Identification of Protein-Protein Interaction Inhibitors Targeting Vaccinia Virus Processivity Factor for Development of Antiviral Agents^{▽†}

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Poxvirus uracil DNA glycosylase D4 in association with A20 and the catalytic subunit of DNA polymerase forms the processive polymerase complex. The binding of D4 and A20 is essential for processive polymerase activity. Using an AlphaScreen assay, we identified compounds that inhibit protein-protein interactions between D4 and A20. Effective interaction inhibitors exhibited both antiviral activity and binding to D4. These results suggest that novel antiviral agents that target the protein-protein interactions between D4 and A20 can be developed for the treatment of infections with poxviruses, including smallpox.

Poxviruses constitute a large family of DNA viruses that infect a wide range of hosts from insects to humans. Smallpox, one of the most devastating infectious diseases affecting humans, is caused by two members of the poxvirus family, *Variola major virus* and *Variola minor virus*. The disease caused 300 to 500 million deaths during the 20th century alone (2, 16). Because of the potential use of smallpox as an agent of bioterrorism together with the high transmission and high mortality rates, *Variola major virus* is designated a category A (high-security/high-priority) agent by the Centers for Disease Control and Prevention. A worldwide vaccination campaign successfully resulted in the global eradication of smallpox. Routine vaccination throughout the United States ceased more than a quarter of a century ago (22). Today, much of the world's population remains unimmunized and susceptible to a reemergence of poxvirus. However, except for the antiviral drug cidofovir (CDV), which was recently approved for the emergency treatment of smallpox, there is currently no drug for the treatment of smallpox (7). Moreover, CDV is approved only for intravenous application and may cause serious renal toxicity. A new drug, ST-246, is currently undergoing clinical trials (11, 21). However, due to the potential emergence of drug resistance, there is a genuine need for a new effective treatment of smallpox and infection by other poxviruses.

Antiviral drugs targeting replication mechanisms have been successfully used as therapeutic agents (5). Unique among other DNA viruses, poxviruses replicate in the host cytoplasm and rely mostly on proteins encoded by the large viral genome of approximately 192 kb (8). Vaccinia virus (VV) is the best-characterized member and the prototype of poxviruses. Ge-

netic and biochemical data reveal a number of vaccinia virus proteins involved in viral replication, and at least six are considered important for replication: DNA polymerase E9; uracil DNA glycosylase D4; A20; a serine threonine kinase, B1; B1 substrate H5; and a nucleotide triphosphatase, D5 (4, 6, 12, 14, 31). Among these, D4 is a ubiquitous enzyme that performs DNA repair activity by removing misincorporated uracil bases from DNA. However, D4 plays an essential role in the replication of vaccinia virus that is not dependent on its enzymatic activity but involves its binding to A20, a unique poxvirus protein, which also binds to E9 (9, 19). Together, the D4:A20:E9 complex forms the processive DNA polymerase holoenzyme, and this trimeric complex is essential for DNA polymerase activity (30).

Although the processivity property of vaccinia virus DNA polymerase has been studied by several laboratories, the biophysical and structural aspects are yet to be characterized. Existing data suggest that D4 does not interact directly with E9 but that its binding to A20 is essential for the formation of the D4:A20:E9 complex. We hypothesized that a blocking of the binding of D4 to A20 would inhibit the polymerase function, and therefore, inhibitors of the D4:A20 protein-protein interaction (PPI) should be effective as antiviral agents. Since these PPI inhibitors target a specific protein-protein interface, they are less likely to interfere with host proteins and enzymes and are expected to be relatively nontoxic. We developed an AlphaScreen-based high-throughput screening (HTS) assay of selected chemical libraries to identify compounds that interfere with the interaction between D4 and A20.

The AlphaScreen is a nonradioactive assay in which the molecular interactions between two binding partners lead to a cascade of chemical reactions to generate an amplified signal (18, 27). The assay uses two beads, the “donor” bead and the “acceptor” bead, each conjugated to one binding partner. When beads conjugated to binding partners are mixed, the beads remain in close proximity if binding partners form a biomolecular interaction. Upon laser excitation, a photosensi-

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tizer in the donor bead converts ambient oxygen to a more excited singlet state, which diffuses across to react with a thioxene derivative in the acceptor bead, thus generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. The fluorophores subsequently emit light at 520 to 620 nm. In the absence of a specific interaction between the binding partners, the beads are not in proximity, and the singlet oxygen molecules produced by the donor bead generate a low signal and remain undetected. The AlphaScreen assay is easily adaptable to high-throughput screening for PPI inhibitors. When incubated in the presence of compounds that disrupt the biomolecular interaction, the signal is lost.

Here, we describe the AlphaScreen assay for the identification of PPI inhibitors that block the D4:A20 interaction. We also show that a selected set of PPI inhibitors possesses antiviral activity against vaccinia virus and cowpox virus (CV) and that representative compounds bind to D4. To our knowledge, this is the first screen describing the identification of PPI inhibitors targeting the D4:A20 interaction.

MATERIALS AND METHODS

Protein samples. Full-length recombinant D4 containing an N-terminal hexahistidine tag (H_6 -D4) was purified by using affinity chromatography on a Talon (BD Biosciences) column by elution with 150 mM imidazole in 50 mM NaH_2PO_4 buffer (pH 7.4), 100 mM NaCl, and 10 mM β -mercaptoethanol (buffer A). The purity of the H_6 -D4 fractions was established with a 12% SDS-polyacrylamide gel.

The coding sequences for the N-terminal 100 amino acid residues of A20 were subcloned into the pMAL-C2 vector (New England BioLabs) and expressed in *Escherichia coli* BL21(DE3) cells as a fusion protein with an N-terminal maltose-binding protein (MBP) tag. The fusion protein was purified on an amylose column (New England BioLabs) by elution with 10 mM maltose in phosphate buffer and then dialyzed against buffer B (buffer A plus 100 mM NaCl). The purity of the fusion protein (referred to as A20 henceforth) was verified on a 10% SDS-polyacrylamide gel.

D4:A20 interaction. The binding of recombinant H_6 -D4 and A20 was analyzed by using a "dual-pulldown assay." Purified H_6 -D4 and A20 were mixed in equimolar amounts in buffer B, and the mixture was applied onto an amylose column to remove any unbound D4. After the column was washed with 10 column volumes of buffer, proteins bound to the column were eluted with 10 mM maltose. The eluted samples were then loaded onto a Talon column, which allowed the removal of any free A20 from the mixture, and the column was washed with 30 mM imidazole in buffer B. Finally, the complex bound to the column was eluted with 200 mM imidazole in the same buffer and analyzed by SDS-PAGE.

AlphaScreen assay. For the AlphaScreen (Perkin-Elmer) assay, A20 was biotinylated by using the EZ-LinkSulfo-NHS-Biotinylation kit (Pierce) according to the manufacturer's protocol. For the preparation of beads, biotinylated A20 was incubated with streptavidin-coated donor beads (DS) at a final concentration of 10 μ g/ml, and H_6 -D4 was mixed with nickel chelate acceptor beads (AN) at a final concentration of 15 μ g/ml for ~1 h at 4°C. All dilutions were made with AlphaLISA buffer (Perkin-Elmer).

High-throughput screening. A high-throughput screen (HTS) was initiated by incubating 30 μ l of H_6 -D4 conjugated with acceptor beads (AN- H_6 -D4). Using a robotic liquid handler, 30 μ l of AN- H_6 -D4 and 0.1 μ l of compound in dimethyl sulfoxide (DMSO) were dispensed into each well, and the mixture was allowed to incubate for 2 to 3 h at room temperature. This was followed by the addition of a 20- μ l donor-bead-bound A20 (DS-A20) suspension with 0.1 μ l of compound in DMSO for 2 to 3 h at room temperature. Each plate included several "negative-control" wells that contained AN- H_6 -D4, DS-A20, and DMSO but no inhibitor. Positive-control wells contained both proteins, both beads, and unbiotinylated A20. One column in each plate contained AN- H_6 -D4 and 20 μ l of buffer containing the DS (without A20); these wells served as the "background control." In general, in each well of one column, A20 (not biotinylated) was included as a "positive inhibitor control." The AlphaScreen reactions at the Institute of Chemistry and Chemical Biology (ICCB) (Harvard Medical School) were read on an automated EnVision multilabel reader

(Perkin-Elmer). A total of 32,944 compounds belonging to the following libraries were screened: ChemDiv3, ChemDiv4, ChemDiv5, ChemDiv6, MMV, Maybridge2, Chembridge3, Maybridge5, Enamine2, Bionet2, Prestwick1 collection, and NINDS.

Cytopathic effect assay. The antiviral activities of selected compounds that were immediately available from chemical vendors were evaluated with a viral cytopathic effect (CPE) assay performed with 96-well plates seeded with 2.5×10^4 cells per well of human foreskin fibroblast (HFF) cells (26). The plates contained a range of drug concentrations and were infected with the Copenhagen strain of vaccinia virus (VV) and the Brighton strain of cowpox virus (CV) at 1,000 PFU per well. After 7 days of incubation, the plates were stained with a crystal violet solution for 4 h, rinsed, allowed to dry for 24 h, and then read on a BioTek plate reader at 620 nm. EC_{50} s (50% effective concentrations) were calculated by standard methods. CDV, which was used as a control, was a gift from Mick Hitchcock at Gilead Sciences.

Four compounds were also evaluated in a plaque reduction assay using BSC-1 cells and the vaccinia virus Western Reserve strain. Among these, compounds 25, 26, and 27 were not included in the CPE assay discussed above. One compound, compound 15, was used in both assays to evaluate the consistency of the results produced by these assays. Confluent BSC-1 cell monolayers were obtained by seeding 2×10^4 cells/well in 300 μ l growth medium overnight in 48-well plates. Cells were infected by adsorbing virus for 1 h in a humidified incubator at 100 PFU/well in 100 μ l growth medium, followed by overnight treatment with inhibitors in a final volume of 300 μ l medium and 1% DMSO. Cells were fixed with 5% formaldehyde in phosphate-buffered saline (PBS) for 6 h at room temperature, followed by 30 min of staining with 5% crystal violet; washed with distilled H_2O ; and air dried overnight. Scaffolds for all compounds used in the viral infectivity assays are shown in the supplemental material. Compounds 1 to 23 and 25 to 27 were purchased from ChemDiv.

Antiviral activities against herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2) were also evaluated by a CPE assay according to methods described previously (15). Briefly, compounds were diluted in 96-well plates containing monolayers of HFF cells. The cells were infected with the E-377 strain of HSV-1 or the MS strain of HSV-2 and incubated for 3 days. Cell monolayers were then stained with crystal violet, and cytopathology was quantified with a microplate reader. Acyclovir (Sigma-Aldrich) and zidovudine (AZT) (Sigma-Aldrich) were used as positive and negative controls in each assay.

Determination of cytotoxicity. Ninety-six-well plates were seeded with HFF cells at a concentration of 2.5×10^4 cells per well. After 24 h, the medium was aspirated, and 125 μ l of each drug concentration in minimal essential medium with 2% fetal bovine serum (FBS) was added to the first row of wells in triplicate. Serial 1:5 dilutions were performed by using the Beckman BioMek liquid handling system. After the addition of compound, the plates were incubated for 7 days in a CO_2 incubator at 37°C. Cell monolayers were stained with crystal violet, and bound dye was used to quantify cell numbers. The concentration of drug that reduced cell viability by 50% (CC_{50}) was then calculated by use of standard methods.

Fluorescence studies for determination of binding to D4. PPI inhibitors belonging to different scaffolds were tested for binding to D4. Compounds that were highly cytotoxic were not used in the assay. Compound 15, which had good EC_{50} values and showed the best selectivity index in the CPE assay, was included in the binding study. The hexahistidine tag was removed by treatment with thrombin, and tag-free D4 was purified. Fluorescently labeled D4 was prepared by using the Alexa Fluor 488 protein labeling kit (Invitrogen), by stirring 1 mg of D4 with the dye in PBS (total volume, 0.5 ml) for 1 h at room temperature. The labeled protein was then separated from excess dye by chromatography on BioGel P-30 fine-size-exclusion resin. Labeled protein was stored in small aliquots at -20°C until use.

Fluorescence studies were carried out with a BioTek Synergy 2 multimode microplate reader using Costar black flat-bottom 96-well plates at wavelengths of 485 and 528 nm for excitation and emission spectra, respectively. We examined the binding of hits belonging to different scaffolds. Data were collected with a total volume of 100 μ l by incubating ~0.6 μ M labeled D4 with compound (final concentration of 0.5 to 4 μ M); DMSO was used as a mock control. Measurements in duplicate were acquired for up to 60 min at 5-min intervals. As a positive control we used a peptide comprising the N-terminal 50 residues of A20 (MTSSADLTNLKELLSLYKSLRFSDSAIEKYNSLVEWGTTSTYWKIGVQKV) at various concentrations (0.5 to 2.5 μ M). As a negative control we used pyrimethamine (Sigma-Aldrich) at a final concentration of 1 to 5 μ M.

FIG. 1. Analysis of protein samples used in the AlphaScreen. SDS-PAGE gel showing purified A20 (MBP fusion), H₆-D4, and the A20:D4 complex. A purified protein sample was boiled with an equal volume of 2× sample buffer containing 1% SDS and subjected to electrophoresis on 12% polyacrylamide gels containing 1% SDS. The gel was fixed in fixing solution containing 50% ethanol and 10% acetic acid, stained with Coomassie blue, and destained in a 5% acetic acid solution. Lane 1, purified A20 (MBP fusion); lane 2, purified H₆-D4; lane 3, A20 and H₆-D4 complex purified by sequential affinity chromatography; lane 4, protein standards, with molecular masses in kilodaltons shown on the right.

RESULTS

Analysis of interactions between recombinant D4 and A20.

Previous studies showed that the N-terminal 25 residues of A20 are essential for interactions with D4 and that binding is enhanced when residues 1 to 50 are used. We expressed the polypeptide representing the N-terminal 100 residues of A20 as an MBP fusion protein (MBP-A20) and purified this fusion protein to homogeneity using affinity chromatography (Fig. 1). Recombinant H₆-D4 was expressed in *E. coli* cells and purified to homogeneity using a single column step. Interactions between these recombinant proteins were verified by isolating the complex of the two proteins using two affinity steps sequentially. As shown in Fig. 1 (lane 3), SDS-PAGE of the sample eluted from the second column step showed two bands representing H₆-D4 and A20 (MBP fusion). The complex was purified by using two affinity columns (amylose and Talon) sequentially to eliminate any excess protein that did not form a complex. The observed difference in the intensities of the bands may be due to differences in the migration patterns and staining properties of the A20-MBP fusion protein and H₆-D4. Using His-tag-free D4, we verified that the tag had no effect on the binding of D4 to A20; similarly, we also confirmed that purified MBP did not bind to H₆-D4 (data not shown).

AlphaScreen assay and identification of PPI inhibitors.

Taking advantage of the histidine tag on H₆-D4, we directly coupled D4 to the nickel chelate acceptor bead. Biotinylated MBP-A20 was conjugated with streptavidin-coated donor beads. By titrating six concentrations of each protein in the range of 0 to 200 nM (at an interval of 25 nM) against each of the six concentrations of the other, followed by measurement of the signal, the optimum concentrations were found to be 25 to 50 nM for H₆-D4 and 25 nM for A20.

In a primary screening of 1,264 compounds representing high-quality inhibitors, we tested these compounds at a single concentration (33 μg/ml). A total of 5 plates were selected

FIG. 2. Assessment of the quality of the AlphaScreen. Calculated Z' factors are plotted against plate number for a representative assay. Z' is calculated using the formula $Z' = 1 - (3\sigma_{c^+} + 3\sigma_{c^-}) / |\mu_{c^+} - \mu_{c^-}|$, where c^+ represents the positive control, c^- represents the negative control, σ is the standard deviation, and μ is the mean value.

from the following libraries: Bionet2, ChemDiv3, Prestwick1 collection, and NINDS custom collection. All assays were done in duplicate. Eight compounds (~0.6% of all compounds tested) showed 90% or more inhibition of protein-protein interaction. Of these, 5 compounds showed at least 95% inhibition. Statistical analysis indicated a good distribution of the readout (luminescence) and a good correlation between replicates. The value of the estimated Z' factor (0.8) for this primary screen showed the suitability of the assay for HTS (32). The Z' factor is a characteristic parameter defining the quality of the assay itself, without the intervention of test compounds, and is used routinely for quality assessment in assay development and optimization. A value of 0.5 or higher for the Z' factor indicates a robust assay. The significance of the Z' factor in the assessment of the quality of HTS was discussed previously by Iversen et al. (20). The formula used for calculating the Z' factor is as follows: $Z' = 1 - (3\sigma_{c^+} + 3\sigma_{c^-}) / |\mu_{c^+} - \mu_{c^-}|$, where c^+ represents the positive control, c^- represents the negative control, σ is the standard deviation, and μ is the mean value.

We then screened a total of 32,944 compounds in an HTS assay using the protocol described above. Analysis of the Z' factor (~0.8) confirmed a good overall quality (20, 32). A typical plot of Z' factors is shown in Fig. 2. Based on the 85% inhibition criteria, a total of 338 hit compounds were identified. These compounds were tested for false-positive hits by using the TruHits kit (Perkin-Elmer) according to the manufacturer's recommendations. False-positive compounds interfere with the HTS assay in ways not related to the screen target. These include singlet-oxygen quenchers, light scatterers, biotin mimetics, and acceptor bead competitors. Based on the results of this test, all suspect compounds and their structural analogs were eliminated. In addition, compounds that are known to belong to toxic classes were eliminated. Finally, a total of 112 compounds were selected as hits (≥85% inhibition) for further analysis.

Analysis of antiviral activity. (i) Inhibition of viral infection.

Upon an examination of the chemical structures, several chemical scaffolds were immediately apparent. A total of 23 compounds representing 11 chemical scaffolds (out of 12 identified [see the supplemental material]) that were immediately available from commercial vendors were tested for the inhibition of

infectivity and cytotoxicity by using a CPE assay. The structures of these compounds are shown in Fig. 3. Cidofovir (compound 24 [structure not shown]), a known inhibitor of vaccinia virus and cowpox virus replication, was used as a positive control. The cytotoxicities of the compounds were measured by a crystal violet assay performed concurrently with the antiviral assays. Several compounds exhibited EC_{50} s (concentrations for 50% inhibition) in the low-micromolar range (Table 1). Compound 15 had EC_{50} values of 4.2 and 4.5 μ M against vaccinia virus and cowpox virus, respectively. The corresponding EC_{90} values (concentration for 90% inhibition) were 7 and 8 μ M, respectively. The selectivity index for this compound was greater than 40.

Four compounds, compounds 15, 25, 26, and 27 (Fig. 3), were also assessed for the inhibition of cellular infection in a plaque reduction assay using a different strain (Western Reserve) of vaccinia virus. Three of these compounds were not tested earlier in the CPE assay. As shown in Fig. 4, the EC_{50} s of these compounds measured in this assay ranged between 2.9 and 13.9 μ M. The EC_{50} of 4.6 μ M for compound 15 agrees well with that determined by our previous assay against the Copenhagen strain.

When tested against herpes simplex viruses 1 and 2, compounds 1 to 8 did not show any measurable antiviral activity, thereby indicating the specificity of these inhibitors for poxvirus.

(ii) Analysis of binding to D4. Inhibitors of the protein-protein interaction between A20 and D4 can bind to “hot spots” on either protein or at the interface. Fluorescently labeled proteins have been successfully used for analyses of protein–small-molecule interactions (23, 24). We tested the binding of selected hits to fluorescently labeled D4. The concentration of D4 for this study (0.6 μ M) was chosen for obtaining a stable signal from a standard curve (data not shown). As a negative control we used an unrelated small organic compound, pyrimethamine (formula weight, 248.71), in the concentration range of 1 to 5 μ M; it showed only minor ($\leq 1\%$ to 2%) changes in the signal, and the observed fluctuations were random (see the supplemental material). To confirm that fluorescence signals were sensitive to the binding of specific molecules to D4, we used a peptide representing the D4-binding sequence of A20. The fluorescence signal was recorded at four different concentrations of the peptide, and the difference in relative fluorescence was dependent on the concentration of the peptide (Fig. 5a).

Of the 24 compounds used in the antiviral assay, we used a number of compounds for studying their binding to D4. Compounds that were highly cytotoxic were not included because of suspected nonspecific binding. Attempts were made to include compounds representing unique scaffolds. The fluorescence intensity was measured at four or five different concentrations of each inhibitor, and the difference in the relative fluorescence at the 30-min interval compared to the starting relative fluorescence at the time point of 0 min [$\Delta(F/F_0)$] was plotted. In Fig. 5b to f we show the change in the fluorescence intensity of labeled D4 upon incubation with various concentrations of compounds 1, 6, 9, 12, and 15. All compounds induced a change (either an increase or a decrease) in the relative fluorescence dependent on the concentration. Conformational changes due to interactions of the PPI inhibitors with D4 may

bring a quenching group into the vicinity of the fluorophore, with a resultant decrease in fluorescence intensity, or it may decrease the solvent exposure of the fluorophore, which will result in an increased intensity (28). However, the precise nature of the conformational changes cannot be predicted without further information about the binding pockets. Additional data supporting the binding of similar compounds to D4 (using ThermoFluor) were presented elsewhere previously (25). All plots were generated with SigmaPlot (Systat Software, Inc.).

DISCUSSION

Therapeutic applications of PPI inhibitors are wide ranging (1, 3, 10). There is growing evidence that small molecules can modulate protein-protein interactions directly via blocking the interaction surface or indirectly by binding to an allosteric site and inducing conformational changes in the target or an associated molecule (17). PPI inhibitors that block the binding of HIV to the host receptor, known as “entry inhibitors,” are already in clinical use (5, 13). Importantly, many of these agents are active against HIV strains resistant to protease inhibitors and reverse transcriptase inhibitors. Therefore, PPI inhibitors can also be effective in combination therapy and as a means to relieve resistance to active drugs. The AlphaScreen provides a platform for the rapid, high-throughput screening of PPI inhibitors (18).

Available data indicate that A20 interacts with both D4 and E9, whereas D4 does not interact directly with E9, and the binding of D4 and A20 is necessary for the processive polymerase activity of E9. We proposed that compounds interfering with the binding between D4 and A20 are expected to block DNA synthesis and viral replication. We present here the first screen to identify small molecules capable of disrupting the D4:A20 interaction. Data presented here provide the proof of concept that inhibitors targeting the processivity factor of vaccinia virus, specifically those inhibiting interactions between D4 and A20, inhibit viral replication.

The versatility of protein-protein interactions offers an attractive choice for a drug target. Drugs targeting protein-protein interactions can not only act as competitive antagonists but also serve as allosteric modulators by interfering with the interaction of one protein with others. In the poxvirus replication apparatus, A20 binds to D4, the DNA polymerase, and a number of other proteins. Although it is not known at this time how the binding of D4 influences the interaction of A20 with other proteins, PPI inhibitors targeting the D4:A20 interaction can have additional effects on A20's association with other proteins. The N-terminal 50 residues of A20 have been shown to be involved in binding to D4. However, the A20-binding regions on D4 have not yet been mapped. We therefore used a recombinant protein representing the N-terminal 100 residues of A20 and full-length wild-type D4 for screening.

We adopted an automated high-throughput format for using the AlphaScreen, but from the outset we used stringent criteria for the selection of active compounds. Instead of random library screening we focused on libraries that are known to contain well-characterized molecules representing diverse chemical classes and, importantly, are rich in drug-like molecules deposited by large pharmaceutical companies. The

FIG. 3. Chemical structures of the compounds studied for antiviral activity. The figure shows the chemical structures of the compounds used in the antiviral activity assay. The numbering scheme is the same as that in Table 1.

TABLE 1. Summary of antiviral activity (CPE) and cytotoxicity assays

Compound ^d (catalog no.)	CC ₅₀ ^a (μM)	Vaccinia virus (Copenhagen strain)		Cowpox virus (Brighton strain)	
		EC ₅₀ ^b (μM)	SI ^c	EC ₅₀ (μM)	SI
1 (4896-3086)	49.8	9.1	5.5	10.3	4.8
2 (4896-3248)	39.2	5.4	7.3	10	3.9
3 (4896-4553)	34.5	9	3.8	9	3.8
4 (5511-0217)	42.5	11	3.9	11	3.9
5 (5704-0768)	38.1	10.4	3.7	10.4	3.7
6 (5994-0466)	196.9	8.2	24	44.7	4.4
7 (6969-1025)	41.7	8.1	5.1	8.5	4.9
8 (4896-3502)	37.5	6.9	5.4	10.6	3.5
9 (5218-0975)	43	8.9	4.8	8.9	4.8
10 (5408-0930)	>286	>286	0	>286	0
11 (6408-0251)	>294	>294	0	>294	0
12 (6408-0282)	42.5	9.3	4.6	9.3	4.6
13 (7170-3701)	>354	>354	0	>354	0
14 (7210-1961)	49.8	11.4	4.4	11.4	4.4
15 (8009-1110)	>198	4.2	>47	4.5	>44
16 (8539-0674)	>221	>221	0	>221	0
17 (C147-0148)	245	51.6	4.7	51.6	4.7
18 (C147-0156)	>258	>258	0	>258	0
19 (C301-2019)	>328	218.4	>1.5	274.1	>1.2
20 (C301-6073)	202.9	41.8	4.9	41.8	4.9
21 (C387-0287)	36.7	8	4.6	8	4.6
22 (C620-0260)	>257	119.9	>2.1	97.3	>2.6
23 (C620-0278)	>265	80.8	>3.3	76	>3.5
24 (CDV)	>358	1.8	>200	4.3	>83

^a CC₅₀, concentration that reduces cell growth by 50% (using crystal violet).^b EC₅₀, concentration required to reduce CPE by 50%.^c SI, selectivity index (CC₅₀/EC₅₀).^d All compounds tested exhibited >85% inhibition of the A20:D4 interaction in the AlphaScreen.

screen was optimized in terms of the concentrations of D4 and A20, bead concentrations, and buffer and incubation conditions to maximize the specificity and signal-to-noise ratio. In particular, the use of AlphaLisa buffer (which contains bovine

serum albumin [BSA] and a low concentration of nonionic detergent) was important for reducing nonspecific binding and improved the signal-to-noise ratio.

In an HTS, the reliability of the active compounds or “hits” depends on the robustness of the assay. The robustness not only implies the reproducibility of the assay but also means that the results are independent of minor changes in the reagents used or in laboratory conditions. Moreover, a high degree of accuracy and sensitivity is critical for the identification of hits. Using both positive and negative controls, statistical analysis of signals, signal-to-noise ratio, correlation between duplicates (if available), hit ratio, and distribution are used to evaluate the quality of assays. A statistical parameter, *Z'*, is routinely used to assess the quality of any assay. A value of the *Z'* factor exceeding 0.5 indicates excellent quality and a robust assay. As shown in Fig. 2 the values of the *Z'* factor calculated for each of the representative 40 plates were >0.7, and the average value was 0.866. The average *Z'* factor value for the entire assay (all plates) was >0.8 (and reached 0.9 and above in some cases). These numbers confirm the high quality of the AlphaScreen method presented here.

We have used a strict criterion for the selection of hits in the AlphaScreen. In the primary screen of 1,264 compounds, the hit rate of 0.6% was consistent with that expected for HTS. For the final HTS we used a minimum 85% inhibition criterion, and compounds that showed <85% inhibition of the D4:A20 interaction were not considered for further evaluation. A TrueHits kit was used to eliminate a few compounds that may have interfered with the assay. Finally, we examined the chemical structures of the active compounds for clearly identifiable similarities. The clustering of active compounds in specific groups with similarity in chemical structures suggested potential structure-activity relationships among the active compounds. More-

FIG. 4. EC₅₀ determination by plaque reduction assay. The plot shows the inhibition of plaque formation (percent) at various concentrations of inhibitors. (Top, left) Compound 15; (right) compound 25. (Bottom, left) Compound 26; (right) compound 27. EC₅₀s and standard deviations for each compound are shown inside the panel. EC₅₀s were obtained by nonlinear regression fitting to a variable-slope, four-parameter, dose-response model using Prism software (GraphPad Software, La Jolla, CA). Experiments were performed in triplicates and repeated twice.

FIG. 5. Plots showing changes in relative fluorescence for the selected PPI inhibitors. The difference in the relative fluorescence at a 30-min interval and the starting relative fluorescence at a time point of 0 min, $\Delta(F/F_0)$, is plotted against the concentration (μM) of selected compounds. F and F_0 are the fluorescence intensities of the sample and the control, respectively. The fluorescence intensity of the control is normalized to 1.0 at each time point. (a) A20 peptide spanning the N-terminal 50 residues; (b) compound 1; (c) compound 6; (d) compound 9; (e) compound 12; (f) compound 15.

over, compounds belonging to scaffolds known to nonspecifically bind DNA or known to be cytotoxic were also eliminated.

The availability of large compound libraries and the development of versatile screening technology allow an unprece-

dent opportunity to use HTS to identify novel hits in a relatively short time. In recent years the number of accessible libraries has increased considerably. Moreover, inventories deposited by pharmaceutical corporations greatly enhanced ac-

cess to large numbers of high-quality compounds rich in drug-like properties. While these advances improve the number and the quality of the hits considerably, it should be taken into consideration that many compounds in these libraries are not available commercially, and custom synthesis may be necessary for acquiring some hits in quantities required for follow-up assays. Based on the commercial availability of compounds, selected hits representing different chemical scaffolds were tested for antiviral activity against poxviruses, vaccinia virus, and cowpox virus. Clearly, the EC_{50} values for a number of compounds are below 10 μ M, while the cytotoxicity of some others did not allow a measurement of their antiviral activities. Importantly, compounds with similar structures had similar EC_{50} s. For example, as shown in Table 1, the EC_{50} values for compounds 1 to 8, which belong to the same chemical class, ranged between 5.4 and 11 μ M against vaccinia virus (Copenhagen strain). Moreover, compound 26, which has the same scaffold (scaffold A [see the supplemental material]), was tested in a separate assay against a different strain of vaccinia virus, and the EC_{50} was determined to be 6.5 μ M. The consistency of the results of the two assays is demonstrated by the nearly identical EC_{50} s of 4.2 and 4.6 μ M for compound 15, which was tested in both assays. Since all compounds used in antiviral assays displayed >85% inhibition in the AlphaScreen, comparative analyses of the two activities are not possible. A number of compounds failed to show any measurable antiviral activity, perhaps resulting from a lack of bioavailability or from cytotoxicity.

The antiviral activities of hits against other members of the poxvirus family were investigated against cowpox virus in the cytopathic effect assay. Significantly, most of the inhibitors that were active against vaccinia virus were also active against cowpox virus, and the measured EC_{50} s for any compound against the two viruses were comparable (except for compound 6), suggesting a similarity in their actions against both viruses. This is expected because of the high degree of homology in the sequences of proteins of various poxviruses. The specificity of the identified hits for poxviruses was shown by their lack of activity against two DNA viruses, HSV-1 and HSV-2. It is remarkable that the EC_{50} s for a number of hits were in the same range as that for CDV. However, CDV has a much superior selectivity index due to its low cytotoxicity. It is expected that the potency, selectivity, and pharmacological profile of the hits from the screen will be improved through modifications optimizing their efficacy for their development as therapeutic agents.

Interactions between D4 and A20 may involve a large surface distributed on the structure of these proteins. The binding pockets or "hot spots" are likely to have a variety of shapes, sizes, and chemical properties. Consistent with this notion, inhibitors identified in the screen cluster into several specific chemical scaffolds, suggesting that inhibitors belonging to different chemical classes recognize specific but different sites on the target. On the other hand, different scaffolds can occupy the same site but may be involved in interactions specific for each scaffold.

The availability of the three-dimensional structure of D4 provides an excellent opportunity to further develop the hits using a structure-based approach (29). Fluorescent spectral analysis was undertaken for the detection of interactions of

various compounds with D4. This analysis by no means provides information about the binding pocket or the nature of interactions between the protein and the ligand, but a dose-dependent change in the fluorescence intensity indicates the potential binding of the small molecule to labeled D4. This assay is fast and requires very small quantities of protein and ligand and therefore is suitable for the preliminary identification of ligands for potential binding with one target partner or another. Compounds that were highly cytotoxic were not used in the binding assay because of suspected nonspecific binding. A concentration-dependent spectral change was verified by using the A20 peptide that binds to D4. Conceivably, a number of identified inhibitors target a pocket(s) on A20, and these compounds are not expected to display binding to D4. In fact, a number of hits showed spectra similar to that obtained for the negative control. On the other hand, five active compounds that were most selective in the cytopathic assay displayed concentration-dependent intensity changes, although the patterns of changes were variable (Fig. 5). The binding studies presented here qualitatively demonstrate a variety of interactions the inhibitors might be involved in when treated with D4 *in vitro*. In a follow-up study, a number of inhibitors identified in our screen were further analyzed and were shown to bind to D4 and inhibit DNA synthesis (25).

The amino acid sequences of both A20 and D4 are highly homologous among members of the poxvirus family. For example, the amino acid sequences for the D4 proteins of vaccinia virus, cowpox virus, smallpox virus, and monkeypox virus are greater than 98% identical, and their A20 sequences are 97 to 99% identical. Therefore, drugs targeting A20:D4 interactions are expected to possess broad-spectrum activity against related poxviruses. It should be noted that HTS has been successfully used to evaluate and identify antiviral compounds directly in cell culture, for example, in the discovery of ST-246. Similar biological screens have longer periods of time required for cell growth. The AlphaScreen provides a rapid screening platform, and hundreds of thousands of compounds can be screened in a day. The screen presented here is target based, and therefore, the identified hits and leads may be relatively amenable to further analyses of the mechanism of action and lead development. The results presented here demonstrate that novel antiviral agents against smallpox and other related viruses can be developed by targeting protein-protein interactions between A20 and D4.

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