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Evaluation of a Peptidomimetic Ribonucleotide Reductase Inhibitor with a Murine Model of Herpes Simplex Virus Type 1 Ocular Disease

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The ribonucleotide reductase (RR) of herpes simplex virus type 1 (HSV-1) is an important virulence factor, being required for neurovirulence, ocular virulence, and reactivation from latency. The RR activity requires the association of two distinct homodimeric subunits, and the association of the subunits is inhibited in the presence of a peptide homologous to the carboxy terminus of the small subunit. A structural analog of the inhibitory peptide (BILD 1263) has been shown to inhibit the replication of HSV-1 at micromolar concentrations in vitro. We used a mouse model of HSV-1 ocular infection to determine the in vivo efficacy of topical BILD 1263. Treatment of HSV-1 KOS-infected mice resulted in significant reductions in the severity and incidence of stromal keratitis and corneal neovascularization. At higher concentrations (5%), BILD 1263 reduced the severity but not the incidence of blepharitis. Treatment with 5% BILD 1263 also reduced viral shedding from the cornea by 10- to 14-fold ($P < 0.001$). In uninfected mice treated with 5% BILD 1263, we found no evidence of corneal epithelial damage, conjunctivitis, or blepharitis, and histopathological studies revealed no changes in the corneas of these mice. These results show that the peptidomimetic RR inhibitor BILD 1263 is effective in preventing disease, has an antiviral effect in vivo, and has little or no toxicity.

Herpes simplex virus (HSV) encodes the enzyme ribonucleotide reductase (RR; EC 1.17.4.1), which is responsible for the conversion of ribonucleoside diphosphates into the corresponding deoxyribonucleotides (32). The functional enzyme is composed of two nonidentical subunits (2, 9, 12, 16, 30, 31). The large subunit (M_r, 140,000) is designated ICP6 (14) or R1, and the small subunit has a mass of 38,000 and is designated R2. The genes encoding both subunits of RR map between 0.562 and 0.597 map units, are translated from 3′ coterminal mRNAs of 5.0 and 1.2 kb, respectively, and are designated the UL39 and UL40 genes, respectively (1, 24, 26).

The viral RR enzyme is clearly a major virulence factor. Cameron et al. (6) were the first to report that the RR mutant ts1222 was avirulent when it was inoculated intracerebrally or intraperitoneally. Jacobson et al. (18) showed that the deletion mutant ICP6Δ grew poorly following corneal inoculation and could not reactivate from latency. More recently, we (5) and others (17, 34) showed that the viral RR is required for ocular virulence.

Fully functional RR requires the association of the R1 and R2 subunits into a complex consisting of two large and two small subunits (32). Dutia et al. (10) and Cohen et al. (7) reported that a synthetic nonapeptide corresponding to the carboxy terminus of the small subunit could inhibit RR activity. It was subsequently shown that the nonapeptide bound to the R1 subunit and inhibited binding of the R2 subunit to form the active holoenzyme (27). However, the nonapeptide was not effective in inhibiting RR in intact cells. Liuzzi et al. (23) recently reported the inhibitory activity of a peptidomimetic compound, BILD 1263, corresponding in structure to the carboxy-terminal 6-amino-acid residues of the R2 subunit (Fig. 1). This compound had a 50% inhibitory concentration in enzyme binding assays of 0.3 nM, was active against HSV in infected cells, with 50% inhibitory concentrations of 3 to 4 μM, and was selective for the viral enzyme.

In this report, we present the results of more extensive in vivo studies on the effectiveness of BILD 1263 with our mouse model of HSV type 1 (HSV-1) ocular disease (3). We show that treatment with BILD 1263 significantly reduced both the severity and the incidence of corneal vascularization and stromal keratitis and that BILD 1263 has significant antiviral activity in vivo. These results suggest that peptidomimetic enzyme subunit association inhibitors of HSV-1 RR may be useful clinically.

MATERIALS AND METHODS

Cell culture and virus. African green monkey kidney cells (Vero cells) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5.0% serum (1:1 [vol/vol] mixture of fetal bovine serum and defined supplemented calf serum; Hyclone, Ogden, Utah), 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml. Cells infected with virus were grown in DMEM with 2% serum. Cells were grown at 37°C in an atmosphere of 5% CO_2. Our laboratory strain of HSV-1 KOS, which, as we have shown previously, causes severe ocular disease but is nonneurovirulent when it is inoculated peripherally (13), was used for all studies. High-titer viral stocks were prepared as we have described previously (3).

Animal inoculation and disease scoring. Four- to 5-week-old female BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were used for all studies. Mice were anesthetized by inhalation of halothane. While under anesthesia, the right cornea was scratched three times vertically and three times horizontally with a sterile 30-gauge needle. A 5-μl drop of DMEM (2% serum) containing 10^6 PFU of HSV-1 KOS was placed on the damaged cornea and was left in place for 30 s. Excess inoculum was removed by adsorption with a sterile swab, and the mice were returned to their cages.

The mice were examined microscopically for ocular disease by using a scoring

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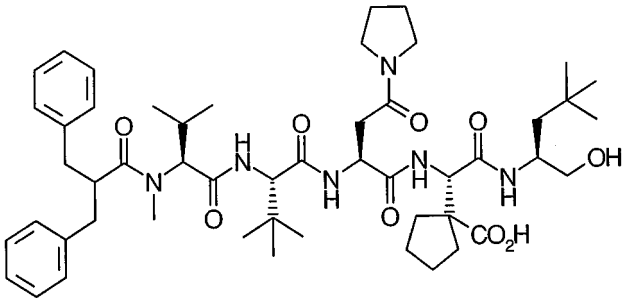

FIG. 1. Structure of BILD 1263.

system that we have described previously (3). Briefly, blepharitis (eyelid inflammation) was scored as follows: 1+, noticeably puffy eyelids; 2+, puffy eyelids with moderate crusting; 3+, eye swollen 50% shut with severe crusting; and 4+, eye totally swollen and crusted shut. Vascularization was scored as follows: 1+, <25% of the cornea involved; 2+, 25 to 50% of the cornea involved; and 3+, >50% of the cornea involved. Stromal keratitis was scored as follows: 1+, cloudiness, some iris detail visible; 2+, iris detail obscured; 3+, cornea totally opaque; and 4+, cornea perforated. A total of 12 mice were used in each group for the scoring of ocular disease. This model is not reliable for scoring epithelial keratitis because of difficulties in distinguishing virus-induced damage from the scarification during infection. The corneas were therefore not stained with fluorescein for scoring ocular disease. The creams were supplied in coded vials, and disease was scored in a masked fashion.

Treatment protocol. The mice were treated six times per day for the first 3 days, beginning 3 to 4 h postinfection (p.i.), and then four times per day for the next 4 days. For mice receiving six treatments per day, treatments were given at 9:00 a.m., 12:00 p.m., 3:00 p.m., 6:00 p.m., 9:00 p.m., and midnight. For mice receiving four treatments per day, treatments were given at 9:00 a.m., 1:00 p.m., 5:00 p.m., and 9:00 p.m. The compound was delivered in a cream consisting of 15% H_2O, 15% white mineral oil, and 70% Aquaphor (Smith and Nephew, Lachine, Quebec, Canada). The compound was ground in the oil and was then mixed with the other ingredients to achieve the final desired concentration (wt/wt). For treatment, the mice were anesthetized by inhalation of halothane, and the cream was applied with a sterile micropipette tip to cover the cornea.

Viral shedding. Mice were infected and treated as described above with either placebo cream or 5% BILD 1263 cream, and on days 1, 2, 3, 6, and 10 p.i., the corneas were washed with 10 μl of DMEM with 2% serum. Samples for virus titer determinations were collected in the morning prior to the onset of treatment to minimize the carryover of BILD 1263 to the titer plates. The washes were transferred to tubes containing 100 μl of medium, and the tubes were stored at −80°C. When all samples had been collected, the amount of infectious virus was determined by plaque assay on Vero cell monolayers. A total of 10 mice were assayed at each time point for each group.

Latency. Mice were infected and treated with 5% BILD 1263 as described above and were then held until 30 days p.i. to allow for the establishment of latency. The trigeminal ganglia were then aseptically removed. Half of the samples were minced, placed in cultures containing Vero cell monolayers, and monitored every other day for 2 weeks for evidence of an HSV-1-induced cytopathic effect. The remaining tissues were disrupted, frozen and thawed three times, serially diluted, and plated on Vero cell monolayers to determine the titer of infectious virus.

Statistical analysis. Analysis of variance was carried out by using Minitab for Windows, release 9.2 (Minitab, Inc., State College, Pa.). Fishers protected least significant difference test was used for pairwise comparisons.

Histopathology. Mice were anesthetized by inhalation of halothane, and the eyes were photographed. The mice were then sacrificed, and the eyes were enucleated and fixed in 10% formalin in phosphate-buffered saline. The eyes were then embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Analyses were carried out on normal eyes, placebo-treated infected eyes, untreated infected eyes, infected eyes treated with 5% BILD 1263, and uninfected eyes treated with 5% BILD 1263. Most eyes were collected at 15 days p.i. to allow for the full development of pathology; however, the eyes used for the analysis of toxicity were collected at 12 h after the last treatment. A total of 10 corneas were examined for each group.

Toxicity. Uninfected mice were treated with 5% BILD 1263 by the protocol described above. At 12 h after the last treatment, the corneas were stained with 1% fluorescein and were examined with a cobalt blue light source for evidence of punctate keratopathy, ulceration, or other epithelial defects. A total of 10 mice were examined.

RESULTS

Effect of treatment on ocular disease. To determine if treatment with BILD 1263 was effective against ocular disease, mice were infected and treated with placebo or 0.1, 1.0, or 5.0% BILD 1263. The results are shown in Fig. 2A to C. Disease development in the placebo-treated group was similar to that observed in untreated infected mice (data not shown). Blepharitis was evident by day 4 in all groups, with scores of approximately 1 (Fig. 2A). Blepharitis continued to increase in the

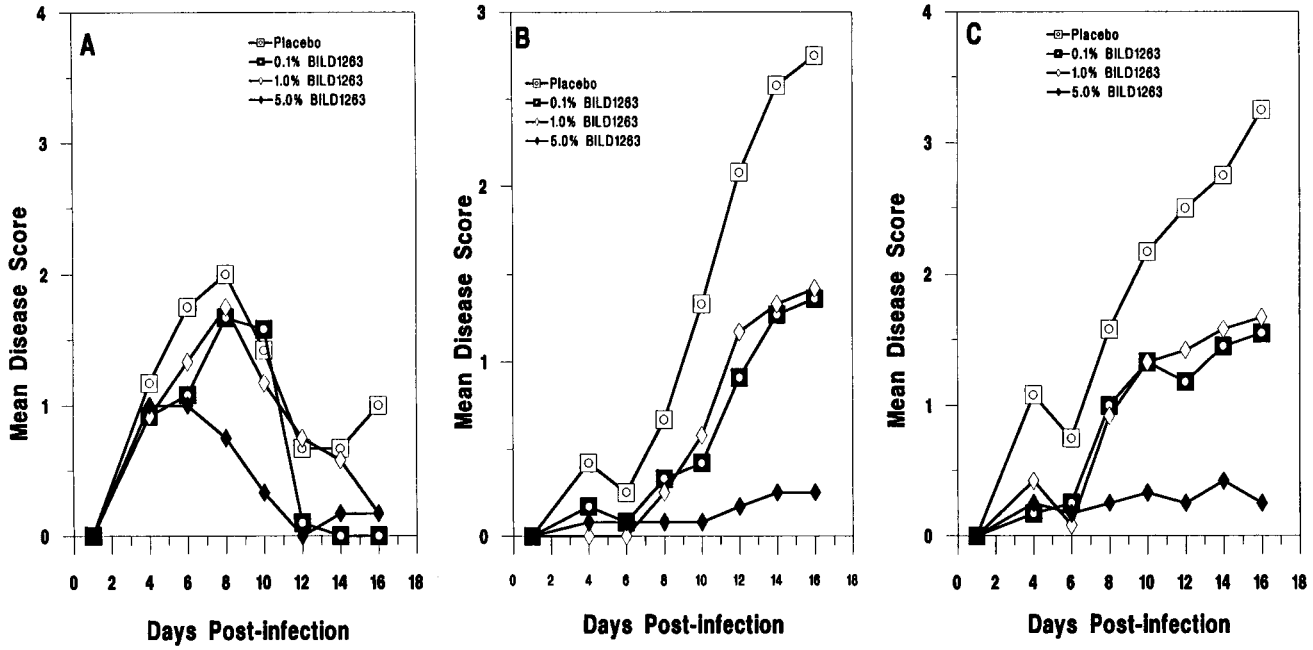

FIG. 2. Effect of BILD 1263 treatment on the severity of HSV-1 ocular disease. Groups of 12 mice each were infected and treated with placebo or 0.1, 1.0, or 5.0% BILD 1263 creams, and the severities of blepharitis (A), vascularization of the cornea (B), and stromal keratitis (C) were scored at various times. Each datum point represents the mean of the individual scores.

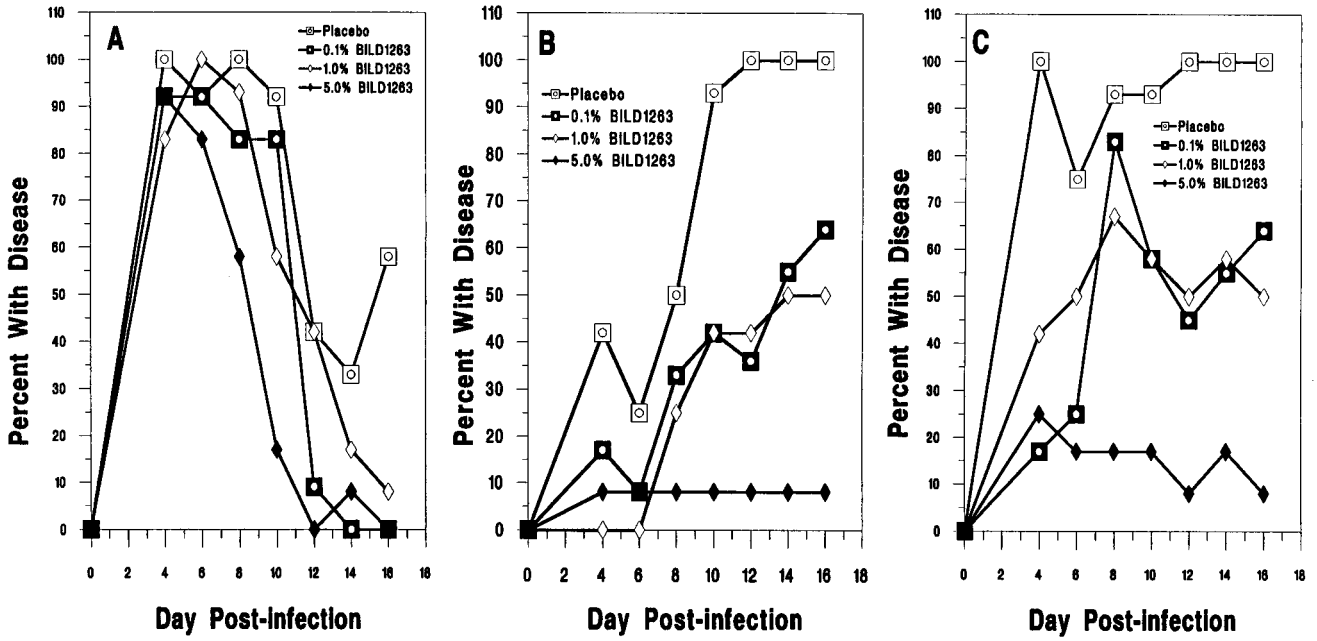

FIG. 3. Effect of BILD 1263 treatment on the incidence of HSV-1 ocular disease. Groups of 12 mice each were infected and treated with cream containing 0, 0.1, 1.0, or 5.0% BILD 1263, and the percentages of mice developing blepharitis (A), corneal vascularization (B), or stromal disease (C) were determined on the indicated days.

groups treated with placebo and 0.1 and 1.0% BILD 1263, peaking on day 8 with scores of approximately 1.75 to 2.2. In the group treated with 5.0% BILD 1263, blepharitis peaked on days 4 and 6 (score, 1.0) and then began to heal. By day 12, blepharitis had essentially healed in the group treated with 5.0% BILD 1263. Healing was evident in the other groups after day 8. Blepharitis healed completely by day 14 in the group treated with 1.0% BILD 1263 and had nearly healed in the group treated with 0.1% BILD 1263 by day 16, when scoring was halted. In the group treated with placebo cream, blepharitis did not heal completely.

Corneal neovascularization was evident by days 4 to 6 in all groups (Fig. 2B). In the group treated with placebo, vascularization continued to increase in severity, reaching a score of 2.75 on day 16, when scoring was halted. In the groups treated with 0.1 and 1.0% BILD 1263, vascularization also increased, but it peaked on day 16 at a score of 1.4 to 1.5, substantially lower than that in the placebo-treated group. In the group treated with 5.0% BILD 1263, vascularization plateaued between days 4 and 10 at a score of 0.1 and then increased to a score of 0.4 on days 14 and 16.

The development of stromal keratitis paralleled vascularization, with corneal clouding evident by day 4 (Fig. 2C). In the placebo-treated group, stromal disease increased in severity, reaching a score of 3.3 on day 16, when scoring was halted. In the groups treated with 0.1 and 1.0% BILD 1263, stromal disease increased in severity, but the disease was reduced compared with that in the placebo group. On day 16, the stromal disease score was 1.6 to 1.75 for the groups treated with 0.1 and 1.0% BILD 1263, whereas it was 3.3 for the group treated with placebo. Stromal disease increased only slightly after day 4 in the group treated with 5.0% BILD 1263, peaking at a score of about 0.25 to 0.4.

Effect on disease incidence. Treatment with BILD 1263 had very little effect on reducing the incidence of blepharitis (Fig. 3A). In the groups treated with placebo and 0.1 and 1.0% BILD 1263, 100% of the mice developed disease. In the group treated with 5% BILD 1263, all but one of the mice developed blepharitis (92%). Peak incidence was reached on day 4 or 6 for all groups. The incidence declined after day 4 in the group treated with 5.0% BILD 1263, reaching a score of 0 on day 12. The decline in the incidence of blepharitis in the other groups was similar and paralleled the disease severity.

For vascularization, BILD 1263 reduced the incidence from 100% in the placebo group to between 50 and 65% in the groups treated with 0.1 and 1.0% BILD 1263 (Fig. 3B). In the group treated with 5.0% BILD 1263, only one mouse (8%) developed vascularization. Treatment reduced the incidence of stromal disease from 100% in the placebo group to approximately 60% in the groups treated with 0.1 and 1.0% BILD 1263 (Fig. 3C). In the group treated with 5.0% BILD 1263, only 25% of the mice developed any evidence of corneal clouding, and the clouding was transient in some of these mice. By day 16, only one mouse (8%) had permanent corneal clouding.

MPDSs. We previously developed a parameter termed mean peak disease score (MPDS) to facilitate the comparison of disease scores between multiple groups (13). The MPDSs for the results are shown in Fig. 4. Statistical analysis of the MPDSs revealed that treatment with 0.1 and 1.0% BILD 1263 did not significantly reduce blepharitis ($P = 0.07$ and 0.1, respectively). However, treatment with 5.0% BILD 1263 had a significant effect ($P = 0.0006$). Treatment with BILD 1263 significantly reduced the MPDSs for vascularization, with P values of 0.002 (0.1% BILD 1263), 0.016 (1.0% BILD 1263), and 2.6×10^{-6} (5.0% BILD 1263). Treatment also significantly reduced stromal disease, with P values of 0.002 (0.1% BILD 1263), 0.03 (1.0% BILD 1263), and 3.4×10^{-6} (5.0% BILD 1263).

BILD 1263 reduced viral shedding from the cornea. To determine if treatment with BILD 1263 had an in vivo antiviral effect, mice were infected and treated with 5.0% BILD 1263 or placebo cream, and the amount of infectious virus shed from the cornea was measured. The results are presented in Fig. 5. Viral shedding from the eyes of the group treated with placebo

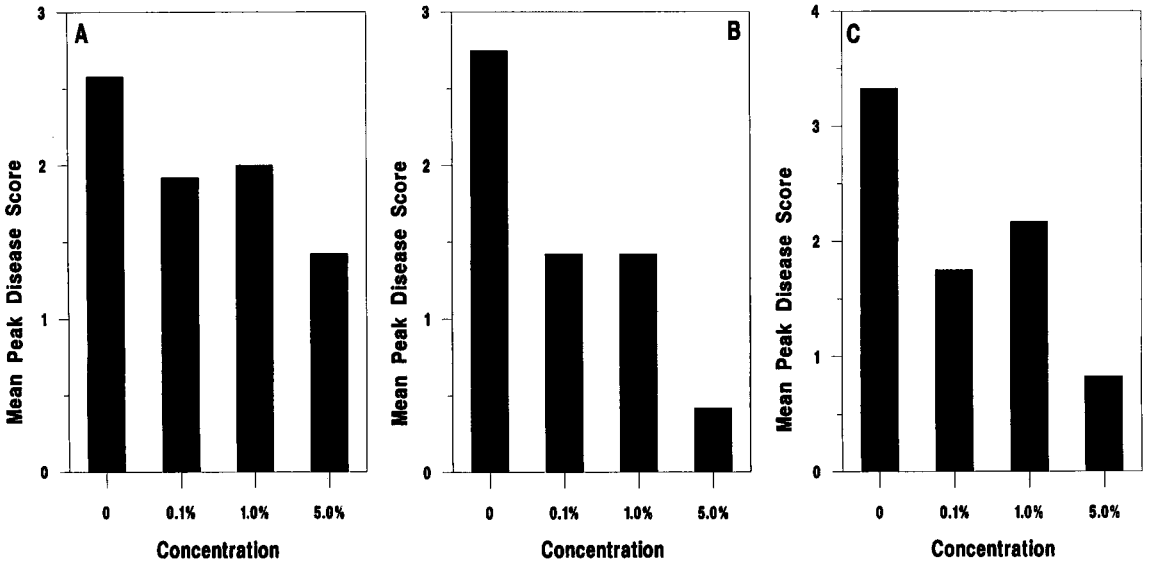

FIG. 4. MPDSs. The most severe disease scores for each mouse were averaged, and the means were plotted. (A) Blepharitis; (B) vascularization; (C) stromal keratitis. Statistical analysis (analysis of variance) of the MPDS was then carried out.

peaked at 4.45×10^4 PFU per eye on day 2 p.i. Viral shedding then declined in the placebo group, and by day 10, no infectious virus was recovered. In the mice treated with 5% BILD 1263, viral shedding peaked on day 1 at 3.95×10^3 PFU per eye, an 11-fold reduction in titer. Viral shedding from the treated eyes declined after day 2 and cleared by day 10. At all times tested, shedding from the 5.0% BILD 1263-treated eyes was 10- to 14-fold lower than that from the placebo-treated eyes. Statistical analysis of the data revealed that on all days tested, the titers in the group treated with 5.0% BILD 1263 were significantly lower ($P < 0.001$) than those in the placebo group.

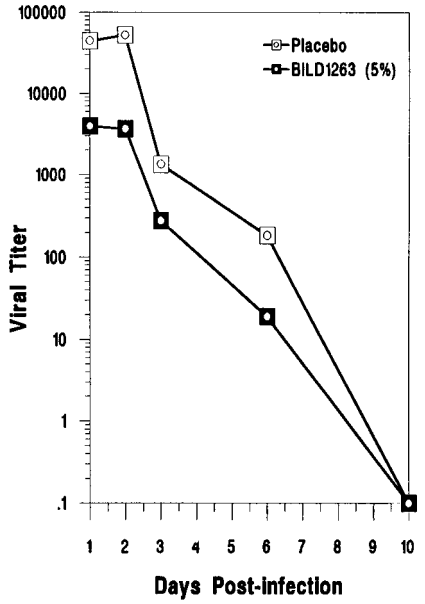

FIG. 5. Effect of treatment on viral shedding from the cornea. Infected mice were treated with 5.0% BILD 1263, and at various times p.i., the corneas were lavaged and the amount of infectious virus was determined by plaque assay on Vero cells.

Effect on latency. The mice treated with placebo and 5.0% BILD 1263 were held until 30 days p.i. The mice were then sacrificed, and the trigeminal ganglia were removed and then the titers of latent virus were determined. None of the samples whose virus titers were determined directly had infectious virus. Of six samples tested for reactivation, six of six (100%) of the placebo-treated and five of six (83%) of the BILD 1263-treated samples showed evidence of reactivation.

Histopathology of corneas. Figure 6 shows representative examples of corneal sections from infected placebo-treated (Fig. 6A) and infected 5% BILD 1263-treated eyes (Fig. 6B) at 15 days p.i. In the infected placebo-treated eyes (Fig. 6A), the cornea was substantially thicker and edematous. The epithelium showed evidence of necrosis and infiltration by polymorphonuclear leukocytes. Intranuclear inclusion bodies typical of HSV-1 infection were also present. There was a stromal infiltrate consisting primarily of polymorphonuclear leukocytes and epitheloid cells. The corneal stroma was vascularized, showing signs of an acute and chronic interstitial keratitis typical of HSV-1 corneal infection. The corneas from infected mice treated with 5% BILD 1263 (Fig. 6B) were normal, with no evidence of inflammation, necrosis, or cells containing inclusion bodies.

In vivo toxicity of BILD 1263. To determine if BILD 1263 was toxic, uninfected mice (10 mice per group) were treated with either placebo cream or 5% BILD 1263, and at the end of the treatment period, the corneas were stained with fluorescein and examined for the presence of epithelial defects. No evidence of corneal damage (e.g., punctate keratopathy) was seen in either placebo- or 5.0% BILD 1263-treated mice (data not shown). Histopathological analysis of the corneas was also carried out, and we found no discernible differences between BILD 1263-treated and normal corneas (data not shown).

DISCUSSION

Previous studies demonstrated that the HSV-1 RR gene is required for virulence (5, 6, 17, 18, 34). It was also shown previously that a peptide consisting of the carboxy-terminal 9 amino acids of the R2 subunit could block the assembly of the

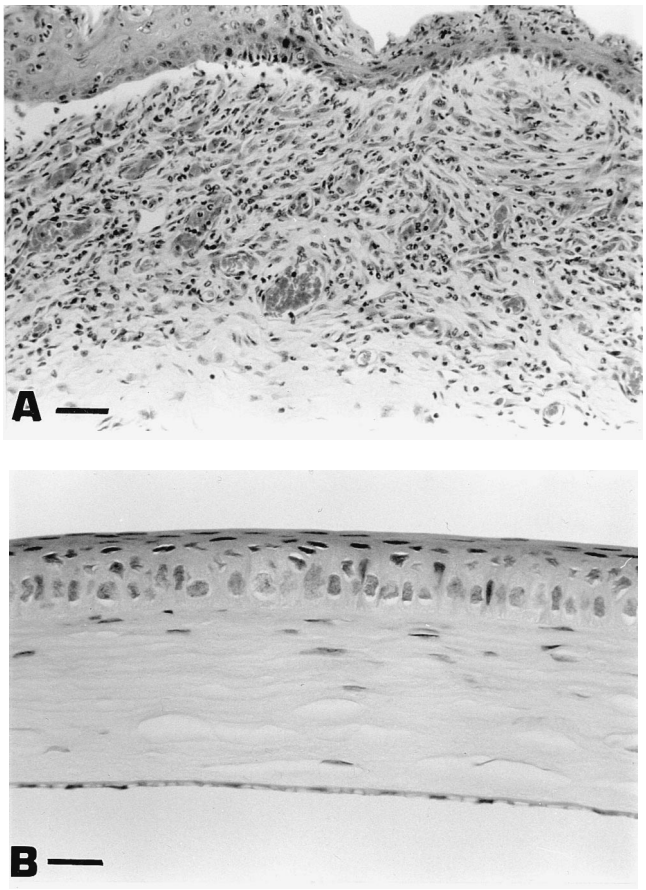

FIG. 6. Histopathological analysis of corneas. (A) Infected, placebo-treated cornea. (B) Infected mouse treated with 5.0% BILD 1263. Bars, 25 μm.

holoenzyme and could subsequently inhibit enzymatic activity (7, 10). When considered together, these results strongly suggest that a peptidomimetic analog of the carboxy-terminal amino acids of the R2 subunit might be an effective antiviral. Liuzzi et al. (23) recently showed that a peptidomimetic compound (BILD 1263) inhibits HSV-1 replication in cultured cells at micromolar concentrations. They also showed that viruses with mutations in thymidine kinase and DNA polymerase could be inhibited by BILD 1263, suggesting that viruses resistant to nucleoside analogs could be inhibited. The results presented in this report provide a detailed analysis of the in vivo effectiveness of BILD 1263 with our previously described murine model (3).

When mice were infected with HSV-1 KOS and treated with various concentrations of BILD 1263, the severity of ocular disease, as measured by scoring blepharitis, corneal vascularization, and stromal keratitis, was reduced in a dose-dependent manner. Even concentrations of 1.0 and 0.1% BILD 1263 significantly reduced the severity of vascularization and stromal disease. Reductions in the severity of blepharitis were also seen with BILD 1263, although only the 5.0% cream had a significant effect. The reduced effectiveness against blepharitis may be explained by the fact that the cream was placed on the cornea and effective concentrations may not have been present in the eyelids. We have previously shown that low concentrations of antiviral agents have less effect on blepharitis than on vascularization or stromal disease (3, 4). Treatment with BILD 1263 also reduced the incidence of corneal vascularization and stromal disease. Although BILD 1263 did not reduce the incidence of blepharitis, it did enhance healing, particularly in the group treated with the 5.0% BILD 1263 cream.

Viral shedding from the cornea was significantly reduced 10- to 14-fold on each day that titers were measured in mice treated with 5.0% BILD 1263, indicating that the compound had an antiviral effect in vivo. In our previous study showing that the viral RR was required for ocular virulence (5), we found that peak titers in the tissues of mice infected with the RR mutant ICP6Δ were reduced 10- to 20-fold, which is within the range that we found with BILD 1263. Although these results suggest that BILD 1263 has approximately the same effect as deletion of the RR1 gene, the results are not directly comparable since titers in tissue, not shedding, were measured in the previous study and we used a 10-fold higher inoculum (10^6 PFU) in the present study.

Our results indicate that significant reductions in disease severity can be achieved, even though viral replication is not completely inhibited. This conclusion is supported by previous studies. In our study with the ICP6Δ virus, replication was reduced and clearance was enhanced (5). In a separate study, treatment with trifluorothymidine (TFT) did not reduce peak titers in tissue but did enhance viral clearance (3), and in a previous study with a phosphorothioate oligonucleotide to the HSV-1 UL13 gene, the mice failed to develop stromal disease, even though viral shedding was reduced only approximately 10-fold yet (4; unpublished data). More recently, we showed that reducing the amount of inoculum has a significant effect on the incidence of HSV-1 ocular infections (21). When considered together, these studies suggest that reducing viral replication 10-fold can significantly affect disease, at least with animal models, and that it is not essential that viral replication be eliminated.

Three previous studies with nucleoside analogs have shown that treatment begun within 4 h of infection can reduce or prevent the establishment of latent infections (3, 11, 22). We found that treatment with 5.0% BILD 1263 did not significantly effect the establishment of latency. However, since the viral RR is required for reactivation from the latent state (3, 18), inhibitors may be useful for prophylactic treatment to prevent reactivation.

With the exception of acyclovir, many of the currently available nucleoside analog antiviral agents exhibit toxicity even when they are used topically (8, 15, 25, 28, 29, 33, 35). Liuzzi et al. (23) reported a selectivity (therapeutic) index of 30 for BILD 1263 in BHK-21 cells. In the present study we have shown that the application of 5.0% BILD 1263 for 7 consecutive days (34 treatments) did not cause punctate keratopathy, conjunctivitis, or blepharitis, indicating that it has little if any toxicity, at least when it is applied topically, which is consistent with the specificity that BILD 1263 has for the viral enzyme compared with that for the host cell RR (23). Histopathological examination of treated corneas also revealed no evidence of toxicity with 5.0% BILD 1263. However, the toxicity studies involved small numbers of mice, and more extensive work might reveal some reaction to the compound.

In our initial characterization of the mouse model, we used commercially available TFT (Viroptic) at doses ranging from 0.01 to 1.0% and showed a dose-dependent effect on eye disease (3). We then calculated the dose that reduced the severity of ocular disease by 50% (the 50% effective dose) and found that it ranged from 0.007 to 0.023%, depending on the disease characteristic. Using similar calculations, we found that the 50% effective doses of BILD 1263 ranged from 0.35 to 1.8% (data not shown). When corrected for the difference in molecular weight, TFT is approximately sevenfold more effective

than BILD 1263. There are a number of caveats in comparing these compounds, however. Both TFT and BILD 1263 give nonlinear dose-response curves (3) (Fig. 5). Both compounds represent completely different types of compounds, and any comparison between nucleoside analogs and peptidomimetic compounds is suspect. Nucleoside analogs and peptidomimetic compounds act on different targets and at different sites in cells and very likely have different pharmacokinetic properties. Pharmacokinetic studies have not been done for BILD 1263, so the actual concentration of BILD 1263 in the eye at any dose is unknown, nor is the turnover of BILD 1263 understood. It is thus impossible to correct or account for these differences. Even if we had tested TFT and BILD 1263 side by side, the comparison would not account for the differences, and it is questionable whether the data would be worth the sacrifice of additional animals. Although we believe that direct comparisons are suspect, the data suggest that TFT has greater activity than BILD 1263. Without pharmacokinetic data, we could only speculate as to the reasons for the nonlinear dose-response seen with BILD 1263.

The testing of idoxuridine and TFT was initially done with rabbit ocular models of HSV-1 keratitis (19, 20). However, rabbits are expensive, and their size requires substantial amounts of test compound. These factors combine to increase the cost of animal testing and affect the number of in vivo studies that can be done. Our mouse model has the advantage of requiring 5 to 10 times less compound on a per-animal basis. In our development of the mouse model, we tested TFT (3) and compared the results obtained with mice with previously published results obtained with rabbits. That comparison showed that the 50% effective dose of TFT with the mouse model ranged from 0.007 to 0.023%, compared with values of 0.0075 to 0.035% with the rabbit models. On the basis of this comparison, the mouse model appears to be equivalent to the rabbit model.

In summary, our studies have shown that the peptidomimetic RR inhibitor BILD 1263 is effective at reducing the severity and incidence of HSV ocular disease. Viral shedding is also reduced by BILD 1263 treatment, indicating an in vivo antiviral effect. Additionally, we have shown that BILD 1263 has little or no toxicity when it is applied topically to the eye. These studies provide further support of the peptidomimetic approach for antiviral agents and provide the basis for further preclinical and clinical studies with BILD 1263.

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