Inhibition of Human Cytomegalovirus Replication by 9-(1,3-Dihydroxy-2-Propoxymethyl)Guanine Alone and in Combination with Human Interferons

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The inhibitory action of 9-(1,3-dihydroxy-2-propoxymethyl)guanine on the replication of human cytomegalovirus was studied. Three laboratory strains (AD-169, Towne, and Davis) and three early passage (<10) clinical isolates were all inhibited in yield inhibition assays. In cultures infected with AD-169, virus yields could be inhibited if the drug was added as late as 3 days after the replication cycle had begun. The effects of the drug were fully reversible during the first 4 days of the viral replication cycle. Viral infectivity and viral DNA synthesis were reduced more than viral protein synthesis. Synergistic antiviral effects were observed with beta-cysteine, and to a lesser extent, with beta-serine recombinant interferons, but only over a narrow range of dose combinations.

The novel guanosine analog, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), is active in vitro against human cytomegalovirus (CMV) (1, 13, 24). Acycloguanosine and other nucleoside analogs have limited activity against human CMV (3, 5, 12, 16, 19, 23). The potent inhibition of CMV replication by DHPG and its minimal effect on uninfected cells suggest that DHPG may be useful for the therapy of human CMV infections. In this report, we describe the in vitro conditions which result in the optimal inhibition of CMV by DHPG, the effect of DHPG on viral polypeptide and DNA syntheses, and the effects of treatment with human recombinant interferon (rIFN) species and DHPG in combination.

MATERIALS AND METHODS

Chemicals. DHPG was provided by Syntex, Inc., Palo Alto, Calif. A 1 mM solution was prepared in Dulbecco minimal essential medium (DMEM) and frozen at -70°C .

IFNs. Partially purified alpha and beta rIFNs (rIFN- α D and rIFN- β , respectively) were supplied by Cetus Corp., Emeryville, Calif. The characteristics of the IFNs have been described previously (6, 29). One form of rIFN- β , like the natural material (supplied by C. Tan), has three cysteine residues in its structure (β -Cys). The other form has serine substituted for cysteine at position 17 of the molecule (β -Ser) (D. Mark et al., *Proc. Natl. Acad. Sci. U.S.A.*, in press). IFN- α was supplied by Kari Cantell. IFN titers were determined by a vesicular stomatitis virus plaque reduction assay on human foreskin fibroblast cells (15).

Cells and viruses. The methods used for culturing and assaying human CMV in human embryonic lung fibroblast (HEL) cells have been described previously (20). CMV AD-169 was used for all experiments unless otherwise indicated.

Assays for antiviral activity. Both yield inhibition and plaque reduction assays were used to evaluate the antiviral action of DHPG. For yield inhibition studies, 35-mm tissue culture plates of HEL cells in multidishes were infected with CMV by adsorption of the virus for 2 h to drained monolayers. DMEM containing 1% fetal calf serum and DHPG at

various concentrations was then added to the tissue cultures. When a multiplicity of infection (MOI) of 0.1 was used, the virus was allowed to replicate for 5 days; replication was continued for multiple cycles (10 to 12 days) if the MOI was <0.1 . Infected cultures were harvested by scraping the cells into the supernatant, pooling duplicate wells, sonicating to disrupt the cells, and then freezing the cells at -70°C . For some experiments, the supernatant and intracellular virus were harvested separately. Samples were assayed for PFU of virus on HEL cells (21). Inhibition was determined by comparing the yields from drug-treated virus-infected cultures with those from untreated virus-infected cultures and was expressed as a percentage of control yields. For plaque reduction assays, the drug was incorporated into the semi-solid overlay. For experiments in which the effects of IFNs and DHPG in combination were tested, cells were first treated overnight with IFN and then infected with CMV. Both drugs were included in the maintenance medium, either DMEM or DMEM with 1% agarose, during virus replication. To evaluate drug interactions, we calculated a combination index (CI) by the formula derived by Spector et al. (28): $\text{CI} = \ln(A) + \ln(B) - \ln(AB) - \ln(VC)$, where A and B are the mean virus yields in the presence of antiviral agents A and B , respectively, AB is the virus yield in cultures treated with both agents, and VC is the virus yield in untreated controls. The $\text{CI} \pm$ standard error (SE) was calculated for each drug combination with data from independent experiments. When the CI was within 2 SEs of 0, the combined drug effects were additive. When the CI was greater than ± 2 SE, the effects were synergistic; when the CI was less than ± 2 SE, the effects were antagonistic. For plaque reduction assays, the drug interactions were assessed by the formula $[(A)(B)]/[AB(VC)]$. When the numerical result was >1 , the effects were synergistic; when the numerical result was equal to 1, the effects were additive; and when the numerical result was <1 , the effects were antagonistic. This formula was not applicable for drug combinations resulting in a 100% inhibition of plaques.

DNA hybridization assay. The hybridization assay for the detection of CMV DNA in tissue specimens was done with a nick-translated, ^{32}P -labeled copy of an *Eco*RI fragment of

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TABLE 1. Action of DHPG on CMV infectivity, DNA synthesis, and polypeptide synthesis

Expt	DHPG concn (μ M)	% Inhibition of:		
		Infectivity ^a	DNA ^b	86-kilodalton polypeptide ^c
1	0.3	45	50	— ^d
2		47	55	—
1	1.0	96	90	14
2		97	71	—
1	3.0	98	92	—
2		99	89	—
1	10.0	99	98	90
2		99	—	—
1	30.0	99	99	—
2		99	—	—
1	100.0	—	—	99

^a Yields from extracts of CMV-infected HEL cells (MOI, 0.1 for all experiments) grown in 35-mm culture dishes were measured by a PFU assay.

^b Measured as the counts per minute in drug-treated infected cultures hybridizable with ³²P-labeled cloned CMV DNA compared with the counts per minute in control infected cultures.

^c CMV-infected cultures, both untreated and treated with DHPG, were labeled with [³⁵S]methionine, extracted, and immunoprecipitated with monoclonal antibody 1G6. Relative amounts of the immunoprecipitated 86-kilodalton viral polypeptide were estimated from autoradiographs. Densitometry tracings were used to determine the area under the peak at each drug concentration (see Fig. 1).

^d—, Not done.

cloned CMV DNA and has been described previously (2).

Assays for viral protein synthesis in infected cell cultures. The methods used for [³⁵S]methionine labeling of CMV-infected cells and immunoprecipitation of extracts for sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been described previously (21). Relative amounts of each immunoprecipitated viral protein were estimated from densitometer tracings of the autoradiogram with a Beckman automated gel scanner. The areas under each peak representing a single band were calculated and compared with those of virus-infected controls to yield the percent reduction at each drug dose.

The synthesis of an 86,000-molecular-weight CMV polypeptide in infected cells was determined by immunofluorescence staining with a murine monoclonal antibody (1G6) (21). Cover slip cultures of CMV-infected cells were acetone fixed 5 days after infection, incubated with 50 μ l of 1G6 immunoglobulin (3.4 μ g/ml) for 30 min at room temperature, and washed three times with warm phosphate-buffered saline. The cover slips were incubated for 30 min with a 1:50 dilution of the F(ab')₂ fragment of fluorescein-treated goat antimouse immunoglobulin (Tago, Burlingame, Calif.), washed, and mounted with buffered glycerol.

RESULTS

Quantitative aspects of the inhibition of CMV by DHPG. The susceptibilities of CMV strains Towne, Davis, and AD-169 and three early passage (<10) clinical isolates to inhibition by DHPG were compared. Tenfold drug concentrations ranging from 0.01 to 10 μ M were used, and the concentration resulting in a 50% reduction in yield was estimated by probit analysis. For AD-169 and Davis, 0.01 μ M DHPG was sufficient to inhibit virus yields by 50%. However, for Towne, 1.0 μ M was necessary to produce the same inhibi-

tion. The clinical isolates were inhibited by 0.13, 0.25, and 0.4 μ M DHPG.

To assure that residual DHPG in the culture supernatant was not inhibiting virus plaque formation, especially at drug concentrations greater than 10, 50, and 100 μ M, DHPG were mixed with ca. 3×10^4 PFU of CMV, representing the approximate yield from 35-mm wells in a typical experiment. Aliquots were diluted and plated for measurement of PFU without removal of the inoculum after adsorption to the cells. PFU titers were 2.9×10^4 in both 50 and 100 μ M DHPG-virus mixtures and 3.3×10^4 in medium-virus mixtures.

Kinetic aspects of the inhibition of CMV by DHPG. The reversibility of the action of DHPG on CMV-infected cultures was studied. Replicate cultures were infected with CMV (MOI, 0.1), and 10 μ M DHPG was added. At daily intervals, the drug was removed from a single set of duplicate cultures by gentle washing three times with phosphate-buffered saline. DMEM was then added, and cultures were reincubated for an additional 5 days. Yields from cultures treated for 1 to 4 days before removal of the drug were not significantly different from those from sham-treated cultures. Specifically, average yields were 72, 66, 74, and 92% of control yields in two separate experiments. However, yields from infected cultures treated for 5 days before removal of the drug were <1% of control yields. As controls for virus yields after removal of the drug, cultures were incubated in drug-free medium for 1, 3, and 5 days, infected with CMV, and then reincubated for 5 days before being harvested. Cultures infected at the beginning of the experiments yielded an average of 2.1×10^5 PFU, as compared with 2.2×10^5 , 1.4×10^5 , and 0.2×10^5 PFU on days 1, 3, and 5, respectively. As an additional control, HEL cells were treated for 5 days with 10-fold dilutions of DHPG ranging from 100 to 0.1 μ M. Cultures were infected with CMV after the removal of DHPG and harvested 5 days later. Yields were 1.2×10^5 PFU/ml in DHPG-treated cultures and 1.4×10^5 PFU/ml in nontreated cultures.

The susceptibility of the CMV replication cycle to interruption by DHPG was tested by adding 0.1, 1, and 10 μ M DHPG at daily intervals from 0 to 5 days after infection. All cultures were harvested for yields 5 days after initial infection. Intra- and extracellular viruses were harvested separately in this experiment. All concentrations resulted in inhibition when the drug was added immediately after infection. However, 1 and 10 μ M DHPG were effective in reducing CMV yields if added 1 day or more after infection. Specifically, intracellular yields were 0.2, 6.5, and 37% of control yields when 10 μ M DHPG was added 1, 2, or 3 days after infection, respectively. A 1 μ M concentration was effective in reducing yields only when added immediately or 1 day after infection (22% of control yields). The reduction in the supernatant virus was parallel to that of the intracellular virus (data not shown).

Effect of DHPG on viral DNA and protein biosyntheses. The amount of CMV DNA in infected cultures incubated with or without DHPG, which was hybridizable with a ³²P-labeled, cloned *Eco*RI fragment of CMV DNA, is shown in Table 1. The percentage of reduction in hybridizable counts was calculated by comparison of drug-treated and untreated CMV-infected cultures. A concentration of 0.3 μ M was sufficient to result in a 50% inhibition of CMV DNA synthesis.

The effect of DHPG on viral protein synthesis was evaluated by both immunofluorescence and immunoprecipitation of [³⁵S]methionine-labeled infected cell extracts. A murine hybridoma antibody, 1G6, which detects an 86-kilodalton

FIG. 1. Effect of DHPG on viral polypeptide synthesis in infected cells, as determined by immunoprecipitation of [35 S]methionine-labeled extracts. Infected cells were treated for 5 days with DHPG. Immunoprecipitation was done with either anti-CMV murine monoclonal antibody 1G6 or a human CMV antibody-positive serum pool. MW, Molecular weight; kD, kilodalton.

envelope glycoprotein and is associated with infectious virus (21), was used as a specific antibody for both immunofluorescence and immunoprecipitation. A human serum pool determined to be seropositive for CMV by complement fixation, neutralization, and radioimmunoassay (20) was also used in immunoprecipitation experiments. CMV-infected cells grown on cover slips in the presence of 10, 1, or 0.1 μ M DHPG were indistinguishable from untreated CMV-infected cells when observed after indirect immunofluorescence staining (data not shown). For immunoprecipitation, infected cells were incubated for 5 days with 100, 10, or 1 μ M DHPG or medium alone before labeling with [35 S]methionine for 4 h. The immunoprecipitation patterns of polypeptides synthesized in the presence of 1 μ M DHPG were not significantly different from those seen in the drug-free, CMV-infected cell extract (Fig. 1). A DHPG concentration of 10 μ M or more was required to result in 90% inhibition of

viral protein synthesis (Table 1). The reduction in viral polypeptide synthesis was not limited to the 86-kilodalton protein, as corresponding reductions were seen in the viral proteins immunoprecipitated by the human serum pool (Fig. 1).

Replication of CMV in cell cultures treated with both DHPG and human IFNs. IFN- α D, rIFN- α , and β -Cys and β -Ser rIFN- β were evaluated in combination with DHPG for the inhibition of CMV yields. Cells were treated overnight with IFN and then infected with CMV at a MOI of 0.1. Medium containing DHPG or IFN or both was added, and the cultures were incubated for 5 days before being harvested. The inhibitory effect was calculated both as a percentage of control culture yields and as a CI. For all yield inhibition experiments, 10-fold dilutions of DHPG (0.01 to 1 μ M) were combined with 3-fold dilutions of IFN (300 to 30 U). Drug or IFN doses resulting in >90% inhibition alone were not

evaluated in combinations. Synergism was observed sporadically (three of six combinations) with β -Cys rIFN- β and DHPG (Table 2). Only one combination (100 U of IFN and 0.01 μ M DHPG) resulted in synergy with β -Ser rIFN- β . The other combinations resulted in additive effects (data not shown). IFN- α and rIFN- α D resulted in only additive effects in combination with DHPG when tested over the range of concentrations used for IFN- β .

DHPG and both rIFN- β species (β -Cys and β -Ser) were tested separately and in combination for the ability to reduce CMV plaque counts. Synergistic effects were observed with β -Cys rIFN- β more consistently for plaque reduction assays than for yield inhibition assays. For example, 30 U resulted in synergism with all concentrations of DHPG used (Table 3). When β -Ser rIFN- β was used in the same concentrations as β -Cys rIFN- β , synergy was detectable only with 10 U of IFN and 1, 0.1, and 0.01 μ M DHPG (data not shown).

DISCUSSION

The replication of both laboratory strains and clinical isolates of CMV was effectively inhibited by DHPG *in vitro*. The action of DHPG against CMV was measured most sensitively by assays of the inhibition of infectious virus or viral DNA. Viral protein synthesis was the biosynthetic event most resistant to DHPG (Table 1). The apparent resistance of CMV to DHPG reported by Smith and co-workers (26) may have resulted from the evaluation of inhibition only by immunofluorescence. The dose of DHPG used in their study (ca. 10 μ M) was insufficient in our experiments to prevent viral protein synthesis by strain AD-169. Our results are in general agreement with those of Mar et al. (11), who studied the effects of DHPG on macromolecular synthesis by the Towne strain. Our results suggest that the selective inhibition of viral functions by DHPG should be considered when assaying body fluids for virus. Specifically, CMV antigen detection alone, without an evaluation of infectious virus or viral DNA in body fluids, may result in a false-negative interpretation of drug action.

Experiments in which DHPG was either added or removed during the course of CMV replication, as well as a lack of viral DNA synthesis in drug-treated cultures, support the idea that replicating DNA is a target for drug action. The

TABLE 3. Combined effects of DHPG and β -Cys rIFN- β on plaque formation by CMV (AD-169)

DHPG concn (μ M)	IFN (U)	PFU ^a	$\frac{[(A)(B)]}{[(AB)(VC)]^b}$	Effect ^c
0	30	33	— ^d	—
	10	60	—	—
	0	150	—	—
0.01	30	29	2.5	S
	10	32	1.4	S
	0	108	—	—
0.1	30	11	3	S
	10	46	1.2	S
	0	144	—	—
1.0	30	1	18	S
	10	9	4	S
	0	80	—	—

^a HEL cells were treated overnight with IFN. After infection, both DHPG and IFN were added to DMEM with 5% agarose before overlay of infected cells. Plaque formation was measured as the average PFU/0.2 ml of inoculum.

^b See text for details.

^c S, Synergism. See text for details.

^d —, Not done.

end of the period during which the effects of DHPG are reversible (3 days) and the inability to interrupt the cycle after 2 days coincide with the beginning of *de novo* viral DNA synthesis (18).

The greater resistance of viral polypeptide synthesis than of either infectivity or viral DNA synthesis to inhibition by DHPG could be explained if the input CMV DNA were to function as a template for transcription and translation in the absence of *de novo* viral DNA synthesis. The synthesis of viral proteins in the presence of inhibitors of viral DNA synthesis has been described previously (9). Another possibility is that DHPG acts as a substrate for CMV DNA polymerase (7, 8, 17) and is incorporated into replicating DNA to terminate the chain, as shown for herpesvirus-infected cells treated with acyclovir (4, 14). The short fragments of viral DNA could then be transcribed and translated to yield viral polypeptide synthesis.

In our experiments, synergism between DHPG and IFN was observed only with the β species, in particular β -Cys. Synergy was demonstrable more consistently in plaque reduction assays than in yield inhibition assays. A similar finding has been reported for the inhibition of CMV by combinations of acyclovir and IFN- α (25), pointing to the importance of the type of assay used in assessing synergy between antiviral drugs. DHPG-IFN- β synergism was observed over a very limited dose range, particularly in yield inhibition assays. This is also consistent with data obtained for IFN- α -acyclovir which showed that an increase in the concentration of the drug in the presence of a constant amount of IFN changed the inhibitory effects from additive to synergistic (25). The lack of synergy between IFN- α and DHPG in inhibiting CMV is in contrast to the findings for acyclovir, a structurally related compound (10, 25, 27). The basis of this difference is unknown. The minimal action of acyclovir alone against CMV makes combination therapy with another antiviral agent necessary to improve possible *in vivo* action. However, DHPG *in vitro* is so active against CMV that it may be preferable to use it alone *in vivo*, thus avoiding any possibility of combined toxicity, as has been observed with IFN and adenine arabinoside (22).

TABLE 2. Combined effects of DHPG and β -Cys rIFN- β on CMV (AD-169) yields

DHPG concn (μ M)	IFN (U)	% of control yields ^a	CI ^b	SE	Effect ^c
0	300	64	— ^d	—	—
	100	60	—	—	—
	30	69	—	—	—
0.01	300	62	0.70	0	S
	100	71	0.35	0.23	A
	30	75	-0.30	0.15	A
	None	100	—	—	—
0.1	300	39	0.65	2.17	S
	100	31	0.80	4.0	S
	30	75	-0.85	-0.31	A
	None	70	—	—	—

^a Yields from drug-treated cultures were compared with those from untreated control cultures. The data presented were calculated from at least two independent experiments.

^b CI = $\ln(\text{IFN}) + \ln(\text{DHPG}) - \ln(\text{IFN} + \text{DHPG}) - \ln(\text{no drug})$.

^c S, Synergistic; A, additive. See text for details.

^d —, Not done.

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