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Selective Anti-Cytomegalovirus Compounds Discovered by Screening for Inhibitors of Subunit Interactions of the Viral Polymerase

Arianna Loregian[1,2] and Donald M. Coen[1,*]
[1] Department of Biological Chemistry
and Molecular Pharmacology
Harvard Medical School
250 Longwood Avenue
Boston, Massachusetts 02115
[2] Department of Histology
Microbiology and Medical Biotechnologies
University of Padua
35121 Padua
Italy

Summary

Better drugs are needed against human cytomegalovirus (HCMV), a pathogen responsible for severe diseases in immunocompromised hosts and newborn children. We investigated whether selective inhibitors of HCMV replication could be discovered by screening for compounds that disrupt the interaction between the accessory subunit of the viral DNA polymerase, UL44, and the C-terminal 22 residues of the catalytic subunit. From ~50,000 small molecules, we identified 5 structurally diverse compounds that not only specifically interfere with this interaction, but also with the physical and functional interaction of UL44 with full-length catalytic subunit. These five compounds also inhibited HCMV replication with sub- to low micromolar potency, and at concentrations up to 500-fold lower than those at which they exhibited cytotoxicity. These compounds represent a promising starting point for the development of anti-HCMV drugs.

Introduction

Protein-protein interactions play a pivotal role in virtually every biological process, including the replication of pathogenic viruses in host cells. Several interactions between viral proteins have been proposed as attractive targets for antiviral drug discovery, as the exquisite specificity of such interactions affords the possibility of interfering with them in a highly selective and effective manner [1]. We have been investigating one such interaction—that between the two subunits of the DNA polymerase of human cytomegalovirus (HCMV) [2–4].

HCMV is a ubiquitous herpesvirus. Although it rarely causes symptomatic disease in immunocompetent individuals, it is responsible for a variety of severe diseases in transplant recipients and in AIDS patients, including pneumonia, gastrointestinal disease, and retinitis [5]. HCMV is also a major cause of congenital malformation in newborn children, often resulting in deafness and mental retardation [6]. Antiviral agents currently approved for the treatment of HCMV infections include ganciclovir (GCV), foscarnet, and cidofovir, all of which inhibit the viral DNA polymerase. GCV, which is the most widely used anti-HCMV drug, and cidofovir are nucleoside analogs, which function as DNA chain terminators, whereas foscarnet inhibits HCMV DNA polymerase through mimicry of the pyrophosphate product of polymerization [7]. However, these drugs are limited by their toxicities, pharmacokinetic drawbacks, and viral resistance issues [8]. Thus, there is considerable need for new anti-HCMV compounds.

One obstacle to the inhibition of protein-protein interactions is that these interactions often involve a large surface area and multiple contacts [9, 10]. However, several studies have shown that relatively few residues within these large surfaces can drive binding [11], and that single substitutions in one subunit of a protein-protein interface can completely disrupt subunit interactions, or nearly so (e.g., [12]). We have previously investigated this issue with the HCMV DNA polymerase, which consists of a 1242 residue catalytic subunit, UL54 [13], and a 433 residue accessory subunit, UL44, which stimulates long-chain DNA synthesis, presumably by increasing processivity [14, 15]. Both UL54 and UL44 are essential for HCMV DNA replication [16–18], and the UL54-UL44 interaction is specific [2]. Additionally, the C-terminal 22 residues of UL54 are both necessary and sufficient for binding to UL44 [3]. A peptide corresponding to these residues can both disrupt the physical interaction between UL54 and UL44 and specifically inhibit the stimulation of UL54 activity by the accessory protein [2]. We found that single substitutions in either UL54 or UL44 can drastically and specifically disrupt the interaction of these two proteins and their ability to synthesize long chains of DNA [3, 4]. Taken together, these observations suggest that a small molecule could specifically inhibit the UL54-UL44 interaction and HCMV replication.

There are only a few examples of small molecules that disrupt protein-protein interactions and exert effects inside cells (reviewed in [19]). However, we were encouraged by the recent identification of a small molecule (BP5) [20], which specifically inhibits the physical interaction between the two subunits of herpes simplex virus (HSV) type 1 DNA polymerase, UL30 and UL42, which are homologous to HCMV UL54 and UL44, respectively. Moreover, BP5 inhibits HSV-1 replication in cell culture, although it is too cytotoxic to contemplate its clinical use against HSV. With the considerable need for new anti-HCMV drugs, the goal of this study was to determine if we could identify, via high-throughput screening, compounds that could specifically inhibit the UL54-UL44 interaction. These studies led to the discovery of several small molecules that are substantially more potent for anti-HCMV activity than for cytoxicity.

Results

Development of an Assay to Identify Small-Molecule Inhibitors of the UL54-UL44 Interaction

To identify small-molecule inhibitors of the UL54-UL44 interaction, we developed an assay based on fluorescence polarization (FP) [21]. In this assay, a peptide corresponding to the C-terminal 22 residues of UL54 (here

*Correspondence: don_coen@hms.harvard.edu

named peptide 1, as in [2]), which was labeled with a fluorophore, is mixed with UL44ΔC290, a truncated protein that retains all known biochemical activities of full-length UL44 [4], fused to glutathione S-transferase (GST-UL44ΔC290). The unbound labeled peptide tumbles relatively rapidly. Thus, if it is excited with polarized light, by the time emission occurs, the polarization of the emitted light is low. However, upon binding to GST-UL44ΔC290, the peptide would be expected to tumble much more slowly, so the emitted light would remain relatively polarized, resulting in an increase in FP, measured as millipolarization units (mP). Thus, addition of increasing amounts of GST-UL44ΔC290 to a solution of 3 nM labeled peptide resulted in increasing FP (Figure 1A). From the data in Figure 1A, a K_d value of ~1 μM was calculated for the interaction between GST-UL44ΔC290 and the UL54 peptide. This value is within 2-fold of that (0.52 μM) previously determined for GST-UL44ΔC290 and the corresponding unlabeled UL54 peptide with isothermal titration calorimetry [3]. As a control, we also examined binding of a mutant GST-UL44ΔC290 (I135A), which is severely impaired for binding to both full-length UL54 and the UL54-derived peptide [4]. Addition of GST-UL44ΔC290 I135A to labeled peptide 1 resulted in only a slight increase in FP at protein concentrations higher than 17.5 μM (Figure 1A). Furthermore, no increase in FP was observed when the labeled peptide was incubated in the presence either of GST or of a fusion between maltose binding protein and the HSV-1 homolog of UL44, UL42 (MBP-UL42ΔC340). Thus, this assay could specifically and quantitatively measure interactions between UL44 and the UL54-derived peptide.

We next investigated whether this assay could detect a known inhibitor of the UL54-UL44 interaction. To this end, a binding reaction of 2.5 μM GST-UL44ΔC290 and 3 nM labeled peptide was titrated with increasing concentrations of unlabeled peptide 1 (Figure 1B). This peptide has been shown to inhibit both the physical interaction between UL54 and UL44 and DNA synthesis by the UL54-UL44 complex [2]. We observed that FP from the UL44-peptide interaction was inhibited by unlabeled peptide 1 with a 50% inhibitory concentration (IC_{50}) of 5 μM. This value is of a similar magnitude to that measured (11 μM) for inhibition of the UL54-UL44 physical interaction by this peptide in an ELISA interaction assay [2]. A peptide corresponding to the last 36 residues of HSV-1 UL30 (peptide A), which has been shown to inhibit the interaction between HSV-1 UL30 and UL42 [22], did not cause a decrease in FP (Figure 1B). Thus, this assay could detect specific inhibition of the interaction between UL44 and the UL54-derived peptide.

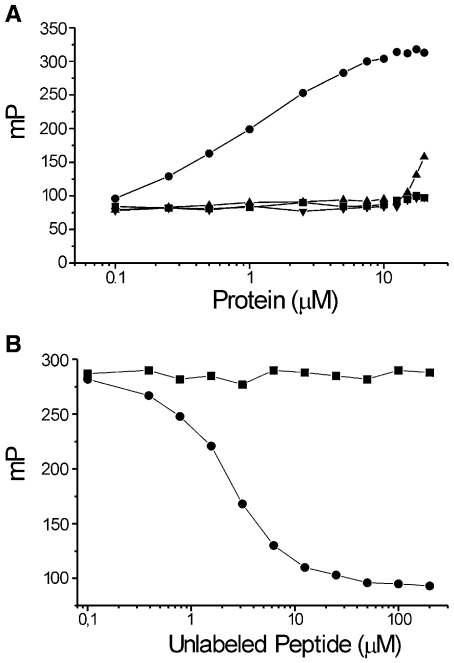

Figure 1. FP Assay

(A) Increasing concentrations of wild-type GST-UL44ΔC290 (circles), of GST-UL44ΔC290 I135A, a mutant UL44 that does not bind UL54 (triangles), of GST (squares), or of MBP-UL42ΔC340 (inverted triangles) were added to 3 nM of a fluorescently labeled peptide corresponding to the C-terminal 22 residues of HCMV UL54 (peptide 1), and FP (as millipolarization units, mP) was measured.

(B) Increasing concentrations of unlabeled peptide 1 (circles), or of peptide A, corresponding to the 36 C-terminal residues of HSV-1 UL30 (squares), were added to reaction mixtures containing 2.5 μM GST-UL44ΔC290 and 3 nM labeled peptide 1, and FP was measured.

High-Throughput Screen

We then used the FP assay and the facilities of the Harvard Institute of Chemistry and Cell Biology (ICCB) to screen the Peakdale (2,816 compounds), Bionet (4,800 compounds), Maybridge (8,800 compounds), and ChemDiv (28,864 compounds) libraries, and 4,348 compounds out of the 50,000 of the Chembridge Microformat library. Thus, we screened a total of 49,628 compounds, each at a concentration of 12.5 μg/ml. As positive controls for inhibition, reaction mixtures of GST-UL44ΔC290 and labeled peptide in the presence of 3 μM or 20 μM of unlabeled peptide 1 were included on each screening plate. The criterion for an active compound was one that exhibited an FP value similar to or lower than that observed in binding reactions of GST-UL44ΔC290 and labeled peptide in the presence of 3 μM unlabeled peptide 1. A total of 143 compounds (approximately 0.3% of the compounds screened) met this criterion. These compounds were then retested in the FP assay with GST-UL44ΔC290 and UL54-derived peptide. They were also assayed, at the same concentration used during the screen (12.5 μg/ml), in an FP assay that was used to discover inhibitors of interactions between HSV-1 DNA polymerase subunits [20]. This assay used MBP-UL42ΔC340 and a labeled HSV-1 UL30-derived peptide [20]. Of the 143 hits, only 25 (0.05% of the compounds screened) were found to specifically inhibit the UL54 peptide-UL44 but not the UL30-UL42 interaction in FP assays. Of the other 118 compounds, 97 inhibited the UL30-UL42 interaction, and 21 were not active in both assays. Among the 25 specifically active compounds, 21 small molecules, which were designated AL1 to AL21 (Figure 2), were commercially available and were purchased for further studies. Among these compounds, two classes with similar structure are recognizable: a class composed of AL1, AL2, AL3, and AL4, and another class composed of AL8 and AL21. The remaining compounds are not obviously similar.

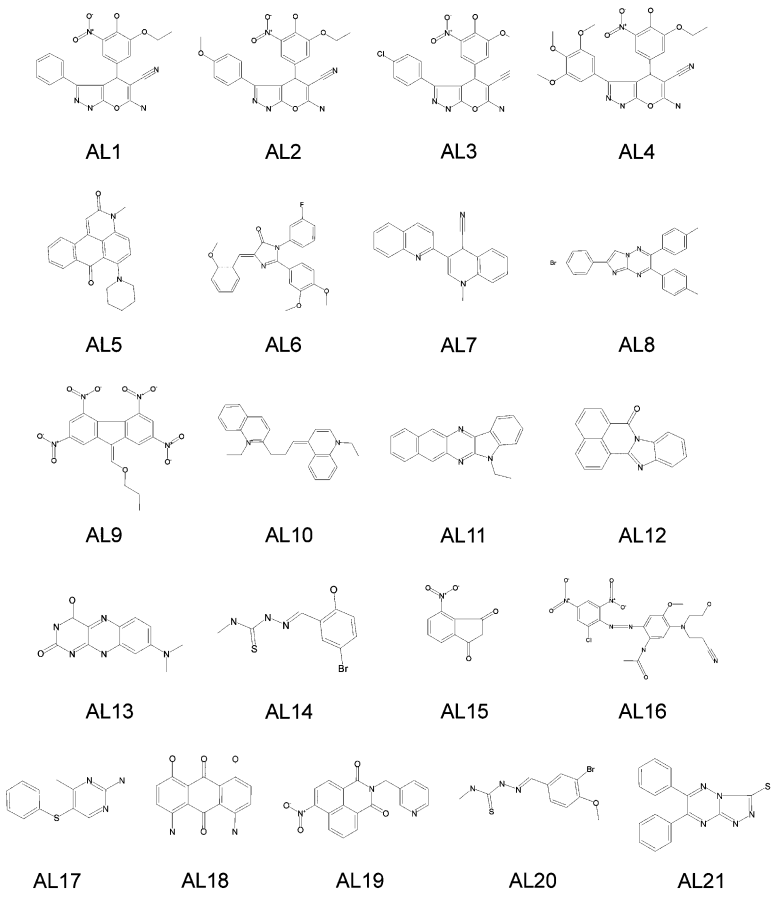

Figure 2. Chemical Structures of 21 Compounds Selected from High-Throughput Screening for Inhibitors of UL44-UL54 Peptide Interaction

None of these molecules was identical to any compound previously identified in screening at the ICCB for inhibition of different protein-protein interactions (C. Shamu, personal communication).

Dose Dependence of Inhibition

Next, we performed dose-response analyses of the inhibition of the UL44-UL54 peptide interaction for each of the 21 compounds in Figure 2. Of these, nine compounds—AL5, AL6, AL8, AL9, AL10, AL11, AL12, AL18, and AL21—reproducibly exhibited a dose-dependent reduction in FP, with IC_{50} values lower than 50 μM (values ranged from 5 to 30 μM; see Figure 3A and Table 1). Two other compounds, AL13 and AL15, inhibited FP in a dose-dependent manner, but with higher IC_{50} values (50 and 60 μM, respectively; Table 1). The remaining 10 compounds did not reproducibly inhibit UL44-UL54 peptide binding in FP assays in a dose-dependent manner (Figure 3A and Table 1). As a control, varying concentrations of the active compounds were also

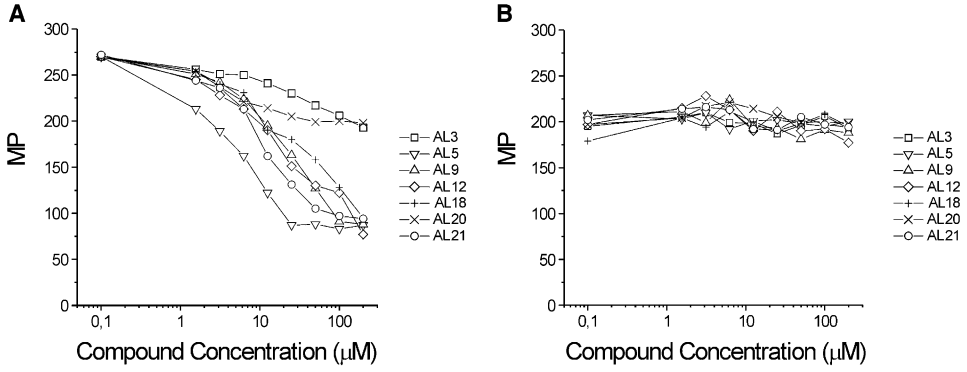

Figure 3. Dose Dependence of Inhibition of FP by Selected Compounds

(A) Increasing concentrations of compound AL3, AL5, AL9, AL12, AL18, AL20, and AL21 were added to reaction mixtures containing 2.5 μM GST-UL44ΔC290 and 3 nM labeled peptide 1, and FP (as millipolarization units, mP) was measured.

(B) Increasing concentrations of compound AL3, AL5, AL9, AL12, AL18, AL20, and AL21, were added to reaction mixtures containing 7 μM MBP-UL42ΔC340 and 5 nM of a fluorescently labeled peptide corresponding to the C-terminal 18 residues of HSV-1 UL30, and FP was measured.

Table 1. Summary of Activities of 21 Compounds, Peptide 1, and GCV

						Viral Yield Reduction		Cytotoxicity		
	Fluorescence Polarization	Long-Chain DNA Synthesis by UL54/UL44	DNA Synthesis by UL54 Alone	ELISA Interaction	Plaque Reduction	ED_{50} (μM)		CC_{50} (μM)		
Compound	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	ED_{50} (μM)	72 hr	120 hr	24 hr	72 hr	120 hr
AL1	>200	>100	>100		>30		50	>200	>200	>200
AL2	>200	>100	95		>30		90	>200	>200	>200
AL3	>200	>100	>100	>200	>30	>100	80	>200	>200	>200
AL4	>200	>40	>100		>30		>100	>200	>200	>200
AL5[a]	**5**	**5**	**>100**	**7**	**2.2**	**1.3**	**1.0**	**110**	**95**	**75**
AL6	30	>40	>100		>30			50	20	20
AL7	>200	>40	>100		>30			>200	60	120
AL8	6	>40	>100		>30			>200	60	60
AL9[a]	**21**	**10**	**>100**	**19**	**10**	**8.0**	**3.1**	**>200**	**60**	**70**
AL10	6	>40	>100		0.1			20	1	3
AL11	6	>40	>100		>30			200	12	10
AL12[a]	**19**	**15**	**>100**	**12**	**1.4**	**1.9**	**3.2**	**>200**	**40**	**35**
AL13	50	>40	>100		10			>200	17	18
AL14	>200	>40	>100		7.0			>200	30	30
AL15	60	>40	>100		>30			>200	57	55
AL16	>200	>40	>100		5.5			18	2	4
AL17	>200	>40	>100		>30			>200	>200	>200
AL18[a]	**30**	**5**	**>100**	**9**	**1.1**	**0.3**	**0.4**	**>200**	**100**	**200**
AL19	>200	>40	>100		4.0			18	1	2
AL20	>200	>100	>100	>200	1.3		4.0	>200	120	200
AL21[a]	**10**	**5**	**78**	**10**	**3.0**	**1.7**	**3.5**	**>200**	**45**	**80**
Peptide 1	5	18	>100	15						
GCV					1.9	0.9	0.8	>200	200	180

Where no value is presented, the measurement was not done.
[a] Data for the five compounds that were active in both biochemical and antiviral assays are highlighted in bold.

tested in the FP assay for inhibition of the HSV-1 DNA polymerase subunit interaction [20]. None of the compounds exhibited a dose-dependent reduction in FP in these assays (Figure 3B and data not shown).

Specific Inhibition of Long-Chain DNA Synthesis Mediated by UL44

To analyze the ability of the compounds to interfere with functional UL54-UL44 interactions, we tested their ability to inhibit long-chain DNA synthesis by UL54 in the presence of UL44 employing a poly(dA)-oligo$(dT)_{12\text{-}18}$ template-primer. In this assay, no long-chain DNA synthesis was detected in the presence of UL54 alone (Figure 4A, lane 1), or of GST-UL44ΔC290 alone (lane 3), while formation of long DNA products was observed when both UL54 and GST-UL44ΔC290 were present (lane 2). An equal concentration (40 μM) of each of the 21 compounds in Figure 2 was initially tested for inhibition of long-chain DNA synthesis by UL54 and UL44. Five compounds (AL5, AL9, AL12, AL18, and AL21) severely decreased formation of long DNA products, while the remaining compounds did not measurably affect long-chain DNA synthesis (data not shown).

To test the dose dependence of inhibition and quantify the effects on long-chain DNA synthesis, different doses of the active compounds (AL5, AL9, AL12, AL18, and AL21) and of four other hits from the screen (AL1, AL2, AL3, and AL20) were tested. As a control for inhibition, various concentrations of peptide 1, which was previously shown to inhibit the rate of incorporation of nucleotides with an IC_{50} of 20 μM [2], were also assayed. These data were quantified by phosphorimager analysis, and the IC_{50} values for inhibition of long-chain DNA synthesis were calculated (Figure 4B and Table 1). Peptide 1 inhibited long-chain DNA synthesis, with an IC_{50} of 18 μM (Figure 4A, lanes 4–8, and Figure 4B). AL5, AL9, AL12, AL18, and AL21 inhibited formation of long DNA products in a dose-dependent manner, with IC_{50} values ranging from 5 μM to 15 μM (Figures 4A and 4B and Table 1). In contrast, AL1, AL2, AL3, and AL20 exhibited little or no inhibition of long-chain DNA synthesis by UL54 and UL44 (Figures 4A and 4B and Table 1).

To determine whether the inhibition of long-chain DNA synthesis could be due to effects on the catalytic activity of the UL54 subunit, rather than on effects on UL44-mediated DNA synthesis, we tested varying concentrations of each of the 21 compounds in Figure 2 for their effects on DNA synthesis by UL54 alone by using a filter binding assay previously described [2]. As summarized in Table 1, only compounds AL2 and AL21 inhibited the activity of UL54 alone, but only at high concentrations (IC_{50}s of 95 and 78 μM, respectively). However, AL21 inhibited the activity of UL54 alone much less potently than the activity of the UL54-UL44 combination (compare IC_{50} values reported in Table 1), suggesting a specific effect on UL44-mediated long-chain DNA synthesis. All other compounds exhibited little or no inhibition of UL54 catalytic activity (IC_{50} values > 100 μM; see Table 1).

Specific Inhibition of the Physical Interaction of UL44 with Full-Length UL54

We next wished to determine, with an ELISA interaction assay, if the compounds that inhibited the UL44-UL54

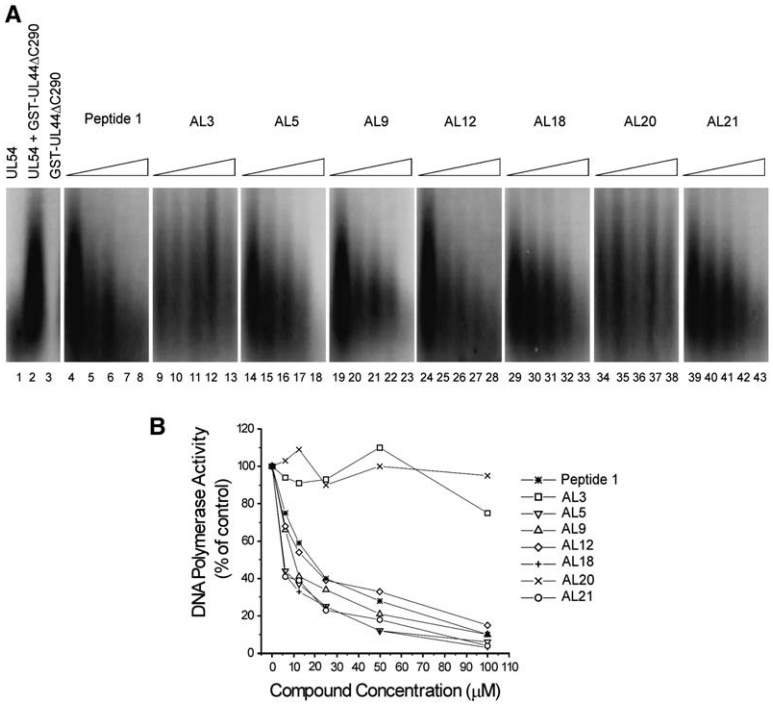

Figure 4. Effect of Selected Compounds on Long-Chain DNA Synthesis by UL54 and UL44

(A) Long-chain DNA synthesis directed by in vitro-expressed UL54 in the presence or absence of purified GST-UL44ΔC290 protein was assayed by measuring the incorporation of labeled [^{32}P]TTP wth a poly(dA)-oligo(dT) template. Examples of long-chain DNA synthesis directed by UL54 alone (lane 1), GST-UL44ΔC290 alone (lane 3), and by UL54 in the presence of GST-UL44ΔC290 with no compound added (lane 2) are shown. Subsequently, long-chain DNA synthesis in the presence of GST-UL44ΔC290 was measured following addition of increasing concentrations (6.25, 12.5, 25, 50, 100 μM) of peptide 1 or of the indicated compounds. The reaction products were visualized by autoradiography following electrophoresis on a 4% alkaline agarose gel.

(B) Autoradiographs such those in (A) were quantified by phosphorimager and the percentage of signal at each concentration of compound relative to that in the absence of added compounds (example given in lane 2 of [A], but different no-compound controls were performed for each experiment) was plotted versus the concentration of each compound tested. Data in (B) represent the means of values from three independent experiments such as that shown in (A).

functional interaction could inhibit the physical interaction of UL44 with full-length UL54. In this assay, binding between purified baculovirus-expressed UL54 and UL44, which was tagged with an EEF epitope, was detected with an antibody that recognizes the epitope tag [2]. This assay was used to test the five compounds that had specifically inhibited long-chain DNA synthesis by UL54-UL44 (AL5, AL9, AL12, AL18, and AL21), two other compounds that had scored as hits in the initial assay (AL3 and AL20), and peptide 1 as a control. Peptide 1 exhibited an IC_{50} for inhibition of the UL54-UL44 interaction of 15 μM, a value similar to that (11 μM) previously determined by using the same assay [2]. Consistent with the results of the long-chain DNA synthesis assay, compounds AL5, AL9, AL12, AL18, and AL21 were able to interfere with the physical interaction between UL44 and full-length UL54, with IC_{50} values ranging from 7 to 19 μM, whereas compounds AL3 and AL20 did not significantly inhibit the UL54-UL44 interaction at concentrations up to 200 μM (Figure 5 and Table 1).

Antiviral and Cytotoxic Activity

We then investigated the antiviral effects of the 21 compounds in human foreskin fibroblast (HFF) cells. First, we tested all 21 compounds in plaque reduction assays, which entail viral infection at low multiplicity. GCV was included in these experiments as a control for inhibition. GCV exhibited a 50% effective dose (ED_{50}) of 1.9 μM, a value in the range of those previously reported (0.43–7 μM [23]). AL5, AL9, AL12, AL18, and AL21, the compounds that inhibited both physical and functional interactions between UL54 and UL44 in biochemical assays, also inhibited plaque formation by HCMV AD169, with ED_{50} values from 1.1 to 10 μM (Figure 6A and Table 1). Although not active in biochemical assays, other compounds, namely AL14, AL16, AL19, and AL20, exhibited activity against virus plaque formation (ED_{50} values < 30 μM; Figure 6A and Table 1).

Next, we tested the effects of selected compounds and of GCV as a control in viral yield assays, which measure the titer of virus progeny produced following infection at relatively high multiplicity. In these assays, the viral titer was measured at two different times points postinfection: at 72 hr, when the release of virus progeny is increasing, and at 120 hr, when the titer of virus progeny after a single cycle of replication reaches peak levels [24]. Virus titers from untreated infected cells were 1.2×10^5 PFU (plaque forming units)/ml and 3.9×10^6 PFU/ml at 72 hr and 120 hr postinfection,

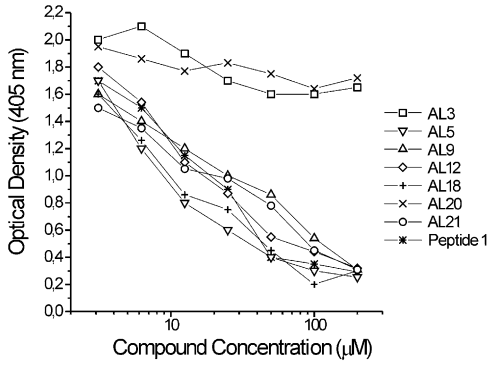

Figure 5. Effect of Selected Compounds on the Physical Interaction of UL44 with Full-Length UL54

The interaction between purified baculovirus-expressed UL54 and UL44 proteins was measured by using an interaction ELISA in the presence of varying concentrations of AL3, AL5, AL9, AL12, AL18, AL20, and AL21, and of peptide 1 as a positive control.

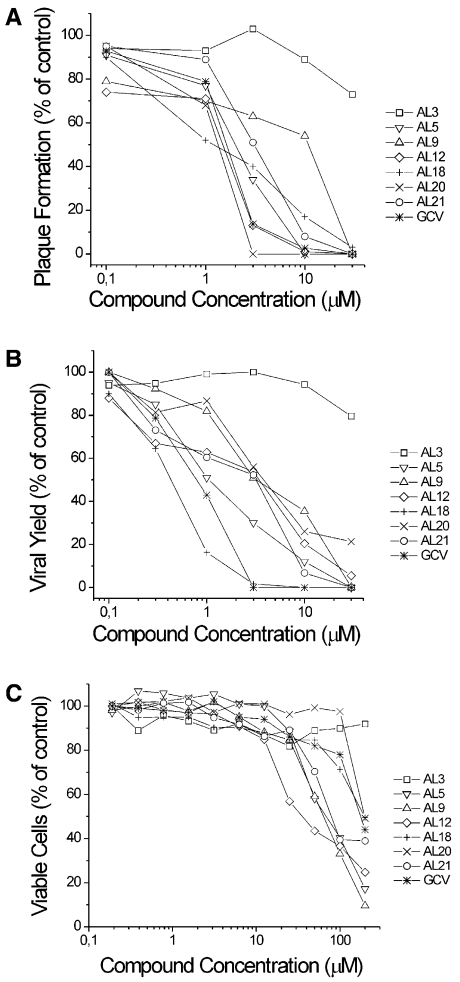

Figure 6. Antiviral Activity and Cytotoxicity of Selected Compounds

(A) The effects of AL3, AL5, AL9, AL12, AL18, AL20, AL21, and GCV as a control, on plaque formation by HCMV strain AD169 were determined in HFF cells.

(B) The effects of AL3, AL5, AL9, AL12, AL18, AL20, AL21, and GCV on the yield of HCMV AD169 in HFF cells were determined at 5 days postinfection in single-cycle growth assays.

(C) The effect of AL3, AL5, AL9, AL12, AL18, AL20, AL21, and GCV on HFF cell viability were measured by using an XTT assay at 120 hr following the addition of compound to cell media. The absorbances measured in the XTT assay were plotted onto a standard curve of number of cells versus absorbance to calculate the percentage of viable cells remaining.

respectively. Compounds AL5, AL9, AL12, AL18, and AL21, which were active in the biochemical assays for inhibition of UL54-UL44 interactions, inhibited virus yield, with ED_{50} values at 72 hr postinfection ranging from 0.3 to 8 μM (Table 1) and with ED_{50} values at 120 hr postinfection ranging from 0.4 to 3.5 μM (Figure 6B and Table 1). GCV exhibited an ED_{50} of 0.9 μM at 72 hr postinfection and of 0.8 μM at 120 hr postinfection in these assays. Of the compounds that were inactive for inhibition in biochemical assays for inhibition of UL54-UL44 interactions, compound AL20 showed significant antiviral activity (ED_{50} = 4.0 μM at 120 hr postinfection), whereas compounds AL1, AL2, AL3, and AL4 exhibited little or no effect on HCMV replication (Figure 6B and Table 1). AL18 reduced virus yields by 4–6 logs, while a 4–5 log reduction in yield was typically observed in samples treated with GCV at similar concentrations. AL5 and AL21 reduced virus yield 4–5 logs, while 3–4 log reductions were observed with AL9, AL12, and AL20.

In parallel, we tested the cytotoxicity of all 21 compounds and of GCV in HFF cells for 24, 72, or 120 hr by using a dye-uptake (XTT; Roche Molecular Biochemicals) assay. The 120 hr assay assessed effects on both viability and proliferation, while the other assays mainly assessed effects on viability. GCV exhibited a concentration that causes a decrease of cell viability of 50% (CC_{50}) around 200 μM (Figure 6C, which shows the 120 hr assay data, and Table 1), a value that is in the range of those previously reported (40–800 μM [23]). Compounds AL6, AL10, AL11, AL13, AL14, AL16, and AL19 exhibited significant cytotoxicity in this assay. Importantly, for compounds AL10, AL13, AL14, AL16, and AL19, the CC_{50} values were similar to the ED_{50}s measured in antiviral assays (Table 1). Thus, the effects of these compounds observed in antiviral assays are likely due to cytotoxicity rather than to specific antiviral activity. In contrast, compounds AL5, AL9, AL12, AL18, AL20, and AL21 showed cytotoxicity at concentrations from 7- to 500-fold higher than those at which they exhibited antiviral activity (Figure 6C and Table 1). Thus, these small molecules appear to exhibit selective anti-HCMV activity. Among these six compounds, five (i.e., AL5, AL9, AL12, AL18, and AL21) also specifically inhibited physical and functional interactions between UL54 and UL44. One compound, AL20, although originally identified in our screen, was not active in biochemical assays of UL54-UL44 interactions. Thus, the mechanism of this compound is likely to be different.

Discussion

In this study, we identified five small molecules with diverse structures that selectively inhibit the physical interaction between the subunits of HCMV DNA polymerase and its functional consequences. These compounds did not detectably inhibit the interaction between the accessory subunit and the C terminus of the catalytic subunit of HSV-1 DNA polymerase. Nor did they inhibit basal DNA polymerase activity of the HCMV catalytic subunit alone, except in one case at high concentrations. These five compounds also exhibited anti-HCMV activity with sub- to micromolar potencies. Importantly, these compounds exhibited much less potent cytotoxic activities than antiviral activities.

Small Molecules that Selectively Inhibit HCMV Polymerase Subunit Interactions

As protein-protein interfaces are crucial to most biological processes and often determine specificity, they can, in principle, be excellent targets for drug discovery. However, while there are numerous reports in the literature of the use of dominant negative proteins, antibodies, or peptides to inhibit protein-protein interactions, there are only a few examples of small "drug-like" molecules that selectively disrupt these interactions. Several factors seem likely to have contributed to our ability to add AL5, AL9, AL12, AL18, and AL21 to that number. Although the UL54-UL44 interaction involves a number

of contacts between UL54 and UL44 [25], only a few of these are crucial. In particular, when either Leu1227 or Phe1231 of UL54 is substituted with alanine, no detectable interaction between UL54 and UL44 is observed [3]. These two residues help form a hydrophobic "plug" that packs against a hydrophobic crevice on UL44 [25]. It is easy to envision a small molecule being able to specifically disrupt this interaction.

It is interesting to compare the compounds identified in this study and BP5, a small molecule that specifically inhibits the interaction between the two subunits of HSV-1 DNA polymerase, UL30 and UL42 [20]. BP5 has more peptide-like qualities than does AL5, AL9, AL12, AL18, or AL21, which include more aromatic properties. This difference may relate to the major difference between the HSV-1 UL30-UL42 interaction, which relies on critical hydrogen bonding interactions [26, 27], and the HCMV UL54-UL44 interaction, which involves hydrophobic interactions [3, 4, 25], even though these interactions both involve the C terminus of each catalytic subunit and the connector loop of the other subunit. It is therefore not surprising that BP5 does not inhibit the HCMV UL54-UL44 interaction [20], while none of the compounds studied here inhibited the HSV-1 UL30 peptide-UL42 interaction (Figure 3B and data not shown). These differences emphasize the highly specific nature of these polymerase subunit interactions, which also share no sequence homology. It is possible that these biochemical differences contribute to the much larger ratios of cytotoxic to antiviral concentrations of the compounds studied here than that observed for BP5.

Selective Inhibitors of HCMV Replication

Because HCMV is such a serious pathogen in newborns and in immunocompromised individuals, and because current drugs have important toxicities and/or pharmacokinetic drawbacks, we were especially heartened to observe relatively potent inhibition of HCMV replication. The potencies observed were similar to those of GCV, the drug of choice for the treatment of most HCMV infections [28]. They are comparable to published values for cidofovir, for which IC_{50} values from 0.5 to 2.8 μM have been described [29], and significantly greater than that of foscarnet, for which IC_{50} values from 23 to 81 μM have been reported [30]. At least as important, these small molecules, without any modifications, exhibit cytotoxic activities similar to those of GCV, and only slightly greater than those reported for foscarnet and cidofovir (600 and 360 μM, respectively [23, 29]). Crystal structures of UL44, either unliganded or bound to the C terminus of UL54, have been solved [25, 31]. This makes feasible studying how the compounds bind to their target in molecular detail and using that information to guide modifications of the compounds in order to increase their antiviral activity and decrease their cytotoxicity.

It is notable that all five of the compounds that inhibited long-chain DNA synthesis by the UL54/UL44 combination exhibited antiviral activity, while 15 out of the 16 compounds that failed to inhibit long-chain DNA synthesis also failed to inhibit HCMV replication. This strong correlation suggests that the anti-HCMV activity of our compounds is likely due to inhibition of viral polymerase subunit interactions inside infected cells. However, further study is needed to determine if this is indeed the antiviral mechanism.

The inhibitors of HCMV DNA polymerase subunit interactions that we have identified may have some theoretical advantages when compared with other classes of anti-HCMV compounds. First, following phosphorylation, drugs such as GCV and cidofovir mimic natural nucleotide substrates of host enzymes, which can lead to toxicities such as mutagenesis, as documented for GCV [32]. The new, non-nucleoside compounds identified here would not be expected to exhibit similar toxicities, although other kinds of toxicities cannot be excluded. Second, GCV, cidofovir, and foscarnet act via inhibition at sites in HCMV DNA polymerase that are conserved among human and viral enzymes. Indeed, mutations that confer resistance to these compounds usually alter such conserved residues [33]. In contrast, compounds that inhibit protein-protein interactions are expected to be highly specific, as protein-protein interactions themselves are highly specific. In the case of herpesvirus DNA polymerase subunit interactions, although the most important residues lie in analogous regions (i.e., the C terminus of the catalytic subunit and the so-called connector loop of the accessory protein), the sequences of these segments differ considerably. Moreover, the side chains of the residues that have been identified as important for HSV-1 UL30-UL42 and for HCMV UL54-UL44 interaction are different, being hydrophilic in the first case [26] and hydrophobic in the second [3, 4]. The differences between HCMV and HSV DNA polymerase subunit interactions herald the prospect that small-molecule inhibitors targeting such interactions could be significantly more specific than most of the drugs currently licensed for anti-herpesvirus chemotherapy. In support of this hypothesis, the small molecules able to block the UL54-UL44 interaction did not detectably inhibit the binding between the subunits of HSV-1 polymerase (Figure 3). Furthermore, the details of the UL54-UL44 interaction differ substantially from those of the human processivity factor, PCNA, and its binding partners [25]. Third, our new compounds, which target sites on the viral polymerase different from those targeted by GCV, cidofovir, and foscarnet, should be active against viruses resistant to these drugs.

Thus, the small molecules that we have identified represent an interesting starting point for the discovery of new, non-substrate-based drugs that may be more potent and specific than currently existing anti-HCMV agents, and that should be active against viral mutants resistant to these compounds. Our results also suggest that screening for inhibitors of viral polymerase subunit interactions could be useful for identifying new compounds active against other viral infections, including those of importance for biodefense.

Significance

HCMV is an important pathogen in the immunocompromised, including transplant recipients and patients with AIDS, and in newborns, where it is a leading infectious cause of birth defects [5]. Although there are several antiviral drugs licensed for the treatment and prophylaxis of HCMV infections, these all suffer from problems with toxicity, drug resistance, and/or

pharmacokinetic drawbacks. Most of these drugs, including the frontline therapy, ganciclovir, work via inhibition of the viral DNA polymerase by binding to active sites that contain residues conserved among human and viral enzymes [7]. In contrast, the residues that comprise the interaction interface between UL54 and UL44 are not conserved with their human counterparts or even with counterparts from other herpesviruses. Thus, a small molecule that specifically inhibits this interaction should avoid the kinds of toxicities observed with current anti-HCMV polymerase inhibitors. It is therefore encouraging that the molecules that we have identified selectively inhibit HCMV polymerase subunit interactions and exhibit sub- to low micromolar potency against HCMV. Even more encouraging are the relatively low potencies of these compounds in assays of cytotoxicity, which distinguish them from the previously identified inhibitor of HSV DNA polymerase subunit interactions. Indeed, even without the benefit of any medicinal chemistry to modify these compounds, their cell-based therapeutic indices are similar to that of ganciclovir. As these compounds are diverse in structure, and the crystal structure of UL44 both unliganded and bound to the C terminus of UL54 have been solved, prospects for modifying at least one of these into a safer, more potent anti-HCMV drug seem promising. Our results also suggest that screening for inhibitors of viral polymerase subunit interactions could be useful for identifying new compounds active against other viral infections, including those of importance for biodefense.

Experimental Procedures

Peptides and Proteins

Unlabeled peptide 1, corresponding to the 22 C-terminal residues of HCMV UL54 [2], and the same peptide N-terminally labeled with the pentafluorofluorescein-derivative Oregon Green 514 (Molecular Probes) were synthesized and HPLC-purified by C. Dahl at the Biopolymers Facility of the Department of Biological Chemistry & Molecular Pharmacology (Harvard Medical School). Peptide A, corresponding to the 36 C-terminal residues of HSV-1 UL30 [22], and a peptide comprising the 18 C-terminal residues of HSV-1 UL30 (peptide E) and N-terminally labeled with Oregon Green 514 were prepared as previously described [20]. The peptides were dissolved in water, and concentrations were determined by quantitative amino acid analysis performed by the Molecular Biology Core Facility, Dana-Farber Cancer Institute.

The wild-type GST-UL44ΔC290 fusion protein, which contains the N-terminal 290 residues of HCMV UL44, the GST-UL44ΔC290 I135A mutant, and GST alone, were purified from *Escherichia coli* BL21(DE3)-pLysS harboring the appropriate plasmid, as previously described [3]. The purification of *E. coli*-expressed MBP-UL42ΔC340 fusion protein, which contains the N-terminal 340 residues of HSV-1 UL42, has been previously reported [26]. Purified baculovirus-expressed HCMV UL54 and UL44 proteins, prepared as previously described [2], were kindly provided by H. S. Marsden (Institute of Virology, Glasgow, UK).

Concentrations of all proteins were determined by using quantitative amino acid analysis at the Molecular Biology Core Facility, Dana-Farber Cancer Institute.

FP Assays

Unless otherwise indicated, 3 nM of Oregon Green-labeled peptide 1 was added to 2.5 μM GST-UL44ΔC290 in 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 0.5 mM EDTA, 150 mM NaCl, 4% glycerol, and 100 μg/ml bovine serum albumin and kept on ice until the mix was distributed into black 384-well plates (Nunc) in a total volume of 40 μl per well. For high-throughput screening, small molecules (5 mg/ml in DMSO) of the Maybridge, Bionet, Peakdale, ChemDiv, and Chembridge Microformat libraries were obtained from the ICCB, and 100 nl of each compound was transferred to individual wells using 384-pin arrays. The 21 compounds that were subsequently purchased for further studies were resuspended in DMSO at a final concentration of 20 mM. All compounds were tested in duplicate. As controls, reaction mixtures containing either labeled peptide 1 only, or GST-UL44ΔC290 plus labeled peptide 1 (no compound), or GST-UL44ΔC290 plus labeled peptide 1 in the presence of 3 μM or 20 μM of unlabeled peptide 1, were included on each screening plate. After 15 min incubation at room temperature, FP values (in millipolarization units) were determined by using an Analyst plate reader (LJL Biosystems).

Selected compounds were also tested in an FP assay used to detect inhibitors of HSV-1 DNA polymerase subunit interactions. The assay was conducted as previously described [20], testing various concentrations of each compound in duplicate.

DNA Polymerase Assays

The effects of compounds were tested in a gel-based assay that measures long-chain DNA synthesis as described previously [3], with 5 μl of in vitro-transcribed and -translated UL54 plus 800 fmol of GST-UL44ΔC290 in the absence or presence of various amounts of each compound. The basal DNA polymerase activity of UL54 in the absence or presence of various amounts of each compound was measured by a filter-based assay, as previously reported [2], but with 5 μl of in vitro-transcribed and -translated UL54, as in the study by Loregian et al. [3]. In both assays, the final concentration of compound-derived DMSO was maintained at 0.5% (v/v) in all samples. In control samples (with no compound), an appropriate volume of pure DMSO was added for a final concentration of 0.5%.

UL54-UL44 Interaction ELISA

This assay was conducted as previously described [2], testing various concentrations of each compound. Briefly, microtiter plates were coated with 0.2 μg of purified baculovirus-expressed UL54 and blocked with 2% bovine serum albumin (Sigma) in PBS for 1 hr. After washing, 0.5 μg of purified baculovirus-expressed UL44, mixed with each compound at different concentrations (maintaining a final 1% DMSO concentration) or with DMSO alone at a final 1% concentration (no compound), was added and incubated for 1 hr at 37°C. Following further washes, the wells were incubated with monoclonal antibody (MAb) YL1/2, which recognizes the EEF epitope inserted at the C terminus of UL44 [2], for 1 hr at 37°C. Plates were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rat antibody (Sigma). After final washes, the chromogenic substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Pierce) in citrate phosphate buffer (pH 4.0) containing 0.01% hydrogen peroxide was added and absorbance was read at 405 nm on a Victor plate reader (Wallac).

Antiviral Assays

Plaque reduction assays were performed essentially as follows: HFF cells were seeded at 2×10^5 cells per well in 24-well plates. The next day, cells were infected with HCMV (strain AD169) at 80 PFU per well in DMEM plus 5% FBS at 37°C. Two hours postinfection, the inocula were removed, cells were washed, and media containing various concentrations of each drug tested, 5% FBS, and 0.6% methylcellulose were added. All drug concentrations were tested at least in duplicate. GCV was obtained from Sigma. After incubation at 37°C for 10–11 days, cell monolayers were stained with crystal violet and plaques were counted.

For yield reduction assays, HFF cells were plated at 2×10^4 cells per well in 96-well plates, incubated overnight, and infected with HCMV AD169 at a multiplicity of infection of 1. After virus adsorption for 2 hr at 37°C, cells were washed and incubated with 200 μl of fresh media containing test compounds at various concentrations. Plates were incubated for 3 or 5 days at 37°C and subjected to one cycle of freezing and thawing, and titers were determined by transferring 100 μl aliquots from each of the wells to a fresh 96-well monolayer culture of HFF cells, followed by 1:5 serial dilution across the plate. Cultures were incubated for 7 days, cells were stained, and the numbers of plaques were determined as described above.

In both assays, the final concentration of compound-derived DMSO was maintained at 1% (v/v) in all samples. In control samples

(with no compound), an appropriate volume of pure DMSO was added for a final concentration of 1%.

Cytotoxicity Assays

HFF cells were seeded at 2×10^4, 4×10^3, or 10^3 cells per well into 96-well plates and, after a 3 hr incubation to allow cell attachment, were treated for 24, 72, or 120 hr, respectively, with various concentrations of each compound in duplicate. The cell numbers seeded were chosen so that untreated cells would yield an absorbance that was near maximal within the linear range of the assay. Cell viability was then determined with an XTT assay (according to the manufacturer's protocol). The values for the 120 hr assay, which started with the smallest number of cells, reflect effects on both proliferation and viability, while the values for the other assays mainly reflect effects on viability. The final concentration of compound-derived DMSO was maintained at 1% (v/v) in all samples. In control samples (with no compound), an appropriate volume of pure DMSO was added for a final concentration of 1%.

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