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Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine against herpesviruses *in vitro* and its mode of action against herpes simplex virus type 1

(antiviral chemotherapy/acyclovir analog/DNA polymerase/thymidine kinase)

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ABSTRACT **A guanosine analog, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG), was found to inhibit herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2, cytomegalovirus, and Epstein–Barr virus replication by >50% at concentrations that do not inhibit cell growth in culture. The potency of the drug against all of these viruses is greater than that of 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir). DHPG was active against HSV-1 growth during the early phase of virus replication and had no activity when added at a later time after infection. Its antiviral activity was irreversible. Thymidine partially neutralized its action. The anti-HSV-1 activity of DHPG was dependent on the induction and the properties of virus-induced thymidine kinase. Virus variants that induced altered virus thymidine kinase and became resistant to acyclovir were still as sensitive to DHPG as the parental virus. DHPG is active against five different HSV variants with induced altered DNA polymerase and resistance to acyclovir.**

Cells infected by herpesviruses, which are important causes of human diseases, undergo many biochemical changes, among which are changes in several virus-specified enzymes that are involved in either deoxynucleotide or DNA metabolism (1). Nucleoside analogs with unique behavior toward such enzymes have been discovered to have selective anti-herpesvirus activity (2–9). Among the nucleoside analogs discovered, 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir), one of the new nucleoside analogs with such activity, was recently licensed in the United States for the treatment of primary genital herpesvirus infection and disseminated herpetic infection. Some of the other compounds are at different stages of clinical development. Recently, an analog of guanosine, 9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine (DHPG), with the structure shown in Fig. 1, was synthesized independently by Syntex (Palo Alto, CA) (10) and Biologics (Toronto, ON, Canada) (11) and was found to have anti-herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) activity. HSV that lacked the ability to induce virally coded thymidine kinase (TK) was found to be resistant to DHPG. Nevertheless, phosphonoacetate-resistant HSV, which is crossresistant to acyclovir, was as sensitive to DHPG as the parental virus, whereas a marginal activity of DHPG against Epstein–Barr virus (EBV) and no activity against human cytomegalovirus (CMV) were reported (11).

In view of the potency and the unique spectrum of antiviral activity of DHPG, we examined: (*i*) the activity of this compound against several unique HSV mutants that had either altered TK or DNA polymerase; (*ii*) its behavior toward HSV-induced TK and its mode of action against HSV-1; and (*iii*) sensitivity of several strains of CMV and EBV to the drug. The results of the studies are reported in this communication.

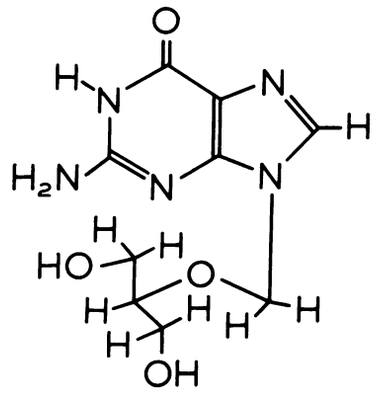

FIG. 1. Structure of DHPG.

EXPERIMENTAL PROCEDURES

Materials. All chemicals used were reagent grade or purer. DHPG, acyclovir, and phosphonoformate (PFA) were gifts from Syntex, Wellcome Research Laboratories (Research Triangle Park, NC), and Astra Laboratories (Södertälje Sweden), respectively. ATP and dThd were purchased from Sigma. [2-^{14}C]-dThd (53 mCi/mmol; 1 Ci = 3.7×10^{10} Bq) was obtained from Schwarz/Mann. [^{3}H]DHPG (16 Ci/mmol) was kindly given by Syntex. The other chemicals or materials were obtained as described (12, 13).

Cells. Cells were grown at 37°C in RPMI 1640 medium (GIBCO) containing 100 μg of kanamycin per ml, supplemented with 5% horse serum for HeLa S_3, 5% fetal bovine serum for Vero cells, and 10% fetal calf serum for P3HR-1 cells. A human fibroblast cell strain, WI-38, was cultivated in modified Eagle's medium containing 100 μg of streptomycin and 100 units of penicillin per ml, supplemented with 15% fetal bovine serum. The WI-38 cells were used between their 22nd and 27th passages.

Virus. Human CMV strain BT1943, isolated from an organ transplant, and strain Towne, isolated from a congenitally infected baby, were received from the Wistar Institute. CMV strain Major isolated from a prostate cell culture was received from

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Abbreviations: DHPG, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; HSV-1 and HSV-2, herpes simplex virus types 1 and 2, respectively; TK, thymidine kinase; EBV, Epstein–Barr virus; CMV, cytomegalovirus; ED_{90}, effective dose, 90%; PFA, phosphonoformate.

F. Rapp. The three strains were used at passages p-4, p-37, and p-29, respectively. Laboratory strains of HSV SC16 parental and TK mutants S1, B3, and Tr7 isolated in the presence of acyclovir at 10 μg/ml, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine at 10 μg/ml, and acyclovir at 1 μg/ml, respectively (14, 15), were gifts of G. Darby and H. Field (Cambridge University, United Kingdom). DNA polymerase mutants (PFA^r, 1a, 2a, 3b, 4b, and 5) were cloned isolates of HSV-1 (KOS strain) grown in the presence of PFA (16). Laboratory strains of virus HSV-1, HSV-2, and all mutants were maintained as described (12).

The Inhibition of Cell Growth. The procedure for establishing the ID_{50} was as described (12).

The Inhibition of Virus Growth: Plaque Reduction Test for CMV. Extracellular fluid was obtained from virus-infected cells (BT1943, Major, and Towne) 3–5 days after the culture reached 100% cytopathic effect. The supernatant fluid containing extracellular virus was divided into aliquots and was frozen at −70°C for plaque-titration reduction tests.

Confluent WI-38 cells seeded in 3.5-mm diameter multi-well Petri dishes were infected with 90–100 plaque-forming units in 0.2 ml of medium. After 2 hr of adsorption at 37°C in 5% CO_2 in air with adequate agitation, modified Eagle's medium containing 6% fetal bovine serum and various concentrations of DHPG was added. After 36 hr of incubation, the medium was replaced with media containing 1% agarose, modified Eagle's medium, 6% fetal bovine serum, and various concentrations of DHPG. The second agarose overlayer was added 6 days after infection. The final reading was made 9–10 days after infection with an inverted microscope.

Assay of the Yield for HSV. To establish the effective dose, 90% (ED_{90}), HeLa S_3 cells were seeded in 25-cm^2 flasks and were used as host cells for virus infection. After a 1-hr adsorption period with virus at 5–10 plaque-forming units per cell, the monolayers were rinsed with phosphate-buffered saline, followed by the addition of 5 ml of growth medium containing various concentrations of drug. The cells were incubated at 37°C for 24 hr and then were stored frozen at −70°C until titration. To establish the effect of time exposure of DHPG on HSV-1, the drug was added and removed at various times after infection. The cells were rinsed at various times to ensure removal of all drug. The cells were incubated at 37°C for 24 hr and then were stored at −70°C until titration. To determine the effect of various nucleosides on DHPG's antiviral activity, each nucleoside was added with DHPG to the infected cells at time 0. The cells then were incubated at 37°C for 24 hr and were stored at −70°C until titration.

Virus titration was carried out as described by Cheng *et al.* (12) with Vero rather than CV-1 cells.

Determination of EBV Genome Copies per Cell. EBV DNA was purified from virus isolated from the supernatant fluids of 12-*O*-tetradecanoylphorbol 13-acetate-induced P3HR-1 cells as described (17). EBV-specific complementary RNA was synthesized *in vitro* (18), and complementary RNA–DNA hybridization was carried out on nitrocellulose filters according to Gillespie and Spiegelman (19), except that $NaDodSO_4$ was present at a final concentration of 0.1% during the hybridization. P3HR-1 cells growing exponentially were treated with DHPG at different concentrations for 5 days. The genome copy numbers were estimated by the method described (18).

TK Preparation and Assays. The enzyme activities were estimated as described (13). HSV-induced TK was purified from infected HeLa (BU25) cells (20) by using affinity column procedures as described (13, 21). To estimate the amount of DHPG phosphorylated, [^{3}H]DHPG (60 mCi/mmol) was used instead of [^{14}C]dThd in the TK assay. The solution used for washing DE81 discs was 1 mM ammonium formate instead of 95% ethanol. DHPG would not adsorb to the disc, whereas its phosphorylated derivatives would. The other conditions of the assay were the same as for the TK assay (13).

RESULTS AND DISCUSSION

Antiviral Activity of DHPG. The effects of DHPG on the growth of HSV-1 and HSV-2, on three strains of human CMV, and on EBV genome replication in P3HR-1 cells, an EBV-producing human cell line, were examined; the results are depicted in Tables 1, 2, and 3. The concentration of DHPG that inhibits 50% of the growth of HeLa S_3 cells was 125 μM. This value is similar to that previously reported (11). DHPG could effectively inhibit the replication of HSV-1 and HSV-2 at noncytotoxic concentrations as reported by Smith *et al.* (11), as shown in Table 1. It could also inhibit growth of three different strains of CMV in WI-38 cells (Table 2) and decrease the EBV genome number in P3HR-1 at the noncytotoxic concentrations (Table 3). These observations are in contrast with the previous report that indicated a lack of activity against EBV and CMV at the noncytotoxic concentration (11). Fluorescent antibody assay and radioimmunoassay were employed previously by other investigators to examine the effect of DHPG on CMV and EBV, respectively. In these assays the expression of virus-specific antigens was examined. Thus, the activity of anti-CMV and EBV growth activity might have been overlooked. It should be stated that DHPG at 50 μM had no inhibitory action against the growth of WI-38, whereas the ID_{50} for P3HR-1 cells in culture was 40 μM.

Activity of DHPG Against HSV-1 or DNA Polymerase Mutants. HSV-1 mutants that induced either altered TK (20) or altered DNA polymerase (16) were examined for their sensitivity to DHPG, the results of which are shown in Table 1. An HSV-1 mutant (B2006) that could not induce TK in infected cells was resistant to DHPG. This result is in agreement with the previous observation (11) and suggests the important role of virus-induced TK in the action of DHPG against HSV-1. Virus mutants (B3, Tr7, S1) that induce an altered TK with no apparent change of induced DNA polymerase in infected cells were still sensitive to DHPG at concentrations far below the 50% cell growth inhibition concentration, in spite of the fact that those mutants were resistant to acyclovir or (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (21). Lack of crossresistance between acyclovir and DHPG was observed with the S1 strain of HSV. Partial crossresistance between acyclovir and DHPG was in-

Table 1. Anti-HSV activity of DHPG and acyclovir

Type	Parental strain	Mutant	Selection reagent	Virus enzyme mutant	ED_{90}, μM DHPG	ED_{90}, μM Acyclovir
HSV-1	KOS	—	—	—	2.0	16
	KOS	PFA^{r}1	PFA	DNA pol	1.0	100
	KOS	PFA^{r}2	PFA	DNA pol	1.0	100
	KOS	PFA^{r}3	PFA	DNA pol	1.8	160
	KOS	PFA^{r}4	PFA	DNA pol	2.6	200
	KOS	PFA^{r}5	PFA	DNA pol	1.5	78
	CL101	B2006	BrdUrd	TK	200	200
	SC16	—	—	—	0.5	3.5
	SC16	S1	Acyclovir	TK	1.0	200
	SC16	B3	BrVdUrd	TK	0.5	2.5
	SC16	Tr7	Acyclovir	TK	16	150
HSV-2	333	—	—	—	2.0	—
	G	—	—	—	2.0	—

DNA pol, DNA polymerase; BrVdUrd, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine.

Table 2. Anti-human CMV activity of DHPG and acyclovir

Strain	ED_{50}, μM*	
	DHPG	Acyclovir
BT1943	1.1	75
Major	4.8	86
Towne	1.0	98

*Concentration required to inhibit 50% plaque formation.

dicated with the Tr7 strain. Thus, mutations producing resistance to acyclovir due to alteration of induced TK do not necessarily lead to resistance to DHPG, in spite of the structural similarity of DHPG and acyclovir.

This laboratory recently isolated several HSV-1 variants (PFA^r1–PFA^r5) with altered DNA polymerase and with no apparent change in induced TK (16). These variants were resistant to acyclovir and PFA in comparison with the parental strain (KOS). The change of behavior of acyclovir triphosphate, the phosphorylated metabolites, and PFA toward DNA polymerase induced by these variants could be responsible for the resistance. When the variants were examined for their sensitivity toward DHPG, they all had sensitivity similar to the parental virus (KOS). Because there was no apparent change of virus-induced TK and the mechanism of action of acyclovir was suggested to be on virus-induced DNA polymerase, the lack of crossresistance between acyclovir and DHPG toward those variants suggested either that acyclovir and DHPG have different effects on viral DNA polymerase or that DHPG may interfere with virus replication by a mechanism differing from that of acyclovir. These observations require further exploration.

Behavior of HSV-Induced TK Toward DHPG. Virus-induced TK was isolated from cells infected by SC16, B3, Tr7, and S1 of laboratory HSV strains. DHPG was examined as the substrate of virus-induced TK; the results are depicted in Fig. 2. DHPG could serve as the substrate of all of the TK isolated; however, the kinetics of DHPG toward different TKs was different. With equivalent TK activity, the optimal DHPG phosphorylation relative to dThd phosphorylation was 35%, 120%, and 80% for SC16, B3, and S1, respectively. The concentrations required to give 50% of optimal activity were 10, 6, and 20 μM for SC16, B3, and S1, respectively. The kinetics of the reaction catalyzed by these virus TKs did not follow the Michaelis–Menten kinetic equation. Substrate inhibition was observed for TK induced by either S1 or SC16. This result may relate to the subunit structure of virus-induced TK, which requires further investigation. In spite of some difference in kinetic parameters of induced TK by SC16, B3, and S1 toward DHPG, the rate of phosphorylation of DHPG was not much different at ≈2 μM DHPG when equal activities of TK were used. Because all three strains of HSV-induced TK activity in cells were within a 3-fold difference (21), it is conceivable that a similar amount of DHPG could be phosphorylated in cells infected by any of those three strains at ≈2 μM DHPG. This could be responsible for the similar sensitivities of the three strains to DHPG. In contrast, the enzyme from Tr7-infected cells seemed to phosphorylate DHPG to a much lesser extent than did the other virus strains when equal TK activities were used for comparison. This lesser degree of the rate of DHPG phosphorylation by Tr7 TK could be responsible for the lesser sensitivity of Tr7 toward DHPG.

Table 3. Effects of DHPG on EBV genome copies in P3HR-1 cells

DHPG, μM	Copies per cell, average no.
0	300
1	180
5	90
10	85
50	50
100	50

P3HR-1 cells were treated with various concentrations of DHPG for 5 days at 37°C. At the end of treatment, cells were harvested and EBV genome copy numbers were determined.

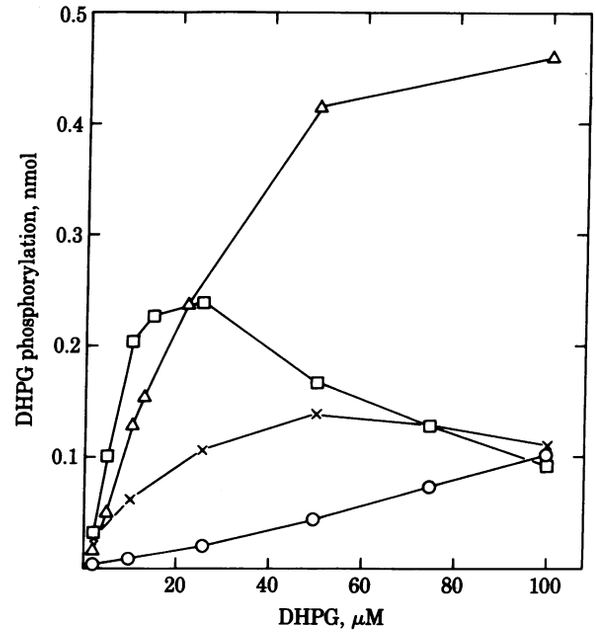

FIG. 2. Phosphorylation of DHPG by HSV-1-induced TK. Virus-induced TK was purified from HeLa (BU-25) cells infected by different strains of HSV (△, B3; □, S1; ×, SC16; and ○, Tr7 (10 ×), as indicated, with the procedures as described (21). The activity of the enzyme used per assay was 4.2 microunits, except that, in the case of Tr7, 42 microunits per assay was used. An enzyme unit is defined as the activity that catalyzes the phosphorylation of 1 nmol of dThd per min at 37°C under our assay conditions (13). Assays were performed at 37°C for 1 hr.

Mode of Action Against HSV-1. HSV-1 (KOS)-infected cells were exposed to DHPG at different times after infection. The drug was active when it was added before infection or at 8 hr after infection. When drug was added at 0 hr after infection and removed at 8 hr after infection, the antiviral activity of DHPG was the same as when infected cells were exposed continuously to DHPG. The antiviral activity could be partially neutralized by adding 5 μM dThd, but not by adding 5 μM guanosine (Table 4). Higher concentrations of dThd could not further neutralize DHPG activity. These results suggest that DHPG exerted its action at an early phase of the virus-infection cycle and

Table 4. Effects of the time of exposure of HSV-1-infected cells to the drug and to the addition of nucleoside on anti-HSV-1 activity of DHPG

Time of exposure to DHPG after infection, hr	Additive	% of control plaque-forming units
—	—	100
0–24	—	11
0–4	—	23
0–8	—	8
8–24	—	98
0–24	dThd, 5 μM	39
0–24	Guo, 5 μM	7

The concentration of DHPG was 2 μM.

that its action is irreversible. Furthermore, the neutralization of antiviral activity by dThd could be related to the decrease of DHPG phosphorylation by virus-induced TK.

In summary, DHPG is active against HSV-1, HSV-2, EBV, and CMV at low concentrations relative to the cytotoxicity; the selective action of DHPG against HSV-1 is virus-TK dependent. The drug has a different spectrum of anti-HSV-1 activity than does acyclovir. The action of DHPG appears to be quite irreversible and thus, the drug has great potential for use in anti-herpesvirus chemotherapy. It should be noted that the mechanism of the selective action against EBV and CMV should be quite different from that against HSV-1 and HSV-2, because there is no definitive evidence that EBV and CMV could induce their specified TKs (1, 22–25). DHPG appears to be at least as inhibitory as acyclovir against EBV replication (26) and much more inhibitory against CMV (27).

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