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The 6-Aminoquinolone WC5 Inhibits Human Cytomegalovirus Replication at an Early Stage by Interfering with the Transactivating Activity of Viral Immediate-Early 2 Protein▽†

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WC5 is a 6-aminoquinolone that potently inhibits the replication of human cytomegalovirus (HCMV) but has no activity, or significantly less activity, against other herpesviruses. Here we investigated the nature of its specific anti-HCMV activity. Structure-activity relationship studies on a small series of analogues showed that WC5 possesses the most suitable pattern of substitutions around the quinolone scaffold to give potent and selective anti-HCMV activity. Studies performed to identify the possible target of WC5 indicated that it prevents viral DNA synthesis but does not significantly affect DNA polymerase activity. In yield reduction experiments with different multiplicities of infection, the anti-HCMV activity of WC5 appeared to be highly dependent on the viral inoculum, suggesting that WC5 may act at an initial stage of virus replication. Consistently, time-of-addition and time-of-removal studies demonstrated that WC5 affects a phase of the HCMV replicative cycle that precedes viral DNA synthesis. Experiments to monitor the effects of the compound on virus attachment and entry showed that it does not inhibit either process. Evaluation of viral mRNA and protein expression revealed that WC5 targets an event of the HCMV replicative cycle that follows the transcription and translation of immediate-early genes and precedes those of early and late genes. In cell-based assays to test the effects of WC5 on the transactivating activity of the HCMV immediate-early 2 (IE2) protein, WC5 markedly interfered with IE2-mediated transactivation of viral early promoters. Finally, WC5 combined with ganciclovir in checkerboard experiments exhibited highly synergistic activity. These findings suggest that WC5 deserves further investigation as a candidate anti-HCMV drug with a novel mechanism of action.

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that infects the majority of the human population. Although it rarely causes symptomatic disease in healthy, immunocompetent individuals, it is responsible for a variety of severe diseases in transplant recipients and in human immunodeficiency virus (HIV)-infected patients, including pneumonia, gastrointestinal disease, and retinitis (8, 27). HCMV is also a major cause of congenital malformations in newborn children, often resulting in deafness and mental retardation (8). Currently, only a few drugs have been approved for the treatment of HCMV infections, i.e., ganciclovir (GCV), foscarnet (FOS), and cidofovir (CDV), all of which inhibit the viral DNA (10). GCV, the most widely used anti-HCMV drug, and CDV are nucleoside analogues that function as DNA chain terminators, whereas FOS inhibits HCMV DNA polymerase through mimicry of the pyrophosphate product of polymerization. These drugs have provided a major advance in anti-HCMV therapy, but they suffer from poor bioavailability, significant toxicity, and limited effectiveness. In addition, the emergence of drug-resistant viral strains is becoming an increasing problem for disease management, and since the approved anti-HCMV compounds have similar mechanisms of action, mutant viruses resistant to one drug are often cross-resistant to others (41). Thus, although other anti-HCMV drugs are in clinical development (i.e., maribavir) (1), new anti-HCMV agents, with good safety profiles and more favorable pharmacokinetic properties, that are directed against targets other than the viral DNA polymerase are still needed.

Recently, we reported the identification of a 6-aminoquinolone (6-AQ), WC5, with potent activity against HCMV (25). Quinolones, whose main structural feature is a 1,4-dihydro-4-oxo-quinolinyl moiety bearing an essential carboxyl group at the C-3 position, were first reported as an important class of broad-spectrum antibacterials able to inhibit prokaryotic type II topoisomerases (2). Later, several quinolone derivatives were shown to possess antiviral activity (31); in particular, some 6-AQs, which are characterized by an amino group at the C-6 position of the bicyclic quinolone ring system, were shown to specifically inhibit HIV replication (9, 40). More recently, some 6-AQs were reported to possess broad-spectrum antiviral

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properties: they were able to inhibit the replication of HCMV in addition to that of HIV (37). Remarkably, in contrast to these broad-spectrum 6-AQs, WC5, which is characterized by a cyclopropyl group at the N-1 position and a 4-(2-pyridyl)-1-piperazine moiety at the C-7 position (see Table 1), exhibited specific anti-HCMV activity, in that it did not significantly affect the replication of other human herpesviruses (i.e., herpes simplex virus type 1 and human herpesviruses 6 and 8) and was ~10-fold less active against murine cytomegalovirus (9, 25). In addition, WC5 demonstrated good activity not only against laboratory strains of HCMV but also against clinical isolates and virus strains resistant to clinically relevant anti-HCMV agents (25). These properties prompted us to pursue our studies on this 6-AQ derivative further.

MATERIALS AND METHODS

Compounds. GCV, FOS, actinomycin D (ActD), and cycloheximide (CHX) were all purchased from Sigma. The phosphorothioate oligodeoxynucleotides (ODN) fomivirsen (also known as ISIS 2922) (4) and CpG 2006 (21) were synthesized by Metabion International AG (Martinsried, Germany). Compounds WC5, WC5E, WM5, WT5, and WC13 were synthesized as described previously (9, 40) and were solubilized in 100% dimethyl sulfoxide (DMSO). By following analogous procedures, the new quinolones WT13 and HC5 were also synthesized; the detailed synthetic procedures are reported in the supplemental material.

Cells and virus. Human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Biotechnologies) supplemented with 10% fetal bovine serum (FBS; Life Biotechnologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Biotechnologies). HEK (human embryonic kidney) 293T cells were grown in DMEM supplemented with 10% FBS and penicillin-streptomycin. The U373-MG UL54-EGFP (clone 2F7) and U373-MG UL112/113-EGFP (clone 1B4) reporter cell lines (22) were maintained in medium containing 0.75 mg/ml neomycin (G418; Gibco-BRL). HCMV strain AD169 was purchased from the American Type Culture Collection (Manassas, VA).

Antiviral assays. Plaque reduction assays and virus yield reduction assays were performed as described previously (18).

Cytotoxicity assays. The cytotoxicities of the 6-AQ compounds in HFF were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) method as described previously (18).

Proteins. Purified baculovirus-expressed HCMV UL54 and UL44 proteins were prepared as described previously (19).

DNA polymerase assays. The effects of WC5 and of FOS (used as a control) on the DNA polymerase activity of HCMV UL54 *in vitro*, in the absence or presence of UL44, were tested by a filter-based assay as described previously (18, 19).

qPCR. To analyze the effect of WC5 on viral DNA synthesis, HFF were seeded at a density of 1.5×10^5 per well in 24-well plates. The next day, cells were infected with HCMV AD169 at a multiplicity of infection (MOI) of 1 or 0.1 PFU/cell and were treated with either 50 μM WC5, 10 μM GCV, or 0.2% DMSO as a control. At 72 h postinfection (p.i.), cells were collected, and total DNA was extracted using the QIAamp DNA extraction kit (Qiagen). The levels of viral DNA were then determined by quantitative real-time PCR (qPCR) and were normalized to the cellular β-globin gene copies as described previously (24).

Attachment assays. The effects of WC5 on virus attachment were assessed by the modification of a procedure previously used to assay herpes simplex virus type 1 (35). Briefly, 100 PFU of HCMV AD169 and various concentrations of WC5 or heparin (from porcine intestinal mucosa; Sigma) were added to HFF monolayers that had been prechilled at 4°C and were incubated for 2 or 4 h at 4°C. Cells were then washed extensively with cold phosphate-buffered saline (PBS) to remove the compound and unattached virus, overlaid with a compound-free medium containing 0.6% methylcellulose, and incubated at 37°C for 10 days. To confirm that incubation at 4°C allowed only viral attachment and not entry, cells to which the virus had been preattached at 4°C were treated for 1 min with acidic citrate buffer (40 mM citrate, 10 mM KCl, 135 mM NaCl [pH 3.0]) to inactivate virus that was attached but had not yet penetrated, and these cells were then incubated with a medium containing 0.6% methylcellulose at 37°C for 10 days. This reproducibly resulted in 100% inhibition of plaque formation (data not shown). Cells were then fixed and stained with crystal violet, and plaques were counted.

Entry assays. One hundred PFU of HCMV AD169 was added to HFF monolayers that had been prechilled at 4°C, and the mixture was incubated for 2 h at 4°C to allow viral attachment. Cells were then washed extensively with cold PBS to remove unattached virus. Various concentrations of WC5 or CpG 2006 as a control were diluted in serum-free medium and added to the cells, and the temperature was shifted to 37°C for 2 h. Cells were then treated for 1 min with citrate buffer (40 mM citrate, 10 mM KCl, 135 mM NaCl [pH 3.0]) to inactivate the virus that had not entered the cells, washed with PBS three times to restore a neutral pH, overlaid with a medium containing 0.6% methylcellulose, and incubated at 37°C for 10 days. Plates were then fixed and stained with crystal violet, and the plaques were counted. The number of plaques derived from cell monolayers incubated at 4°C with the virus but not treated with WC5 was taken as 100%.

Time-of-addition and time-of-removal studies. For time-of-addition studies, HFF were seeded at 10^4 per well in 96-well plates, incubated overnight, and infected the next day with HCMV AD169 at an MOI of 1. After virus adsorption for 2 h at 37°C, the viral inocula were removed, and the end of viral adsorption was considered the zero point of the time course. At 0, 1, 2, 6, 9, 12, 24, 32, 48, 56, 60, 72, 78, and 84 h p.i., the cell medium was replaced with a medium containing 50 μM WC5 or 10 μM GCV. The plates were incubated at 37°C for a total of 100 h after infection; at this point, cells were subjected to one cycle of freezing and thawing, and the amount of viral progeny of each well of the microtiter plates was determined by titration on fresh HFF cultures.

For time-of-removal studies, HFF were seeded at 10^4 per well in 96-well plates, incubated overnight, and infected the next day with HCMV AD169 at an MOI of 1. Following a 2-h adsorption, cells were incubated with a medium containing 50 μM WC5 or 10 μM GCV. At 1, 2, 6, 9, 12, 24, 32, 36, 48, 58, 72, 78, 84, and 96 h following the addition of the drug, the medium was removed and was replaced with a drug-free medium after three washes. The plates were incubated for a total of 100 h after infection, and then the virus titer in each sample was determined as described above.

In both assays, data at each point are expressed as the percentage of the control data (the virus titer of the no-drug control at the last time point, i.e., 84 h p.i. for time-of-addition and 96 h p.i. for time-of-removal studies).

RT-PCR assays. HFF were seeded at 6×10^5 per well in 6-well plates. The next day, cells were infected with HCMV AD169 at an MOI of 0.1 PFU/cell and were treated with 50 μM WC5 or with 0.2% DMSO as a control. Infected control samples treated with ActD (5 μg/ml), CHX (75 μg/ml), or FOS (750 μM) as inhibitors of immediate-early (IE), early (E), and late (L) transcription, respectively, were included. Cells were collected at 16 and 48 h p.i., and total RNA was extracted using the SV total RNA isolation system (Promega). Then cDNA synthesis was carried out from 1 μg of total RNA using 5 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate (dNTP), 100 U of RNase inhibitor, 1.25 μM random hexamers, and 150 U of murine leukemia virus (MuLV) reverse transcriptase (RT) (all reagents were from Applied Biosystems) in a final volume of 100 μl. PCR was then performed using 2 μl of cDNA, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 20% DMSO, 0.5 μM each primer (Invitrogen), and 1.25 U of AmpliTaq Gold (Applied Biosystems) in a 25-μl reaction volume. The following primers were used: for *UL122*, 5′-ATGTGGATGGCTTGTATTAAG-3′ (forward) and 5′-CTGGTCAGCCTTGCTTCTAGT-3′ (reverse); for *UL123*, 5′-CAAGAGAAAGATGGACCCTGA-3′ (forward) and 5′-TCGGCCCCCAGAATGTAC-3′ (reverse); for *UL54*, 5′-CACGATGAATTCATGTTTTTCAACCCG-3′ (forward) and 5′-CACACGGACCTCGTACACGGG-3′ (reverse); for *UL84*, 5′-CACGATGAATTCATGCCACGCGTCGACCCC-3′ (forward) and 5′-TTCCACCTGCAGTTAGAGATCGCCGCAGACC-3′ (reverse); for *UL75*, 5′-ATATGCCTCGATGTCTTTTTG-3′ (forward) and 5′-CTGGTGATGTCGGTGATCTGG-3′ (reverse); for *UL55*, 5′-TCAGAGTCTTTCAAGTGTCGG-3′ (forward) and 5′-ATATGAACGTGAAGGAGTCGC-3′ (reverse). Analysis of the cellular β-actin transcript with primers 5′-ACGCCTCTGGCCGTACCACTG-3′ (forward) and 5′-CTTTTAGGATGGCAAGGGACT-3′ (reverse) was included as a control. PCR amplification was then carried out, and each PCR product was analyzed by DNA electrophoresis, followed by staining with ethidium bromide.

Western blot analysis. HFF were seeded at 1.5×10^5/well in 24-well plates. The next day, cells were infected with HCMV AD169 at an MOI of 0.1 PFU/cell and were treated with 50 μM WC5 or with 0.2% DMSO as a control. Infected samples treated with either 5 μg/ml ActD, 75 μg/ml CHX, or 750 μM FOS were included. Cells were collected at 24, 48, and 72 h p.i. and were lysed in 300 mM NaCl, 500 mM Tris-HCl (pH 8), 0.5% NP-40, and protease inhibitors (Complete protease inhibitor cocktail tablets; Roche) for 30 min on ice. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were then electroblotted onto a polyvinylidene difluoride (PVDF)

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membrane (Bio-Rad Laboratories). The blots were blocked for 1 h at room temperature with 5% skim milk in PBS and were then incubated overnight at 4°C with the following antibody: a mouse monoclonal antibody (MAb) against HCMV IE1 and IE2 (clone E13; dilution, 1:500; Argene Biosoft), mouse anti-HCMV UL44 (clone 10-C50; 1:1,000; Fitzgerald Industries International), or mouse anti-HCMV UL55 (1:1,000; Novus Biologicals). Immunocomplexes were detected with a goat anti-mouse immunoglobulin antibody (Ab) conjugated to horseradish peroxidase (diluted 1:2,000; Santa Cruz Biotech) and were visualized by enhanced chemiluminescence (LiteAblot Extend long-lasting chemiluminescent substrate kit; Euroclone). The immunoblots were then stripped and reincubated overnight at 4°C with a mouse anti-human β-actin Ab (1:5,000; Sigma). The immunocomplexes were then detected as described above.

IE2-dependent cell-based assays. For inhibition studies of the IE2 transactivating activity in HCMV-infected cells, the U373-MG UL54-EGFP and U373-MG UL112/113-EGFP cell lines were seeded onto glass coverslips in 6-well plates at a density of 3×10^5 cells/well. The next day, monolayers were infected with HCMV AD169 (MOI, 0.1 or 0.5 PFU/cell) and were then treated with either 50 μM WC5, 5 μM fomivirsen, or 0.2% DMSO as a control. The samples treated with fomivirsen were also pretreated with 5 μM fomivirsen for 1 h prior to infection and during infection. At 48 h p.i., the coverslips were fixed for 15 min with 4% paraformaldehyde in PBS. Following washes with PBS, cells were mounted using mounting fluid (70% glycerol in PBS) and were imaged using a Leica TCS-NT/SP2 confocal microscope with a 20× objective. Images were digitally analyzed with Leica software.

To confirm the inhibition of IE2 transactivating activity, 293T cells at a density of 4×10^5/well were seeded onto glass coverslips in 6-well plates and were incubated overnight at 37°C. The next day, cells were transiently transfected using the calcium phosphate precipitation method with 1 μg of each plasmid. The pUL54-EGFP, pUL112/113-EGFP, and pSGIE86 plasmids used for transfection have been described previously (22). After incubation for 3 h, the transfection mixtures were removed, and medium containing 50 μM WC5, 5 μM fomivirsen, or 0.2% DMSO as a control was added to the cells. At 24 h posttransfection, cells were fixed and imaged as described above.

Drug combination studies. To evaluate the combined inhibitory effects of WC5 and GCV on HCMV replication, a checkerboard combination study was performed using plaque reduction assays. HFF were incubated at 37°C overnight and were then infected with HCMV AD169. Following a 2-h adsorption, compounds were added to quadruplicate samples. The use of a 6-by-6 grid on a 48-well plate allowed the evaluation of WC5 at concentrations of 0, 0.05, 0.1, 0.5, 1, and 2 μM in combination with GCV at 0, 0.1, 0.5, 1, 2, and 4 μM. After a 10-day incubation, cells were fixed and stained with crystal violet, and plaques were counted. The data were analyzed by the isobologram method according to the methods of references 12 and 36. In this analysis, the 50% effective concentrations (EC_{50}s) were used for the calculation of the fractional inhibitory concentration (FIC) as follows: FICx = (EC_{50} of compound X in combination)/(EC_{50} of compound X alone); FICy = (EC_{50} of compound Y in combination)/(EC_{50} of compound Y alone). When the FIC index, which corresponds to the sum of the FIC values of the combined compounds (FIC index = FICx + FICy), is equal to 1, the combination is additive; when the FIC index is between 1.0 and 0.5, the combination is partially synergistic; when it is <0.5, the combination is synergistic; when it is between 1.0 and 2.0, the combination is partially antagonistic; and finally, when it is >2.0, the combination is antagonistic.

RESULTS

SAR studies of WC5. In a previous report (25), we compared the anti-HCMV activity and cytotoxicity of WC5 with those of two 6-AQ analogues, WM5 and WT5, which maintain 4-(2-pyridinyl)piperazine at the C-7 position but bear a methyl and a *tert*-butyl moiety, respectively, instead of the cyclopropyl group at the N-1 position (Table 1). Among these compounds, WC5 exhibited the highest selectivity index, having potent antiviral activity coupled with low cytotoxicity (Table 1) (25). This observation highlighted the key role of the N-1 substituent in modulating compound potency and selectivity. In order to acquire further structure-activity relationship (SAR) information, and in an attempt to increase the potency of WC5, some structural modifications were planned. In particular, compound HC5 was synthesized, in which the amino group at C-6 was replaced by a hydrogen atom. The 6-hydrogen derivative maintained low cytotoxicity but exhibited a 6-fold decrease in antiviral activity (Table 1), indicating that the amino group in the C-6 position is important for preserving potent anti-HCMV activity. This is in agreement with recent reports on other 6-hydrogen-1-methylquinolones, which were found to be inactive against HCMV (39). To explore the importance of the carboxylic substituent at the C-3 position, the anti-HCMV activity of WC5E, the ethyl ester analogue of the lead compound, WC5 (9), was also tested. This compound was completely devoid of anti-HCMV activity (Table 1), suggesting that the 3-carboxylic function likely plays a major role in the recognition of the target involved in the mechanism of antiviral activity. Then we turned our attention to the C-7 position of the quinolone nucleus. It was previously reported that the 6-AQ derivative WC13, characterized by a benzothiazolpiperazinyl moiety at the C-7 position, exhibited potent anti-HCMV activity (25, 37). However, this compound also had high cytotoxicity, likely due to broad-spectrum transactivation-interfering properties (25, 37). In an attempt to preserve the potency of WC13 while decreasing its cytotoxicity, we planned the synthesis of WT13, in which the N-1 *tert*-butyl group, which would confer low toxicity (25), was coupled with the C-7 benzothiazolpiperazinyl moiety, which would impart high antiviral activity. Compound WT13 maintained good anti-HCMV activity, confirming the suitability of the benzothiazolpiperaziny moiety, but unfortunately, this was coupled with high cytotoxicity, a pattern analogous to that shown by the N-1 cyclopropyl derivative WC13 (Table 1).

Taken together, the SAR information derived from the small series of analogues synthesized showed that WC5 possesses the most suitable pattern of substitutions around the quinolone scaffold for conferring potent and selective anti-HCMV activity. Thus, subsequent studies were focused on the further characterization of WC5 activity.

The anti-HCMV activity of WC5 does not rely on inhibition of viral DNA polymerase. We previously showed that WC5 retains its activity against HCMV strains resistant to DNA polymerase inhibitors (GCV, CDV, acyclovir [ACV], and FOS) (25), which suggested that the antiviral activity of WC5 does not likely entail inhibition of viral DNA polymerase. However, it has been reported that some compounds structurally related to 6-AQ, i.e., 4-oxo-dihydroquinoline carboxamides, which are active against both GCV-resistant and CDV-resistant HCMV strains, also show activity against HCMV DNA polymerase (7, 28). Thus, to investigate whether WC5 might inhibit the HCMV DNA polymerase through a mechanism different from that of GCV, CDV, ACV, and FOS, we tested the effects of the compound on the activity of the viral enzyme *in vitro*. The enzyme activity in the presence of serial dilutions of WC5 or of FOS as a control was evaluated by measuring the incorporation of [^{3}H]dTTP into a poly(dA)-oligo$(dT)_{12–18}$ template-primer by a purified, baculovirus-expressed DNA polymerase catalytic subunit (UL54) both in the absence and in the presence of the accessory subunit (UL44). As expected, FOS inhibited the DNA polymerase activities of both UL54 alone and the UL54/UL44 complex in a dose-dependent manner, with 50% inhibitory concentrations (IC_{50}s) of 3.1 μM and 5.5 μM, respectively (Fig. 1). In contrast, WC5 showed very weak inhibitory activity against both UL54 alone

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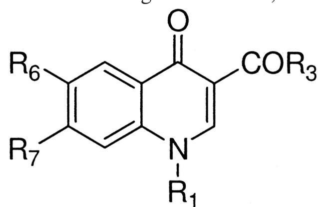
TABLE 1. Chemical structures, antiviral activities against HCMV, and cytotoxicities of quinolone derivatives



Compound	Chemical composition[a]				Antiviral activity[b]	Cytotoxicity[c]	SI[d]
	R_1	R_3	R_6	R_7			
WC5[e]	*c*-Pr	OH	NH_2		0.9 ± 0.2	431 ± 61	479
WM5[e]	Me	OH	NH_2		0.7 ± 0.2	54 ± 15	77
WT5[e]	*t*-But	OH	NH_2		36.7 ± 15.2	≥475	≥13
HC5	*c*-Pr	OH	H		5.6 ± 1.3	390 ± 37	70
WC5E	*c*-Pr	O-Et	NH_2		>50	409 ± 46	>8
WC13[e]	*c*-Pr	OH	NH_2		0.02 ± 0.05	4.5 ± 3.9	225
WT13	*t*-But	OH	NH_2		0.18 ± 0.1	5.8 ± 2.3	32
GCV[e]					1.9 ± 0.2	550 ± 75	289

[a] Me, methyl; *c*-Pr, cyclopropyl; *t*-But, *tert*-butyl; Et, ethyl.
[b] Expressed as the EC_{50} (in micromolar concentrations), defined as the concentration of the compound that inhibits 50% of plaque formation, as determined by plaque reduction assays against HCMV AD169 in HFF. Reported values are means ± standard deviations of data derived from at least three independent experiments performed in duplicate.
[c] Expressed as the CC_{50} (in micromolar concentrations), defined as the concentration of the compound that produces 50% cytotoxicity as determined by MTT assays in HFF. Reported values are means ± standard deviations of data derived from at least three independent experiments performed in quadruplicate.
[d] SI, selectivity index, determined as the ratio between the CC_{50} and the EC_{50}.
[e] Data for this compound were reported in reference 25.

and the UL54/UL44 complex (Fig. 1), exhibiting IC_{50}s (>100 μM) much higher than the EC_{50} observed in plaque reduction assays in HCMV-infected cells (0.9 μM) (Table 1) (25). Thus, it seems unlikely that the anti-HCMV properties of this 6-AQ derivative are due to inhibition of the polymerization activity of the viral DNA polymerase.

Effects of WC5 on viral DNA synthesis. Given the ability of quinolones to interact with protein-nucleic acid complexes (2, 30), the possibility existed that WC5 might inhibit HCMV DNA replication either by affecting the activity of a component of the DNA replication complex other than the viral polymerase or of a protein(s) indirectly involved in virus genome replication (26, 29) or by interfering with DNA maturation. To test this hypothesis, the effect of WC5 on viral DNA synthesis in HCMV-infected cells was quantitatively determined by qPCR at 72 h p.i. (Fig. 2). GCV was used as a control, and as expected from its mode of action, it strongly inhibited HCMV DNA replication. Similarly, 98% inhibition of viral DNA synthesis was observed in the presence of WC5. Thus, WC5 appears to prevent viral DNA synthesis, suggesting that it likely interferes with an event of the HCMV replication cycle that precedes or takes place during virus DNA replication.

The antiviral activity of WC5 depends on the MOI. To characterize further the antiviral activity of WC5, we performed yield reduction experiments in HFF infected with HCMV at an MOI of 1 PFU/cell. Interestingly, in these assays WC5 showed an activity almost 10-fold lower (EC_{50}, 8.6 ± 2.1 μM) than that exhibited in plaque reduction assays (EC_{50}, 0.9 ± 0.2 μM) (Table 1) (25). One possible explanation was the different MOIs used in the two assays. To examine the effects of MOI on the anti-HCMV activity of WC5, yield reduction assays were performed at three different MOIs (0.01, 0.1, and 1 PFU/cell). Dose-response curves for WC5 at the three MOIs tested were very different, with significantly lower antiviral activity observed at an MOI of 1 PFU/cell than at an MOI of 0.01 PFU/cell (see Fig. S1 in the supplemental material). To

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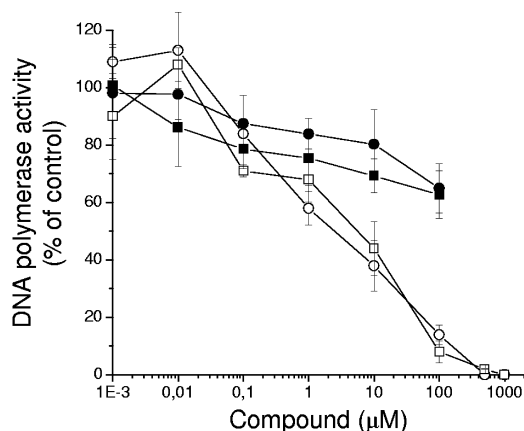

FIG. 1. WC5 does not significantly inhibit the activity of HCMV DNA polymerase *in vitro*. The effects of increasing concentrations of WC5 (filled symbols), and of FOS as a control (open symbols), on the activity of the purified, baculovirus-expressed HCMV DNA polymerase catalytic subunit UL54 in the absence (circles) and in the presence (squares) of the accessory subunit UL44 were determined by filter-based DNA polymerase assays. The data shown are means ± standard deviations (error bars) for four independent experiments.

explore this MOI dependency further, we compared the effects of MOI on the antiviral activity of WC5 with those on GCV activity. In repeated experiments, there was no statistically significant difference among the EC_{90}s of GCV at three different MOIs (Table 2), as determined by paired *t* tests (P, $\geq$0.05). This result is in agreement with previous observations that GCV does not act in an MOI-dependent manner (17, 36). In contrast, there were significant differences among the EC_{90}s of WC5 at all MOIs tested (Table 2), with the greatest difference between the EC_{90}s of WC5 at MOIs of 1 and 0.01 PFU/cell (P, 0.005), as expected. These data indicate that the antiviral activity of WC5, unlike that of GCV, is highly

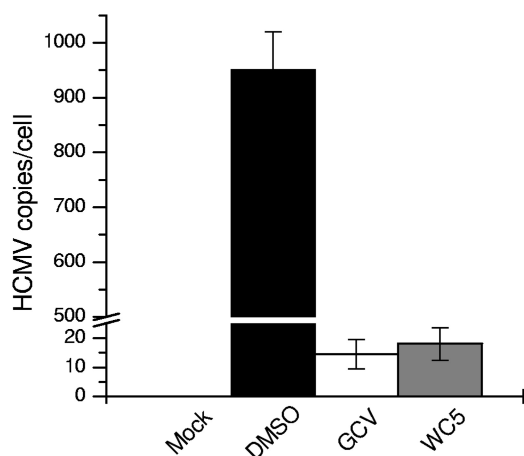

FIG. 2. WC5 prevents viral DNA synthesis in HCMV-infected cells. HFF were either infected with HCMV AD169 (MOI, 0.1) or mock infected (Mock) and were then treated with either WC5, GCV, or DMSO as a control. At 72 h p.i., total DNA was extracted, and qPCR was performed with appropriate IE2 and β-actin primers. HCMV genomic copies were normalized to the cellular β-actin gene copies. Data shown are means ± standard deviations (error bars) for three independent experiments performed in duplicate.

TABLE 2. Effects of MOI on antiviral activities of WC5 and GCV

MOI (PFU/cell)	EC_{90} (μM)[a]	
	WC5	GCV
1	51.9 ± 11.4	0.8 ± 0.3
0.1	34.7 ± 9.4	1.0 ± 0.1
0.01	8.5 ± 1.7	1.0 ± 0.2

[a] Defined as the concentration of the compound that inhibits 90% of infectious virus yield as determined by yield reduction assays in HFF infected with HCMV AD169 at different MOIs. Reported values are means ± standard deviations of data derived from at least three independent experiments performed in duplicate.

dependent on the viral inoculum, as shown for other early-acting antiherpetic compounds whose antiviral activity was MOI dependent (6, 13, 15). Thus, this finding suggests that WC5 might act early in the virus replication cycle.

WC5 does not affect the attachment and entry of HCMV. We therefore investigated whether WC5 might affect the initial phases of the virus life cycle, i.e., attachment and/or entry. To examine the effects of WC5 on the viral adsorption stage, we tested the inhibition of plaque formation when WC5 was present for 2 or 4 h only during viral attachment at 4°C (a condition that is known to allow virus adsorption only). As a positive control, we assayed the susceptibility of viral attachment to heparin, which is a polyanionic molecule chemically similar to heparan sulfate moieties located on the cell surface and has been shown to inhibit the attachment of HCMV (16). As expected, heparin was able to inhibit virus attachment potently (EC_{50}, 0.15 μM [Fig. 3A]). In contrast, WC5 did not significantly reduce the binding of HCMV virions to HFF.

To test the effects of WC5 on viral entry, we preattached the virus to cells at 4°C in the absence of drug, added various concentrations of WC5, and then incubated the mixtures at 37°C for 2 h to permit entry. The ODN CpG 2006, which has been shown to block HCMV entry (21), was included as a control. After virus that had not entered was inactivated with acidic citrate buffer, plaques were allowed to form and were then counted. As shown in Fig. 3B, WC5 did not significantly affect the entry of HCMV (EC_{50}, >25 μM), while CpG 2006 exhibited inhibitory activity. Thus, these results indicate that WC5 does not block either the adsorption of the virion particle to the host cell or virus entry.

Time-of-addition and time-of-removal studies. To determine the phase of the HCMV replication cycle that is affected by WC5, experiments were performed in which 50 μM WC5, or 10 μM GCV for purposes of comparison, was added to infected cells at different times p.i., and the HCMV titer was determined at 100 h p.i. Such compound concentrations were selected because they were able to completely inhibit HCMV replication in HFF but were not cytotoxic (25). Viral replication was fully blocked when WC5 was added up to ~30 h p.i. (Fig. 4A). Increasing viral titers were observed when WC5 was added from ~30 h to 84 h p.i. GCV, on the other hand, markedly reduced virus yield when it was added up to 56 to 60 h p.i., when HCMV DNA replication occurs (38), but it produced lower or no reductions in viral titers when added at 72 h p.i. or later (Fig. 4A). These results suggest that WC5 likely targets a process occurring earlier than DNA synthesis.

Studies to examine the effect of removal of WC5 at various

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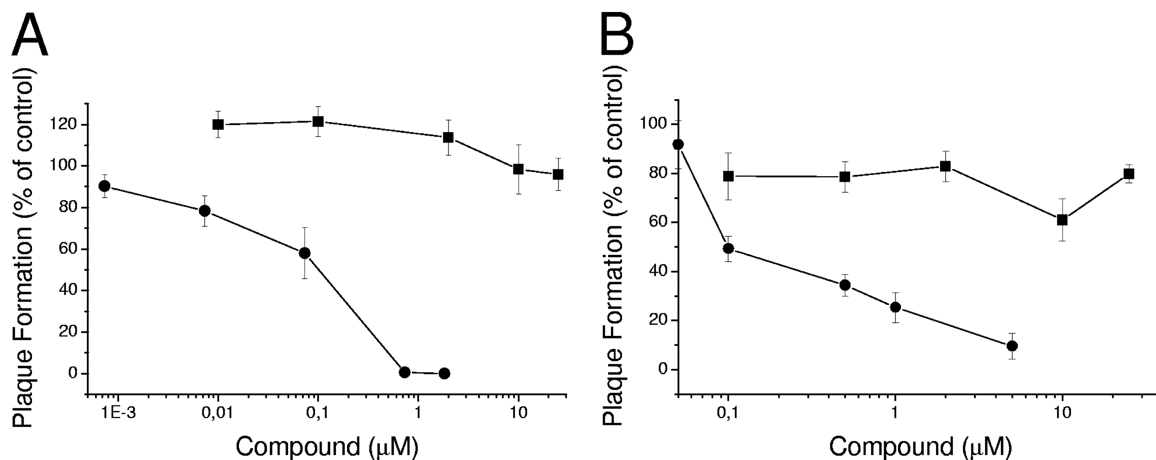

FIG. 3. WC5 does not interfere with the adsorption and entry of HCMV. (A) Effects on attachment. HCMV AD169 was allowed to attach to HFF at 4°C in the presence of various concentrations of WC5 (■) or heparin (●) as a control. (B) Effects on entry. HCMV AD169 was attached to cells at 4°C; unattached virus was removed; and the cells were incubated for 2 h at 37°C in the presence of various concentrations of WC5 (■) or ODN CpG 2006 (●) as a control. The data shown in both panels are means ± standard deviations for three experiments carried out in duplicate.

times p.i. were performed to further elucidate when the 6-AQ derivative acts in the virus lytic cycle. HFF monolayers were infected with HCMV AD169 and were treated with 50 μM WC5 or 10 μM GCV. The compounds were then washed from the cultures at various times p.i. up to 96 h, and the amount of infectious virus released was determined at 100 h p.i. As shown in Fig. 4B, removal of GCV prior to viral DNA synthesis resulted in a partial block of HCMV replication, with complete inhibition observed from 48 h p.i. In contrast, WC5 appeared to possess diminished activity if it was removed as early as 12 h p.i., and it completely blocked HCMV replication from 36 h p.i. These observations are consistent with the results of the time-of-addition studies, which showed that WC5 acts before GCV in the HCMV lytic cycle.

Effects of WC5 on HCMV gene transcription and protein expression. To gain deeper insight into the mechanism of WC5 antiviral activity, its effects on the transcription of selected viral immediate-early (IE), early (E), and late (L) genes were studied using RT-PCR analysis. Transcripts were selected as representatives of their kinetic class, irrespective of any specific role in HCMV replication. HFF were infected with HCMV and were treated with 50 μM WC5. The RNA synthesis inhibitor ActD, the protein synthesis inhibitor CHX, and the viral DNA polymerase inhibitor FOS were included in order to temporally categorize each transcript. RNA extracts were prepared at 16 and 48 h p.i. (within the first viral replication cycle), and RT-PCR amplification of HCMV transcripts and human β-actin mRNA was performed on the same cDNA sample. The

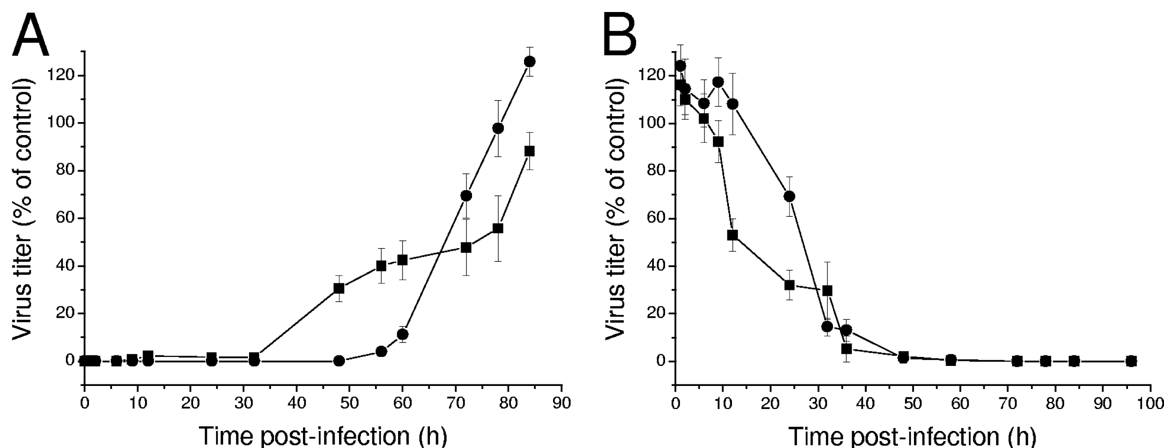

FIG. 4. Effects of addition and removal of WC5 at different time-points after HCMV infection. (A) Effects of time of addition of WC5 and GCV on HCMV yield. HFF were infected with HCMV AD169 at an MOI of 1 and were then treated with WC5 (■) or GCV (● at 0, 1, 2, 6, 9, 12, 24, 32, 48, 56, 60, 72, 78, or 84 h p.i. The antiviral activity of each compound is expressed as a percentage of the virus titer in the untreated infected samples. (B) Effects of time of removal of WC5 or GCV on HCMV yield. HFF were infected with HCMV AD169 at an MOI of 1 and were treated with WC5 (■) or GCV (●). At 1, 2, 6, 9, 12, 24, 32, 36, 48, 58, 72, 78, 84, or 96 h p.i., the cells were rinsed to remove the compound, and fresh medium was added; infected cultures with no compound were treated identically to serve as controls. In both panels, the data shown are means ± standard deviations for three experiments carried out in duplicate.

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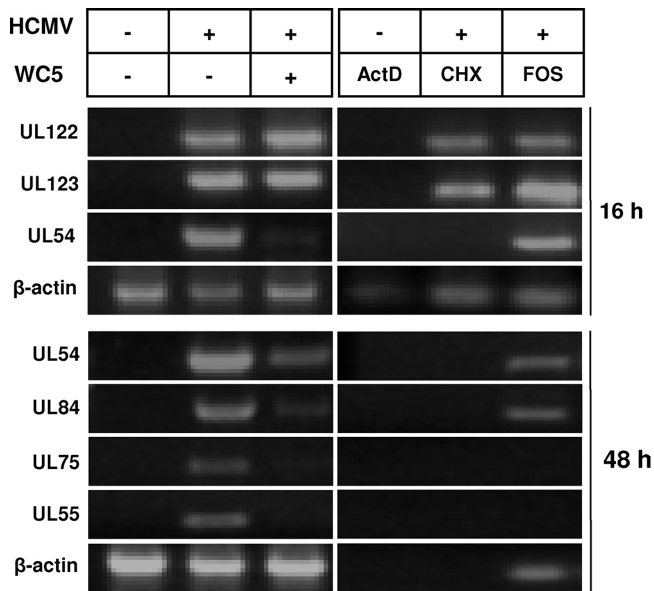

FIG. 5. WC5 inhibits transcription from viral E and L genes but not from viral IE genes. HFF were either infected with HCMV AD169 (MOI, 0.1) or mock infected and were treated with WC5 or DMSO as a control. Infected cell samples treated with ActD, CHX, and FOS were included as controls for inhibition of IE, E, and L gene expression, respectively. At 16 and 48 h p.i., total RNA was extracted and reverse transcribed. PCRs were then carried out with primers for the *UL122* and *UL123* (IE), *UL54* and *UL84* (E), and *UL55* and *UL75* (L) genes. Human β-actin was included as a control. PCR products were analyzed in ethidium bromide-stained agarose gels.

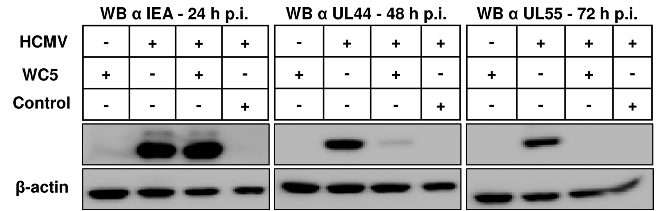

FIG. 6. Effects of WC5 on the expression of HCMV IE, E, and L proteins. HFF were either infected with HCMV AD169 (MOI, 0.1) or mock infected and were then treated with WC5 or with DMSO as a control. At 24, 48, and 72 h p.i., whole-cell extracts were prepared, fractionated by SDS-PAGE, and analyzed by Western blotting (WB) with anti-IEA (IE1 and IE2), anti-UL44, and anti-UL55 antibodies. Controls for inhibition of gene expression were ActD (24 h), CHX (48 h), and FOS (72 h). Immunodetection of cellular β-actin was also included as an internal control.

time points chosen for RNA extraction exceed those generally used for studying superinduction of HCMV IE mRNA (see, e.g., reference 14); however, the 16- and 48-h time points enabled us to detect at the same time IE/E and E/L gene expression, respectively, as already reported in studies of other anti-betaherpesvirus compounds (see, e.g., references 11 and 28). Based on their decreased levels following incubation with ActD, CHX, or FOS, the selected HCMV transcripts were categorized as IE (UL122 and UL123), E (UL54 and UL84), or L (UL55 and UL75). No IE, E, or L mRNAs were present in the ActD-treated samples, as expected, while WC5 had no inhibitory effect on the transcription of the IE genes *UL122* and *UL123* (Fig. 5). In contrast, the levels of the two early transcripts, UL54 and UL84, were clearly reduced following incubation with WC5 as well as with CHX, while no significant effect of FOS was observed on the transcription of these genes. Both WC5 and FOS strongly inhibited the expression of the *UL55* and *UL75* late genes.

We next examined the effects of WC5 on overall HCMV protein synthesis. For this purpose, HFF were infected with HCMV and were then incubated with WC5 or 0.2% DMSO as a control, and the expression of viral IE, E, and L proteins was evaluated by both Western blotting and immunofluorescence at various times p.i. Infected control samples treated with ActD, CHX, or FOS as inhibitors of IE, E, and L gene expression, respectively, were included. Western blot analysis after 24 h (Fig. 6, left) showed strong expression of the IE proteins p72 and p86, which was completely inhibited by CHX, whereas WC5 had no influence on the expression of these proteins. At 48 h p.i., the expression of the early protein UL44 was not completely inhibited but was strongly reduced by WC5 (Fig. 6, center). The HCMV late protein UL55 was not detected in the presence of WC5 or of FOS at 72 h p.i. (Fig. 6, right). Similar effects were observed when cells were stained with antibodies against the same IE (p72/p86), E (UL44), and L (UL55) proteins (data not shown).

Taken together, these results demonstrate that WC5 targets an early phase of the viral replicative cycle that follows the transcription and translation of IE genes and precedes those of E and L genes.

Effects of WC5 on HCMV IE2 transactivating activity. The results presented above suggested that WC5 might act by inhibiting the activity of IE proteins. HCMV IE genes encode the transcriptional regulators IE1 (p72) and IE2 (p86), which are required for the subsequent expression of E and L genes (23). In particular, the IE2 protein has been shown to play a crucial role in the transactivation of viral E and L genes (23). To analyze the effects of WC5 on IE2-mediated E gene transactivation in HCMV-infected cells, we employed a cell-based assay (22) in which the expression of the enhanced green fluorescent protein (EGFP) reporter gene is driven by the *UL54* or *UL112*/*UL113* E gene promoter, both of which are transactivated by IE2 protein during HCMV replication (3, 34, 42). U373-MG cells stably transfected with a plasmid expressing the UL54-EGFP or UL112/113-EGFP reporter were infected with HCMV at an MOI of 0.1 or 0.5 and were subsequently treated with WC5 or with fomivirsen (ISIS 2922) as a reference compound. Analysis of EGFP expression in control HCMV-infected cells (MOI, 0.1) at 48 h p.i. showed a specific fluorescence signal (Fig. 7A). As expected, the HCMV-induced EGFP expression was almost completely prevented by treatment of indicator cells with fomivirsen, an antisense oligodeoxynucleotide designed to block IE2 expression (4). A pronounced inhibitory effect was also observed when infected cells were treated with WC5 (Fig. 7A). Similar effects were detected in cell monolayers infected with HCMV at an MOI of 0.5 (data not shown).

To confirm that the effects observed in HCMV-infected cells could be ascribed to inhibition of IE2 transactivating activity, 293T cells were transiently cotransfected with the UL54-EGFP or UL112/113-EGFP reporter plasmid and with an expression vector for IE2 (pSGIE86); then they were treated with either

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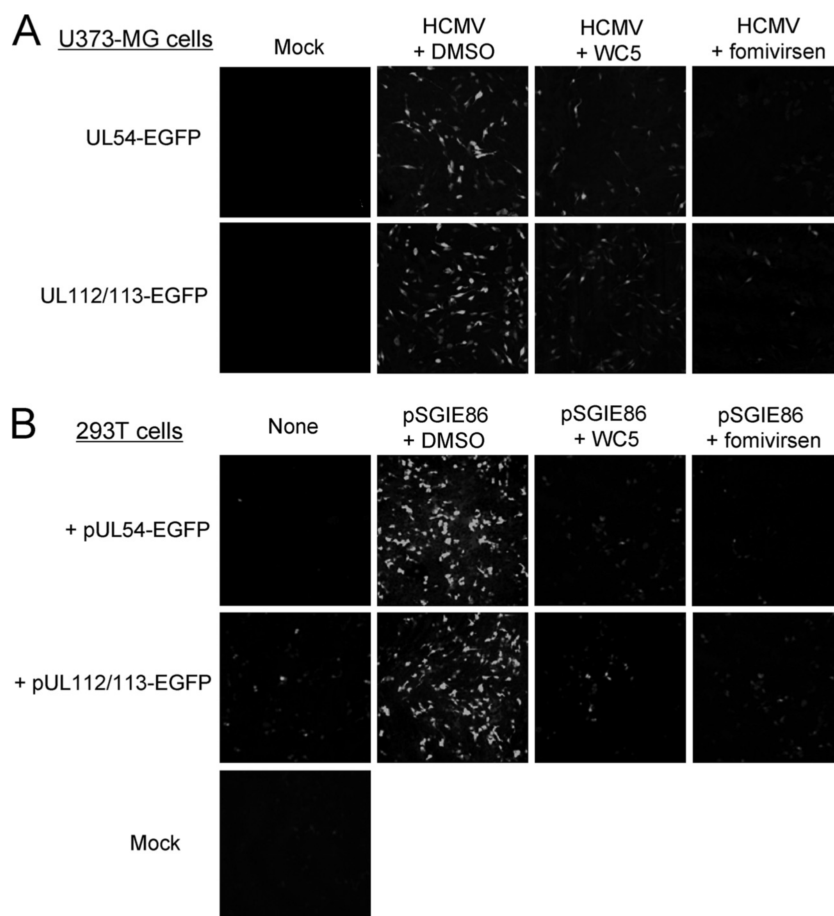

FIG. 7. WC5 inhibits the transactivation of E promoters by IE2. (A) U373-MG cells stably transfected with the pUL54-EGFP or the pUL112/113-EGFP plasmid were either infected with HCMV AD169 (MOI, 0.1) or mock infected. Cells were then treated with either WC5, fomivirsen, or DMSO as a control. At 48 h p.i., the cells were examined by confocal fluorescence microscopy to evaluate the number of EGFP-expressing cells. (B) 293T cells either were transiently transfected with the UL54-EGFP or the UL112/113-EGFP reporter plasmid alone or in combination with pSGIE86 or were mock transfected (Mock). Cells were then treated with WC5, fomivirsen, or DMSO as a control. At 24 h after transfection, the cells were analyzed for EGFP expression by confocal fluorescence microscopy. Representative images are shown for both panels.

WC5, fomivirsen, or 0.2% DMSO as a control. A significant increase in EGFP expression was observed in DMSO-treated cells (Fig. 7B). Treatment with fomivirsen reduced the IE2-induced EGFP fluorescence to background levels in both UL54-EGFP- and UL112/113-EGFP-expressing cells (Fig. 7B). Consistent with the results from assays in HCMV-infected cells, WC5 also markedly reduced EGFP expression (Fig. 7B). When different concentrations of WC5 were tested, an EC_{50} of ~8 μM for inhibition of IE2 transactivation was determined (B. Mercorelli and A. Loregian, unpublished results).

Taken together, these results strongly suggest that WC5 interferes with the IE2-mediated transactivation of E gene promoters.

Drug combination studies. Since WC5 appeared not to share the same target as GCV, we then wanted to investigate whether a combination of the two compounds would result in synergistic activity against HCMV replication. The combined effects of WC5 and GCV on the replication of HCMV AD169 in HFF were evaluated by plaque reduction assays and were then analyzed by an isobologram method (12). Figure 8 is an isobologram representing the WC5-GCV interaction; in this figure, the solid line represents the theoretical plot for an additive interaction between the two compounds (which is the expression of an independent antiviral effect), while the broken line represents the theoretical plot for a significant synergistic interaction. Since the FIC values obtained for several of the combinations of WC5 and GCV were located under the plot representing synergism (FIC index, <0.5), we concluded that the two compounds interact in a strongly synergistic manner, each reinforcing the other's anti-HCMV activity. Importantly, WC5, when combined with GCV and tested for its 50% cytotoxic concentration (CC_{50}) toward growing HFF, proved additive rather than synergistic (data not shown), suggesting that the highly synergistic antiviral activity was not due to an increased CC_{50} but, plausibly, the result of interference with two different steps of the viral replicative cycle.

DISCUSSION

WC5 has recently emerged, among a series of 6-AQ derivatives tested for their anti-HCMV activities, as an antiviral

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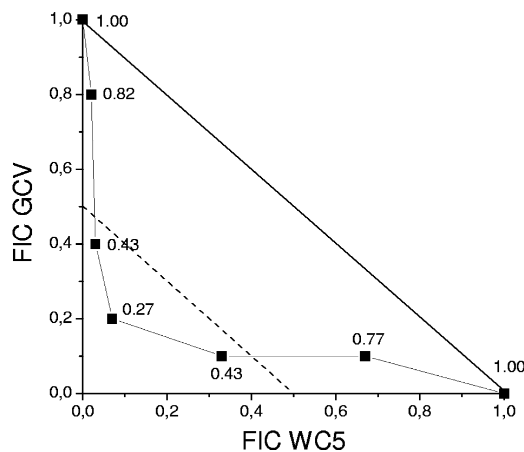

FIG. 8. Effects of combinations of WC5 and GCV on HCMV replication. HFF were infected with HCMV AD169 and were then treated with different concentrations of WC5 either alone or combined with different concentrations of GCV. The data for each compound are reported as the fractional inhibitory concentration (FIC), calculated by dividing the EC_{50} of compound X in combination with compound Y by the EC_{50} of compound X alone. The solid and broken lines represent the unit lines for FICs equal to 1 and 0.5, respectively. The FIC index, which is the FIC of compounds X and Y combined, is given for every combination of concentrations. A FIC index between 0.5 and 0.9 is suggestive of partial synergism, whereas a FIC index of <0.5 indicates significant synergism. The results are from a single experiment performed in quadruplicate, representative of two independent experiments.

agent with an *in vitro* activity equal to or greater than that of GCV against both laboratory strains and clinical isolates of HCMV (25). Intriguingly, a striking feature of WC5 is its specificity for HCMV, as it has essentially no or little inhibitory activity against other human or animal herpesviruses (25). In stark contrast, other 6-AQs have been reported to possess broad-spectrum antiviral properties, as they can inhibit HIV and murine CMV in addition to HCMV replication (37). Since the specific antiviral activity of WC5 might provide an interesting platform for future clinical applications, in the present study the nature of the anti-HCMV activity of this compound has been further examined.

Our previous observation that HCMV strains showing resistance to GCV, ACV, CDV, or FOS are still sensitive to WC5 (25) suggested that the HCMV DNA polymerase could not serve as a target for this 6-AQ derivative. Indeed, when WC5 was examined for its effects against the HCMV DNA polymerase catalytic subunit, UL54, in *in vitro* DNA polymerase assays, no significant inhibitory activity was observed (Fig. 2). Therefore, it was concluded that most likely WC5 does not directly target UL54 activity. However, it should be kept in mind that HCMV DNA synthesis in infected cells requires the interaction of UL54 with the DNA polymerase accessory protein UL44, which binds double-stranded DNA (dsDNA) with high affinity and stimulates UL54-mediated DNA polymerase activity (19, 20). Thus, by including this protein in the reaction mixture of the DNA polymerase assays, we also investigated whether WC5 might indirectly inhibit HCMV DNA polymerase by interfering with the activities of UL44, e.g., through disruption of its interaction with UL54, as reported for other compounds (18), or through inhibition of its DNA-binding activity. Our data indicate that most likely the DNA polymerase accessory protein can also be excluded as a potential target for WC5. Of note, in contrast to WC5, structurally related 4-oxo-dihydroquinoline carboxamides were highly active against the HCMV DNA polymerase and exhibited broad-spectrum antiherpesvirus activity (7, 28).

Experiments to test the effects of WC5 on HCMV DNA replication showed that it prevents viral DNA synthesis, suggesting that it might act by inhibiting a protein(s), other than the viral DNA polymerase, directly or indirectly involved in virus genome replication (26, 29). However, time-of-addition and time-of-removal studies then revealed that WC5 targets an early event in the HCMV replicative cycle that takes place prior to viral DNA replication. In fact, compared to the reference compound GCV, which targets the viral DNA polymerase, WC5 was able to inhibit HCMV replication when added earlier in infection than the DNA synthesis inhibitor. In addition, the inhibition was not reversible, suggesting either tight-binding inhibition of a viral target or inhibition of an early viral event required for subsequent replication steps. Among the first events that take place during the virus replication cycle, a block of viral adsorption or penetration could be excluded based on our results that no significant inhibition of HCMV infection was observed when WC5 was present only during the attachment or entry phase.

In a pattern typical of herpesviruses, HCMV gene expression is temporally regulated after infection and can be divided into immediate-early (IE), early (E), and late (L) phases (27). Therefore, we next analyzed both viral gene transcription and protein expression throughout the virus replicative cycle in WC5-treated cells. Our results clearly pointed to a step of the virus replicative cycle that follows the expression of IE antigens and precedes that of E and L antigens as the target for WC5. Since it has been shown that the IE2 protein plays a major role in regulating the expression of viral E and L genes (23), we hypothesized that WC5 might interfere with the IE2 transactivating activity. We show here that WC5 does inhibit IE2-mediated transactivation of two different HCMV E promoters. Importantly, we previously reported that WC5 did not affect GFP expression driven by cellular and other viral promoters (25). Of note, consistent with the results of the studies on the effects of WC5 on HCMV gene transcription and expression (Fig. 6 and 7), WC5 also did not interfere with the transactivation of the HCMV IE promoter (25). Thus, the inhibition of IE2 transactivating activity does not stem from broad-spectrum transactivation-interfering properties such as those possessed by other 6-AQs (37). Further exploratory work, such as the isolation and characterization of WC5-resistant viruses, will be required in order to provide a better understanding of the molecular basis of the anti-IE2 activity of WC5 and to demonstrate the link with its highly specific anti-HCMV activity. However, as reported in earlier studies with compounds that inhibit HCMV at an early step in replication (see, e.g., references 6 and 36), the isolation of drug-resistant mutants might not be feasible. Indeed, after 6 months of passage in cell culture according to the procedure described by Biron et al. (5) no virus resistance against WC5 has been detected yet (B. Mercorelli and A. Loregian, unpublished results).

Whatever the molecular mechanism may be, the inhibition of IE2 activity could prove to be beneficial at more than one

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level. There is compelling evidence that IE2 plays a direct role in the pathogenesis of HCMV infection by inducing a broad dysregulation of host gene expression. This leads to changes in host cell physiology and contributes to HCMV-induced cell cycle alterations, immunomodulation, and proinflammatory responses (32). Thus, molecules that reduce IE2 expression (such as fomivirsen) or inhibit IE2-dependent activities may be effective in blocking the virus-induced pathological phenomena at an early stage of infection. Moreover, inhibitors of IE2 could be of particular importance for the treatment of patients who do not respond to the currently used inhibitors of viral DNA replication (33). Although a number of compounds with anti-HCMV activity have been reported to act at early stages of virus replication, their targets are still mostly unknown, and fomivirsen, the sole approved anti-HCMV drug specifically designed against IE2, is no longer marketed.

In addition, since WC5 and GCV act against different viral targets, the use of these two compounds in combination could potentiate the effect of each compound alone. Indeed, our drug combination studies showed that when WC5 and GCV are used in combination, inhibition of viral replication is achieved at lower drug concentrations than when the drugs are used individually (Fig. 8). Thus, WC5 and GCV exhibit strong synergistic antiviral activity without significantly increased toxicity. Combination therapy with a reduced GCV dose may result in similar synergistic effects, providing a means to treat HCMV infectious diseases while reducing drug toxicity and the emergence of drug-resistant mutants.

In summary, this is the first study—to our knowledge—showing that a potent anti-HCMV compound acts by inhibiting the IE2 transactivating activity; thus, this compound has a target clearly different from that of the anti-HCMV drugs currently approved for clinical use. This observation, together with our previous finding that WC5 is not cross-resistant with ganciclovir, acyclovir, cidofovir, and foscarnet (25), may offer an important advantage in the potential clinical use of this compound, since drug-resistant HCMV strains often emerge after long-term treatment with antiherpetic drugs. Furthermore, the observation that WC5 shows synergism upon cotreatment with ganciclovir opens up the possibility for drug combinations, although this is not common practice in herpesvirus therapy. Thus, WC5 deserves further investigation as a candidate anti-HCMV drug.

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