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Inhibition of Human Cytomegalovirus in Culture by Alkenyl Guanine Analogs of the Thiazolo[4,5-*d*]Pyrimidine Ring System

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A series of alkyl and alkenyl guanine analogs containing a thiazolo[4,5-*d*]pyrimidine ring system were prepared by reaction of the appropriate alkyl halide with the sodium salt of the heterocycle. In preliminary antiviral efficacy evaluations against laboratory strains of both human cytomegalovirus (HCMV) and herpes simplex virus types 1 and 2, it was determined that two of the compounds (T70072 and T01132) were more active and less toxic in stationary-phase cell monolayers than were the other derivatives tested. T01132 and T70072, which have 2-pentenyl and 3-methyl-2-butenyl moieties attached to position 3 of the 5-aminothiazolo[4,5-*d*]pyrimidine-2,7-dione, respectively, were then more extensively evaluated for anti-HCMV activity. The concentrations of T01132 and T70072 required to inhibit HCMV by 50% in plaque reduction assays were ~0.5 and 6.8 μM, respectively. These two compounds inhibited the growth of KB, MRC-5, or Vero cells at concentrations of 75 to 150 μM, depending upon the cell line. In bone marrow progenitor cells T01132 was slightly less toxic than ganciclovir (DHPG). The 50% inhibitory concentrations of T01132 against clinical isolates and DHPG-resistant strains of HCMV were approximately the same as those obtained for laboratory strains of HCMV (~0.5 μM). When tested in combination with DHPG, the resultant antiviral activity was determined to be additive but not synergistic. Experiments performed using variations of the viral multiplicity of infection (MOI) demonstrated that T01132 was more active than DHPG at a low MOI (0.002 or 0.02). However, when a higher MOI (0.2 or 2.0) was used, DHPG was more efficacious than T01132. In experiments in which drug was added at various times post-viral infection, T01132 was most effective when added within the first 24 h post-HCMV infection while DHPG was able to protect cells in this assay system when added up to 48 h postinfection, indicating that T01132 is exerting its antiviral effect on events leading up to and possibly including viral DNA synthesis. The data presented in this report suggest that the antiviral activity of alkenyl-substituted thiazolopyrimidine derivatives may represent a mechanism of action against herpesviruses alternative to that of classical nucleoside analogs such as acyclovir or DHPG.

One of the most widely used drugs today for human cytomegalovirus (HCMV) infections is ganciclovir (DHPG) (3, 8, 10). DHPG is a potent inhibitor of most human and animal herpesviruses in culture, whereas much higher concentrations are needed to inhibit growth of uninfected cells. However, DHPG is toxic to bone marrow progenitor cells in culture, which was predictive of DHPG's adverse effects in vivo in that most clinical toxicity is myelosuppression (16). This problem is most apparent in patients in need of long-term therapy such as those with AIDS or HCMV retinitis and transplant recipients. Another concern for patients on long-term DHPG therapy is the development of DHPG-resistant strains of HCMV (9, 12). The second anti-HCMV drug approved for use against HCMV retinitis in AIDS patients is foscarnet. This compound is a broad-spectrum antiviral agent with observed activity against all known human herpesviruses (7). However, treatment with foscarnet has resulted in nephrotoxicity, hypocalcemia, and seizures in some patients (11). Therefore, additional efficacious, nontoxic drugs for use against HCMV infections are needed.

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Several base-modified nucleoside derivatives of the purine analog 5-amino-3-β-D-ribofuranosylthiazolo[4,5-*d*]pyrimidine-2,7-dione have been synthesized and found to possess antiviral activity in vivo against a variety of RNA and DNA viruses, presumably via stimulation of the host immune system (13, 17, 27, 29). Recently we synthesized a series of derivatives of 5-aminothiazolo[4,5-*d*]pyrimidine-2,7-dione (7-thia-8-oxoguanine) and assayed them for activity against HCMV in culture (14). Several of these compounds were active against HCMV but had various degrees of in vitro toxicity. We report here on the antiherpesvirus activity of the two most active compounds synthesized in this series.

MATERIALS AND METHODS

Cell cultures, viruses, and plasmids. The routine growth and passage of Vero, BSC-1, and KB (a human oral epidermoid carcinoma cell line) cells were performed in monolayer cultures using minimal essential medium with either Hanks or Earle salts supplemented with 10% calf serum, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. Cultures of diploid human foreskin fibroblast (HFF) and MRC-5 cells were routinely grown in minimal essential medium with Earle salts supplemented with 10% fetal bovine serum without antibiotics. Cells were passaged at 1:2 to 1:10

dilutions according to conventional procedures (24, 25). HFF and MRC-5 cells were passaged only at 1:2 dilutions.

The Towne strain, plaque-purified isolate P_o, of HCMV was kindly provided by Mark Stinski, University of Iowa. The AD169 strain of HCMV was obtained from the American Type Culture Collection. HCMV strains P8 (DHPG susceptible) and D16 (DHPG resistant) were obtained from K. Thompson (Loyola University). The HCMV strains 17517 (DHPG susceptible) and 48041 (DHPG resistant) were obtained from K. Biron (Burroughs Wellcome). The KOS strain of herpes simplex virus type 1 (HSV-1) was provided by Sandra K. Weller, University of Connecticut. HSV-1 strain 17 and HSV-2 strain HG52 were obtained from D. J. McGeoch (University of Glasgow). Virus titers were determined with monolayer cultures of HFF or BSC-1 cells (21).

The plasmid pRL43a, which contains a portion of the HCMV Towne strain immediate-early gene loci (18), was kindly provided by G. Hayward at Johns Hopkins University. The recombinant plasmid pGR18, which contains the HSV thymidine kinase gene (23), was kindly provided by G. Reyes (Ingenex, Inc.).

Chemicals and reagents. For the synthesis of the two alkenyl derivatives of the heterocycle discussed in this report (compounds T70072 and T01132), commercially available alkenyl bromides (Aldrich) were used. The heterocycle was synthesized by the method described by Baker and Chatfield (2). The radioisotope [α-^{32}P]dATP was obtained from Amersham. The Random Primed DNA Labeling kit and RNase A were purchased from Boehringer Mannheim. Phosphonoacetic acid was purchased from Sigma. DHPG was obtained from Syntex, and acyclovir (ACV) was obtained from Burroughs Wellcome.

Antiviral assays. The effects of compounds on the replication of HCMV, HSV-1, and HSV-2 have been measured by plaque reduction and cytopathic effect (CPE) assays, enzyme-linked immunosorbent assays (ELISAs), and viral yield reduction assays.

(i) HCMV plaque reduction assays. Two different plaque reduction assays were employed to determine the anti-HCMV efficacy of the test compounds in culture. The first assay used MRC-5 cells and the AD169, P8, and D16 strains of HCMV. Plaque reduction experiments using MRC-5 cells were performed in quadruplicate as described by Barnard et al. (4).

The second assay procedure, using HFF cells and the Towne, 17517, and 48041 strains of HCMV, was performed as previously described (21, 33). Drug effects were calculated as a percentage of reduction in the number of plaques in the presence of each drug concentration compared with the number observed in the absence of drug.

(ii) HCMV yield assay. HCMV viral yield reduction assays were performed as previously described (21). HFF or MRC-5 cells were plated in 96-well cluster dishes at a concentration of 12,500 cells per well and incubated overnight. The next day, the medium was shaken out and the cultures were inoculated with HCMV at a multiplicity of infection (MOI) of 0.5 to 1 PFU per cell. After virus adsorption, virus inoculum was replaced with 0.2 ml of fresh medium containing test compounds. Plates were incubated at 37°C for 7 days and then subjected to one cycle of freezing at −76°C and thawing at 37°C to disrupt the cells. Aliquots from each well were transferred to a fresh 96-well monolayer culture of HFF cells. The contents were mixed and serially diluted (1:3) across the secondary plate. Each column of the original primary plate was diluted across a separate plate in this manner. Cultures were incubated, plaques were enumerated, and titers were calculated as described above.

(iii) HCMV ELISA. MRC-5 cells were plated in 96-well cluster dishes (15,000 cells per well). After overnight incubation in culture medium at 37°C, selected drug concentrations in quadruplicate and HCMV (strain Towne) at an MOI of 0.002 were added to each well. Following a 7-day incubation at 37°C, the medium was removed and plates were fixed with 200 μl of 95% ethanol per well for 15 min. The ethanol was then removed, and the wells were rinsed with 200 μl of distilled water, dried, and blocked for 30 min with (per well) 200 μl of a combination of 10% calf serum and 0.05% Tween in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (HBS-T) (24). The blocking agent was then removed, and the wells were rinsed with HBS-T. The primary (murine anti-HCMV) and secondary (peroxidase-conjugated rabbit anti-mouse) antibodies were obtained from Dako Corp. and used as recommended by the manufacturer. After removal of the secondary antibody, the plates were rinsed twice for 1 min each time and twice for 5 min each time with HBS-T and then developed for 15 min in, per well, 150 μl of 0.1 M citrate buffer, pH 5.0, containing 0.04% hydrogen peroxide and 125 μg of 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma) per ml. The developing reaction was stopped by the addition of 50 μl of 2 N H_2SO_4 per well, and the A_{450} and A_{570} were determined. Drug effects were calculated as a percentage of the reduction in absorbance in the presence of each drug concentration compared with absorbance obtained with virus in the absence of drug.

(iv) HSV-1 ELISA. An ELISA also was employed to detect the antiviral effects of T01132 on HSV-1 strain KOS. Ninety-six-well cluster dishes were planted with 10,000 BSC-1 cells per well in 200 μl of culture medium per well. After overnight incubation at 37°C, selected drug concentrations (in quadruplicate) and HSV-1 at a concentration of 100 PFU per well were added. Following a 3- to 5-day incubation at 37°C, the medium was removed and plates were blocked with 200 μl of a combination of 10% calf serum and HBS-T per well. After 30 min, the blocking agent was removed and the wells were rinsed with HBS-T. Horseradish peroxidase-conjugated rabbit anti-HSV-1 antibody in HBS was added and incubated on a rocker for 1 h at room temperature. Detection of HSV-1 specific proteins was performed as described for the HCMV ELISA.

(v) HSV-1 and HSV-2 CPE assays. Vero cells were plated at 4×10^4 cells per well in a 96-well microtiter dish in 0.1 ml of culture medium 24 h before infection with virus at an MOI of 0.001. The virus was allowed to adsorb to the cells for 10 min at 37°C. The virus-containing medium was then removed, and the cells were rinsed three times with fresh medium. Finally, 100-μl aliquots of fresh medium containing the various dilutions of test compounds were added to each well. Plaques were observed 24 h postinfection, and the degree of CPE was scored 40 to 48 h postinfection. ACV was used as a standard in all HSV assays.

(vi) Combination study. The HCMV ELISA protocol was adapted for use in combination studies. On each plate, a grid of wells (10 by 6 wells) was used for different combinations of 10 concentrations of T01132 and 6 concentrations of DHPG. Five plates were used to produce replicates for each combination of the two drugs. The combination experiments were performed as described by Prichard and Shipman (20) and Prichard et al. (19). Plates were incubated and developed as described above for the HCMV ELISA.

Data analysis. Dose-response relationships were constructed by linearly regressing the percent inhibition of parameters derived in the preceding sections against log drug concentrations. Fifty-percent inhibitory (IC_{50}) concentrations were calculated from the regression lines. Samples containing positive controls (ACV for HSV-1 or HSV-2, DHPG for HCMV, and 2-acetylpyridine thiosemicarbazone for cytotoxicity) were used

in all assays. Results from sets of assays were rejected if inhibition by the positive control deviated from its mean response by >1.5 standard deviations. The analysis of the data obtained from the combination drug study was performed with the MacSynergy program as described by Prichard and Shipman (20).

Inhibition of viral DNA synthesis. MRC-5 cells were seeded at 1.8×10^5 cells per well into 6-well plates. The following day the culture medium was replaced with 2 ml of minimal essential medium–2% fetal bovine serum containing either 40 μM T01132 or 150 μg of phosphonoacetic acid per ml. Following overnight incubation at 37°C, the cells were infected with HCMV (Towne) at an MOI of 0.4. Cells were infected with virus (2 h at 37°C), rinsed three times with complete medium, and then cultured with 2 ml of minimal essential medium–2% fetal bovine serum containing the amount of drug indicated above. DNA was harvested either 1 h or 3 days postinfection by standard extraction and purification procedures (15).

Purified DNAs were denatured, immobilized on Nytran membranes, and pretreated in a buffer containing 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate (SDS) for 30 min at 42°C. The filter membranes were then prehybridized at 42°C for 6 h in a buffer containing 6× SSC, 5× Denhardt's solution, 1% SDS, 50% formamide, 1% nonfat dry milk, and 100 μg of both denatured salmon sperm DNA and yeast tRNA per ml. The plasmid pRL43a (HCMV immediate-early genes) or total extracted cellular (MRC-5) DNA was radiolabeled with [α-^{32}P]dATP and the random priming kit, purified with nick columns (Pharmacia), and added to the prehybridization buffer (5.3×10^6 cpm/ml). The hybridization reactions proceeded for 16 to 18 h at 42°C, at which time the filters were washed (twice with 2× SSC–0.1% SDS buffer for 10 min each time at room temperature and then twice with 0.1× SSC–0.1% SDS buffer for 15 min each time at 50°C), dried, and either processed for autoradiography or quantitated with a Betascope blot analyzer (Betagen).

For HSV-2 analysis, DNA was extracted from Vero cells 14 h postinfection with HSV-2 (MOI of 1.0). Total extracted DNA was then analyzed for the presence of HSV-2 DNA by using either pGR18 (HSV-2 thymidine kinase gene) or total Vero cell DNA as a molecular probe.

Cytotoxicity. Cytotoxicity as measured by inhibition of cell growth was measured with Vero or MRC-5 cells by a CellTiter 96 Aqueous Non-Radioactivity Cell Proliferation Assay (Promega). Cytotoxicity assays were performed using a 4-day incubation of test compound with cells plated at 5,000 cells per assay. Each assay was performed in quadruplicate. The resultant data were averaged, graphed, and then used to calculate the median toxic concentration for the compound tested.

The effects of compounds during two population doublings of KB cells were determined by crystal violet staining and spectrophotometric quantitation of dye eluted from stained cells as described earlier (19).

T01132 and T70072 were also tested for their toxic effects on human bone marrow cells as previously described by Sommadossi et al. (30). Human bone marrow cells were collected by aspiration from the posterior iliac crest of healthy volunteers and treated with heparin, and the mononuclear population was separated by Ficoll-Hypaque gradient centrifugation. Cells were washed twice in Hanks balanced salt solution and counted with a hematocytometer, and their viability was assessed by trypan blue exclusion (should be >98%). Following isolation and purification, the cells were grown in a double agar layer (approximately 10^5 cells per plate) in the presence of 1 U of erythropoietin for the erythroid burst-forming units (BFU-E) cell studies (30) or in the presence of 75 to 100 U of granulocyte-macrophage colony-stimulating factor (Genzyme, Boston, Mass.). DHPG was used as a toxicity control in these assays.

FIG. 1. Structural representation of two alkenyl derivatives of 7-thia-8-oxoguanine: T01132 (left) and T70072 (right).

RESULTS

Compound design and synthesis. A series of 3-alkyl (or alkenyl) guanine analogs containing a thiazolo[4,5-*d*]pyrimidine ring system were prepared by reaction of the appropriate alkyl halide with the sodium salt of the heterocycle (2). Compounds were purified by column chromatography or recrystallization or both. The compounds were assayed for purity by ^{1}H nuclear magnetic resonance, UV, and infrared spectroscopy and by elemental analysis. The structures of the two compounds used in the following antiviral assays are presented in Fig. 1.

Antiviral plaque reduction and CPE assays. Compounds T70072 and T01132 were evaluated for their ability to inhibit HCMV, HSV-1, and HSV-2 production in acute infection assays in culture. A compilation of the antiviral profiles of T70072, T01132, DHPG, and ACV against these viruses is presented in Table 1. The results of the anti-HCMV experiments demonstrated that T01132 was more effective at inhibiting HCMV plaque formation than DHPG; the IC_{50} was approximately 0.5 μM for experiments performed using HFF cells, while the IC_{50}s obtained for T70072 and DHPG were 6.8 and 10.8 μM, respectively (Table 1). Results obtained in various HSV assays determined that T01132 was also an effective inhibitor of HSV-1 and HSV-2 (Table 1). T01132 was further evaluated for its effect against DHPG-resistant mutants and primary isolates of HCMV (Table 1). Two DHPG-resistant strains of HCMV were employed. One strain (D16) has been reported to contain a mutation which mapped to the viral DNA polymerase gene (32), while the second mutant strain used (48041) has a mutation within the UL97 open reading frame which is reported to encode the protein kinase used in the phosphorylation of DHPG (31). Compared with DHPG, T01132 had superior activity against all laboratory, drug-resistant, and clinical isolate strains of HCMV tested (Table 1). For all HCMV strains tested, T01132 was at least 10-fold more effective than DHPG at inhibiting virus-induced plaque formation.

Cytotoxicity studies. The ability of T01132 and T70072 to inhibit cell growth was monitored with log-phase growing Vero, MRC-5, KB, and human bone marrow progenitor cells. In Vero, MRC-5, or KB cells the observed median toxic concentrations were between 75 and 150 μM for T01132 and between 80 and 124 μM for T70072 (Table 2). The observed toxic effects of these compounds on bone marrow progenitor cells were the same as, or less than, those of DHPG, depending on the growth factor used in the assay (Table 2).

Measurement of inhibition of viral protein synthesis. The antiviral activity observed for T01132 in the plaque reduction and CPE assays was confirmed by an assay designed to monitor

TABLE 1. Antiherpesvirus activity of alkenyl derivatives of 7-thia-8-oxoguanine

Virus and strain	Culture/assay system	IC_{50} (μM)[a] of:			
		T70072	T01132	DHPG	ACV
HCMV					
AD169	MRC-5/plaque reduction	16.0	<4.0 (0.07)	8.4 (0.78)	ND
P8 (DHPG susceptible)[b]	MRC-5/plaque reduction	ND	<0.4 (0.02)	3.2 (0.65)	ND
D16 (DHPG resistant)[b]	MRC-5/plaque reduction	ND	<0.4 (0.05)	44.0 (2.98)	ND
Towne	HFF/plaque reduction	6.8	0.56	10.8	ND
Towne	HFF/ELISA	ND	1.00	10.0	ND
17517 (DHPG susceptible)[b]	HFF/plaque reduction	ND	0.16	6.8	ND
48041 (DHPG resistant)[b]	HFF/plaque reduction	ND	0.16	50.4	ND
HSV-1					
17	Vero/CPE	22.4	2.12	ND	0.5
KOS	BSC-1/ELISA	ND	4.4	ND	2.0
HSV-2 strain HG52	Vero/CPE	22.4	<1.00	ND	4.4

[a] The numbers in parentheses are selected standard deviation values. For experiments in which the IC_{50}s were below the lowest concentration tested, the standard deviation is given for the lowest concentration tested. Values listed for assays performed in HFF cells are the averages from two or more experiments performed in duplicate. ND, not determined.

[b] D16 is reported to carry a mutation in the HCMV polymerase gene (32), while strain 48041 contains a mutation in the UL97 (protein kinase) open reading frame (31). P8 and 17517 are clinical isolates of HCMV.

viral protein synthesis. In this assay the inhibition of the HCMV Towne strain was monitored by quantitating the presence of virus protein by using virus-specific antibodies in an ELISA format. The anti-HCMV results obtained by this assay reinforced the results obtained by the plaque reduction assay in that a dose-dependent inhibition of viral protein was observed, with an IC_{50} of approximately 1 μM in the ELISA versus 0.56 μM in the plaque reduction assay (Table 1).

The same type of experiment was used to confirm the antiviral activity of T01132 against HSV-1 strain KOS. The observed HSV-1 activity of T01132 in the ELISA (IC_{50} of 4.4 μM) was approximately the same as that obtained by the CPE assay (IC_{50} of ~2.0 μM) for the inhibition of HSV-1 strain 17 (Table 1).

Viral yield assays. T01132 and T70072 were used in a single-cycle assay format to determine the inhibition of HCMV (Towne) replication in MRC-5 cells. The anti-HCMV inhibitory profiles for these two compounds were found to be similar to those obtained by the plaque reduction assay and ELISA, with IC_{50}s of approximately 0.2 and 2.0 μM for T01132 and T70072, respectively (data not shown).

Variations in viral MOI. T01132 was added to HFF cells immediately after infection with various MOIs of HCMV (Towne). At 7 days postinfection, the culture plates were subjected to one round of freezing and thawing and the virus titer was determined as described in Materials and Methods. The results of this assay showed that T01132 was capable of reducing viral titers when cells were initially infected with an MOI of 0.002 or 0.02, with IC_{90}s of approximately 0.15 and 0.5 μM, respectively (Fig. 2). However, when a higher MOI (0.2 to 2.0) was used in the initial infection, the observed IC_{90}s rose to approximately 90 μM (Fig. 2). These data are in contrast to those obtained with DHPG in the same experiment. The IC_{90}s of DHPG were approximately the same at all MOIs tested (Fig. 2).

TABLE 2. Analysis of cellular cytotoxicity of T70072, T01132, and DHPG

Cell line	TC_{50}[a] (μM) of:		
	DHPG	T70072	T01132
Vero	270.4 (17.3)	124	153.6 (26)
MRC-5	>500	132	93.4 (21)
KB		80	75
Bone marrow[b]			
CFU-GM	12.3 (3.2)	127.0 (112.5)	>100
BFU-E	4.6 (1.6)	93.5 (52.3)	14.0 (9.0)

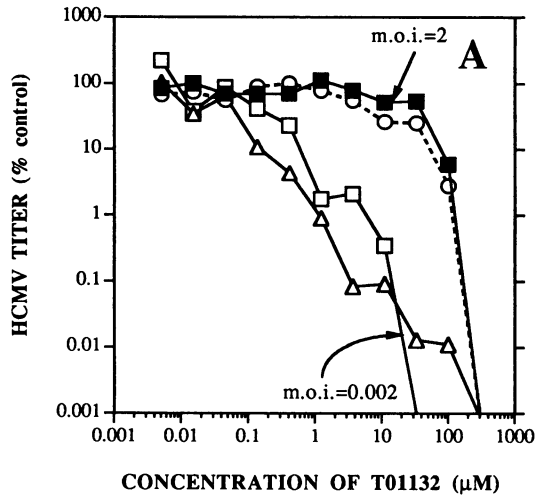
[a] Drug dose required to inhibit log-phase growing cells by 50%. All values listed are the average results of three or four experiments. Selected standard deviation values are in parentheses.

[b] Bone marrow assays were performed using either granulocyte-macrophage colony-stimulating factor (CFU-GM)- or BFU-E assay-stimulated human bone marrow mononuclear cells.

Time of drug administration experiments. To define the point of drug intervention during the viral life cycle, a single-cycle assay in which an MOI of 1 or greater is used is usually employed. However, the activity of T01132 was found to vary with the level of infectious virus used; therefore, we performed a limited timing experiment in which a low MOI was used and the virus titer was monitored after at least two rounds of viral replication. In this assay format, the effects of timing of administration of T01132 during the first round of virus replication would be observed as a decrease in overall virus titer obtained during a subsequent round of infection. The presence of virus was monitored 7 days post-initial infection. The results of this experiment demonstrated that T01132 was capable of maintaining its full antiviral activity when added up to 24 h post-viral infection (Fig. 3) while DHPG (as predicted) maintained most of its activity even when added during the second round of replication (Fig. 3).

In addition, we performed a timing experiment in which a low MOI (0.0002) was used and the virus titer was monitored 3 days postinfection. Although the virus titers were low, the data indicated that T01132 could be added up to 24 h postinfection while DHPG maintained most of its antiviral activity when added up to 48 h postinfection (data not shown).

T01132 in combination with DHPG. A study which examined the combined activity of compound T01132 and DHPG was performed using the ELISA format. The highest concentrations of T01132 and DHPG used in this experiment were 33 and 100 μM, respectively. The data from this experiment were plotted as a three-dimensional graph to produce a dose-response surface. Subsequent analysis of the data yielded a flat surface. Because the result was a plane, with neither peaks nor valleys, the analysis established that there was neither synergy nor antagonism; therefore, the antiviral activity of T01132 and



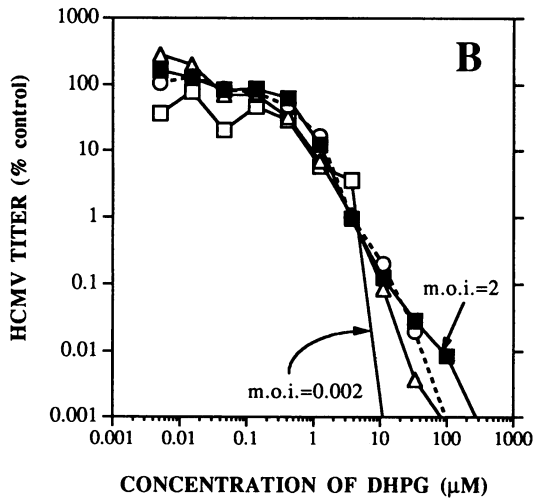
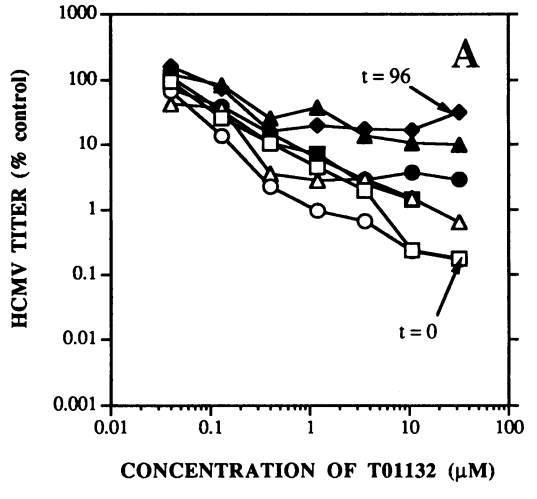


FIG. 2. Effects of the MOI on the inhibition of HCMV replication by T01132 and DHPG. Selected MOIs of HCMV (0.002 [△], 0.02 [□], 0.2 [○], and 2.0 [■]) were used to infect HFF cells treated in a dose-response fashion with either T01132 (A) or DHPG (B). Seven days after infection, cells were freeze-thaw lysed and the titer of virus present in the cell lysate was determined in 96-well plates.



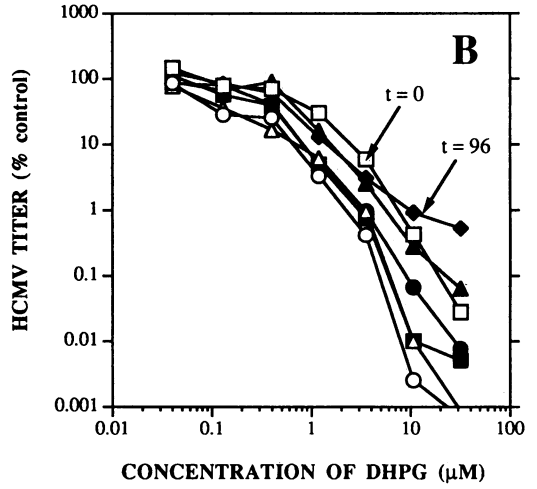


FIG. 3. Effect of time of drug addition on the inhibition profile of T01132. In a dose-response fashion either T01132 (A) or DHPG (B) was added to HFF cells infected with HCMV at an MOI of 0.02 at various times after virus infection (0 h [□], 12 h [○], 24 h [△], 36 h [■], 48 h [●], 72 h [▲], and 96 h [◆]). Seven days postinfection, the cells were freeze-thaw lysed and the titer of the virus present in the cell lysate was determined in 96-well plates.

DHPG when coadministered was additive (data not shown). The concentrations of drugs used in this study were below the observable toxic levels of the individual drugs on stationary-phase HFF cells. When administered together, no synergistic or antagonistic toxicity was observed (data not shown).

Inhibition of viral DNA. Total DNA was isolated from either HCMV- or HSV-2-infected cells at a time which would allow for only one cycle of infection (see Materials and Methods). The nucleic acids were then immobilized on a Nytran filter membrane and probed for the presence of viral DNA. After quantitation of the radioactive virus-specific signal, the filter membranes were washed and rehybridized with cellular DNA to verify the uniform application of extracted DNA to each slot. The viral DNA was normalized to the level of detected cellular DNA (Table 3). There was no detectable decrease in cellular DNA at the time points examined for either virus (data not shown). In these assays T01132 was able to significantly reduce the amount of viral DNA recovered from HSV-2 and HCMV-infected cells. The degree of inhibition was greater in the HSV-2-infected Vero cells than in the HCMV-infected MRC-5 cells (Table 3). This observation may reflect the fact that a relatively high MOI (~0.4) of HCMV was used in this assay.

DISCUSSION

Guanosine analogs of the thiazolo[4,5-*d*]pyrimidine ring system, such as 7-thia-8-oxoguanosine, have been reported to possess in vivo activity against a variety of RNA and DNA viruses (17, 26, 28). Surprisingly, no direct in vitro antiviral activity in virus-infected cells was observed with these compounds (5). Therefore, the in vivo antiviral activity of 7-thia-8-oxoguanosine was attributed to immunomodulatory effects, especially interferon induction (27, 29).

TABLE 3. Inhibition of HCMV and HSV-2 DNA synthesis in a single-cycle assay[a]

Compound	% Inhibition of viral DNA	
	HCMV	HSV-2
None	0	0
T01132 (40 μM)	60	90
Phosphonoacetic acid (150 μg/ml)	93	98.8

[a] Total DNA was extracted 14 h (HSV-2) or 3 days (HCMV) postinfection and analyzed for the presence of viral DNA by slot blot hybridization. Values were normalized by quantitating the amount of cellular DNA in each slot.

Recently, we began to synthesize the acyclic derivatives of 7-thia-8-oxoguanine to test the utility of the thiazolo[4,5-*d*]pyrimidine ring system, which potentially could exert its effects directly on the virus as well as indirectly via immunomodulation. In the course of this effort it became clear that the most active, and least toxic, derivatives tested were those compounds with an alkenyl group attached to position 3 of the ring system (Fig. 1).

The most active derivative of 7-thia-8-oxoguanine, T01132, was found to be a potent inhibitor of HCMV in tissue culture assays. The antiviral activity (IC_{50}) noted for T01132 against HSV-2 was comparable to that of ACV (Table 1). However, the dose-response curve in the HSV-2 assay was shallow so that the IC_{90} obtained for T01132 was less efficacious than that obtained for ACV (data not shown). In initial anti-HCMV evaluations (performed using a low MOI of HCMV) T01132 was approximately 10-fold more active than either T70072 or DHPG and had a therapeutic index on the order of 200 to 300. When compared with DHPG in bone marrow cytotoxicity studies, T01132 was superior to DHPG independent of the growth factor used in the assay. There was no difference in the antiviral profile when T01132 was used to inhibit clinical isolates, laboratory strains, or protein kinase or DNA polymerase DHPG-resistant mutants of HCMV, which suggests that T01132 has a mechanism of action different from that of DHPG. However, when cells were infected with various MOIs of HCMV, T01132 became significantly less active as the level of input virus increased (MOI of 0.2 or above).

It is unlikely that T01132 or T70072 is directly phosphorylated by the virus-encoded kinases (UL97 or HSV thymidine kinase) in the infected cells because of the nature of their side chains. Recently Ashton et al. (1) synthesized a series of ACV and DHPG derivatives in which the acyclic side chains were conformationally constrained in their flexibility by the internal incorporation of a cylcopropane ring or unsaturation but maintained hydrophilic moieties at the termini of the side chains. The authors concluded that there was no absolute correlation of either triphosphate formation or polymerase inhibition with in vitro antiviral activity against HSV-1. In fact, several compounds were found to be not phosphorylated at all by HSV thymidine kinase in their assay systems and yet exhibited modest antiviral activity (1). Conceivably their compounds as well as T01132 and T70072 may be phosphorylated by other cellular enzymes, or their antiviral activity may be unrelated to phosphorylation or DNA polymerase inhibition. For example, inhibition of *S*-adenosylhomocysteine (SAH) hydrolase may be a target for these compounds. However, the 9-(3-methylbutenyl) derivative of adenine, which has the same side chain as T70072, while reported to be mildly inhibitory of bovine liver SAH hydrolase, was over 1,000-fold less effective than the known SAH hydrolase inhibitor Neplanocin A at inhibiting vaccinia virus in culture (6). The acyclic compounds prepared during this study, being guanine analogs, may not be inhibitors of SAH hydrolase. Thus, the mechanism of action of the alkyl derivatives of 7-thia-8-oxoguanosine is open to speculation at this time. A recent report described the anti-HCMV activity of certain nonphosphorylatable derivatives of 7-deazaadenine-7-thiocarboxamide (9-methyl, 9-propyl, and 9-allyl) (22). It was surmised that the thioamide moiety of these analogs provides antiviral activity without the requirement for phosphorylation of a side chain hydroxyl group. The fact that 7-thia-8-oxoguanine derivatives containing phosphorylatable moieties [e.g., 7-thia-8-oxoguanosine and 9-(6-hydroxy-*n*-hexyl)-7-thia-8-oxoguanine] (14, 17) were found to be inactive against HCMV in culture suggests that the observed antiviral activity of T01132 and T70072 is mediated via a unique mechanism.

Thus, the observations presented in this report indicate that T01132 and DHPG have different mechanisms of action, making these alkenyl derivatives of 7-thia-8-oxoguanine an interesting class of compounds to study. However, because T01132 does not inhibit HCMV under culture conditions in which an HCMV MOI of 0.2 or 2.0 is used, it is unlikely that this compound will be efficacious in individuals with a high viral burden. At this time, experiments are in progress to isolate and characterize HCMV mutants resistant to T01132. If the antiviral target for T70032 and T01132 can be identified and proves to be unique, then a class of antiviral agents against this target may be rationally designed.

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