Novel Class of Thiourea Compounds That Inhibit Herpes Simplex Virus Type 1 DNA Cleavage and Encapsidation: Resistance Maps to the UL6 Gene

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In our search for novel inhibitors of herpes simplex virus type 1 (HSV-1), a new class of thiourea inhibitors was discovered. N-[4-[3-(5-Chloro-2,4-dimethoxyphenyl)-thioureaido]-phenyl]-acetamide and its 2-fluoro-benzamide derivative inhibited HSV-1 replication. HSV-2, human cytomegalovirus, and varicella-zoster virus were inhibited to a lesser extent. The compounds acted late in the replication cycle by impairing both the cleavage of concatameric viral DNA into progeny genome length and the packaging of the DNA into capsids, indicative of a defect in the encapsidation process. To uncover the molecular target of the inhibition, resistant HSV-1 isolates were generated, and the mutation responsible for the resistance was mapped using marker transfer techniques. Each of three independent isolates had point mutations in the UL6 gene which resulted in independent single-amino-acid changes. One mutation was located in the N terminus of the protein (E121D), while two were located close together in the C terminus (A618V and Q621R). Each of these point mutations was sufficient to confer drug resistance when introduced into wild-type virus. The UL6 gene is one of the seven HSV-1 genes known to play a role in DNA packaging. This novel class of inhibitors has provided a new tool for dissection of HSV-1 encapsidation mechanisms and has uncovered a new viable target for the treatment of herpessviral diseases.

The herpesvirus family has many members that are human pathogens and make a significant contribution to morbidity and mortality associated with viral diseases. Based on criteria such as host cell specificity, oncogenicity, length of replication cycle, and genome arrangement, the herpesviruses have been divided into alpha-, beta-, and gammaherpesviruses (31). The alphaherpesviruses herpes simplex virus (HSV) types 1 and 2 latently infect nerve cells, HSV-1 is primarily associated with herpes labialis, and HSV-2 is associated with herpes genitalis, but both types have been associated with both diseases (28, 39, 47). In immunocompetent adults, these diseases often recur due to reactivation of the virus from the latent state. HSV infections of immunocompromised patients such as transplant and AIDS patients are often chronic and fatal. Current therapy for HSV disease consists of nucleoside analogs such as acyclovir (ACV) and valacyclovir, a prodrug of ACV, and penciclovir (PCV) and its prodrug, famciclovir. ACV and PCV are selectively phosphorylated by the viral thymidine kinase in HSV-infected cells, followed by further phosphorylation to the triphosphate by cellular kinases. Triphosphorylated ACV and PCV are both inhibitors of the viral DNA polymerase, and ACV also acts as a chain terminator when incorporated into the nascent viral DNA chain (4, 13). Drug resistance can occur in chronic infections, where replication is ineffectively curtailed by the immune system. Recently increasing numbers of drug-resistant HSV strains have been isolated from immunocompromised people. The mechanism of resistance of most ACV-resistant isolates is associated with thymidine kinase alterations, but some have mutations associated with the viral DNA polymerase (9, 10, 27). It is clear that alternative treatment options which have new mechanisms of action are needed. This will enable dual therapy and provide alternatives for patients with drug-resistant infections.

The life cycle of herpesviruses is a highly regulated process (31). After entry of the virus into the cell, the nucleocapsid migrates to the nucleus, where the viral DNA is deposited and transcription of so-called immediate-early genes occurs. The resulting immediate-early proteins initiate transcription of early genes, some of which encode proteins from the viral DNA replication machinery. Viral DNA is then replicated in what is believed to be a rolling-circle mechanism, resulting in concatameric DNA. At approximately the same time, the assembly of viral procapsids commences in the nucleus. During the process of encapsidation, the progeny viral DNA is cleaved to monomeric forms, and in a closely coupled process, the monomeric DNA is packaged into these immature capsids. Encapsidated genomes then migrate out of the nucleus, acquire a lipid envelope containing viral glycoproteins, and leave the cell (15, 32). Each of these steps in the replication cycle, in theory, could be inhibited by small-molecule therapeutics.

We have discovered a new class of compounds in our search for novel HSV inhibitors. We describe here two thiourea molecules that inhibit HSV through a novel mechanism. Instead of inhibiting the replication of viral DNA, we show that these compounds prevent the cleavage and packaging of viral DNA. Seven HSV-1 genes have been shown to be involved in the encapsidation process, ULs 6, 15, 17, 25, 28, 32, and 33 (1, 2, 3, 8, 20–22, 25, 26, 35, 41, 46, 48). When HSV viruses containing
loss-of-function mutations in these encapsidation genes are used to infect cells, the viral progeny DNA fails to be packaged into capsids.

Experiments with laboratory-generated mutations resistant to the compounds described here indicated that resistance was associated with mutations in the UL6 open reading frame. The UL6 product is a 75-kDa protein that is a component of capsids (24). UL6 deletion mutants are defective in both DNA packaging and cleavage (20, 24).

The results presented here suggest that the encapsidation process in general, and the UL6 gene product in particular, are valid targets for antitherpesvirus chemotherapy.

MATERIALS AND METHODS

Cells and viruses. Vero cells were cultured in Dulbecco's minimal essential medium (DMEM; Mediatech, Herndon, Va.) supplemented with 5% fetal bovine serum, 5% calf serum (CS), penicillin, streptomycin, and 1-glutamine. HSV-1 strain Patton was obtained from R. Hyman (The Pennsylvania State University College of Medicine, Hershey, Pa.). HSV-1 strain E137 and HSV-2 strains 12 and 186 were obtained from C. P. Cerini (Wyeth Lederle Vaccine Research, Pearl River, N.Y.). HSV types 1 and 2 were propagated on Vero cells in the presence of 2% CS. Varicella-zoster virus (VZV) strain Ellen was obtained from the American Type Culture Collection (no. 1367-VR) and propagated in human foreskin fibroblast (HFF) cells in DMEM with 2% CS.

The construction and isolation of recombinant mutant human cytomegalovirus (HCMV), designated RV12174, containing the US3 polyadenylation sequence and the US2-US1 flanking sequences, was performed as previously described (17). Plasmid pUC-US3-US4 was constructed. Sequencing of this plasmid contains the 1.706-kb ApaI-ApaI fragment (HCMV AD169 bases 194647 to 194741 [GenBank accession number X17403]) containing the US3 flanking sequences and the US2 promoter, the β-glucuronidase gene, and the 2.075-kb ApaI-ApaI-ApaI fragment (HCMV AD169 bases 194108 to 192033, containing the US3 polyadenylation sequence and the US2-US1 flanking sequences). Following plasmid linearization and cotransfection with HCMV wild-type DNA, the proper genomic organization of the mutant HCMV was verified by DNA blot hybridization analysis (data not shown) as previously described (17).

Clones. CL-253824 was obtained from Alfred Bader Chemical Company (catalog no. S68470-8). WAY-150138 was prepared in a three-step procedure described previously (37). Plasmid pUC-US3-US4 was constructed. Sequencing of this plasmid contains the 1.706-kb ApaI-ApaI fragment (HCMV AD169 bases 194647 to 194741 [GenBank accession number X17403]) containing the US3 flanking sequences and the US2 promoter, the β-glucuronidase gene, and the 2.075-kb ApaI-ApaI-ApaI fragment (HCMV AD169 bases 194108 to 192033, containing the US3 polyadenylation sequence and the US2-US1 flanking sequences). Following plasmid linearization and cotransfection with HCMV wild-type strain AD169 genome DNA, plaques containing β-glucuronidase-expressing virus were picked and purified. The proper genomic organization of the mutant HCMV was verified by DNA blot hybridization analysis (data not shown) as previously described (17).
same Ascl fragment from 132h5. Also, the XhoI-HindIII fragment from pBSwt was replaced with the same fragment from pBS30, and the AgeI-Ascl fragment was replaced with the same fragment from pBSR. All subcloning of the mutations was confirmed by sequencing.

Electron microscopy. Vero cells grown in T175 flasks were infected with wt HSV-1 or 253R virus at an MOI of 2 in the absence or presence of CL-253824 (30 μg/ml) and grown for 24 h. Cells were harvested by scraping, washed twice, and fixed sequentially with 3% glutaraldehyde and 0.8% OsO4, both in 0.1 M sodium cacodylate (pH 7.5). During subsequent dehydration in graded ethanol and propylene oxide, the cells were stained with 50% saturated uranyl acetate in 50% ethanol. They were embedded in Poly/Bed 812/Araldite 502 (Polysciences, Inc. Warrington, Pa.). Thin sections were stained with Reynolds' lead citrate (30) and examined in a JEOL 100CX electron microscope using an accelerating voltage of 60 kV.

RESULTS

During an automated screen of a proprietary chemical library for molecules with antitherpesvirus activity, a compound, CL-253824, with modest activity against HSV-1 was discovered (Fig. 1). The 50% inhibitory concentration (IC50) against HSV-1 (Patton) in an ELISA assay was 3 μg/ml (7.9 μM) (Table 1). Antiviral activity was slightly less against another HSV-1 strain (E377; IC50, 9.5 μg/ml) and HSV-2 strains 12 and 186 (IC50, 11 and 27 μg/ml, respectively). CL-253824 had even less inhibitory activity against HCMV, VZV, and other non-herpesviruses tested (influenza virus, respiratory syncytial virus, and parainfluenza virus type 3; data not shown). In an effort to increase the antiviral activity, a 2-fluoro-benzamido analogue (WAY-150138; Fig. 1) was synthesized, which had a more than 10-fold increased potency (IC50, 0.2 μg/ml [0.43 μM]) against HSV-1 Patton (Table 1). Activity against HSV-2 and HCMV was not improved as dramatically as against HSV-1 (two- to sixfold), but no increase in activity was seen against VZV.

Effect of time of inhibitor addition on virus yield. To investigate the mode of action of this new class of thioamide HSV-1 inhibitors, an experiment was performed in which the compounds were added at different times after infection. Vero cells were infected with HSV-1, and CL-253824 or WAY-150138 was added at 2-h intervals from 0 to 10 h.p.i. Virus was harvested from all plates at 24 h.p.i. and counted (Table 2). When added at 0 or 2 h.p.i., CL-253824 reduced the viral yield by almost 37-fold and WAY-150138 by over 750-fold; when the compound was added at 4 h.p.i. or later, virus yield was still significantly inhibited. In contrast, these compounds had no effect on virus yield when added at 6 h.p.i. or later. Viral DNA synthesis in HSV-1-infected cells starts around 3 h after infection (32) (see also Fig. 2). If a compound added after viral replication has started still has an inhibitory effect, it must affect a process later in the viral life cycle. Thus, these results suggested that the compounds did not inhibit DNA replication, but likely affect a later step in the viral life cycle, such as late protein synthesis, DNA processing, or capsid maturation. To further confirm the lack of DNA replication inhibition, viral DNA isolated at various times postinfection from mock-treated and 

![FIG. 1. Structures of CL-253824 (R = H) and WAY-150138 (R = 2-fluoro phenyl).](image)

![FIG. 2. Time course of DNA cleavage. (A) Representation of the HSV-1 genome, indicating the positions of internal joint BamHI-K and the terminal BamHI-P- and -S fragments. (B) Time course of DNA synthesis and cleavage in infected, untreated cells. Vero cells were infected at an MOI of 5 with HSV-1 and harvested at 0, 2, 4, 6, 8, 10, or 24 h.p.i. (lanes 1 to 7). Viral DNA was extracted, digested with BamHI, separated on a 1% agarose gel, and analyzed by Southern blotting using the BamHI-K fragment, which spans the cleavage joint, as a probe. The positions of BamHI fragments K (joint) and S and P (termini) are indicated. The image has been chosen intentionally dark to show the presence of cleavage products at 6 h.p.i. (C) Effect of WAY-150138 on DNA cleavage. Vero cells were infected at an MOI of 5 with HSV-1. WAY-150138 (10 μg/ml) was added at 0 to 10 h.p.i. (lanes 1 to 6). Lane 7 had no compound added. Viral DNA was harvested at 24 h.p.i. Viral DNA was extracted, digested with BamHI, and analyzed by Southern blotting using the BamHI K fragment as a probe. The image has been chosen intentionally dark to show the presence of cleavage products at 4 h.p.i.](image)

<table>
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<th>Table 1. Activity of CL-253824 and WAY-150138 against HSV-1, HSV-2, HCMV, and VZV*&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>HCMV</td>
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<td>VZV</td>
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<sup>a</sup>IC50 values were determined by growing several herpesvirus strains in the presence of various concentration of compound. Virus replication was determined by ELISA as described in Materials and Methods. <sup>b</sup>IC50, highest concentration tested.

* Vero cells were infected with HSV-1 in the presence of CL-253824 (30 μg/ml) or WAY-150138 (10 μg/ml) throughout the experiment (0 h), or compound was added at later times after infection (2 to 10 h.p.i.). Virus was harvested at 24 h.p.i., and the reduction in yield was determined by plaque titration. The cells in the 24-h time point were not exposed to compound. The fold reduction of yield compared to the 24 h.p.i. untreated sample is listed. ND, not determined.
CL-253824-treated infected cells was analyzed by slot blot hybridization. Viral DNA from 10^4 cells was loaded on a slot blot with a titration of purified HSV DNA and probed with a radiolabeled HSV-1 EcoRI-A fragment. Quantitation of the resulting autoradiograph by densitometry showed the presence of 10 ng of DNA in CL-253824-treated cells versus 13 ng in untreated cells at 14 h.p.i. At 18 h.p.i. the amount of viral DNA in treated versus untreated cells was 20 versus 18 ng (data not shown). The effects of the compounds on protein synthesis were examined, but no differences with untreated infected cells were detected (data not shown).

**Inhibition of viral DNA cleavage.** One of the processes in the viral life cycle which starts between 4 and 6 h.p.i. is capsid maturation (32). During capsid maturation, the newly replicated viral DNA is cleaved into unit length and packaged into capsids (15, 32). To analyze the kinetics of viral DNA cleavage, Vero cells were infected with HSV-1, and viral DNA was harvested at various times after infection. The DNA was then analyzed by Southern blot for assessment of total DNA synthesis and cleavage.

To facilitate cleavage analysis, DNA was digested with BamHI restriction enzyme prior to gel electrophoresis. The HSV-1 genome contains inverted repeats bordering each of two unique DNA components (L and S). After cleavage, each unit-length genome has internal repeats at the junction of the L and S components (i.e., the joint) and two terminal repeats (i.e., the molecular ends of each genome) (Fig. 2A). The DNA blot was probed with the BamHI K fragment, which spans the joint and is partly repeated in each of the terminal BamHI fragments S and P. Viral DNA synthesis was initially detected after 4 h.p.i., and terminal fragments were observed at 6 h.p.i. (Fig. 2B). Thus, the time of initial DNA cleavage is similar to the time when the compounds fail to inhibit virus (Table 2).

To confirm this, Vero cells were infected with HSV-1, and WAY-150138 was added at 2-h intervals and maintained for 24 h, at which time DNA was harvested and analyzed by DNA blot (Fig. 2C). When the compound was added prior to 4 h.p.i., substantial amounts of viral DNA were detected, but no free termini were observed, indicating that the progeny DNA had not been cleaved. When compound addition was delayed to 6 h.p.i., DNA cleavage was detected, indicating that the process that the compound inhibited had presumably already commenced. Viral DNA replication was not affected even when WAY-150138 was present from the start of infection (0 h.p.i.), as indicated by the presence of uncleaved (i.e., joint) DNA.

**Effect of inhibitors on capsid morphology.** The cleavage of viral DNA and the packaging of the DNA into capsids are closely linked. The lack of viral DNA cleavage is usually accompanied by the accumulation of immature (empty) B-type capsids in the nucleus of infected cells and a decrease in the number of mature, DNA-containing C capsids (1, 15). We examined the morphology of the HSV-1 capsids in infected cells treated with CL-253824 by electron microscopy. Representative electron micrographs of the cells show that while both immature B capsids and mature C capsids could be found in untreated cells (Fig. 3A), no mature C capsids were found in any of the compound-treated cells that we examined (Fig. 3B). The large cluster of capsids in the nucleus shown in Fig. 3B, a common feature of HSV-1 infection (32), was also observed in untreated HSV-1-infected Vero cells. HSV-1 can produce up to 100 infectious progeny virus particles per cell (45). With a yield reduction of up to 750-fold (Fig. 2), it was not surprising that we could not detect any C capsids in treated cells.

**Selection of resistant virus.** To unravel the mechanism of action of the compounds at the molecular level, HSV-1 was serially passaged in Vero cells in the presence of inhibitor, resulting in the generation of compound-resistant mutants. Three independent mutants were purified: one resistant to CL-253824 (253R) and two resistant to WAY-150138 (138R/S5 and 138R/S30). All isolates grew with wild-type kinetics (Fig. 4). All isolates were resistant to high (10 times the IC50) concentrations of inhibitor and cross-resistant to the other compound (data not shown).

**Characterization of resistant virus.** Initial characterization of the resistant viruses consisted of analysis of the capsid morphology by electron microscopy and DNA cleavage analysis by DNA blot. When Vero cells were infected with HSV-1 253R in either the absence or presence of CL-253824, examination by electron microscopy revealed no differences. Mature C capsids were detected in both the absence (Fig. 3C) and presence (Fig. 3D) of compound. The morphology of the capsids was indistinguishable from that of capsids found in untreated wild-type virus (Fig. 3A).

The extent of DNA cleavage in Vero cells infected with 253R in the presence of CL-253824 (Fig. 5a) or WAY-150138 (Fig. 5b) was also analyzed. In the presence or absence of compounds, DNA termini were detected in cells infected with resistant viruses (lanes 3 and 4). In contrast, in cells infected with wild-type HSV-1 DNA, termini were not detected in the presence of compound (lanes 2).

**Identification of the resistance gene.** To date, at least seven HSV gene products have been implicated in participating in the encapsidation process. These include UL6, 6, 15, 17, 25, 28, 32, and 33. To determine which HSV gene carried the mutation that conferred resistance to our inhibitors, marker transfer experiments were performed. To facilitate this, EcoRI restriction fragments of the resistant viruses were subcloned and individually cotransfected into Vero cells with infectious wild-type DNA. After the cells were allowed to recover for 24 h, either CL-253824 or WAY-150138 selection was applied. In all cases, only the EcoRI-D fragment conferred resistance (i.e., allowed viral growth), whereas no virus growth was observed with any other fragment (Table 3). The EcoRI-D fragment was further subcloned, and the marker transfer procedure was repeated. The smallest fragment tested that conferred resistance to the compounds was the 4-kb BglII-ScaI subfragment (see Fig. 6) (Table 3). The BglII-ScaI fragment contains the entire UL6, UL7, and UL8 open reading frames (ORFs) and the 3′ end of the UL5 gene. UL5 and UL8 are part of the helicase-primase complex which is involved in DNA replication (11, 42). HSV-1 UL7 has an unknown function, but its homologue in bovine herpes virus has been shown to be a nonessential cytoplasmic protein (38). UL6 was the most interesting candidate, since its involvement in the encapsidation process has been documented (20, 24, 25). At this point, the 4-kb BglII-ScaI fragments were sequenced in their entirety, revealing that each resistant isolate contained one point mutation in the UL6 gene (Table 4). The mutation in HSV-1 138R/S30 resulted in a change of glutamic acid 121 into aspartic acid, a conservative amino acid change since both are negatively charged. The other two viruses had mutations at the C terminus; in 253R, alanine 621 was mutated to a valine, and in 138R/S5, glutamine 621 was changed to an arginine. The mutation in 138R/S5 resulted in the most drastic change, from an uncharged glutamine to a positively charged arginine.

The ultimate proof that each of these mutations conferred resistance to the compounds was shown by subcloning a minimal fragment containing the mutation into the wild-type BglII-ScaI fragment. The 210-bp XhoI-HindIII fragment of the 138R/S30 BglII-ScaI fragment, the 231-bp AscI fragment of 138R/S5, and the 953-bp AgeI fragment of 253R were separately subcloned into a plasmid containing the wild-type BglII-ScaI fragment.
fragment, and the resulting plasmids were cotransfected with wild-type HSV-1 DNA. Each of these fragments enabled the viruses to grow in the presence of compound (Table 3), whereas the wild-type BglII-ScaI fragment did not. Therefore, three independent point mutations in the UL6 gene were each sufficient to confer the compound-resistant phenotype.

**DISCUSSION**

We have presented here two members of a novel class of compounds that are potent inhibitors of HSV-1. These compounds inhibit HSV-1 by interfering with the virus encapsidation process. These inhibitors therefore block HSV replication through an entirely novel mechanism compared to the nucleoside drugs currently used in the clinic to treat HSV infections.

The encapsidation of HSV DNA occurs when preformed viral capsids receive progeny DNA. The replicated viral DNA exists as polygenomic concatamers in the infected cell nucleus. During the process of encapsidation, these concatamers are cleaved in such a way that a DNA molecule of one unit length is packaged into a preformed, immature capsid. When this process goes awry, concatameric, uncleaved viral DNA and
immature, empty capsids accumulate. The timing of the events during herpesvirus encapsidation is poorly understood. More knowledge has been acquired on the encapsidation of bacteriophage genomes due to the powerful genetics in those systems. In the double-stranded DNA bacteriophages such as lambda and T4, an encapsidation complex is assembled on the concatameric DNA and the first end of the progeny genome is generated by endonucleolytic cleavage. This protein-DNA complex then binds to the preassembled procapsid (prohead) at the portal vertex; DNA is subsequently packaged and cleaved again. The encapsidation protein complex mainly consists of a heterodimeric terminase and phage structural components such as the portal proteins (5, 7). From genetic analysis, it is known that for HSV, at least seven genes are involved in the encapsidation process, ULs 6, 15, 17, 25, 28, 32, and 33 (1, 2, 8, 20, 21, 25, 26, 35, 41). All seven genes are essential for growth of the virus, and known loss-of-function mutations in most of these genes result in a very similar phenotype: concatameric DNA and empty capsids accumulate. Exactly this phenotype was observed after treatment of HSV-1-infected cells with the compounds described here. To dissect the exact mode of action, we generated HSV-1 virus resistant to the compounds and mapped the location of the resistance-conferring mutation. Analysis of the smallest fragments that conferred resistance to the compounds revealed three independent point mutations in the UL6 ORF, a member of the encapsidation gene group.

For some of the HSV-1 encapsidation gene products, additional information is emerging that allows further dissection of the DNA-packaging process. The UL32 and UL17 proteins might have early roles in the process in that they appear to have an effect on the localization of capsids and capsid proteins to replicative sites (21, 40). The UL15 and UL28 gene products have a potential role in the cleavage of the progeny DNA, based on the following findings: UL15 has homology with gp17, one of the terminase genes of bacteriophage T4 (12), and is the best candidate to date to perform this function for HSV, perhaps in conjunction with the UL28 protein. The involvement of UL28 in DNA cleavage is suggested by the data published by Bogner et al. (6), implicating the HCMV homologue of UL28, HCMV UL56, in DNA-binding and nuclease activity. In addition, there are data which suggest that UL15 and UL28 interact: some forms of UL15 protein products fail to localize to B capsids when the UL28 protein is not expressed (36, 48).

### Table 3. Marker rescue efficiencies of restriction fragments derived from resistant viruses in the presence of the compounds

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Plating efficiency</th>
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<tr>
<td>EcoD</td>
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</tr>
<tr>
<td>BglK</td>
<td>0.58</td>
</tr>
<tr>
<td>BamHI-9</td>
<td>0.16</td>
</tr>
<tr>
<td>BamHI-3.5</td>
<td>0</td>
</tr>
<tr>
<td>Del BglII</td>
<td>0</td>
</tr>
<tr>
<td>BglII-ScaI</td>
<td>0.03</td>
</tr>
<tr>
<td>BS5XH</td>
<td>0.01</td>
</tr>
<tr>
<td>BS30Asc</td>
<td>0.32</td>
</tr>
<tr>
<td>BSR-Age</td>
<td>0.26</td>
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</tbody>
</table>

* 138 virus fragments were plated in the presence of WAY-150138 (30 μg/ml), and 253 virus fragments were plated in the presence of CL-253824 (30 μg/ml). Fragments are explained in Fig. 6. BS5XH, BS30Asc, and BSR-Age are the XhoI-HindIII fragment of 138/5, AscI fragment of 138/30, and AgeI fragment of 253, respectively, cloned into BglII-ScaI wild-type fragment. Plating efficiency is expressed as the number of plaques in the presence of compound divided by the number of plaques in the absence of compound. n.t., not tested.

**FIG. 4.** Growth analysis of wild-type and resistant viruses. Vero cells were infected with wild-type (●) or resistant HSV-1 Patton isolates 253R (■), 138/5 (▲), and 138/30 (○) at an MOI of 0.05 to 0.1 and harvested at various times between 0 and 24 h.p.i. Virus yields were determined by plaque assays and plotted.

**FIG. 5.** Analysis of DNA cleavage in resistant virus-infected cells. (a) Vero cells were infected at an MOI of 3 with wild-type (wt, lanes 1 and 2) or 253 virus (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of CL-253824 (75 μg/ml) for 14 h. (b) Vero cells were infected at an MOI of 5 with wild-type (wt, lanes 1 and 2) or 138 R/30 virus with WAY-150138 (10 μg/ml) (lanes 3 and 4) and cultured for 24 h. Viral DNA was harvested, digested with BamHI, separated on a 1% agarose gel, and analyzed by Southern blotting using the BamHI K fragment, which spans the cleavage joint (see Fig. 2A), as a probe. The positions of BamHI fragments K (joint) and S and P (termini) are indicated.
could be explained by recent results from Koslowski et al. (18) that indicate that UL28 can mediate the nuclear import of the UL15 protein. Only one of the encapsidation genes does not seem to play a role in DNA cleavage. HSV-1 defective in UL25 is able to cleave DNA, but the DNA fails to be packaged (22). Also, in cells infected with this UL25-defective virus, there is an increase in empty or A-type capsids, which has been interpreted to indicate that the UL25 protein plays a role in stabilizing the DNA-filled capsids (22). This indicates that the UL25 protein has a role late in the encapsidation process. Apart from being essential for DNA packaging, very little was known about the UL33 protein (2). Just recently, Reynolds et al. (29) reported that the UL33 protein is a 19,000-Da protein that localizes to the cytoplasm and replication compartments in the nucleus, but could not be detected in purified virions or capsids.

The UL6 protein is associated with B and C capsids and is a component of the mature virion (24, 48), and like the UL28 protein, UL6 influences the cellular localization of UL15 proteins (36, 48). By uncovering a new class of encapsidation inhibitors, we may have found a way to analyze the role of the UL6 protein in this process. Three independent point mutations, two close to each other near the C terminus and one close to the N terminus of UL6, each causing an amino acid change in the UL6 ORF (Table 4), can each render HSV-1 resistant to the inhibitors. Two of the mutations are conservative (E121D and A618V) and cause only a change in the size of the amino acid side chain, but one mutation (Q621R) is more drastic, acquiring a charged and bulkier side chain. Each of these mutations conferred the same level of resistance to high concentrations of compound. Limits to compound solubility did not allow us to test whether a combination of these mutations into the same UL6 gene increased the level of resistance. We have also not tested the effect of a combination of these mutations on the ability of the UL6 gene to support viral growth. One can speculate that the two areas of UL6 that carry mutations come together in the three-dimensional structure of the protein to form interaction surfaces with the compounds. Determination of the significance of these mutations will have to wait until more information about the UL6 protein becomes available.

Another class of Herpesviridae inhibitors has recently been reported which have a mechanism of action similar to that of the compounds described herein. Certain benzimidazole ribosides inhibit HCMV effectively through interfering with the encapsidation process (19, 44). These inhibitors also cause the accumulation of immature capsids and uncleaved DNA in the infected cells, but maximal resistance to these inhibitors maps to two different encapsidation genes, UL89 and UL56, homologues of the HSV-1 genes UL15 and UL28, respectively (19). One of these benzimidazole ribosides, BDCRB (43), indeed inhibited HCMV in our assays (IC₅₀, 1.6 μM), but not HSV-1 or -2 (data not shown). Our inhibitors appear to be most potent against HSV-1 and less so against HSV-2 and HCMV.

The level of similarity between HSV-1 UL6 (strain Patton) and its HCMV homologue UL104 (strain AD169) is 21% (data not shown). This might indicate that the actual function of these proteins is conserved but that the points of contact of the proteins with the compounds are probably not. However, the level of similarity between the UL6 gene products from HSV-1 and HCMV is 84.5%, yet there is a 10-fold difference in sensitivity to WAY-150138. The amino acids which are mutated in the resistant HSV-1 isolates (Table 4) are conserved in HCMV-2 UL6 (by comparison to strain HG52, GenBank accession number Z86099; data not shown). It is possible that the HCMV-2 strains that we tested have amino acid substitutions at those positions. Alternatively, the compounds may have a larger interaction interface with UL6 outside the mutated residues, which includes amino acids not conserved between HSV-1 and -2. More detailed knowledge of the molecular interaction between the compounds and the UL6 protein is necessary to understand the decreased potency against HSV-2 and HCMV.

Protein-protein interactions are likely to be crucial for the formation of an encapsidation complex. These interactions can influence the encapsidation process at various levels: (i) the correct subcellular localization of the protein components; (ii) assembly of the encapsidation protein complex; (iii) interaction of the proteins with DNA; (iv) interaction of the encapsidation proteins with the capsid proteins; and (v) stabilization of the DNA-capsid complex. Each of these steps is a viable target for the action of novel inhibitors. Our compounds have demonstrated that DNA packaging is a novel, viable target for chemotherapy of HCMV. Further studies are required to determine how the thiourea inhibitors affect UL6 in the encapsidation process.

**TABLE 4. UL6 mutations resulting in resistance**

<table>
<thead>
<tr>
<th>UL6 isolate</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>138⁮/³⁰</td>
<td>G363T</td>
<td>E121D</td>
</tr>
<tr>
<td>253⁮³⁰</td>
<td>C1853T</td>
<td>A618V</td>
</tr>
<tr>
<td>138⁮/⁵</td>
<td>A1862G</td>
<td>Q621R</td>
</tr>
</tbody>
</table>

*a Positions are numbered from the beginning of the UL6 reading frame.
REFERENCES


