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# Resistance of Herpes Simplex Virus Type 1 to Peptidomimetic Ribonucleotide Reductase Inhibitors: Selection and Characterization of Mutant Isolates

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**Herpes simplex virus (HSV) encodes its own ribonucleotide reductase (RR), which provides the high levels of deoxynucleoside triphosphates required for viral DNA replication in infected cells. HSV RR is composed of two distinct subunits, R1 and R2, whose association is required for enzymatic activity. Peptidomimetic inhibitors that mimic the C-terminal amino acids of R2 inhibit HSV RR by preventing the association of R1 and R2. These compounds are candidate antiviral therapeutic agents. Here we describe the in vitro selection of HSV type 1 KOS variants with three- to ninefold-decreased sensitivity to the RR inhibitor BILD 733. The resistant isolates have growth properties in vitro similar to those of wild-type KOS but are more sensitive to acyclovir, possibly as a consequence of functional impairment of their RRs. A single amino acid substitution in R1 (Ala-1091 to Ser) was associated with threefold resistance to BILD 733, whereas an additional substitution (Pro-1090 to Leu) was required for higher levels of resistance. These mutations were reintroduced into HSV type 1 KOS and shown to be sufficient to confer the resistance phenotype. Studies in vitro with RRs isolated from cells infected with these mutant viruses demonstrated that these RRs bind BILD 733 more weakly than the wild-type enzyme and are also functionally impaired, exhibiting an elevated dissociation constant ($K_d$) for R1-R2 subunit association and/or reduced activity ($k_{cat}$). This work provides evidence that the C-terminal end of HSV R1 (residues 1090 and 1091) is involved in R2 binding interactions and demonstrates that resistance to subunit association inhibitors may be associated with compromised activity of the target enzyme.**

Herpes simplex viruses (HSV) cause a wide spectrum of acute and recurrent diseases in humans. The disease manifestations are generally mild for immunocompetent individuals (e.g., mucocutaneous infections, such as herpes labialis and genital herpes) but may be life threatening for immunodeficient patients (e.g., encephalitis). HSV encodes its own ribonucleotide reductase (RR), which catalyzes the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides and is thus instrumental in increasing the deoxyribonucleoside triphosphate (dNTP) pools to the levels required for optimal viral DNA replication in infected cells. The importance of this viral enzyme in virulence and in reactivation of the virus from latency in animal models has been demonstrated in several studies (2, 4, 17, 19, 36). Antiviral agents directed towards inhibition of the HSV RR may therefore be valuable therapeutic agents for the treatment of HSV infections. The HSV RR is composed of two distinct homodimeric subunits, R1 (140 kDa) and R2 (38 kDa), whose association in an $\alpha_2\beta_2$ complex is essential for enzymatic activity. The C terminus of the small subunit, R2, is critical for subunit association (14, 21). Earlier studies have demonstrated that a nonapeptide derived from the carboxy-terminal sequence of the HSV R2 subunit is an inhibitor of RR activity (9, 13) and mediates its activity by preventing the association of the R1 and R2 subunits (26, 29, 33). We have developed novel peptidomimetic inhibitors based on the amino acid sequence of the R2 C terminus. This new class of inhibitors is directed specifically against HSV RR and inhibits enzymatic activity by preventing the formation of the active RR complex (24, 31, 32). BILD 1263, a representative of this class of inhibitors, was shown to inhibit HSV type 1 (HSV-1) and HSV-2 strains (including acyclovir-resistant strains) in vitro and to reduce the severity of HSV-induced keratitis in mice (25). The antiviral activity of this class of inhibitors results from the inhibition of viral RR activity. The treatment of HSV-infected cells with the viral RR inhibitor BILD 1263 has been demonstrated to provoke a dramatic suppression of the HSV-2-induced dNTP pools in infected serum-starved cells (25), which as a result can no longer support viral replication.

The development of resistance to antiviral drugs is a significant problem, particularly in immunocompromised patients. Several reports have described the emergence of clinically significant drug-resistant infections in organ transplant and AIDS patients. Acyclovir-resistant HSV and varicella-zoster virus as well as ganciclovir-resistant cytomegalovirus strains have been isolated repeatedly from these patient populations (for a review, see reference 5). Given the novel mode of action of peptidomimetic RR subunit association inhibitors, it was of interest to determine if resistance against these inhibitors could be generated and to identify the mechanism by which resistance may occur. In this paper, we describe the selection and characterization of HSV-1 mutants with decreased sensitivity to BILD 733, a peptidomimetic RR inhibitor closely related to BILD 1263. The development of resistance resulted from mutational changes in the C-terminal end of the large subunit of HSV RR, further confirming the mechanism-based antiviral activity of these inhibitors and implicating the R1 C-terminal end in interactions with the R2 subunit. Moreover, we show that resistant RR enzymes are compromised in their enzymatic activities. The mechanisms by which resistance occurs and the consequences of these results for potential devel-

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opment of resistance to peptidomimetic subunit association inhibitors of HSV RR are discussed.

## MATERIALS AND METHODS

**Materials.** [*methyl*-$^3$H]thymidine, [$^{14}$C]CDP, [$^{35}$S]methionine, and [$\alpha$-$^{32}$P]dATP were purchased from Dupont NEN Research Products (Boston, Mass.). HSV-1 R1 (pRI 4) and R2 (pRI 9) plasmids were a generous gift from R. Ingemarson, Umea University, Umea, Sweden (18). The cycle sequencing kit was purchased from Pharmacia Biotech (Baie d'Urfé, Québec, Canada). Anti-gC antibody C11 was graciously donated by J. Glorioso, Pittsburgh University, Pittsburgh, Pa.

**Antiviral compounds.** BILD 733 (31) and BW348U87 (34) were synthesized at Bio-Méga/Boehringer Ingelheim Research Inc. Acyclovir was purchased from Burroughs Wellcome, and the sodium salt was prepared in our laboratories. Stock solutions of these inhibitors were prepared in 100% dimethyl sulfoxide and then diluted in BBMT medium (3) as described below for the viral replication assay.

**Cells.** BHK-21 (C13) cells (ATCC CCL10) and Vero cells (ATCC CCL81) were grown in $\alpha$-minimum essential medium ($\alpha$-MEM) (Gibco) supplemented with 8% fetal bovine serum (FBS) (Gibco). For serum starvation, BHK-21 cells were first plated in $\alpha$-MEM containing 8% FBS and incubated at 37°C with 5% $CO_2$. After 6 h, the concentration of FBS was reduced to 0.5% and the cells were serum starved for 3 days.

**Development of BILD 733$^r$ mutant viruses.** HSV-1 strain KOS (a generous gift from D. M. Coen, Harvard Medical School, Boston, Mass.) was serially passaged (multiplicity of infection [MOI] of between 0.01 and 0.1 PFU per cell) in serum-starved BHK-21 cells in the presence of increasing concentrations (15 to 150 $\mu$M) of the RR inhibitor BILD 733 (passages 1 to 8 were performed in the presence of 15, 25, 45, 45, 60, 60, 100, and 150 $\mu$M BILD 733, respectively). The viral progeny was harvested when the infected cells exhibited approximately 90% cytopathic effect. Resistant isolates (designated KOS-733$^r$) were plaque purified from virus pools on Vero cells (three times) in the presence of BILD 733. Their resistant phenotype was determined by using the viral replication assay as described below.

**Viral replication and cell viability assays.** Serum-starved BHK-21 cells were infected with wild-type KOS or KOS-733$^r$ viruses at an MOI of 0.01 PFU per cell (for colorimetric assay) or 0.1 PFU per cell (for enzyme-linked immunosorbent assay [ELISA]) in defined BBMT medium (3). Following 1 h of virus adsorption, the HSV-infected cells were washed with BBMT medium and then incubated with appropriate concentrations of BILD 733 in BBMT medium. After 28 h of incubation at 37°C, the infected cells were harvested (for colorimetric assay) or fixed (for ELISA), and the infectious virus produced was quantified on BHK-21 cells by a colorimetric (neutral red uptake) titration assay (22) or by an ELISA detecting HSV-1 glycoprotein C with monoclonal antibody C11. To obtain the 50% effective concentration ($EC_{50}$), a plot of the percentage of inhibition of viral replication (relative to a control without drug) versus drug concentration was constructed. The cytotoxicity of BILD 733 for serum-starved BHK-21 cells was determined with the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) (12) under the same cell culture assay conditions used for the replication assay.

**Single-step growth analyses and [$^3$H]thymidine incorporation studies.** Serum-starved BHK-21 cells were infected with wild-type KOS or with the KOS-733$^r$ isolate from passage 5 or 8 (KOS-733$^r$ p5 or p8, respectively) at an MOI of 10 PFU per cell in $\alpha$-MEM–2% inactivated FBS. Following 1 h of virus adsorption, the infected monolayers were washed once with medium and then incubated at 37°C for various periods of time. Viral yields were estimated at several time points by standard plaque assay on Vero cells. In parallel to the viral yield estimations, [$^3$H]thymidine incorporation was monitored by 30-min pulse-labeling with 10 $\mu$Ci of [$^3$H]thymidine per ml in $\alpha$-MEM–2% inactivated dialyzed FBS. Cells were lysed with 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–10 mM NaCl–0.1% sodium dodecyl sulfate (SDS). The lysate was spotted onto Whatman GF/C filters and precipitated with 1 N HCl (27). Labelled DNA was then quantitated by liquid scintillation.

**Marker rescue and sequencing.** Infectious DNA isolated from cells infected with KOS-733$^r$ p5 was cotransfected into Vero cells by calcium phosphate precipitation (16) with DNA fragments encoding the R1 subunit, the R2 subunit, or smaller fragments of R1. The smaller fragments of R1 were generated by either a *Pst*I-*Hin*dIII or a *Bgl*II-*Hin*dIII digestion of the plasmid containing the R1 sequence (pRI 4) and subsequent purification of the individual fragments by electrophoresis. Individual clones were assayed for their sensitivities to BILD 733 in a viral replication assay as described above. Sequences encoding the C-terminal ends of the KOS-733$^r$ p5 and p8 R1 subunits were amplified by PCR with the primers 5′-CCTGTTCACCAACCTGTTCA-3′ and 5′-CGTGGGACG GCGACGACAGT-3′. DNAs extracted from Vero cells infected with KOS-733$^r$ p5 and p8 isolates were amplified by standard PCR protocols and a PCR Core Kit purchased from Boehringer Mannheim Canada Ltd. The PCR-amplified DNA was then sequenced by dideoxy sequencing with the above-described primers and a third primer, 5′-CCTACGTCGACCATAGCCAA-3′. Nucleotide changes in the KOS-733$^r$ p5 and p8 R1 sequences were identified by comparing the sequences with that of wild-type KOS R1 sequenced in the same manner.

**Reintroduction of mutations into the R1 gene.** Plasmids containing the KOS-733$^r$ p5 and p8 mutations were generated as follows. DNA fragments containing the *Bgl*II-to-*Hin*dIII region of R1 were isolated by PCR amplification of DNA from the original viral isolates. The PCR-amplified DNA was digested with *Bgl*II and *Hin*dIII and used to replace the equivalent region in pRI 4, creating pRI 4-S and pRI 4-LS. For the mutation of Pro-1090 to Leu (P1090L) present in KOS-733$^r$ p8, the *Bgl*II-to-*Hin*dIII fragment containing the mutation was generated by PCR-directed mutagenesis of wild-type HSV-1 KOS DNA (11) and subsequently transferred to pRI 4 as described above to generate pRI 4-L. The pRI 4-S, pRI 4-L, and pRI 4-LS plasmids were then used to transfer the mutations to wild-type HSV-1 KOS by marker rescue (20). The sequences of the modified portions of these plasmids were verified by dideoxy sequencing before use in the marker transfer experiments.

**RR enzyme preparation and enzymatic and binding assays.** RR for enzymatic assays was partially purified from HSV-1-infected BHK-21 cells as described elsewhere (8). Infections were performed at an MOI of 10 PFU per cell with wild-type KOS or mutant viruses containing resistance mutations engineered in the R1 coding sequence (A1091S, P1090L, and A1091S/P1090L). RR was also extracted from cells infected with KOS-733 p5 and p8 isolates.

Binding of BILD 733 to R1 was measured by using a solid-phase binding assay described previously (21). Briefly, a nonneutralizing monoclonal antibody specific for the R1 subunit was bound to the wells of 96-well microtiter plates, and extract containing R1 was incubated in the wells for 2 h. After washing, solutions containing a fixed concentration of a radiolabelled tracer and serial dilutions of BILD 733 were incubated in the wells to compete for the bound R1. The 50% inhibitory concentration ($IC_{50}$) was calculated as the concentration of BILD 733 giving 50% displacement of the tracer. To compensate for weak binding of the tracer by two of the mutant subunits (A1091S and A1091S/P1090L), a fourfold-higher concentration of tracer was used in experiments to determine $IC_{50}$s for these mutants. Moreover, in the case of A1091S, it was necessary to add 5 $\mu$g of extract per well, whereas 0.5 to 1.0 $\mu$g of extract was sufficient for binding assays with KOS, P1090L, and A1091S/P1090L extracts. In all cases, the observed $IC_{50}$s are expected to be close to true $K_i$ values.

The kinetic parameters $k_{cat}$ and $K_d$ were determined in the presence of saturating concentrations of substrate (CDP). The parameters are dependent on estimates of R1 and R2 concentrations. Concentrations of R2 and relative amounts of R1 in the crude extracts were estimated from Western blot (immunoblot) analyses. The absolute concentration of wild-type KOS R1 was determined by measuring the enzymatic activity in the presence of BILD 733. For a potent inhibitor such as BILD 733, 50% of enzyme activity is inhibited when the concentration of BILD 733 equals approximately two-thirds of the concentration of R1 dimers (data not shown). These estimates of concentrations yielded consistent values. RR activity was measured by using [$^{14}$C]CDP as described previously (21). The procedure was modified slightly by performing reactions at 25°C rather than 37°C and using a total CDP concentration of 10 $\mu$M rather than 54 $\mu$M. The $K_m$ for CDP is less than 1 $\mu$M (1). Observed rates of ribonucleotide reduction were converted to units of (moles of dCDP)/[(minutes)(moles of R1)]. Assay mixtures contained from 0 to 1.6 $\mu$M purified R2 dimers in addition to the R1 and R2 present from the extract. The purification of the recombinant R2 subunit protein has been described elsewhere (28). Data for rate versus R2 concentration were fit to a mathematical model for tightly binding ligands (since [R1] $\approx$ $K_d$) by using the nonlinear regression program GraFit (version 3.0; Erithacus Software Ltd., Staines, United Kingdom):

$$k_{obs} = \frac{(K_d + [R2] + k_{cat}) - \sqrt{(K_d + [R2] + k_{cat})^2 - 4[R2]k_{cat}}}{2}$$

It was assumed that, for saturating concentrations of CDP, the observed rate of ribonucleotide reduction is proportional to the concentration of R1-R2 complex.

## RESULTS

**Selection of HSV-1 with resistance to BILD 733.** In order to generate resistant variants, HSV-1 strain KOS was passaged eight times (MOI of 0.01 to 0.1 PFU per cell) on serum-starved BHK-21 cells in the presence of increasing concentrations (15 to 150 $\mu$M) of the RR subunit association inhibitor BILD 733. The structure and in vitro antiviral activity of BILD 733 have been described previously (31). Since viral RR is not required for HSV replication in exponentially growing cells, because of preexisting dNTP pools (15), serum starvation of BHK-21 cells was used throughout this study to decrease cellular dNTP pool levels and therefore maximize the requirement for viral RR. The viral pools harvested after each passage were analyzed for their resistance phenotype by using a viral replication assay (colorimetric viral yield assay) described in Materials and Methods. Figure 1 illustrates the resistance phenotypes of viral pools obtained after 5 and 8 passages in the presence of BILD

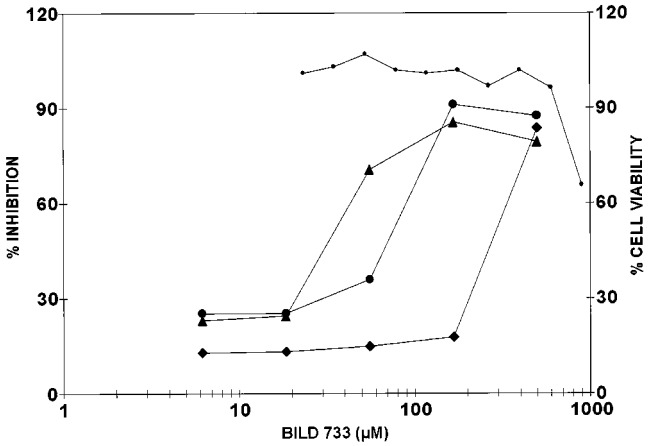


FIG. 1. Resistance phenotypes of viral pools after drug selection. The $EC_{50}$s with the antiviral compound BILD 733 were determined for wild-type KOS (▲) and the viral pools from passages 5 (●) and 8 (◆) on serum-starved BHK-21 cells by the colorimetric viral yield assay (see Materials and Methods). Cell viability (●) under identical conditions was measured by the MTT cytotoxicity assay.

733. In these experiments, the viral pools from passages 5 and 8 were shown to be, respectively, three- and ninefold less sensitive to BILD 733 than wild-type KOS, with respective $EC_{50}$s of 90 and 285 μM. Figure 1 also shows the results of an MTT cell viability assay, which indicate that concentrations of BILD 733 as high as 600 μM can be used without any significant toxic effects being detected on serum-starved BHK-21 cells. Under these conditions, a 50% toxic concentration of 1,000 μM was obtained for BILD 733. As a control for development of resistance in these experiments, wild-type KOS virus was similarly passaged in the presence of increasing concentrations of acyclovir. Viral pools at passages 5 and 8 were shown to exhibit 11- and 50-fold resistance, respectively, to acyclovir (data not shown), confirming that acyclovir resistance can be readily selected under these experimental conditions.

**Resistance phenotypes of purified isolates.** In order to characterize BILD 733 resistance, several isolates were plaque purified from the passage 5 and 8 viral pools which had exhibited differential resistance to the drug, and two selected BILD 733$^r$ isolates (KOS-733$^r$ p5 and p8) were evaluated further. The results in Table 1 show that the plaque-purified isolates had levels of resistance similar to those observed with the original viral pools. $EC_{50}$s of 90 and 310 μM were obtained for BILD 733 against the passage 5 and 8 isolates, respectively. To determine if the development of resistance to the RR inhibitor BILD 733 affects the susceptibility of the viral isolates to other inhibitors of HSV, the sensitivities of wild-type KOS and KOS-733$^r$ isolates to acyclovir and BW348U87 (34) were determined (Table 1). As expected, the $EC_{50}$ of BW348U87, an RR inhibitor with a mechanism of action (i.e., iron chelation) distinct from that of the subunit association inhibitor BILD 733, was unchanged for KOS-733$^r$ isolates compared with wild-type KOS. In contrast, both KOS-733$^r$ p5 and p8 displayed a moderate but reproducible hypersensitivity to the antiviral effects of acyclovir. It has been reported that RR-deficient HSV-1 mutants exhibit hypersensitivity to acyclovir in cell culture (7). It is therefore possible that the moderate increase in susceptibility of BILD 733$^r$ viruses to acyclovir that we have observed is an indication that the mutant viruses encode an impaired RR activity.

TABLE 1. $EC_{50}$s of inhibitors against wild-type KOS and KOS-733$^r$ viruses

| Compound | $EC_{50}$ (μM)[a] | | |
|---|---|---|---|
| | KOS | KOS-733$^r$ p5 | KOS-733$^r$ p8 |
| BILD 733 | 32 | 90 | 310 |
| Acyclovir | 4.6 | 1.9 | 2.0 |
| BW348U87 | 12 | 12 | 12 |

[a] The dose required for 50% inhibition of viral replication was obtained from graphs in which percentage of inhibition of viral replication was plotted against drug concentration.

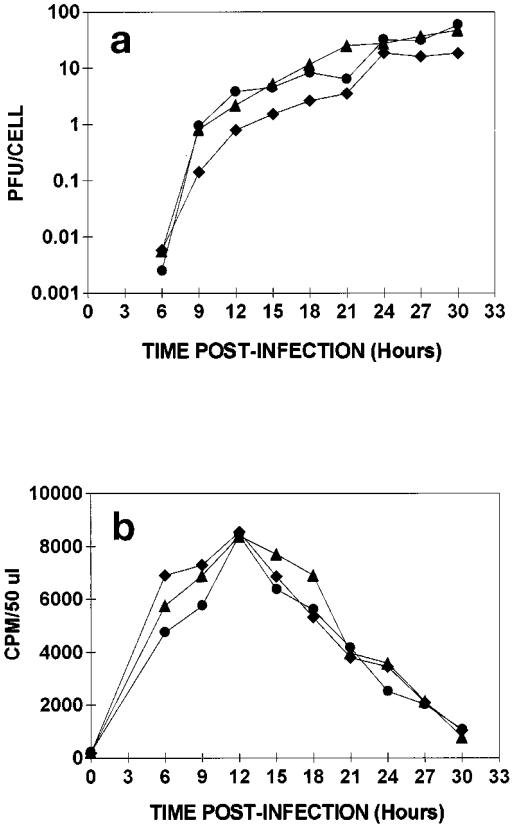


FIG. 2. Growth properties of BILD 733-resistant isolates. Serum-starved BHK-21 cells were infected at a high MOI with wild-type KOS (▲) and BILD 733$^r$ isolates KOS-733$^r$ p5 (●) and p8 (◆), and then viral replication was monitored at different times postinfection. Viral yields (a) were determined by plaque assay, and DNA replication (b) was monitored by incorporation of [$^3$H]thymidine.

**Growth properties of KOS-733$^r$ isolates.** Single-step growth analyses were performed in order to study the ability of the resistant isolates to replicate in cell culture (Fig. 2a). Serum-starved BHK-21 cells were infected at a high MOI, and virus yields were estimated at different times postinfection (up to 30 h). The results in Fig. 2a show that viral yields and kinetics of production of infectious virus similar to those of the wild-type KOS were obtained with both the KOS-733$^r$ p5 and p8 isolates. Incorporation of [$^3$H]thymidine was also monitored in similar infections in order to determine the kinetics and levels of DNA synthesis in cells infected with the viral mutants compared with those infected with KOS. These studies were conducted under serum starvation conditions, therefore, the background level of incorporation of [$^3$H]thymidine by mock-infected cells is very low (256 cpm/50 μl), and total incorporation in infected cells is a measure of viral DNA synthesis. The results in Fig. 2b show that the levels and kinetics

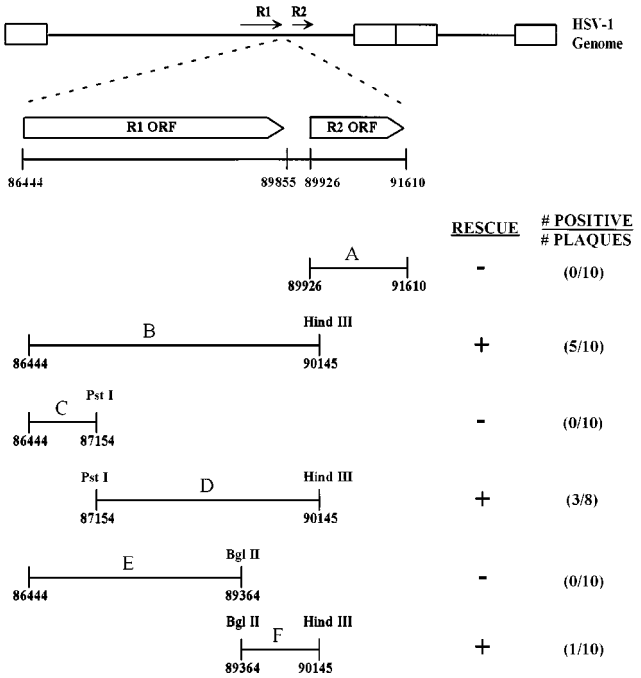


FIG. 3. Marker rescue of mutant KOS-733$^r$ p5. The exact locations of the DNA fragments used in the marker rescue experiments are indicated below the fragments and refer to their positions in the HSV-1 strain 17 sequence (30). The ability of each fragment to rescue a wild-type sensitivity into the KOS-733$^r$ p5 virus, indicated by a + or −, and the frequency of recombination, in number of positive rescues per number of plaques assayed, are shown to the right of each fragment.

of viral DNA synthesis were similar in wild-type KOS-, KOS-733$^r$ p5-, and KOS-733$^r$ p8-infected cells. Results similar to those for wild-type KOS were also obtained when levels of expression of representative immediate-early (ICP4), early (UL42), and late (gC) proteins were monitored by Western blot analyses (data not shown). It therefore appears that in these experiments, the BILD 733$^r$ isolates possess in vitro growth properties indistinguishable from those of the parental virus. Given the mode of action of this class of RR subunit association inhibitors, it is possible to envisage resistance occurring by alternative mechanisms. To address the possibility that overexpression of one or both subunits of HSV RR could be responsible for the observed resistance phenotype, we examined the levels of expression of both R1 and R2 proteins in cells infected with the different viruses. Protein levels were monitored by [$^{35}$S]methionine labelling and SDS-polyacrylamide gel electrophoresis as well as Western blot analyses. No significant differences from results with wild-type KOS were observed in the levels of expression of either R1 or R2 subunits in cells infected with the BILD 733$^r$ mutants (data not shown).

**Location of mutations associated with resistance phenotype.** Since this class of peptidomimetic RR inhibitors was designed to bind with high affinity to the R1 subunit and compete for binding of the R2 subunit, it was likely that the mutations conferring the resistance phenotype would be located in one or both of the RR subunits. In order to localize the mutations contained in the mutant viruses, various restriction enzyme fragments of wild-type viral DNA spanning UL39 and UL40, which encode the RR R1 and R2 subunits, respectively, were used to marker rescue wild-type BILD 733 sensitivity into the resistant isolates (Fig. 3). After the initial transfections, individual plaques were isolated and each of the clones was tested for BILD 733 sensitivity in the viral replication assay described above. The DNA fragments were scored positive for marker rescue if any of the clones from that transfection exhibited wild-type sensitivity to BILD 733. None of the R2-containing cloned fragments were positive for rescue. However, clones obtained from fragment B, containing the whole R1-coding sequence, and from subfragments D and F were all positive for marker rescue of the wild-type phenotype. The frequencies of rescue were 50, 37.5, and 10% for fragments B, D, and F respectively. The smallest DNA fragment capable of rescuing the wild-type phenotype, fragment F, consisted of 493 nucleotides at the C terminus of the R1 gene and 219 nucleotides from the N terminus of the R2 gene. This result, combined with the lack of rescue by the DNA fragments containing R2-coding sequences, suggested that the resistance mutations were most likely located within the C terminus of the R1 gene. The nucleotide sequences of the C-terminal 493 nucleotides of the R1 genes from the mutant viruses were therefore determined and compared with the sequence of wild-type HSV-1 KOS (Fig. 4). KOS-733$^r$ p5 contained a single mutation of guanosine to thymidine at nucleotide 89714 of the HSV-1 genome, corresponding to an alanine-to-serine mutation at position 1091 of the large RR subunit. DNA sequence analysis of the same region of KOS-733$^r$ p8 revealed the same mutational change and an additional mutation from cytidine to thymidine at nucleotide 89712, corresponding to an amino acid change of proline 1090 to leucine. No other mutations were detected within this portion of the R1 gene in either mutant virus DNA.

**Marker transfer of the resistance-associated mutations into a wild-type KOS background.** Marker rescue and sequence data demonstrated the emergence of two distinct mutations in the BILD 733-resistant isolates. In order to establish the contribution of each of these mutations to the BILD 733 resistance phenotype exhibited by KOS-733$^r$ p5 and KOS-733$^r$ p8, plasmid DNAs containing the mutations A1091S and P1090L sin-

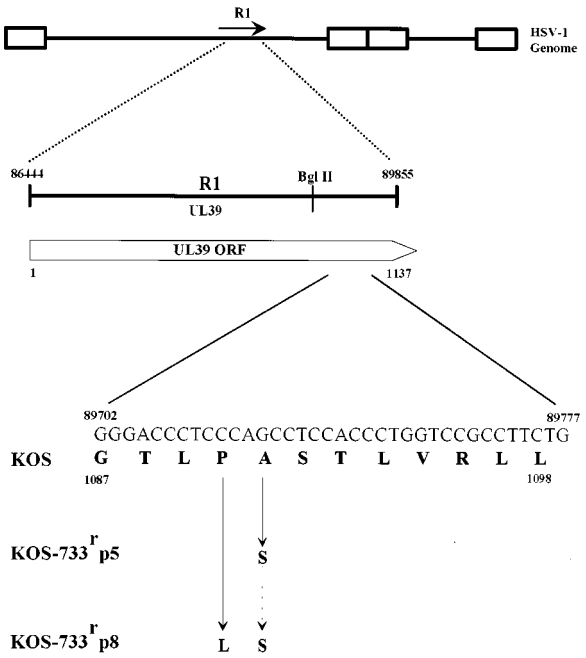


FIG. 4. Nucleotide sequences of the regions surrounding the mutations in mutants KOS-733$^r$ p5 and KOS-733$^r$ p8. The predicted amino acid sequence for wild-type HSV-1 KOS is shown below the nucleotide sequence. The amino acid changes present in each of the mutant viruses are indicated by the arrows. The nucleotide numbers above the sequences are from reference 30.

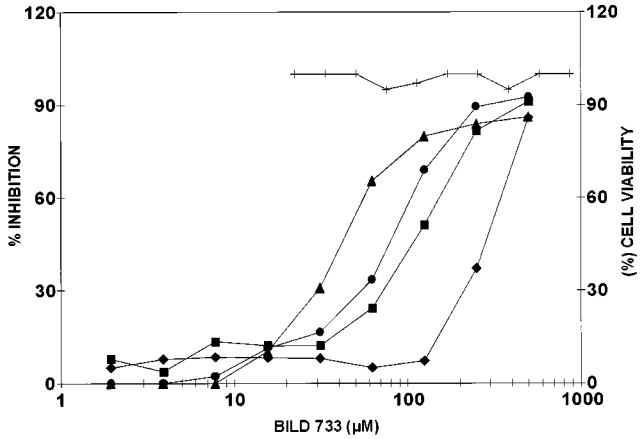


FIG. 5. Resistance phenotypes of viruses with reintroduced mutations. Individual mutations A1091S (●), A1091S/P1090L (◆), and P1090L (■) were reintroduced into KOS (▲), and reconstructed viruses were tested for their resistance phenotypes in the replication assay (ELISA). Cell viability (+) under identical conditions was measured by the MTT cytotoxicity assay.

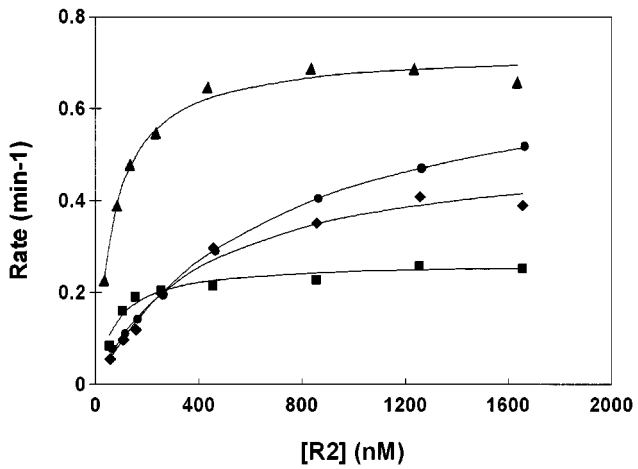


FIG. 6. Characterization of mutant RR activities. For each mutant, the rate of substrate conversion [moles of dCDP (minute)$^{-1}$ (mole of R1 dimers)$^{-1}$] is plotted as a function of R2 dimer concentration. Datum points represent experimental values for KOS (▲), A1091S (●), A1091S/P1090L (◆), and P1090L (■) RR enzymes. Curves represent theoretical fits to the data as described in Materials and Methods. The data shown are from a single experiment, but duplicate experiments gave the same values within experimental error.

gly or in combination were transfected into Vero cells along with infectious HSV KOS DNA. Viral clones were isolated by plaquing, and the successful introduction of mutations into the viral DNA was verified by dideoxy sequencing of the corresponding region of the viral genome. The isolates containing the mutations were tested for BILD 733 resistance by using the viral replication assay (ELISA). Each of the reconstructed virus mutants (A1091S and A1091S/P1090L) exhibited $EC_{50}$s similar to those of the original resistant isolates in which the mutations were identified (Fig. 5). Moreover, on evaluation of the virus isolate containing the single P1090L mutation, it was also found to possess threefold resistance to BILD 733. This mutation was never seen by itself in sequence analysis of the BILD 733-resistant viral pools from various passages (data not shown). These results indicate that the mutations identified at the C terminus of the KOS-733$^r$ p5 and p8 R1 subunits are both necessary and sufficient to confer the resistance phenotype. Mutations at two independent positions in a small region of R1 therefore appear to confer low-level (approximately threefold) resistance to the drug BILD 733. Moreover, viruses containing mutational changes at both positions (1090 and 1091) exhibit higher levels of resistance (ninefold).

**Characterization of the mutant RR enzyme.** Since the resistance mutations mapped to the coding sequence of R1, we expected that the viral drug resistance phenotype was the result of weakened binding of BILD 733 to mutant R1 subunits. To confirm this, studies were performed on RR extracted from cells infected with wild-type and reconstructed mutant viruses. The affinities of the wild type and each of the mutant R1 subunits for BILD 733 were measured by competitive displacement of a radiolabelled tracer ligand in a solid-phase binding assay (see Materials and Methods). The A1091S mutation resulted in a significant (25-fold) increase in the $IC_{50}$ of BILD 733, from 0.4 nM for the wild type to 10 nM for the A1091S mutant R1 (all values are averages from four experiments). As expected, the $IC_{50}$ was even larger for the A1091S/P1090L mutant (100-fold increase, to 40 nM). The P1090L mutation caused a smaller but still significant increase in $IC_{50}$ (2.5-fold, to 1.0 nM). Increased $IC_{50}$s of BILD 733 with mutant R1 subunits are consistent with the finding of resistance in the viral replication assay. The difference in $IC_{50}$ for the two single mutants, despite their similar $EC_{50}$s (Fig. 5), may be explained by other effects of these mutations on enzyme activity, as discussed below.

HSV requires a functional RR enzyme for optimal growth in serum-starved cells (15), and the growth kinetics of BILD 733-resistant viruses were indistinguishable from those of wild-type KOS. However, since both the A1091S and A1091S/P1090L mutants are hypersensitive to acyclovir, we reasoned that optimal viral growth in vitro might still be possible with a moderately impaired RR. We speculate that the A1091S and P1090L mutations might weaken the affinity of the mutant R1 for R2, since R2 presumably binds at the same site on R1 as the inhibitor BILD 733 (26, 29, 33). These mutations might also reduce the intrinsic reductase activity of R1 in the holoenzyme, perhaps by causing a conformational change at the R1-R2 interface, which would reduce the efficiency of electron transfer between the subunits.

With this in mind, both R1-R2 affinity and intrinsic R1 reductase activity were determined for each mutant by measuring reductase activity in the presence of different concentrations of R2, and the data were fit to an equation with two parameters, the dissociation constant ($K_d$) and the maximum possible reaction rate ($k_{cat}$), obtained at saturating R2 concentrations. For each enzyme extract, the observed rate constant for reduction was plotted as a function of R2 concentration (Fig. 6). The lowest R2 concentration value in each curve represents the amount contributed from the protein extract; higher concentrations of R2 in enzyme reactions were achieved by adding pure, recombinant R2. In the concentration range shown, all of the mutant RRs were less active than KOS RR. Values of $K_d$ and $k_{cat}$ are given in Table 2. The value of $k_{cat}$ is dependent on the estimated concentration of R1, as described

TABLE 2. Kinetic parameters for viral RRs

| Viral RR | $k_{cat}$ (min$^{-1}$) | $K_d$ (nM) |
|---|---|---|
| KOS | 0.70 | 80 |
| A1091S | 0.71 | 800 |
| P1090L | 0.29 | 90 |
| A1091S/P1090L | 0.53 | 410 |

in Materials and Methods. These values indicate that the A1091S mutant was less active than KOS principally because the mutant R1 binds more weakly to R2. P1090L R1, in contrast, binds R2 as well as KOS R1 but was less active at all concentrations of R2 because $k_{cat}$ was reduced. The double mutant P1090L/A1091S had intermediate properties: the $k_d$ value was higher than that of KOS or P1090L R1 but lower than that of A1091S R1, and the $k_{cat}$ value was also between those of A1091S and P1090L R1. It was shown above that BILD 733 binds more weakly to the A1091S than to the P1090L R1, despite the fact that the inhibitor is equally effective at inhibiting the growth of the two mutant viruses. This finding is explained by the greatly increased $K_d$ for R2 binding to A1091S R1 compared with P1090L R1, since the effectiveness of the inhibitor for enzyme inhibition is determined by the relative affinity of BILD 733 binding in competition with the natural subunit, R2.

The slight hypersensitivity of the resistant mutants to acyclovir is also explained by these activity measurements. We believe that R2 is present at low micromolar concentrations in infected cells (23). Examination of the R2 saturation curves in Fig. 6 indicates that in this concentration range, all three mutant RR enzymes are approximately twofold less active than KOS RR. This moderate effect may not impair virus growth but could be responsible for the increased sensitivity of these mutant viruses to acyclovir (Table 1). Reduced reductase activity will decrease levels of deoxynucleotides in cells, and acyclovir triphosphate, which competes with the dGTP pools for binding to the viral DNA polymerase, will thus be preferentially incorporated into the DNA.

Activity measurements, as well as BILD 733 binding assays, were also carried out with extracts from cells infected with the original BILD 733-resistant viruses isolated from the selection experiments (data not shown). The results were similar to those described above and shown in Table 2, further confirming that the specific mutations identified here were responsible for the observed resistance phenotype of the enzyme.

## DISCUSSION

In this paper, we describe the isolation and characterization of HSV-1 variants with resistance to BILD 733, which is a representative of a novel class of peptidomimetic HSV RR subunit association inhibitors. Consistent with the mode of action of these inhibitors, the resistance mutations were mapped to two amino acid positions in the large subunit (R1) of the RR enzyme. Moreover, these mutations were found to be both necessary and sufficient to confer the resistance phenotype to reconstructed virus mutants. A resistant virus variant isolated after five passages in the presence of the drug contained a single amino acid change (Ala to Ser), at position 1091 of the R1 protein, which conferred threefold-reduced sensitivity to BILD 733. The development of higher levels of resistance after eight passages was associated with the emergence of a virus variant containing an additional mutation (Pro to Leu) at the adjacent amino acid position (1090) in the R1 subunit. This virus exhibited ninefold resistance to the drug and displayed the maximum level of resistance observed in the selection experiments with RR subunit association inhibitors. These levels of resistance are significantly lower than those obtained for acyclovir (passage 5, 11-fold; passage 8, 50-fold) in parallel studies (data not shown). Although we have not characterized the acyclovir-resistant viruses from our own experiments, many reports have shown that a variety of alterations in the viral thymidine kinase gene (deletion or single point mutations) or mutations in the DNA polymerase gene are responsible for the resistance phenotype (for reviews, see references 5 and 6). Although the cytotoxicity of BILD 733 at high concentrations may preclude the possibility of obtaining levels of resistance higher than we have obtained in these in vitro experiments, it is possible that the resistance observed in these experiments may be limited to moderate levels as a consequence of the mode of action of these inhibitors. As indicated above, these peptidomimetic RR inhibitors prevent the association of the two RR subunits by mimicking the C-terminal end of R2. Resistance mutations which result in weaker binding of the C terminus mimetic BILD 733 to the R2 binding site on R1 may therefore be expected to have detrimental consequences for natural R1-R2 interactions and result in impaired activity of the mutant enzyme. It is possible that the selection of mutations which would more radically reduce inhibitor-R1 binding and thus provide greater levels of drug resistance may be prohibited because of their detrimental effect on RR enzymatic activity and consequences for viral growth.

Single-step growth analyses and DNA synthesis studies with serum-starved cells indicated that the BILD 733-resistant virus variants that we selected in vitro were not significantly impaired in their growth in cultured cells. Despite these observations, however, both the KOS-733$^r$ p5 and p8 viruses exhibited greater sensitivity to acyclovir than the parental virus. It has been previously reported that ICP6Δ, an HSV-1 RR null mutant, is hypersensitive to acyclovir (7). It was proposed that in ICP6Δ-infected cells, the accumulation of lower levels of dNTP because of the absence of a viral RR activity favors preferential incorporation of acyclovir triphosphate into the viral DNA. We propose that a similar phenomenon is occurring in cells infected with the KOS-733$^r$ isolates and that while the RR enzymes of the BILD 733-resistant viruses are capable of supporting viral replication at wild-type levels in cultured cells, they fail to generate dNTP pools as high as those in cells infected with the wild-type virus. In support of this hypothesis, in vitro enzymatic studies of RRs extracted from virus-infected cells confirmed that the enzymes encoded by both of the mutants had activities lower than that of the wild type when assayed at R2 concentrations which are judged to best reflect the conditions in tissue culture cells. The A1091S mutation affects primarily the ability of R1 and R2 dimers to form the required $\alpha_2\beta_2$ tetramer, while the enzyme containing the P1090L mutation is thought to form tetramers with normal efficacy but to have lower intrinsic activity.

The precise location of the R2 binding site on HSV R1 is presently unknown. However, given that the BILD 733 inhibitor binds to the R1 subunit by mimicking the C-terminal end of R2, we assumed that mutations conferring resistance to the inhibitor may be localized in R1 at or close to residues which interact with R2. Previous studies using truncated R1 proteins have identified two regions in R1, residues 349 to 373 and 996 to 1137, which may be implicated in R1-R2 interactions (10). The present identification of resistance mutations at positions 1090 and 1091 of R1 confirms that this second region is important for interactions with R2 and provides evidence for more precise localization of the R2 binding site on R1 in the HSV enzyme. Moreover, examination of the X-ray crystal structure of the *Escherichia coli* R1 subunit and the sequence alignments of HSV and *E. coli* R1 proteins indicates that the mutations we have identified may be located in a region corresponding to an α-helix in *E. coli* R1 which is directly involved in binding interactions with a 20-residue peptide corresponding to the C-terminal end of R2 (35). In the absence of structural data for the HSV R1 protein, the results from the present study provide the first direct indication that a region located at the C-terminal end of HSV-1 R1 (including residues P-1090

and A-1091) is involved in R1-R2 interactions and provide useful information for more precise identification of the R1-R2 binding site on HSV R1.

In summary, we have demonstrated that mutations localized near the C terminus of the HSV-1 R1 subunit (residues 1090 and 1091) are responsible for conferring low to moderate levels of resistance towards the RR inhibitor BILD 733. Although the KOS-733$^r$ mutants can replicate in tissue culture as well as wild-type virus does, their RRs are somewhat impaired. It will be interesting to study the pathogenicity of these mutant viruses in animal models of virus infection, particularly with respect to their neuropathogenicity, and their ability to reactivate from latency. Since neuronal cells are arrested in $G_0$ and therefore possess very low dNTP pools, it is expected that the function of the virus-encoded RR will be of paramount importance in these cells. Experiments are in progress to evaluate the neurovirulence of the BILD 733$^r$ isolates in mice following intracranial inoculation and their ability to reactivate from latency in explanted trigeminal ganglia. In addition, ongoing studies which involve both in vitro and in vivo characterization of mutants resistant to several structurally diverse peptidomimetic RR inhibitors will provide further understanding of the mechanism by which HSV becomes resistant to this new class of antiviral agents and may provide useful information concerning the potential clinical significance of HSV resistance to RR inhibitors.

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