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9-(3,4-Dihydroxybutyl)guanine, a New Inhibitor of Herpesvirus Multiplication

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A new compound, 9-(3,4-dihydroxybutyl)guanine, has been synthesized and its antiherpes activity determined. 9-(3,4-Dihydroxybutyl)guanine was selectively phosphorylated by herpes simplex virus thymidine kinase and had a high affinity for this enzyme, with an inhibition constant of 1.5 μM. In cell culture, replication of different strains of herpes simplex virus types 1 and 2 was inhibited to the extent of 50% by 4 to 18 μM (*RS*)-9-(3,4-dihydroxybutyl)guanine. The (*R*)-enantiomer of this compound was more inhibitory than the (*S*)-enantiomer. Herpesvirus DNA synthesis was selectively inhibited by (*RS*)-9-(3,4-dihydroxybutyl)guanine in infected cells, and a low cellular toxicity was observed. (*RS*)-9-(3,4-Dihydroxybutyl)guanine had a therapeutic effect when applied topically to guinea pigs with cutaneous herpes simplex type 1 infections and to rabbits with herpes keratitis. Oral treatment of a generalized herpes simplex type 2 infection in mice had a therapeutic effect.

Two principally different types of herpesvirus inhibitors have recently been designed. One utilizes herpes simplex virus (HSV) thymidine kinase (TK) to selectively phosphorylate the inhibitor which, after phosphorylation to a triphosphate, interferes with viral DNA synthesis. Both these steps are competitive reactions which depend on cellular concentrations of thymidine (dThd) and nucleoside triphosphates. Examples of this type of inhibitor are acyclovir (ACV) (11, 24) and bromovinyldeoxyuridine (4, 8). The other principle for herpesvirus inhibitors can be exemplified by the noncompetitive inhibition of herpesvirus DNA polymerase by foscarnet (phosphonoformate) (12, 15, 23) and phosphonoacetate (5, 18, 20, 21). In this case, the cellular concentrations of dThd and nucleoside triphosphates do not interfere with the antiherpes activity (25).

A nucleoside analog that is useful as an antiherpes drug should preferably contain a normal base to decrease the risk of mutagenic effects and, in addition, if incorporated into DNA, should not allow chain elongation. ACV is a compound fulfilling these requirements (11, 24), but its ability to compete with dThd is rather low (16; A. Larsson, S. Alenius, N.-G. Johansson, and B. Öberg, Antiviral Res., in press), which should lead to decreased efficacy if a high dThd concentration is present in the infected organ.

A series of new nucleoside analogs has been synthesized in which a high affinity for herpesvirus TK, a normal base, and a low probability of internal incorporation into DNA have been attempted. One of these, 9-(3,4-dihydroxybutyl)-guanine (DHBG [Fig. 1]), was found to have the desired properties of an antiherpes agent. This paper describes the antiviral effect of this compound in cell culture, its cellular toxicity, phosphorylation by different kinases, probable mechanism of action, and therapeutic efficacy in animal models.

MATERIALS AND METHODS

Materials. (*RS*)-DHBG and the (*R*)- and (*S*)-enantiomers were synthesized as will be described elsewhere (manuscript in preparation). [*methyl*-^{3}H]dThd (specific activity, 77.2 Ci/mmol) and [γ-^{32}P]ATP (specific activity, >5,000 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass., and carrier-free P_i (1 mCi/ml) was obtained from the Radiochemical Centre, Amersham, England.

Purification of TK. Vero cells were grown in roller bottles (625 cm^2) and infected with HSV type 1 (HSV-1) strain C42 at a multiplicity of 0.5 to 1 PFU per cell and incubated at 37°C for 16 h (70 to 90% cytopathic effect). The HSV-1-infected or uninfected Vero cells were harvested and treated before the final application to the CH-Sepharose-dThd affinity column as described by Cheng and Ostrander (6). The application and separation of HSV-1 and cellular cytosol TK on the affinity column were carried out by a procedure described previously (14). HSV-1 TK was extracted at about 40 to 60 μM dThd, and the cellular cytosol TK was extracted at about 80 to 100 μM dThd. The combined fractions containing TK activity were dia-

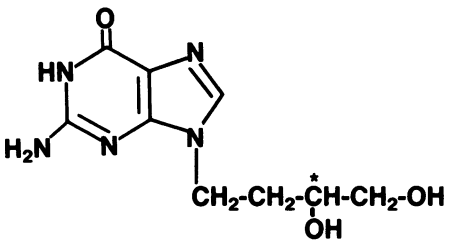

FIG. 1. Structure of DHBG.

lyzed and concentrated as described previously (14).

TK assay. For the enzymes used in this study, 1 U of activity was defined as the amount of enzyme that could catalyze the formation of 1 pmol of product per min at 37°C in a standard assay. Samples containing enzymes were assayed in 100 μl of reaction mixture containing 150 mM Tris-hydrochloride (pH 7.5), 2 mM ATP, 2 mM $MgCl_2$, 3 mM dithiothreitol, and 10 μM [^{3}H]dThd. After incubation at 37°C for 30 min, 50 μl of the reaction mixture was added to Whatman DE 81 paper disks (2.3 cm) and washed twice with water and three times with ethanol. The dried paper disks were counted in 3 ml of Econofluor scintillation solution.

The kinetic studies were performed with 2 U of HSV-1 TK or Vero cell cytosol TK. The incubation period was 5 min at 37°C, and the reaction mixture was the same as that used for the standard TK assay, with different amounts of [^{3}H]dThd. The K_m value for dThd and the K_i values for the tested compounds were determined by linear regression analyses of the data obtained, which were plotted in double reciprocal plots. The nomenclature of Cleland (7) was used. To determine the ability of the test compounds to act as substrates for the TKs, we applied the procedure of Dobersen and Greer (10) and used 100 μl of reaction mixture containing 150 mM Tris-hydrochloride (pH 7.5), 0.5 mM [γ-^{32}P]ATP, 2 mM $MgCl_2$, 3 mM dithiothreitol, 250 μM test compound, and TK.

Cells and viruses. Vero cells (CCL81) were grown and used for plaque assays as described previously (13, 25). Most of the HSV-1 and HSV-2 strains used have been described previously (13, 15, 28).

The method used to determine viral and cellular DNA synthesis by ^{32}P labeling and isodensity gradient centrifugation has been reported previously (18, 19).

Effects on cell proliferation were determined as described previously (27). The test compound was added to actively growing Vero, 3T3 (CCL92, mouse embryo) or Flow 5000 (human embryo) cells and incubated for 48 h. Cell numbers and volumes were measured by use of an electronic cell counter (Analys-instrument AB, Stockholm, Sweden).

Animal experiments. The method for inoculating the backs of guinea pigs with HSV-1, as well as the scoring system used to determine the therapeutic effect of treatment, have been described previously (3). The herpes keratitis model, in which rabbits are inoculated in the cornea with HSV-1, has been described previously (2). In the experiments with systemic herpes infections, NMRI mice weighing 14 to 15 g each were inoculated intraperitoneally with 10^4 PFU of HSV-2 strain 91075 as described by Kern et al. (17).

RESULTS

Phosphorylation of DHBG by TKs. The phosphorylation rates by viral and cellular TK of (*RS*)-, (*R*)-, and (*S*)-DHBG at concentrations of 250 μM each were determined and compared with that of dThd (Table 1). Owing to the sensitivity of the assay for measuring transfer of γ-^{32}P from [γ-^{32}P]ATP to the tested compound, the K_m values could not be determined. The (*S*)-enantiomer showed a lower amount of product formed, 46% of that obtained when dThd was used as substrate, whereas the amount of product formed for (*RS*)- and (*R*)-DHBG was about 74%. The explanation for the same rates of phosphorylation of (*RS*)- and (*R*)-DHBG is that the high concentration used was at the saturating level, and at this concentration the lower phosphorylation rate of (*S*)- in (*RS*)-DHBG was not detectable. No phosphorylation was detectable when cytosol TK was used for these compounds.

The affinity for HSV-1 TK and cytosol TK was determined in enzyme kinetic experiments. (*RS*)-DHBG was a competitive inhibitor of dThd phosphorylation, with a K_i value of 1.5 μM for HSV-1 TK (Fig. 2). By replotting the data from the double-reciprocal plot, the K_{is} (slope) and K_{ii} (intersept) values could be determined (Fig. 2). No difference in the affinity for the (*R*)- and (*S*)-enantiomer was detectable. When cytosol TK was used, (*RS*)-, (*R*)-, and (*S*)-DHBG all showed a K_i of >250 μM (Table 1). These results show that DHBG has a high and preferential affinity for HSV-1 TK as compared with cytosol TK and that DHBG can act as an alternative substrate for viral TK with a high phosphorylation rate.

Inhibition of herpesvirus multiplication in cell culture by DHBG. The inhibition of HSV-1 and HSV-2 multiplication in cell cultures was determined at different concentrations of (*RS*)-DHBG, and the 50% inhibitory doses are shown in Table 2. No major difference could be seen

TABLE 1. Cell-free phosphorylation of DHBG by TKs[a]

Compound	Relative rate of phosphorylation		K_i	
	HSV-1 TK	Cytosol TK	HSV-1 TK	Cytosol TK
(*RS*)-DHBG	74.7 ± 16.5	<5	1.53 ± 0.33	>250
(*R*)-DHBG	73.4 ± 10.5	<5	1.47 ± 0.21	>250
(*S*)-DHBG	46.0 ± 8.5	<5	1.69 ± 0.32	>250

[a] The assay was carried out as described in the text; results shown are mean (± standard deviation) values from five different determinations. The relative rate of phosphorylation for dThd was 100% for both HSV-1 TK and cytosol TK, and its K_m was 0.41 ± 0.11 for HSV-1 TK and 1.31 ± 0.13 for cytosol TK.

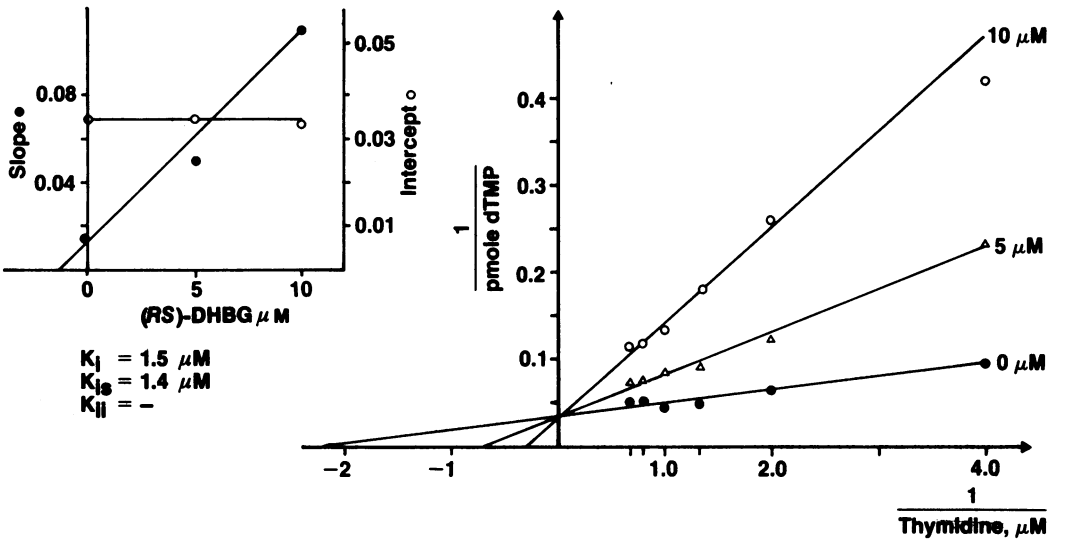

FIG. 2. Inhibition of dThd phosphorylation by (*RS*)-DHBG. dThd was used as the varied substrate in this reaction with purified HSV-1 TK and (*RS*)-DHBG as inhibitor. The assay is described in the text.

between the inhibition of HSV-1 and HSV-2, and it was evident that different HSV strains exhibited different susceptibilities to (*RS*)-DHBG. Strains that lacked viral TK, such as HSV-1 C915 TK−, or were resistant to ACV (C42, ACV^r) were also resistant to (*RS*)-DHBG. A 50% inhibition of HSV-1 C42 was seen with 6 μM (*RS*)-DHBG, 4 μM (*R*)-DHBG, and 12 μM (*S*)-DHBG. The inhibition by (*RS*)-DHBG could be reversed by dThd but not by deoxyuridine (data not shown).

Effect of DHBG on cellular and viral DNA synthesis. The inhibition of cellular and HSV-1 DNA synthesis in infected and uninfected cells by (*RS*)-DHBG is shown in Fig. 3. At a concentration of 2.5 μM (*RS*)-DHBG, HSV-1 DNA synthesis was inhibited by 49% and cellular DNA synthesis by 17%. When the HSV-1 DNA synthesis was totally inhibited at 100 μM (*RS*)-DHBG, the cellular DNA synthesis was inhibited by 84%. However, the DNA synthesis in uninfected cells was not inhibited at this concentration, and at 500 μM (*RS*)-DHBG, the DNA synthesis was inhibited by only 7%.

TABLE 2. Inhibition of HSV plaque formation by (*RS*)-DHBG[a]

Virus strain	ID_{50} (μM)
HSV-1 C42	6
HSV-1 7935-72	4
HSV-1 KJ502	11
HSV-1 C915 TK$^-$	>200
HSV-1 C42 ACV^r	>200
HSV-2 72	12
HSV-2 91075	12
HSV-2 B4327	18

[a] The plaque assay was carried out as described in the text. The 50% inhibitory dose (ID_{50}) was calculated from dose-response curves with seven different (*RS*)-DHBG concentrations.

Cellular toxicity of DHBG. The effects of (*RS*)-DHBG on cellular proliferation were determined at concentrations of 100 and 500 μM (Table 3). At 500 μM, (*R*)- and (*RS*)-DHBG gave 50% inhibition and (*S*)-DHBG gave 15% inhibition of Vero cell multiplication, whereas no effect on cell volume was observed (data not shown).

Animal experiments with DHBG. The therapeutic effect of (*RS*)-DHBG on HSV-1 cutaneous infection in guinea pigs is shown in Fig. 4. A 5% (wt/wt) (*RS*)-DHBG water-based cream was applied topically to infected skin areas, and the daily lesion score is shown for drug- and placebo cream-treated areas. (*RS*)-DHBG reduced the lesion score by 55%, and no skin irritation was observed.

Topical treatment of herpes keratitis in rabbits showed that 3% (wt/wt) (*RS*)-DHBG in an occulentum simplex formulation rapidly reduced the keratitis score (Fig. 5). No irritation was observed in uninfected eyes treated with the 3% (wt/wt) (*RS*)-DHBG formulation.

Mice inoculated intraperitoneally with HSV-2 were treated orally with 150 mg of (*RS*)-DHBG per kg of body weight per day for 10 days (Fig. 6). (*RS*)-DHBG reduced the mortality rate by 70%, and no toxicity was observed.

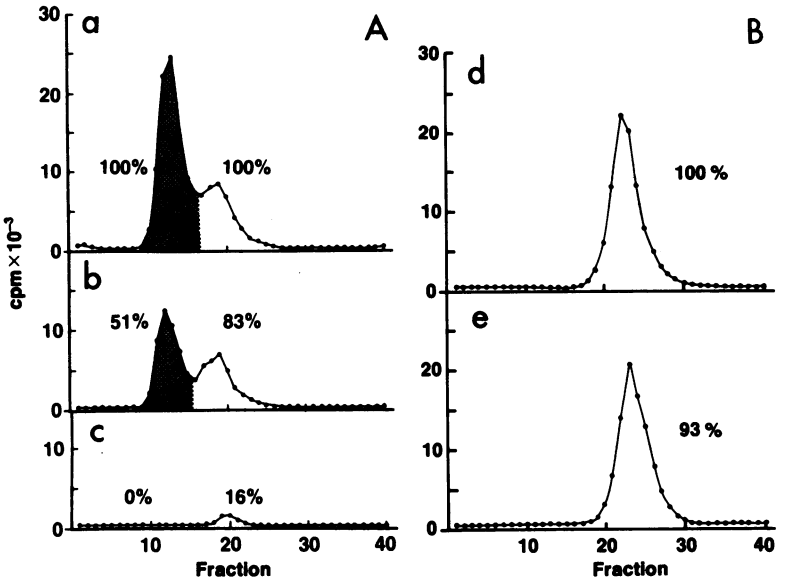

FIG. 3. Inhibition of cellular and HSV-1 DNA synthesis by (*RS*)-DHBG. Viral and cellular DNA were labeled with ^{32}P and separated as described in the text. The shaded area denotes viral DNA. [^{3}H]dThd-labeled DNA from HSV-1-infected or uninfected and untreated cells was used as internal density marker (data not shown). (A) HSV-1-infected Vero cells: (a) control; (b) 2.5 μM (*RS*)-DHBG; (c) 100 μM (*RS*)-DHBG. (B) Uninfected Vero cells: (d) control; (e) 500 μM (*RS*)-DHBG.

DISCUSSION

The three principal ideas behind the search for a new antiherpes agent are that it should be a nucleoside analog with a normal base, should compete well with dThd in selective phosphorylation by HSV TK, and should not be incorporated internally into DNA. These criteria appear to be met by the newly synthesized compound DHBG.

The phosphorylation of DHBG is a selective process carried out only by HSV TK (Table 1). The observed K_i value for DHBG was about 115-fold lower than that observed for ACV, which was 173 μM (Larsson et al., in press). A small difference was seen between the rates of phosphorylation of the enantiomers, and it was highest for (*R*)-DHBG.

TABLE 3. Effect of DHBG on cell proliferation

Cell	Compound	% inhibition by concn (μM) of:	
		100	500
Vero	(*RS*)-DHBG	22	50
	(*R*)-DHBG		52
	(*S*)-DHBG		15
Flow 5000	(*RS*)-DHBG		53
3T3	(*RS*)-DHBG	29	48

In cell culture, both HSV-1 and HSV-2 multiplication (Table 2) were inhibited at concentrations of DHBG considerably lower than that causing inhibition of cell proliferation (Table 3). The decreased inhibition of HSV strains either lacking TK or with a TK resistant to ACV shows that phosphorylation by HSV TK is an obligatory step in the inhibition of HSV multiplication by DHBG. A selective inhibition of HSV DNA synthesis in infected cells (Fig. 3) suggests that a phosphorylated form of DHBG selectively inhibits the viral DNA synthesis by analogy with inhibition by ACV (19) and foscarnet (18). It remains to be seen whether DHBG will act as a chain terminator.

It should be noted that a compound such as foscarnet, which is not affected by the presence of dThd (25), is considerably more effective for treatment of cutaneous HSV-1 infection in guinea pigs than is (*RS*)-DHBG or ACV, although it is less active in cell cultures (1). A high dThd concentration has been observed in guinea pig skin (J. Harmenberg, personal communication), and it would be interesting to determine the dThd content of different organs in animals, including humans, since this could influence the efficacy of antiherpes drugs phosphorylated by viral TK.

A good therapeutic activity was found for (*RS*)-DHBG against herpes keratitis in rabbits (Fig. 5) and against a systemic herpes infection

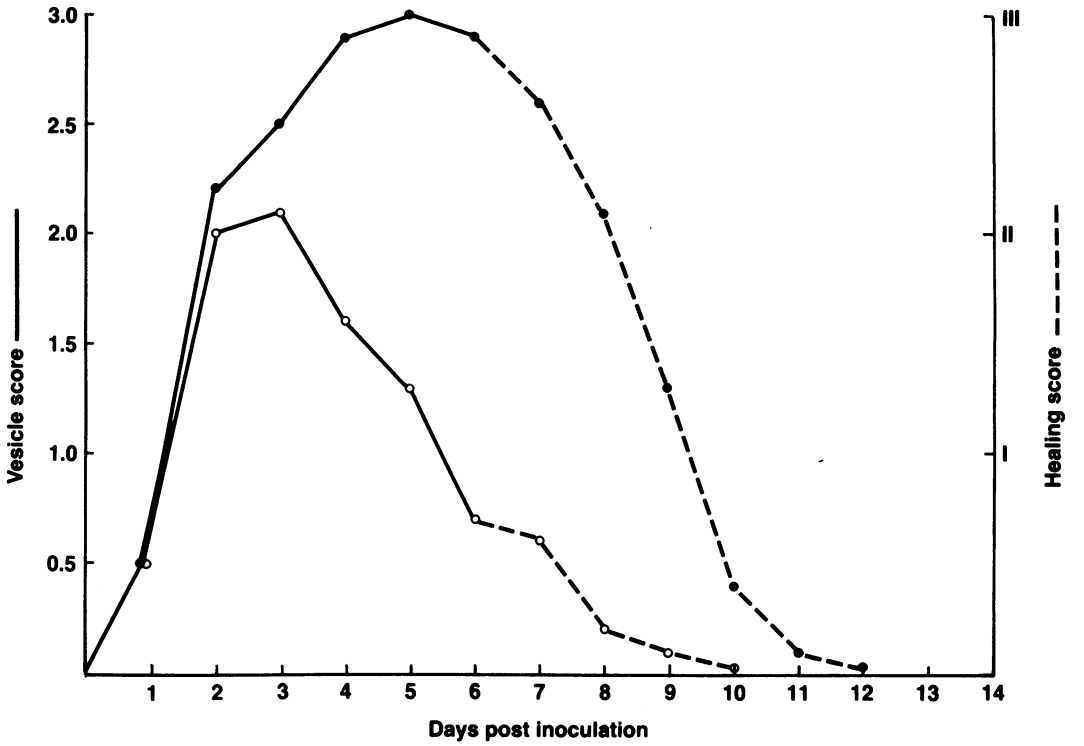

FIG. 4. Therapeutic effect of (*RS*)-DHBG on cutaneous HSV-1 infection in guinea pigs. Topical treatment with HSV-1 7935-72 started 24 h post-inoculation with 20 μl of cream and consisted of four daily applications for 5 days. Each point represents the average score of 12 treated skin areas. The cumulative score for the placebo-treated areas was 19.8 ± 3.8, and the corresponding score for (*RS*)-DHBG treated areas was 9.0 ± 3.8. The difference is statistically significant ($P < 0.001$; Student's *t* test). ●, Placebo cream; ○, 5% (wt/wt) (*RS*)-DHBG cream.

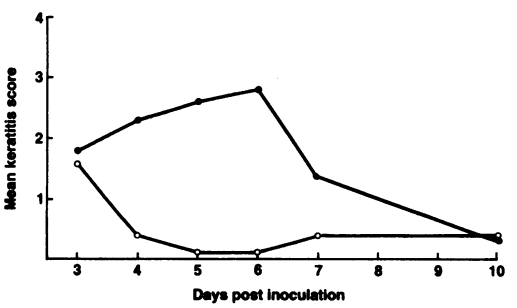

FIG. 5. Therapeutic effect of (*RS*)-DHBG on herpes keratitis in rabbits. Rabbits were inoculated with HSV-1 C42 and treated topically with 100 μl of occulentum simplex containing 3% (wt/wt) finely ground drug. The treatments started 3 days post-inoculation and consisted of four daily applications for 5 days. The animals were scored daily, and each point represents the average of eight eyes. A statistically significant difference in score between placebo and treated eyes with (*RS*)-DHBG was observed on days 4, 5, and 6 ($P < 0.001$) and also on day 7 ($P < 0.01$; Student's *t* test). ●, Placebo ointment; ○, 3% (*RS*)-DHBG ointment.

in mice (Fig. 6) when the drug was given topically and orally, respectively.

In addition to HSV-1 and -2, varicella zoster virus but not cytomegalovirus, which lacks TK, was inhibited by (*RS*)-DHBG (B. Wahren, personal communication).

Some nucleoside analogs with acyclic side chains, such as ACV (11, 24), 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine (26), erythro-9-(2-hydroxy-3-nonyl)adenine (22), and (*S*)-(2,3-dihydroxypropyl)adenine (9) have previously been investigated as antiherpes agents. The present study shows that other compounds of this type have antiviral activity.

A detailed analysis of the differences between (*R*)- and (*S*)-DHBG and their efficacies as compared with those of other compounds has yet to be carried out, but the present results show that DHBG has a definite potential as an antiherpes agent. The high affinity of DHBG for HSV TK will make this compound effective even in the presence of dThd.

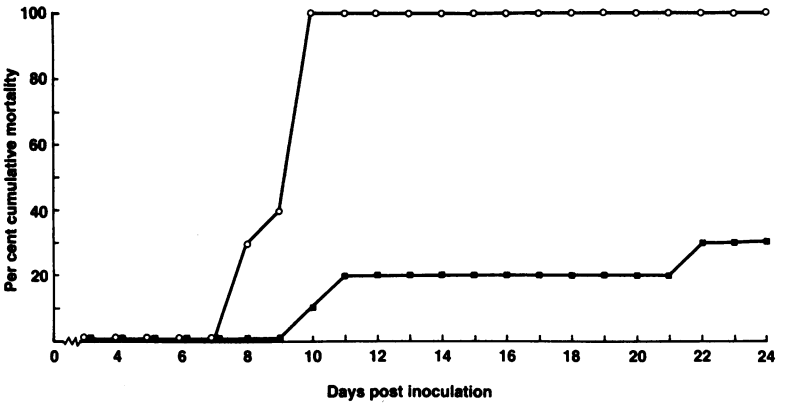

FIG. 6. Therapeutic effect of (*RS*)-DHBG on systemic HSV-2 infection in mice. Mice inoculated with HSV-2 91075 were treated orally with 2 × 75 mg/kg per day, starting 1 h after inoculation and continuing for 10 days. Placebo-treated animals received phosphate-buffered saline. Ten mice were used in each group. An increased survival was observed in the group treated with (*RS*)-DHBG ($P < 0.01$; Fisher's exact test). ●, Placebo treatment; ○, 150 mg of (*RS*)-DHBG per kg of body weight per day.

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