Antiviral Activity of a Selective Ribonucleotide Reductase Inhibitor against Acyclovir-Resistant Herpes Simplex Virus Type 1 In Vivo

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Received 17 December 1997/Returned for modification 31 March 1998/Accepted 1 May 1998

Herpes simplex virus (HSV) encodes a ribonucleotide reductase (RR) that catalyzes the synthesis of the deoxyri-bonucleotide precursors required for DNA synthesis. This enzyme shares many similarities with the host cellular RR but differs markedly from the latter in terms of primary amino acid sequences and allosteric regulation (24, 26, 35). Both RRs consist of two subunits (26, 35). The larger subunit contains redox-active thiols that provide the hydrogen for nucleotide reduction (24, 29, 31, 35). The smaller subunit contains a binuclear μ-oxo bridged iron center associated with a tyrosyl free radical (1, 17, 26, 32, 35). The carboxy terminus of the RR small subunit is critical for subunit association and for the catalytic activity of the enzyme (3, 13, 26). Although HSV RR is not required for virus replication in exponentially growing cells, it is necessary in nondividing cells (18) and is required for the full expression of pathogenicity of HSV in animal models of primary infection (3, 20, 21) and for reactivation from latency (20, 21). Therefore, HSV RR represents an interesting target for antiviraltherapies (3, 13, 21, 26). It has been reported that nonselective RR inhibitors, such as 2′-fluoromethylene-2′-deoxyctydine and A1110U, potentiate the activity of acyclovir (ACV) both in vitro and in vivo against wild-type and ACV-resistant HSV infections (6, 14, 28, 39). However, these RR inhibitors have not been shown to be useful as monotherapy, and therefore, the combination therapy has not been clearly validated due in part to the lack of specificity and the toxicity of these agents (14, 26, 28, 39). Our group has developed a class of selective HSV RR subunit association inhibitors that act as mimics of the carboxy terminus of the small subunit (4, 27, 30). We have recently reported the in vivo activity of one of the earliest lead compounds, BILD 1263, as a monotherapy against HSV type 1 (HSV-1)-induced ocular disease in mice (4, 27). In the present study, we examined the effects of BILD 1633 SE, a more potent HSV RR inhibitor, against cutaneous ACV-resistant HSV-1 infections in the athymic nude mouse model when BILD 1633 SE was used either alone or in combination with ACV. The athymic nude mouse was chosen because ACV-resistant HSV-1 fails to induce significant disease in normal mice (3, 14). A lack of functional T cells renders athymic nude mice more susceptible to cutaneous infections by ACV-resistant HSV-1 and more suitable for drug evaluation (14, 15). In addition, since ACV-resistant HSV infections cause significant disease mainly in immunocompromised patients (7, 8, 23), information obtained from these immunodeficient animals may be clinically relevant (14, 15). Our results indicate that BILD 1633 SE is effective in the treatment of ACV-resistant HSV infections in this model when it is used either alone or in combination with ACV. Therefore, selective inhibition of HSV RR by peptidomimetic subunit association inhibitors may offer a potential alternative topical therapy for the treatment of ACV-resistant HSV infections in humans.
MATERIALS AND METHODS

Viruses. Four different laboratory strains of HSV-1 were used in the present study. These include two wild-type strains, HSV-1 F and KOS, and two ACV-resistant strains, dlspTk (11) and PAA5 (9, 16). Both wild-type HSV-1 strains have been described elsewhere (27). The ACV-resistant, thymidine kinase (TK)-deficient HSV-1 strain dlspTk and the DNA polymerase mutant PAA5 were kindly provided by D. Coen (9, 11). Virus stocks were routinely grown in Vero (African green monkey kidney) cells, and virus titers were determined by a standard plaque assay with confluent Vero cells.

Compounds. The preparation of BILD 1633 SE was based on our previously published procedure (30). The [α-(R)-methyl-3-cyclohexyl]-propionyl functionali ty of the N-terminal part of BILD 1633 SE is introduced in the last stage of the synthesis by condensation of the corresponding acid chloride.

In vitro antiviral assay. Antiviral assays were performed essentially as described previously (27). Baby hamster kidney (BHK)-21/C13 (ATCC CCL 10) cells were seeded in 96-well culture plates at a density of 5,000 cells per well in a minimum essential medium containing 8% (vol/vol) fetal bovine serum (Gibco), and the plates were incubated at 37°C with 5% CO2. After 6 h, the concentration of fetal bovine serum was reduced to 0.5% (vol/vol) and the cells were serum starved for 3 days. Serum-starved cell monolayers were infected with HSV-1 F, KOS, PAA5, and dlspTk at a multiplicity of infection of 0.05 in defined medium (5). The defined medium (5) consisted of F-12 medium, Dulbecco’s modified Eagle medium, and Bgjb medium (0.3; 1; vol/vol) with 2 g of bovine serum albumin per liter, 2.38 g of HEPES per liter, 50 mg of garamycin per liter, 100 μg of tetracycline per liter, 1 μg of insulin per liter, 0.4 μg of triiodothyronine per liter, 0.2 μg of parathyroid hormone per liter, 10 μg of glucagon per liter, 0.1 μg of epidermal growth factor per liter, 0.2 μg of fibroblast growth factor per liter, and 10 μg of transferrin per liter. After 1 h, the HSV-infected cells were rinsed with defined medium and were then incubated with test compounds for 24 h for strains F and KOS and 44 h for strains PAA5 and dlspTk. The extent of replication was assessed by a novel enzyme-linked immunosorbent assay (ELISA). The cells were fixed with 0.063% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min and were blocked with 0.5% casein in PBS for 1 h. Thereafter, mouse monoclonal antibody C11 that recognizes the HSV-1 late glycoprotein C (41) was added to each well for 2 h. After the cells were washed three times with PBS containing 0.1% Tween 20, the bound monoclonal antibody was detected with sheep anti-mouse immunoglobulin G horseradish peroxidase for 1 h in the dark. The plates were washed three times with PBS and once with 0.1 M sodium citrate (pH 4.5). ortho-Phenylendiaminedi hydriodochrome (Gibco) was used as a substrate for 30 min in the dark, and color development in individual wells was monitored at 450 nm with a Titertek microplate spectrophotometer. For inhibition studies, compounds were tested in threefold serial dilutions. All topical treatments were started at 3 h postinoculation and continued for 10 days (four times per day between 8:30 a.m. and 5:30 p.m.). In some experiments, ACV was administered orally in drinking water immediately following inoculation. Topical lesions and systemic diseases were observed daily. The following criteria were applied to score topical lesions: 0, no lesions; 1, discrete vesicles; 2, two or more open lesions; 3, separate ulcerations; and 4, zoster band formations. The onset and time course of topical lesions are dependent on the titer of the inoculum. Under current experimental conditions, topical lesions became visible within 2 to 3 days and peaked within about 10 to 13 days; this was dependent on the titer of the inoculum. Although some degree of spontaneous regression occurred in animals infected with PAA5’s mutants, topical lesions persisted over the whole duration of experiment. Only a few (fewer than 10%) of the PAA5-infected mice developed systemic disease (disseminated infections, neurological and physical abnormalities, and mortality) following the appearance of severe topical lesions. Therefore, systemic disease was not used for drug evaluation. Topical lesion data are presented in terms of the mean and the standard error of the mean (SEM). Daily lesion scores and the areas under the curve (AUC) of the lesion scores are compared for statistical significance by the analysis of variance followed by Student-Newman-Keuls multiple comparisons with SAS software (SAS Institute, Cary, N.C.). A P value of <0.05 was considered statistically significant.

RESULTS

Comparative in vitro activities of BILD 1633 SE and ACV against several strains of HSV-1. Figure 1 shows the structure of the HSV RR subunit association inhibitor BILD 1633 SE. This compound inhibits HSV RR with a 50% inhibitory concentration of 3 nM, as determined by a competitive binding assay (24). Like previously published inhibitors in this class, it does not affect the activity of the human RR at a concentration up to 250 μM, on the basis of the enzyme assay. Therefore, this compound represents a highly selective HSV RR inhibitor. As indicated in Table 1, BILD 1633 SE is about 10 times more potent than ACV in inhibiting the replication of the wild-type strains HSV-1 F and KOS (EC50 = 0.4 μM) and is about 100 times more potent then ACV against both ACV-resistant strains. In addition, this compound is about three times more active against the ACV-resistant mutant PAA5 than against both wild-type strains and the dlspTk HSV-1 strains. The 50% cytotoxic concentration of BILD 1633 SE is 14 μM, as measured by the in vitro MTT assay, yielding a selectivity index of 35. Compared with the potency of BILD 1263 published previously (3, 27), BILD 1633 SE is about 5 to 10 times more potent in vitro against all the viruses studied (Table 1) and in vivo against HSV-1 infection in the mouse ocular model (unpublished data).

<table>
<thead>
<tr>
<th>HSV-1 strain</th>
<th>BILD 1633 SE EC50 (μM)</th>
<th>ACV</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>0.35 ± 0.10</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>KOS</td>
<td>0.43 ± 0.06</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>dlspTk</td>
<td>0.46 ± 0.09</td>
<td>60.2 ± 12.9</td>
</tr>
<tr>
<td>PAA5</td>
<td>0.14 ± 0.02</td>
<td>17.2 ± 2.9</td>
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*Values represent the means ± standard deviations from at least 10 determinations.

In vivo drug combination studies. The antiviral activity of BILD 1633 SE in combination with ACV against wild-type and ACV-resistant HSV-1 was assessed by the 96-well ELISA described above. Evaluation of drug interactions was performed by the isobole method (40). In this method, selected concentrations of BILD 1633 SE were tested in combination with various doses of ACV, and average EC50 of ACV were determined from duplicate dose-response curves. These values were used to calculate FECAV (ACV), which represents the ratio of the concentration of ACV required to inhibit HSV-1 replication by 50% in the presence of a fixed concentration of BILD 1633 SE to the concentration of ACV required to inhibit HSV-1 replication by 50% in the absence of BILD 1633 SE. The isobologram representation is obtained by plotting FECAV (ACV) as a function of the ratio of the fixed concentration of BILD 1633 SE to the EC50 of BILD 1633 SE in the absence of ACV. In this representation when experimental datum points for the drugs used in combination fall on the hypotenuse, the effects of the two drugs are additive. If experimental datum points fall below the theoretical line for noninteracting drugs, the effects of the two drugs are considered synergistic.

Animals. Athymic nude mice (female nu/nu CD1 mice from Charles River Canada, St. Constant, Quebec, Canada) at 5 to 6 weeks of age were used for all experiments. Animals were housed in microisolator cages inside semigird isolators with sterile food, water, and bedding. All manipulations were carried out within class II-type safety cabinets (Nuaire, Plymouth, Minn.), according to the protocols approved by the Canadian Council on Animal Care (Ottawa, Ontario, Canada).

Cutaneous inoculation. Animals were inoculated with ACV-resistant HSV-1 dlspTk or PAA5’s mutants under halothane anesthesia by scarification and rubbing 30 μl of the desired virus stock on an area of about 1 cm2 on each side of the dorsal skin for 10 s.

Treatments and drug evaluation. Both ACV and BILD 1633 SE were topically administered in a cream vehicle containing 16% DMSO, 5% linoleic acid, 16% Cremophor EL, and 63% polyvinyl alcohol (25% solution in 100 mM HEPES buffer pH 8.5)). All topical treatments were started at 3 h postinoculation and continued for 10 days (four times per day between 8:30 a.m. and 5:30 p.m.). In some experiments, ACV was administered orally in drinking water immediately following inoculation. Topical lesions and systemic diseases were observed daily. The following criteria were applied to score topical lesions: 0, no lesions; 1, discrete vesicles; 2, two or more open lesions; 3, separate ulcerations; and 4, zoster band formations. The onset and time course of topical lesions are dependent on the titer of the inoculum. Under current experimental conditions, topical lesions became visible within 2 to 3 days and peaked within about 10 to 13 days; this was dependent on the titer of the inoculum. Although some degree of spontaneous regression occurred in animals infected with PAA5’s mutants, topical lesions persisted over the whole duration of experiment. Only a few (fewer than 10%) of the PAA5-infected mice developed systemic disease (disseminated infections, neurological and physical abnormalities, and mortality) following the appearance of severe topical lesions. Therefore, systemic disease was not used for drug evaluation. Topical lesion data are presented in terms of the mean and the standard error of the mean (SEM). Daily lesion scores and the areas under the curve (AUC) of the lesion scores are compared for statistical significance by the analysis of variance followed by Student-Newman-Keuls multiple comparisons with SAS software (SAS Institute, Cary, N.C.). A P value of <0.05 was considered statistically significant.
in vivo antiviral effect of BILD 1633 SE was highly reproducible, as verified by three additional independent experiments that showed reductions of the AUC values of the lesion scores of 60, 81, and 61%, respectively (n = 12 for both the vehicle- and the drug-treated groups; P was < 0.05 for all experiments). The dose-dependent effect of topical BILD 1633 SE against HSV-1 PAAr5-induced topical lesions in athymic mice is shown in Fig. 4C and D.

Combination therapy with oral ACV and topical BILD 1633 SE against HSV-1 PAAr5 infection. Since concomitant administration of two compounds by the same route may lead to chemical and/or physical interactions of the compounds, we administered ACV and BILD 1633 SE by two different routes. Figure 5 shows the effect of oral ACV supplied continuously in drinking water. When ACV was administered for 10 days in drinking water at a concentration of 1 mg/ml, no protection from HSV disease was seen (Fig. 5A and B). However, maximum protection was achieved with a concentration of 3 mg/ml (daily dose, 871 ± 49 mg/kg of body weight), resulting in a reduction of the AUC of the lesion score by 48%. This protection was similar to that achieved with topical ACV treatment, as described above. Increasing the ACV concentration to 5 mg/ml in drinking water (daily dose, 1,391 ± 66 mg/kg) did not change the behaviors or the body weights of the treated mice.

To determine the effectiveness of the combination therapy in the treatment of HSV-1 PAAr5-induced topical lesions, ACV was administered orally in drinking water at a concentration of 1.5 mg/ml in combination with 0.8% BILD 1633 SE in a topical cream. As illustrated in Fig. 6, treatment with ACV alone did not significantly affect the infection, while treatment with BILD 1633 SE alone reduced the mean disease score significantly for 7 days starting on day 4. Combination therapy was more effective than therapy with each agent alone. As shown in Fig. 6, the AUC values and the mean disease score for the combination of ACV and BILD 1633 SE were reduced.
significantly on days 8 to 11 compared to those for either treatment alone.

Monotherapy and combination therapy with ACV and BILD 1633 SE against HSV-1 dlspk infection. Reproducible and persistent topical lesions were induced by inoculating athymic nude mice with $10^7$ PFU/site, as described in Materials and Methods. (A) Mean lesion scores. (B) AUC of the lesion scores. The AUCs of the lesion scores were presented as means ± SEMs ($n = 12$). * $P < 0.05$ compared with the results for the vehicle group; † $P < 0.05$ compared with the results for the vehicle and 0.8% BILD 1633 SE groups.

5% BILD 1633 SE for 10 days reduced the AUC by 51% ($P < 0.05$) (Fig. 8). The combination therapy of oral ACV (5 mg/ml) and topical BILD 1633 SE (5% cream) was more effective than treatment with either agent alone (Fig. 8). Only a few animals in this group developed observable vesicles.

FIG. 4. Effects of BILD 1633 SE and ACV against HSV-1 PAAr5 infection. Animals were cutaneously inoculated with $10^7$ PFU/site, as described in Materials and Methods. (A and B) BILD 1633 SE and ACV were applied in 5% topical formulation. (C and D) BILD 1633 SE was applied four times a day at concentrations of 0, 0.8, 2, and 5%. The AUCs of the lesion scores are presented as means ± SEMs ($n = 12$). * $P < 0.05$ compared with the results for the vehicle group; † $P < 0.05$ compared with the results for the vehicle and 0.8% BILD 1633 SE groups.

DISCUSSION

The present study reports for the first time an effective therapy against ACV-resistant HSV infections in athymic nude mice with a selective HSV RR subunit association inhibitor, BILD 1633 SE. Consistent with its in vitro antiviral properties, BILD 1633 SE was more effective against HSV-1 PAAr5-induced lesions than against HSV-1 dlspk-induced lesions, with inoculum-dependent AUC reductions ranging from 67 to 96% and 28 to 51%, respectively. Although BILD 1633 SE was less effective against HSV-1 dlspk-induced infections, it should be

FIG. 5. Antiviral effects of ACV in drinking water against HSV-1 PAAr5 infection. Animals were cutaneously inoculated with $10^7$ PFU/site, as described in Materials and Methods. ACV was given in the drinking water at 0, 1, 3, and 5 mg/ml. (A) Mean lesion scores. (B) AUC of the lesion scores. The AUCs of the lesion scores are presented as means ± SEMs ($n = 12$). * $P < 0.05$ compared with the results for the vehicle group.

FIG. 6. Antiviral effects of oral ACV and topical BILD 1633 SE against HSV-1 PAAr5 infection. Animals were cutaneously inoculated with $10^7$ PFU/site, as described in Materials and Methods. ACV was given in the drinking water at a dose of 1.5 mg/ml. BILD 1633 SE (0.8%) was applied topically four times per day. (A) Mean lesion scores. (B) AUC of the lesion scores. The AUCs of the lesion scores are presented as means ± SEMs ($n = 24$). * $P < 0.05$ compared with the results for the vehicle groups.
HSV-1

(A) Mean lesion scores. (B) AUCs of the lesion scores. ACV transiently (were applied topically four times per day. (A) Mean lesion scores. (B) AUCs of the lesion scores. ACV only transiently reduced the mean lesion score on days 13 to 15 compared with the scores for the no-treatment group (n = 12) and the vehicle group (n = 24). BILD 1633 SE more effectively reduced the mean lesion score (n = 24; P < 0.05 on days 8 to 24). The AUCs of the lesion scores are presented as means ± SEMs. *, P < 0.05 compared with the results for the vehicle groups.

Inhibition of HSV RR has previously been suggested as a potential strategy for therapy against both ACV-sensitive and ACV-resistant HSV infections (3, 13, 21, 26). However, a significant in vivo efficacy of specific RR inhibitors against ACV-resistant HSV infections has, to our knowledge, not been reported. Spector et al. (36, 39) have demonstrated the in vitro and limited in vivo antiviral effects of thiosemicarbazone derivatives, a series of compounds that disrupt the integrity of the free radical and/or iron center of the small subunit of the enzyme. Since the human RR also contains similar chemical features, those compounds do not appear to be very selective against HSV RR (26, 36). In addition, the in vivo efficacy of this class of RR inhibitors seems to be limited to potentiating the effects of ACV rather than acting as effective monotherapeutic antiviral agents (14, 28, 39). In a different study, Bridges et al. (6) have investigated the possibility of treating cutaneous HSV infections with an inhibitor of mammalian RR, MDL 101731. Although those investigators demonstrated the potent in vitro activity of this compound against both ACV-sensitive and ACV-resistant HSV, they failed to observe in vivo activity against ACV-resistant HSV infections (6). The lack of efficacy of these agents as monotherapy has, in part, been attributed to poor tissue penetration and to the fact that the viral RR may be dispensable in these infections because of elevated deoxynucleoside triphosphate (dNTP) pools. Our results clearly demonstrate that specific HSV RR inhibitors are effective as monotherapy in the athymic nude mouse model, implying that the viral RR is important for pathogenesis in this model.

Studies of ACV in combination with RR inhibitors have been designed previously to increase the efficacy of ACV. A number of studies have demonstrated that decreasing the dGTP pool levels with RR inhibitors increases the apparent potency of ACV by increasing the ACV triphosphate/dGTP ratio (36–38). Mechanism-based synergy between an RR inhibitor and ACV is also supported by the demonstration that HSV RR null mutants are hypersensitive to ACV (10). As expected, the combination of ACV and BILD 1633 SE also synergistically inhibited the replication of wild-type HSV strains and the ACV-resistant strain dl/sptk. However, both drugs acted only in an additive manner against the polymerase mutant, ACV-resistant strain PAA5, consistent with previous observation with nonspecific RR inhibitors (28). The precise reason for the lack of synergy of BILD 1633 SE and ACV in vitro against the HSV-1 polymerase mutant PAA5 is unclear. It has been suggested that ACV-resistant polymerase mutants display lower affinities for normal dNTPs in their HSV DNA polymerase (19) than that in the wild-type virus. Therefore, a decrease in the dNTP concentration below the $K_{m}$ should occur at a lower concentration of an RR inhibitor and should enhance its antiviral effect. Because of this increased efficacy of BILD 1633 SE alone against this mutant virus, the synergistic effect of the combination may be less pronounced and is therefore difficult to observe experimentally in vitro. Nevertheless, the in vivo data indicate that combination therapy with ACV with BILD 1633 SE is more than additive against both ACV-resistant mutants. Thus, combination therapy may not have to be synergistic in vitro to achieve more than additive in vivo effects.

Although ACV has generally been successful in treating HSV infections, chronic therapy with this drug in patients who are immunocompromised due to AIDS, organ transplantation, or cancer chemotherapy leads to an increase in the viral burden and the possible emergence of resistance. It has recently been reported that ACV-resistant mutants are responsible for HSV pneumonia, progressive whitlow, meningoencephalitis, and mucocutaneous dissemination in AIDS patients (7, 8, 22–24, 34). The unfulfilled clinical needs call for continued investigation to identify novel therapeutic antiviral agents. In the present study, BILD 1633 SE reduced both
**REFERENCES**


