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Identification of a Small Molecule that Inhibits Herpes Simplex Virus DNA Polymerase Subunit Interactions and Viral Replication

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Summary

The interaction between the catalytic subunit Pol and the processivity subunit UL42 of herpes simplex virus DNA polymerase has been characterized structurally and mutationally and is a potential target for novel antiviral drugs. We developed and validated an assay for small molecules that could disrupt the interaction of UL42 and a Pol-derived peptide and used it to screen ~16,000 compounds. Of 37 "hits" identified, four inhibited UL42-stimulated long-chain DNA synthesis by Pol in vitro, of which two exhibited little inhibition of polymerase activity by Pol alone. One of these specifically inhibited the physical interaction of Pol and UL42 and also inhibited viral replication at concentrations below those that caused cytotoxic effects. Thus, a small molecule can inhibit this protein-protein interaction, which provides a starting point for the discovery of new antiviral drugs.

Introduction

Protein-protein interactions are crucial to most, if not all, biological processes. Because these interactions also often determine specificity, selective disruption of these interactions should, in principle, be an excellent strategy for drug discovery. However, there are only a few examples of small molecules that disrupt protein-protein interactions and exert effects inside cells (reviewed in [1, 2]). The paucity of such compounds derives in part from a reluctance to target protein-protein interactions for drug development based on the perception that these interactions entail large, flat interfaces that cannot be readily disrupted by small molecules. Although this perception may be accurate in a number of cases, there are important counterexamples that emphasize the value of structural and mutational analyses in choosing protein-protein interactions to target for drug discovery [1, 2].

The interaction between the two subunits of herpes simplex virus (HSV) type 1 DNA polymerase, the catalytic subunit UL30 (Pol) and the processivity subunit UL42, has been characterized structurally and mutationally [8, 14, 17] and may represent a target for new anti-HSV drugs. In immunocompetent adults, HSV causes a spectrum of diseases including debilitating genital infections and sight-threatening ocular infections [3]. In newborns and immunosuppressed individuals such as patients with AIDS, HSV infections are even more severe. Safe, effective antivirals against HSV, notably the nucleoside analog acyclovir (ACV) whose triphosphate inhibits the viral polymerase, have been developed and are widely used. However, ACV is not highly potent (active at μM concentrations), and there are HSV infections for which it is not particularly efficacious. Moreover, ACV-resistance is a problem, especially in the immunosuppressed [4, 5]. For these and other reasons, it would be desirable to develop safe, effective anti-HSV agents with mechanisms different from that of ACV. Interestingly, nearly all ACV-resistance *pol* mutations map in regions encoding motifs that are conserved with human cellular DNA polymerases (reviewed in [6]). Thus, ACV appears to exploit only rather subtle differences between viral and cellular polymerases.

In contrast, the interaction between Pol and UL42 is much more distinct, with no detectable sequence homology between the Pol C terminus, which interacts with UL42, and cellular or even other herpesvirus polymerases. Several lines of evidence indicate that this interaction is required for viral replication. In vitro, UL42 is not required for catalysis by Pol but is necessary for long-chain DNA synthesis [7]. Moreover, mutants of Pol or UL42 that are specifically impaired for subunit interactions exhibit drastic defects in long-chain DNA synthesis in vitro and are unable to complement the replication of their corresponding null mutant viruses in cells [8–10]. Peptides corresponding to the extreme C terminus of Pol, which is both necessary and sufficient for interaction with UL42 [8, 9, 11–13], can inhibit long-chain DNA synthesis mediated by UL42 [11, 14, 15]. One such peptide, when fused to *E. coli* enterotoxin B, inhibits HSV replication in cell culture [16]. Thus, the Pol-UL42 interaction appears to be a valid target for new anti-HSV drugs.

The crystal structure of UL42 bound to the C-terminal 36 residues of Pol has been elucidated to 2.7 Å [17]. The interaction entails a fairly large interface (1087 $Å^2$ of the peptide). However, a peptide corresponding to the C-terminal 18 residues binds with similar affinity (K_d = 1–2 μM), as does one corresponding to the C-terminal 36 residues [8]. Moreover, mutational analyses of particular Pol and UL42 residues that participate in hydrogen-bonding interactions have shown that each of these residues is crucial for the interaction [8]. As interruption of a few hydrogen bonds is sufficient to disrupt the Pol-UL42 interaction, it seemed possible that a small molecule might be able to do the same. We therefore developed and validated a high-throughput screen for compounds that could disrupt polymerase subunit interactions and applied it to a library of small molecules. Among these, we have identified a molecule that specifically inhibits Pol-UL42 interactions and has antiviral activity.

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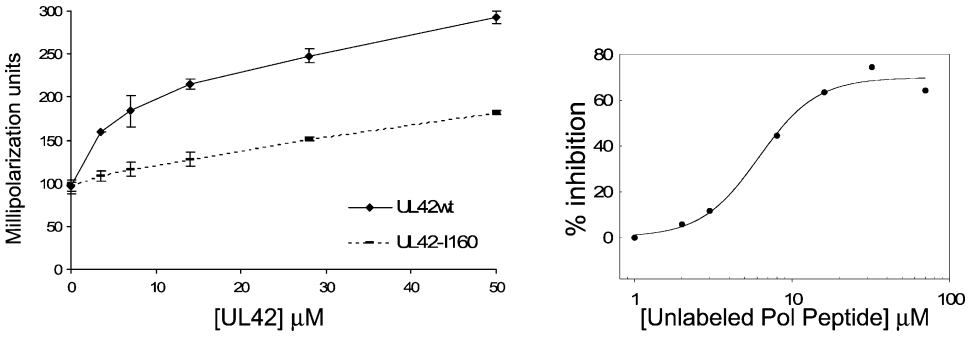

Figure 1. Validation of the FP Assay

(A) Varying concentrations of MBP-UL42 (UL42wt, solid line) or a mutant MBP-UL42 that does not bind to HSV Pol (UL42-I160, dotted line) were added to 1 nM of a fluorescently labeled peptide corresponding to the C-terminal 18 residues of Pol, and FP was measured.

(B) Varying concentrations of unlableled peptide A, which corresponds to the C-terminal 36 residues of Pol, were titrated into reactions containing 7 μM UL42 and 1 nM labeled peptide, and FP was measured. The percent of inhibition of FP relative to no peptide A (0%) and free peptide (100%) is plotted versus the concentration of peptide A.

Results and Discussion

Development and Validation of an Assay to Identify Small Molecule Inhibitors of the Pol-UL42 Interaction

We developed an assay for the Pol-UL42 interaction based on fluorescence polarization (FP), a format that facilitates high-throughput screening [18, 19]. In this assay, a peptide corresponding to the C-terminal 18 residues of Pol, which was labeled with the fluorophore Oregon Green, was mixed with UL42 fused to maltose binding protein (MBP-UL42), a fusion protein that retains all known biochemical activities of UL42 [10, 21, 23]. The free-labeled peptide tumbles relatively rapidly. Thus, if it is excited with polarized light, its polarization is low by the time emission occurs. However, upon binding to MBP-UL42, the peptide would be expected to tumble much more slowly, so the light emitted would remain relatively polarized, resulting in an increase in FP.

Titration of MBP-UL42 into a solution of 1 nM labeled peptide resulted in two phases of increasing FP, with steeper increases at lower concentrations, followed by shallower increases at higher concentrations (Figure 1A). (These shallower increases were essentially eliminated when more highly purified MBP-UL42 was used [C.C., A. Loregian, and D.M.C., unpublished data]; however, purifying to this extent decreased the yield of protein so that it became difficult to use the assay for high-throughput screening.) MBP-UL42 did not result in increasing FP with a labeled peptide derived from the C terminus of human cytomegalovirus (HCMV) Pol (C.C., A. Loregian, and D.M.C., unpublished data). As a control, we also titrated a mutant MBP-UL42 (I-160), which is severely impaired for binding to Pol and Pol-derived peptides [8, 10]. This resulted in only the shallow increases in FP seen at high concentrations with the wild-type protein, which we infer are due to nonspecific binding. Analysis of the data in Figure 1A, including subtraction of the nonspecific component, revealed a K_d value of 5 μM for the interaction between MBP-UL42 and the peptide. This value is close to that (~2 μM) for MBP-UL42 and the corresponding unlabeled 18 residue Pol peptide determined previously in our laboratory by using isothermal calorimetry [8]. (When more highly purified MBP-UL42 was used in the FP assay, the K_d value was, indeed, ~2 μM [C.C., A. Loregian, and D.M.C., unpublished data].) Thus, this assay could specifically and quantitatively measure interactions between UL42 and the Pol-derived peptide.

We next investigated whether this assay could detect a known inhibitor of Pol-UL42 interactions—a peptide corresponding to the last 36 residues of Pol (peptide A) that has been shown to inhibit long-chain DNA synthesis by Pol and UL42 [14, 15]. A binding reaction of 7 μM MBP-UL42 and 1 nM labeled peptide was titrated with increasing concentrations of unlabeled peptide A (Figure 1B). FP from the UL42-peptide interaction was inhibited by peptide A with a 50% inhibitory concentration (IC_{50}) of 6 μM. This value is similar to that observed for inhibition of long-chain DNA synthesis by this peptide [14, 15]. An unlabeled peptide corresponding to the C terminus of HCMV Pol [22] did not inhibit FP from the UL42-peptide interaction, even at concentrations as high as 50 μM (data not shown). Thus, this assay could detect specific inhibition of interactions between UL42 and the Pol-derived peptide.

High-Throughput Screen

We then used the FP assay to screen a library of 16,320 compounds, each at a concentration of 10 μg/ml. Our initial criterion for an active compound was one that resulted in an FP value of >3 standard deviations below the mean FP for all the compounds. 98 compounds (0.6% of the compounds screened) met this criterion. Of these, however, 61 turned out to be false positives due to, for example, intrinsic fluorescence of the small molecule. Thus, the screen identified 37 hits to pursue (0.2% of library).

Of the 37 hits, we identified nine (0.06%) that were the most promising based on their reproducible inhibition of FP (Figure 2). These fell into six structural classes of which three have two members each (BP2 and BP7, BP3 and BP9, and BP6 and BP8), and three have one member each (BP1, BP4, and BP5). BP1 and BP5 each

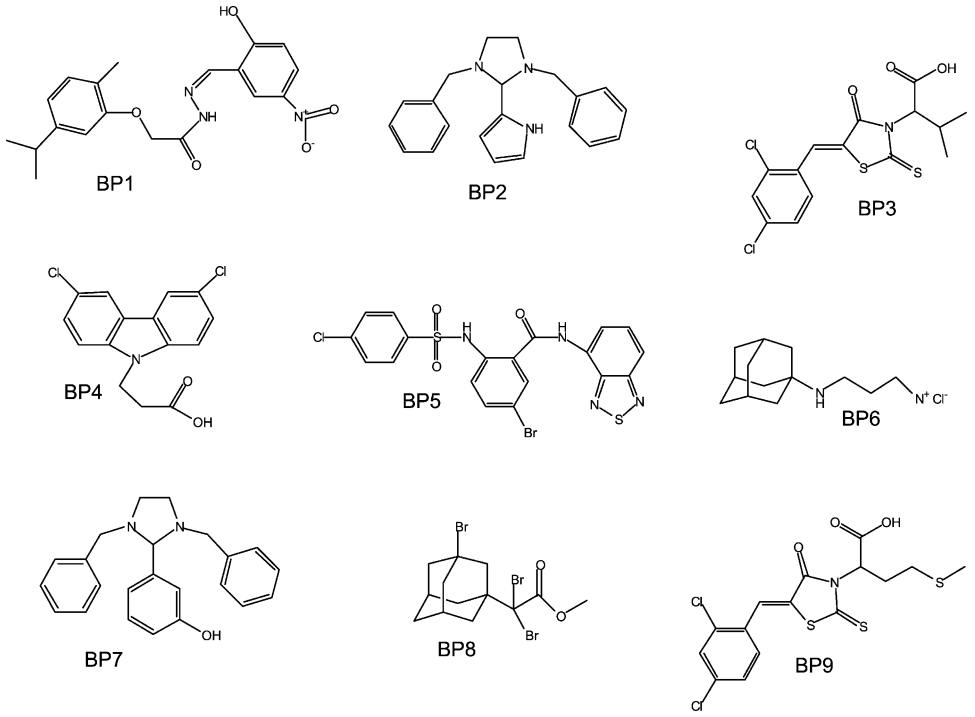

Figure 2. Chemical Structures of Nine Hits from Screen for Inhibitors of UL42-Pol Peptide Interactions
The compounds are designated as BP1–BP9.

contain a peptide bond. None of these molecules was identical to any from the same library that were most potent for inhibition of a different protein-protein interaction, that of Bak BH3-Bcl-x_L [20]. Interestingly, BP3 and BP9 are similar to one class of these Bak BH3-Bcl-x_L inhibitors but differ in the number of halogens, while BP5 resembles a second class of Bak Bak BH3-Bcl-x_L inhibitors but is distinct in terms of the orientation of the peptide bond and in containing a thiadiazol in the place of a phenyl. Thus, the screen identified a small number of distinct inhibitors.

Dose Dependence of Inhibition

For further studies, the nine compounds and a Bak BH3-Bcl-x_L inhibitor that resembles BP5 (BH3I-2, [20]) were reordered from the supplier and subjected to mass spectroscopy (MS) analysis. Although impurities were detected, the expected peak predominated for each compound (see Supplemental Data for MS and high-performance liquid chromatography plots for BP5). In an initial study, we performed dose-response analyses of the inhibition of FP from the UL42-Pol peptide interaction for each of the nine compounds in Figure 2. Of these, five compounds—BP1, BP3, BP4, BP5, and BP9—reproducibly exhibited a dose-dependent reduction in FP, with IC_{50} values between 10 and 40 μM (Figure 3 and Table 1). Two structurally related pairs of compounds (BP2 and BP7, and BP6 and BP8) did not reproducibly inhibit FP in a dose-dependent manner (not shown). BH3I-2 did not detectably reduce FP at concentrations as high as 40 μM (Figure 3; lack of solubility precluded testing higher concentrations).

Specific Inhibition of Long-Chain DNA Synthesis Mediated by UL42

We next tested the nine compounds for the ability to interfere with Pol-UL42 interactions in a functional assay—UL42-dependent long-chain DNA synthesis by Pol (Figure 4). In this assay, which utilizes a poly(dA) template and oligo(dT) primers [21], Pol alone adds only one or a few bases (lane 1), while Pol plus UL42 adds many bases, leading to incorporation into longer products that can be visualized following alkaline agarose gel electrophoresis (lane 2). As previously observed [14, 15], peptide A inhibited long-chain DNA synthesis (lanes 3 and 4). We initially tested each compound from the screen at 30 μM. At this concentration, BP1 (lane 5) and BP3 (lane 7) severely decreased long-chain DNA synthesis. This concentration of BP5 (lane 9) and BP9 (not shown) also markedly decreased long-chain DNA synthesis. BP4 exhibited more modest inhibition. BP2, BP6, BP7, and BP8 (lanes 6, 10, 11, and 12) exerted even less inhibition of long-chain DNA synthesis, consistent with their lack of dose-dependent inhibition of FP. It is possible that these hydrophobic compounds scored as hits in the high-throughput assay due to an effect on the fluorophore rather than on the protein-peptide interaction.

To quantify the effects of the four most active compounds (BP1, 3, 5, and 9), different doses of the active compounds were tested for their effects on long-chain DNA synthesis, as illustrated here only for BP9 (Figure 4, lanes 13–17). These data were quantified by phosphorimage analysis, as described [14], or by the binding of radiolabeled products to DEAE filters. Both assays

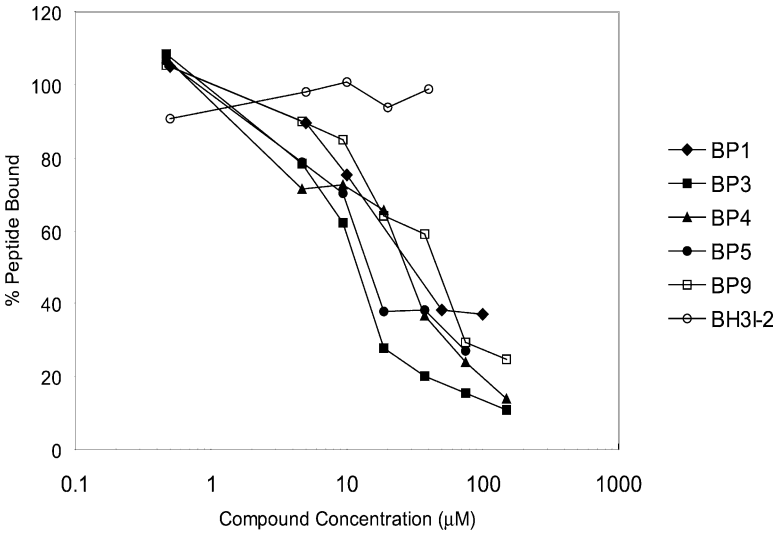

Figure 3. Dose-Dependence of Inhibition of FP

Varying concentrations of BP1 (filled diamonds), BP3 (squares), BP4 (triangles), BP5 (filled circles), BP9 (open squares), and BH3I-2 (open circles) were titrated into reactions containing 7 μM UL42 and 1 nM labeled peptide, and FP was measured. The values are plotted relative to the amount of signal with no compound added (100% peptide bound) and no UL42 (0% peptide bound) versus the concentration of the compounds.

yielded similar results. The IC_{50}s of the active compounds for inhibition of long-chain DNA synthesis were all ~30 μM (Table 1).

The inhibition of long-chain DNA synthesis could be due to effects on the catalytic activity of the Pol subunit rather than effects on a UL42-mediated activity. We therefore tested varying concentrations of the four active compounds on DNA synthesis by Pol alone by using a filter binding assay. As summarized in Table 1, the structurally related compounds BP3 and BP9 inhibited the activity of Pol alone, at least as potently as they inhibited long-chain DNA synthesis mediated by UL42. Thus, inhibition by these two compounds was not specific for UL42-mediated activity. However, BP1 and BP5, both of which share peptide-like features, inhibited activity of Pol alone much less potently (Table 1), indicating a specific effect on UL42-mediated long-chain DNA synthesis.

Specific Inhibition of Interaction of UL42 with Full-Length Pol

Although both BP1 and BP5 had been shown to inhibit the interaction of UL42 with the Pol-derived peptide, we wished to determine if they could inhibit the interaction of UL42 with full-length Pol by using a pull-down assay. In this assay, full-length Pol was expressed as a radiolabeled protein by using in vitro translation in reticulocyte lysates. When mixed with MBP-UL42, the radiolabeled Pol (UL30) was retained on amylose columns that bind the MBP fusion partner (Figure 5A, lane 1). Radiolabeled Pol alone did not bind to an amylose column (lane 6) nor to a glutathione-S-transferase fusion to the human cytomegalovirus (HCMV) homolog of HSV UL42, UL44 (lane 14), as previously observed [22]. Moreover, the catalytic subunit of HCMV DNA polymerase, UL54, did not bind to MBP-UL42 (lane 7), although, as reported [22], it did bind to UL44 (lane 8). Thus, the interaction of HSV Pol with UL42 in this assay is specific.

Unfortunately, we were unable to study BP1 by using this assay or cell-culture-based assays (see below) due to its lack of solubility under the conditions of these assays. However, BP5 retained solubility under these conditions. BP5 inhibited the Pol-UL42 interaction in a dose-dependent manner, with an IC_{50} of about 4 μM (Figure 5A, lanes 1-5, and Figure 5B). However, it had little or no effect on the interaction of HCMV Pol with GST-UL44 (Figure 5A, lanes 8–12, and Figure 5B). Thus, BP5 specifically inhibits the HSV Pol-UL42 interaction.

BP5 Exhibits More Potent Antiviral Than Cytotoxic Activity

Because of the considerable evidence that the interaction between Pol and UL42 is required for viral replication, we investigated the antiviral effect of BP5 by using two assays in Vero cells: plaque formation (Figure 6A), which entails a low multiplity of infection, and yield (Figure 6B), which measures the amount of infectious virus produced following a single cycle of viral replication

Table 1. Potencies of Selected Compounds in Biochemical Assays

	IC_{50} (μM) for:		
Compound	Fluorescence Polarization (UL42 and Pol Peptide)	Long Chain DNA Synthesis (Pol/UL42)	DNA Synthesis by Pol Alone
Peptide A	5	2[a]	50[a]
BP1	35	25	>100
BP3	12	30	25
BP5	15	26	>100
BP9	48	25	25

[a] Data from [15].

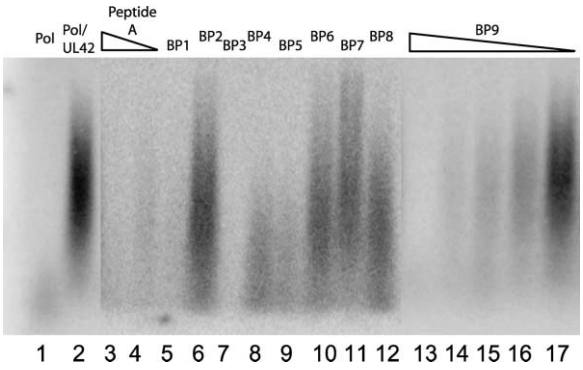

Figure 4. Effect of Compounds on Long-Chain DNA Synthesis by Pol and UL42

Assays were performed with a poly(dA) template and an oligo(dT) primer and labeled TTP. The reaction products were analyzed by alkaline agarose electrophoresis and autoradiography. Lane 1, HSV Pol alone; lanes 2–17 HSV Pol plus UL42 either without any added candidate inhibitor (lane 2), with 40 or 20 μM peptide A (lanes 3 and 4, respectively), with 30 μM BP1, BP2, BP3, BP4, BP5, BP6, BP7, or BP8 (lanes 5–12, respectively), or with 100, 75, 50, 25, or 12 μM BP9 (lanes 13–17, respectively).

initiated at a high multiplicity of infection. BP5 inhibited plaque formation by wild-type HSV-1 strain KOS with a 50% effective dose (ED_{50}) of 2 μM and inhibited virus yield with an ED_{50} of 0.3 μM. Similar results were obtained with another HSV-1 strain (not shown).

In parallel, we tested the cytotoxicity of BP5 in Vero cells by using an assay in which, in the presence of an electron-coupling reagent, only viable cells convert a compound (XTT) to a form that can be measured colorimetrically. BP5 exhibited cytotoxicity in this assay (Figure 6C) but at concentrations substantially higher than those at which it exhibited antiviral activity (IC_{50} ~20 μM). Thus, BP5 appears to exhibit selective anti-HSV activity.

Implications and Prospects

The primary goal of this study, which stems from previous work showing that interruption of a few hydrogen bonds is sufficient to disrupt HSV polymerase subunit interactions [8], was to identify a small molecule that can specifically inhibit these interactions in vitro. From a library of ~16,000 molecules, we found a compound, here termed BP5, that inhibits the binding of UL42 to both a Pol-derived peptide and full-length Pol, and inhibits the functional consequences of the Pol-UL42 interaction, long-chain DNA synthesis. This compound was not identified in a screen of the same library for inhibition of another protein-protein interaction [20], it did not detectably inhibit the binding of the subunits of a homologous herpesvirus polymerase, and it did not potently inhibit the activity of HSV Pol alone. Thus, BP5 is a

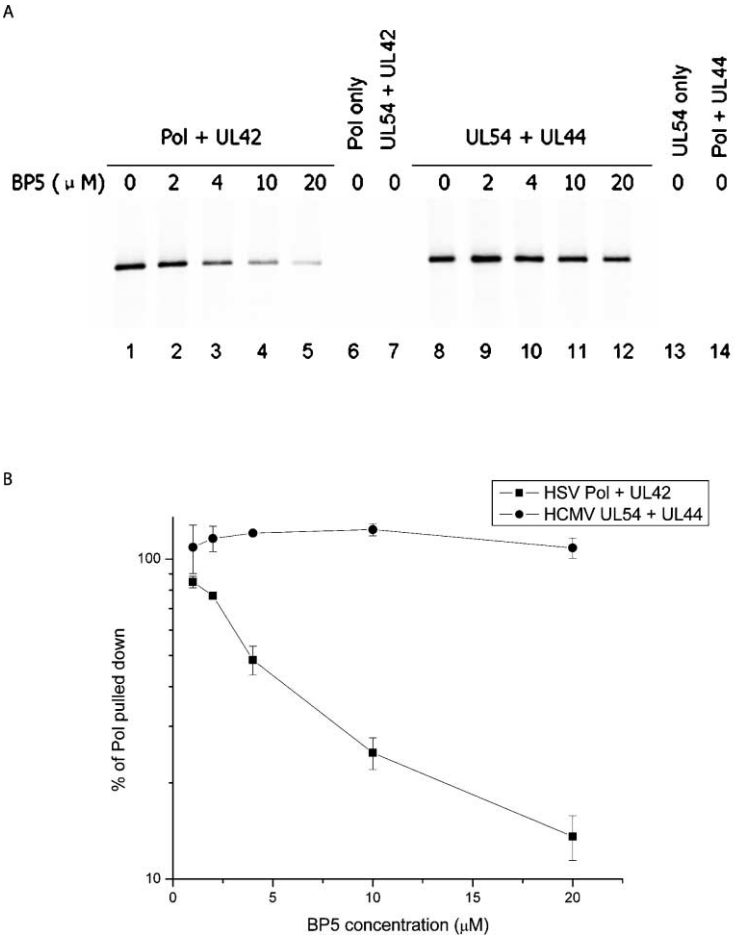

Figure 5. BP5 Inhibits the Interaction of Full-Length HSV Pol and UL42

(A) Cell-free translated HSV Pol (lanes 1–6, 14) or HCMV polymerase catalytic subunit (UL54, lanes 7–13) were mixed with either MBP-UL42 (UL42, lanes 1–5), a GST fusion to the HCMV homolog of UL42 (UL44, lanes 8–12, 14), or with no protein (lanes 6 and 13), and the indicated concentrations of BP5. The reactions in lanes 1–7 were bound to and eluted from an amylose column, and the reactions in lanes 8–14 were bound to and eluted from a gluathione column and analyzed by SDS polyacrylamide gel electrophoresis and autoradiography.

(B) Autoradiographs such as those in (A) were quantified by phosphorimager and the percentage of signal relative to that in the absence of BP5 was plotted versus BP5 concentration (HSV Pol/UL42, squares; HCMV UL54/UL44, circles). The data shown are the average of two experiments ± the standard error of the mean.

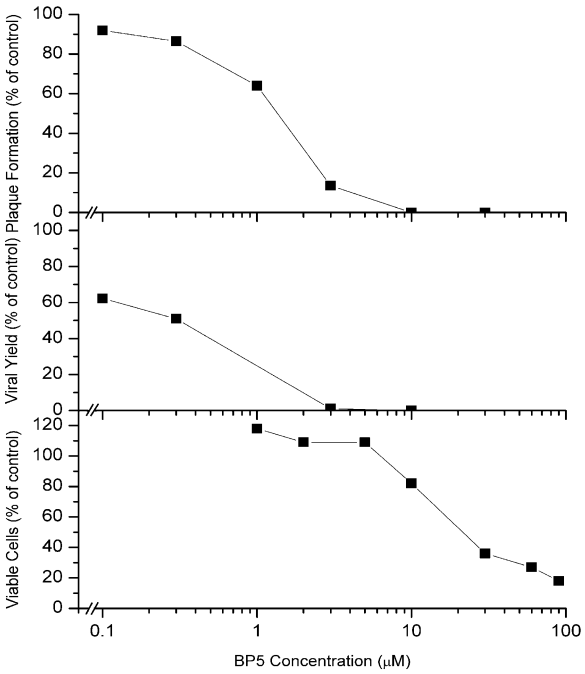

Figure 6. Antiviral Activity and Cytotoxicity of BP5

(A) The effect of BP5 on plaque formation in Vero cells by HSV-1 strain KOS.

(B) The effect of BP5 on the yield of KOS in Vero cells in single-cycle growth assays.

(C) The effect of BP5 on Vero cell viability as measured by an XTT assay. The absorbances measured in the XTT assay were plotted onto a standard curve of numbers of cells versus absorbance to measure the number of viable cells remaining.

specific inhibitor of Pol-UL42 interactions in vitro and joins a short but expanding list of small molecules that can specifically abrogate protein-protein interactions [1, 2]. To our knowledge, it is the first small molecule not derived from a peptide that interferes with interactions between viral proteins.

We do not know whether BP5 binds to UL42 or to the C terminus of Pol, whether it inhibits competitively by binding to the interaction interface, or whether it inhibits allosterically. The mechanism of action of BP5 may be of interest in understanding inhibitors of protein-protein interactions generally. Regardless of mechanism, the identification of BP5 proves the concept that HSV polymerase subunit interactions can be specifically disrupted by a small molecule.

Based on previous studies [9, 10, 12, 16], we predicted that a small molecule that inhibits Pol-UL42 interactions would have antiviral activity. It was pleasing then to observe that BP5 exhibits more potent antiviral than cytotoxic activity. The potency of BP5 in our antiviral assays was greater than in the in vitro assays. This may reflect lower concentrations of polymerase subunits in cells relative to those employed in the biochemical assays. The antiviral potency of BP5 is in the same range as that of ACV, but it is much more toxic. We are presently attempting to identify more potent, less toxic derivatives of BP5.

We do not know whether BP5's antiviral activity is, indeed, due to its inhibition of polymerase subunit interactions. Clearly, it could exert antiviral activity via any number of other mechanisms. To investigate the mechanism of antiviral action, we have recently isolated an HSV mutant resistant to BP5 (C.C. and D.M.C., unpublished results). This indicates that the compound acts via a virus-specific mechanism. However, the mutant's degree of resistance is limited and our ability to analyze the mechanism of resistance is made difficult by the cytotoxicity of BP5 at higher concentrations. More potent, less toxic derivatives of BP5 could aid this effort. Regardless, BP5 is likely to provide a useful tool for investigating the importance of Pol-UL42 interactions during DNA synthesis in vitro and in infected cells. BP5 also represents an interesting starting point for the discovery of drugs that may be more potent than ACV and that should be active against viral mutants resistant to currently existing drugs such as ACV.

Significance

Protein-protein interactions are crucial to most, if not all, biological processes. Because these interactions also often determine specificity, selective disruption of these interactions should, in principle, be an excellent strategy for drug discovery. However, there are only a few examples of small molecules that disrupt protein-protein interactions and exert effects inside cells. Such interactions could provide targets for new drugs against herpes simplex virus (HSV), which causes a spectrum of diseases that are especially severe in newborns and immunosuppressed individuals, such as patients with AIDS. Although antivirals against HSV, such as acyclovir (ACV), are widely used, new drugs are needed, especially for combatting ACV-resistant infections. A protein-protein interaction that has been characterized structurally and mutationally is that between the catalytic subunit UL30 (Pol) and the processivity subunit UL42 of the HSV DNA polymerase. It has been shown that individual Pol and UL42 residues are crucial for the interaction, suggesting that a small molecule might be able to disrupt the interaction. We therefore developed and validated a high-throughput screen for compounds that could disrupt polymerase subunit interactions and applied it to a library of small molecules. Among these, we have identified a molecule that specifically inhibits Pol-UL42 interactions, both physically and functionally. To our knowledge, this is the first small molecule not derived from a peptide that interferes with interactions between viral proteins. Moreover, this molecule has antiviral activity that is more potent than its cytotoxic activity. This small molecule is likely to provide a useful tool for investigations of the Pol-UL42 interaction and its role in viral DNA replication. It also represents an interesting starting point for the discovery of new anti-HSV drugs that should be active against viral mutants resistant to currently existing drugs such as ACV.

Experimental Procedures

Peptides and UL42

Peptide A, corresponding to the 36 C-terminal amino acids of HSV Pol [15], and a peptide comprising the 18 C-terminal amino acids of

HSV Pol that is 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 and Molecular Pharmacology (Harvard Medical School). Recombinant MBP-UL42 protein was expressed as a truncated form ending at residue 340 (ΔC340) and purified by amylose column chromatography, as described [8]. For assays of interaction with full-length Pol, the protein was additionally purified by ssDNA-agarose chromatography as described [8]. The protein was concentrated by using spin columns (BioRad, Centricon) when necessary, and stored at −80°C until further use. After thawing, the purification buffer was exchanged to low fluorescent grade reagents (PanVera), and any maltose was removed simultaneously by using 10-DG Bio-Gel columns (BioRad).

FP Assays

1 nM of Oregon Green-labeled peptide was added to 7 μM MBP-UL42ΔC340 in 50 mM TrisHCl (pH 7.5), 1 mM DTT, 0.5 mM EDTA, 150 mM NaCl, 4% Glycerol and 100 μg ml^{-1} bovine γ globulin and kept on ice until the mix was distributed into black 384-well plates (LJL Biosystems) in a total volume of 20 μl per well. All chemicals were low fluorescent grade (PanVera). Small molecules (5 mg/ml in DMSO; ChemBridge) were obtained from the Harvard Institute of Chemistry and Cell Biology (ICCB, Harvard Medical School), and 40 nl of each compound was transfered to individual wells using 384-pin arrays (Genetix). To ensure comparable data, internal reference values (peptide only, no compound, no DMSO) were included on each screening plate. After incubation between 5 and 45 min at room temperature, the FP values were determined by using an Analyst plate reader (LJL Biosystems) at the ICCB. Small molecules for further testing were obtained from ChemBridge.

DNA Polymerase Assays

Selected compounds were tested for their ability to inhibit either the activity of Pol alone by using an oligo(dT) primer (Roche Molecular Biochemicals) and a poly(dA) template (Amersham Pharmacia) or UL42-mediated long-chain DNA synthesis by Pol [14, 15, 21] on the same primer-template. Reaction mixtures (50 mM TrisHCl [pH 7.5], 100 mM ammonium sulfate, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 4% glycerol, 40 μg/ml BSA, 100 μg/ml primer/template, 50 μM α-^{32}P-TTP [111 TBq/mmol]) contained 200 fmol of HSV Pol, kindly provided by K. Kumura-Ishii of this laboratory, either none or 400 fmol of HSV MBP-UL42, and varying amounts of compound in a final volume of 25 μl. Reactions were carried out at 37°C for 5 min (UL42 present) or 25 min (no UL42). For analysis of incorporated radioactivity, 10 μl of each reaction was placed onto a DE81 filter disc. After three washes in 5% Na_2HPO_4 and once in 95% ethanol, the radioactivity was measured by liquid scintillation. For analysis of product sizes, the reactions were stopped by adding 5 μl of alkaline loading buffer (2 mM EDTA, 50 mM NaOH, 2.5% glycerol, 0.025% bromcresol green) and placing them on ice, and were then loaded onto a 4% alkaline agarose gel. After electrophoresis, gels were dried overnight and exposed to phosphorescence screens (BioRad), and the radioactivity was quantified by using a phosphorimager (BioRad). IC_{50} values from these data were calculated as described [14].

Binding of Radiolabeled Pol to UL42

MBP-UL42Δ340, or as a control, GST-UL44Δ290 [22] (kindly provided by A. Loregian of this laboratory) was preincubated for 30 min at 25°C. with or without the indicated concentrations of BP5 in 493 μl binding buffer (30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 10% glycerol for UL42 and 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 30% glycerol for UL44). Then 7 μl of reticulocyte lysate containing either HSV Pol (UL30) or HCMV UL54, which was expressed and radiolabeled in vitro from plasmids pING-UL30 [24] or pRSET-Pol (kindly provided by P. Ertl) by using the TNT-coupled reticulocyte lysate system (Promega), were added. Incubations were continued for 2 hr at 4°C. Reactions were then loaded onto 200 μl amylose or gluathione columns, respectively. The flow-through fractions were collected and reloaded three times. The columns were then washed three times with the appropriate binding buffer containing Triton X-100 and NP40 (0.5% each) and varying concentrations of BP5, and eluted with binding buffer containing 10 mM maltose or 15 mM glutathione, respectively. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

Assays of Antiviral Activity

Plaque reduction assays of the antiviral activity of BP5 in Vero (African green monkey kidney) cells were performed essentially as described [25] with the following modifications: virus was adsorbed in a volume of 1 ml for 2 hr. The inocula were then removed, and medium containing methylcellulose and various concentrations of BP5 was added. Plaques were counted after crystal violet staining after 2 days. As a control, the concentration of DMSO corresponding to that present in the medium containing the highest concentration of BP5 tested was assayed and found not to reduce plaque formation. Yield reduction assays were perfomed at 37°C in 24-well plates that were seeded with 1.5×10^5 Vero cells in 0.75 ml medium 1 day before infection. The medium was removed and inoculated with HSV-1 strain KOS at a multiplicity of infection of 5 plaque-forming units/cell in 0.2 ml medium. Following adsorption for 2 hr, the inoculum was removed, the cells were rinsed with medium, and then 0.2 ml medium containing various concentrations of BP5 or DMSO (which had no effect) was added. After 24 hr incubation, the plates were frozen and thawed twice, and the infectious virus present in each well was titrated by plaque assay.

Assay of Cytotoxic Activity

Vero cells were seeded at 5×10^4 cells per well into 96-well plates and treated with various concentrations of BP5 for 24 hr. in quadruplicate. Cell viability was then determined with an XTT assay (Roche Molecular Biochemicals) according to the manufacturer's protocol by using a Victor plate reader (Wallac).

Supplemental Data

Supplemental Data including three figures are available at http://www.chembiol.com/cgi/content/full/11/5/647/DC1/.

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