharmacokinetic parameters of the three analogs and of BCV are summarized in Table 7. The concentration-time curves in plasma after i.v. administration of the analogs showed a biexponential decline, whereas the curves for BCV showed a triexponential decline (10). The half-lives associated with the largest portion of the AUCs for BCV, 3HM-HBG, (±)2HM-HBG, and 2EN-HBG (Table 7) were 19.7, 10.4, 6.8, and 6.4 min, respectively, and their plasma clearances were 21.1, 38.4, 44.1, and 59.6 ml/min per kg, respectively.

Biochemical studies. The affinities of 3HM-HBG, (±)2HM-HBG, and 2EN-HBG for purified HSV-1 thymidine kinase were determined in enzyme kinetic experiments. The analogs were competitive inhibitors of thymidine phosphorylation, and K_i values are shown in Table 8. The phosphorylation rates by the viral thymidine kinase were determined at concentrations of 250 μM for each analog and compared with that of 250 μM thymidine (Table 8). The results show that the guanosine analogs all have a high affinity for the viral thymidine kinase and can also act as alternative substrates, although the relative substrate efficiencies differ from each other. The guanosine analogs were not substrates for cellular cytosol thymidine kinase (Table 8).

Another enzyme assumed to be involved in phosphorylation of acyclic guanosine analogs is cellular guanylate kinase (27). The enzyme kinetic constants of the guanosine analog monophosphates for this enzyme are shown in Table 8. Thus, when the relative substrate efficiencies for the viral thymidine kinase and cellular guanylate kinase are compared, BCV and BCV monophosphate, respectively, are better substrates than the other acyclic guanosine analogs and their monophosphates, respectively.

The ability of the guanosine analogs to inhibit viral DNA synthesis was measured by nucleic acid hybridization with a spot test (16). The IC_{50}s obtained for HSV-1 C-42 DNA synthesis in Vero cells were 0.18 μM for 3HM-HBG, 0.13 μM for (±)2HM-HBG, and 0.9 μM for 2EN-HBG. The values correspond well to the IC_{50}s found in the plaque reduction test for this virus strain (Table 2).

TABLE 5. i.p. treatment of systemic HSV-1 C-42 infections in mice with guanosine analogs

Compound	Dose (mg/kg per day)[a]	No. of deaths/no. of mice treated	Mean no. of days to death ± SD
Placebo		15/16	7.9 ± 0.8
BCV	5	5/10	9.2 ± 0.4[b]
	10	0/10[b]	
	20	0/10[b]	
3HM-HBG	10	3/10[b]	9.3 ± 0.6[b]
	20	0/10[b]	
(±)2HM-HBG	10	4/10	9.0 ± 2.9
	20	6/10	8.6 ± 0.8[b]
	50	3/10[b]	9.6 ± 1.2[b]
	100	3/10[b]	8.0 ± 1.7
2EN-HBG	50	8/10	8.1 ± 1.9
	100	6/10	9.1 ± 0.8[b]

[a] See Materials and Methods for administration mode.
[b] Result is significantly different from results with placebo-treated mice ($P < 0.05$ [χ^2 test, mortality; Student's t test, mean days to death]).

TABLE 6. Treatment in mice of systemic HSV-2 91075 infections with guanosine analogs[a]

Treatment route and compound	Dose (mg/kg per day)[b]	No. of deaths/no. of mice treated	Mean no. of days to death ± SD
i.p.			
Placebo		15/16	8.2 ± 2.2
BCV	10	2/10[c]	11.0
	25	2/10[c]	13.0
	50	1/10[c]	13
3HM-HBG	10	7/10	10.1 ± 2.7[c]
	20	2/10[c]	14.5
	50	0/10[c]	
(±)2HM-HBG	10	9/10	8.7 ± 1.0
	20	10/10	10.9 ± 2.9[c]
	50	14/15	9.3 ± 2.9
	100	15/15	8.9 ± 1.6
2EN-HBG	10	9/10	8.9 ± 0.8
	20	10/10	8.4 ± 1.8
	50	9/10	8.3 ± 1.8
Oral[d]			
BCV	400	3/16[c]	13.7 ± 2.1[c]
3HM-HBG	400	3/15[c]	9.8 ± 3.2
(±)2HM-HBG	400	11/15	8.1 ± 0.9

[a] Of 16 infected untreated mice, 15 died; mean day to death was 8.2 ± 2.2.
[b] See Materials and Methods for administration mode.
[c] Result is significantly different from placebo-treated control ($P < 0.05$ [χ^2 test, mortality; Student's t test, mean days to death]).
[d] In drinking water; dose estimated from daily intake of water.

DISCUSSION

To determine the effects of structural modifications of BCV on anti-HSV activity, a number of analogs with different acyclic side chains were synthesized (19). Of these,

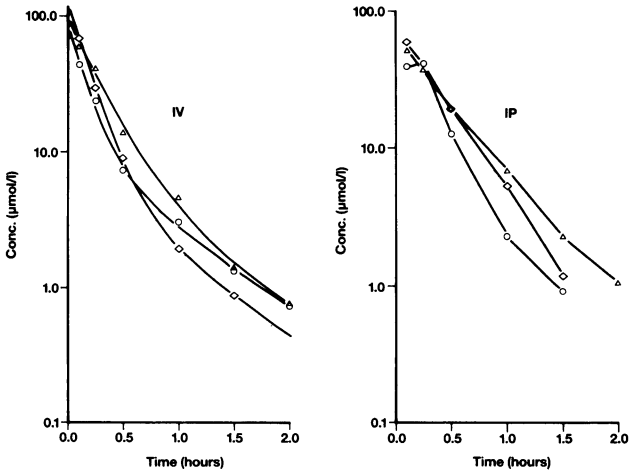


FIG. 1. Mean levels of guanosine analogs in plasma in male mice. Mice were given a single dose of 62.8 μmol of guanosine analog per kg of body weight by the i.v. route or i.p. route. △, 3HM-HBG; ◇, 2EN-HBG; ○, 2EN-HBG (all fitted to a two-compartment model). Each point represents the mean value for n = 5. For detection limits, see text. For kinetics of BCV, see reference 10.

TABLE 7. Mean pharmacokinetic parameters for the guanosine analogs in mice[a]

Compound	$AUC_{0-\infty}$ i.v. (μmol · h/liter) (range)	% AUC during phase: α	β	γ	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	Clearance (ml/min per kg) (range)	% Oral bioavailability
BCV	49.5 (44.9–55.5)	30	55	15	6.4	19.7[b]	21.1 (18.9–23.4)	13 (12.4–16.5)
3HM-HBG	27.3 (26.7–29.5)	75	25		10.4	33.3	38.4 (35.6–39.3)	20 (14.6–23.2)
(±)2HM-HBG	23.8 (21.2–26.3)	80	20		6.8	30.8	44.1 (39.9–49.5)	19 (16.0–20.5)
2EN-HBG	18.4 (18.1–21.5)	60	40		6.4	31.7	56.9 (49.0–58.1)	50 (43.5–54.8)

[a] Drugs were administered i.v. or orally at a dose of 63 μmol/kg of body weight, and five animals for each sampling time and route of administration were included. $AUC_{0-\infty}$, Area under the concentration-time curve from 0 h to infinity; $t_{1/2}$, half-life.
[b] The $t_{1/2\gamma}$ was 143.4 min.

3HM-HBG, (±)2HM-HBG, and 2EN-HBG were rather active inhibitors of HSV replication in cell cultures. As noted previously (10) with related guanosine analogs, the antiviral effects were dependent on cell type and virus strain, yet 3HM-HBG, (±)2HM-HBG, and 2EN-HBG were usually more active than BCV as inhibitors of replication of HSV-1 but not of HSV-2. It remains to be investigated why, for example, drug concentrations 150 to 500 times higher were needed to inhibit plaque formation in guinea pig cells to the same extent as in mouse cells. Like BCV and HBG, the three new analogs had high affinities for the HSV-1-induced thymidine kinase but not for a cellular thymidine kinase. In addition, they were substrates for this enzyme and not for the cellular enzyme, and in cells infected with thymidine-kinase-deficient strains of HSV, an antiviral effect was not observed unless high concentrations of the drugs were used.

These results point to the crucial role of the viral thymidine kinase in the antiherpesvirus effects of 3HM-HBG, (±)2-HM-HBG, and 2EN-HBG. Another enzyme assumed to be involved in phosphorylation of acyclic guanosine analogs is the cellular guanylate kinase (27). Indeed, the monophosphates of 3HM-HBG, (±)2HM-HBG, and 2EN-HBG were also substrates for this enzyme. It remains to be investigated whether triphosphates of these nucleoside analogs are formed in the infected cells and whether the triphosphates inhibit the viral DNA polymerase, as previously shown for BCV and other acyclic guanosine analogs (7, 13, 15, 17, 31, 33). Such a mechanism of action is likely, however, as in infected cells viral DNA synthesis was inhibited at concentrations of the nucleoside analogs giving antiviral effects.

The cellular toxicities of the acyclic guanosine analogs were generally low in all cell types studied. This indicates that the acyclic nucleoside analogs at concentrations under 50 μM did not affect cellular processes necessary for cell growth. For comparison, addition of 35 μM of thymidine reduces the growth of L1210 cells by 50% after 45 h of incubation (24). When the anti-HSV effect was compared with the anticellular effect on a concentration basis for different cell lines (Table 3), high therapeutic indices were found for all compounds in SIRC and HEL cells, whereas lower values were found in BHK and GP 1405 cells. The differences in toxicity among the cell lines were not due to differences in growth rates, media, or other experimental conditions but might reflect inherent differences among the cell lines.

The appearance of chromosomal breaks at concentrations that are growth inhibitory suggests that the toxicity of acyclic guanosine analogs may involve effects on the synthesis of DNA. The type of chromosomal effects, chromatid breaks, might be expected if the compounds can be incorporated into DNA or at least interfere with DNA synthesis in a way that results in the premature termination of DNA synthesis. This has been shown to occur in cells expressing HSV thymidine kinase with acyclovir and 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG), which can be incorporated into DNA after metabolism to triphosphates in these cells (4, 14). Indeed, 0.5 to 1 mM acyclovir caused chromatid breaks to the same extent as BCV (data not shown). Natural nucleosides at high concentrations (about 1 mM) also caused chromosome aberrations, possibly due to imbalances in nucleotide pools, and all kinds of structural aberrations were observed (2). This is in marked contrast to the almost exclusive induction of chromatid breaks by BCV, 3HM-HBG, and (±)2HM-HBG as shown here and by acyclovir as shown by Clive et al. (5), suggesting different genotoxic mechanisms for the guanosine analogs and natural nucleosides.

TABLE 8. Comparison of enzyme kinetic parameters for guanosine analogs and guanosine analog monophosphates in the thymidine kinase and guanylate kinase reactions

Substrate	HSV-1 C42 thymidine kinase		Cell thymidine kinase (Vero cells)		GMP kinase[a]	
	% Phosphorylation rate[b]	K_i (K_m) (μM)	% Phosphorylation rate[b]	K_i (K_m) (μM)	K_m (μM)	V_{max} (μmol/min per mg)
Thymidine	100	0.4	100	1.3		
BCV	73	1.5	<5	>250		
3HM-HBG	9	1.5	<5	>250		
(±)2HM-HBG	17	0.6	<5	>250		
2EN-HBG	48	7.0	<5	>250		
dGMP					120	3.2
BCV-MP					62	1.5
3HM-HBG-MP					260	1.0
(±)2HM-HBG-MP					120	0.1
2EN-HBG-MP					220	0.3

[a] Hog brain guanylate kinase (see Materials and Methods).
[b] Relative to phosphorylation of thymidine.

It is of interest that, whereas 3HM-HBG, (±)2HM-HBG, and 2EN-HBG had similar IC_{50}s for HSV-1 C-42 plaque formation in mouse cells, the compounds differed in efficacy in the i.p. treatment of mice infected systemically with this HSV-1 strain. This difference can in part be explained by differences in the pharmacokinetic properties of these drugs, such as the plasma clearances and the half-lives associated with the largest portion of the AUCs. The effects of these two parameters are also reflected in the amount of drug obtained in blood over a period of time, as shown by the AUC values (Table 7). For example, the AUC of 3HM-HBG is 1.5 times that of 2EN-HBG. The inactivity of (±)2HM-HBG and especially of 2EN-HBG is not surprising, since these drugs persist in the body only for a short time due to extremely short half-lives and large plasma clearances.

Nevertheless, the i.p. administration of 10 mg of 3HM-HBG per kg gives levels of the drug in serum exceeding the IC_{50} in mouse cells (0.1 μM) only during the 3 h after injection, whereas the twice-daily administration of this dose was sufficient to give full protection. This phenomenon was also observed for BCV (10), and we assumed (6) that the high rate of phosphorylation of BCV and the stability of BCV triphosphate in infected cells (33) make the antivirally active compound (BCV triphosphate) persist in infected cells, even though levels of BCV in serum have declined below the IC_{50} value. A similar trapping mechanism was proposed to explain the potent effects in vivo of DHPG (17, 30).

When mice were inoculated i.p. with HSV-1 or HSV-2, BCV, 3HM-HBG, and (±)2HM-HBG appeared to be more effective against the HSV-1 infection than against the HSV-2 infection (Tables 5 and 6). This reflects the activities in cell culture studies. The inferior efficacies of (±)2HM-HBG and 2EN-HBG relative to 3HM-HBG were observed not only in systemic HSV-1 infections but also in the treatment of HSV-2-infected mice and in topical treatment of cutaneous HSV-1 infections in guinea pigs. In fact, the results obtained with infected animals show that 3HM-HBG, (±)2HM-HBG, and 2EN-HBG were not more efficacious than BCV.

The synthesis and some properties of 3HM-HBG were recently reported independently by two other groups (25, 34). Also, in their studies, it was shown that the drug was more active in inhibiting HSV-1 plaque formation than HSV-2 plaque formation, which was in contrast to results obtained with the congener of 3HM-HBG, DHPG (12). The fact that 3HM-HBG, (±)2HM-HBG, and 2EN-HBG are active against HSV-1 but are less active against HSV-2 limits their utility. However, when investigating the effects of the drugs against herpesviruses other than HSV, it was observed (B. Wahren, unpublished results) that (±)2HM-HBG inhibited several strains of varicella-zoster virus at an IC_{50} of 0.3 to 5.0 μM. Further investigations are required to determine the clinical potential of this guanosine analog as an anti-varicella-zoster drug.

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