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Isolation and characterization of herpes simplex virus type 1 resistant to aminothiazolylphenyl-based inhibitors of the viral helicase-primase

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Abstract

The aminothiazolylphenyl-containing compounds BILS 179 BS and BILS 45 BS are novel inhibitors of the herpes simplex virus helicase-primase with antiviral activity in vitro and in animal models of HSV disease. To verify the mechanism of antiviral action, resistant viruses were selected by serial passage or by single-step plaque selection of HSV-1 KOS in the presence of inhibitors. Three resistant isolates K138^{r}3, K22^{r}5, and K22^{r}1 were found to be 38-, 316-, and 2500-fold resistant to BILS 22 BS, a potent analog of BILS 45 BS. All three viruses had growth properties in vitro similar to wild-type HSV-1 KOS but they were sensitive to acyclovir. Cutaneous and intra-cerebral inoculation of mice with K22^{r}1 or K22^{r}5 resulted in pathogenicity equivalent to that of HSV-1 KOS. Both isolates were fully competent for reactivation from latency following corneal inoculation. Helicase-primase purified from cells infected with resistant viruses showed decreased inhibition in an in vitro DNA-dependent ATPase assay that correlated well with antiviral resistance. Marker transfer experiments and DNA sequence analysis identified single base pair mutations clustered in the N-terminus of the UL5 gene that resulted in single amino acid changes in the UL5 protein. Taken together, the results indicate that helicase-primase inhibitors prevent HSV growth by inhibiting HSV helicase-primase through specific interaction with the UL5 protein.

Keywords: HSV; Helicase-primase; Aminothiazolylphenyl; Resistance; Antiviral

1. Introduction

The herpes simplex virus (HSV) type 1 encodes a heterotrimeric helicase-primase that is composed of the UL5, UL8, and UL52 gene products with DNA helicase, DNA-dependent ATPase and DNA primase activity (Crute et al., 1989; Crute and Lehman, 1991). All three subunits of the complex are required for viral DNA replication in vivo and are essential for virus growth (Zhu and Weller, 1992a; Goldstein and Weller, 1988; Carmichael and Weller, 1989). UL5 contains seven motifs conserved among superfamily I helicases (Zhu and Weller, 1992b) and the primase active center is localized within the UL52 subunit (Dracheva et al., 1995). UL8 modulates these activities, facilitates nuclear uptake of the complex and interacts with other DNA replication proteins (Tenney et al., 1993; Marsden et al., 1996, 1997).

We reported previously on novel aminothiazolylphenyl-containing inhibitors of the HSV helicase-primase (Crute et al., 2002). As exemplified by BILS 179 BS and BILS 45 BS, these compounds inhibit all three enzymatic activities of the helicase-primase and exhibit potent antiviral activity in cell culture and in several animal models of HSV disease (Crute et al., 2002; Duan et al., 2003). Here, we describe the isolation and characterization of HSV-1 mutants resistant to helicase-primase inhibitors. Antiviral resistance was mediated by single amino acid substitutions near the N-terminus of the UL5 protein. These amino acid changes resulted in viral mutants that exhibited in vitro and in vivo replication and pathogenicity profiles similar to that of the parental strain HSV-1 KOS. Combined, these results demonstrate that mutations within UL5 confer resistance to aminothiazolylphenyl-based inhibitors and confirm that helicase-primase is the target of antiviral action.

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2. Materials and methods

2.1. Cell culture, viruses, and animals

Cell culture reagents and media were obtained from Gibco BRL (Burlington, ON). Cells were from American Type Culture Collection (ATCC) (Rockville, MD). Vero cells (African green monkey kidney cells) (ATCC CCL81) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml kanamycin sulfate. Baby hamster kidney (BHK)-21/C13 (ATCC CCL10) cells were grown in α-MEM medium instead of DMEM medium. All cells were grown at 37 °C in an atmosphere of 5% CO_2. HSV-1 KOS and ICP6Δ have been described previously (Jacobson et al., 1989; Duan et al., 2003). Virus stocks were routinely grown in Vero cells and virus titers were determined by plaque assay on confluent Vero cells as described below. Balb/c mice were obtained from Charles River Canada Inc. (St-Constant, PQ, Canada). SKH-1 hairless mice were obtained from a breeding colony maintained at Boehringer Ingelheim (Canada) Ltd., Research and Development. All experiments involving animals were conducted according to protocols approved by an Institutional Animal Care Committee and the Canadian Council on Animal Care.

2.2. Reagents and antiviral compounds

[α-^{32}P]GTP (specific activity, 3000 Ci/mmol) was purchased from Dupont NEN Research Products (Boston, MS). Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and acyclovir (ACV) were purchased from Sigma (St. Louis, MO). BILS 103 BS and BILS 45 BS were synthesized in-house according to protocols described previously (Crute et al., 2002; Duan et al., 2003). The synthesis of BILS 22 BS and BILS 138 BS will be described elsewhere. The chemical structures of the new inhibitors used in this study are shown in Fig. 1. All compounds were dissolved in DMSO and then diluted with cell culture medium to yield 1% final DMSO. All stock compound solutions were filter sterilized through 0.22 μm Millex-GV filters (Millipore, Bedford, MA).

2.3. Antiviral assays

The antiviral activity of compounds was determined with an ELISA or a plaque reduction assay (Duan et al., 2003). For the ELISA, BHK-21/C13 cells were seeded in 96-well culture plates (Corning, Cambridge, MA) at a density of 3000 cells per well in α-MEM medium and incubated to reach 90% confluency. Cells were infected with wild-type or resistant HSV-1 KOS at a multiplicity of infection (MOI) of 0.1. After 1 h, the HSV-infected cells were rinsed with cell culture medium and then incubated for 24 h in α-MEM medium containing 2% (v/v) FBS and increasing concentrations of test compounds. Then the cells were fixed and the extent of replication was assessed by ELISA as described previously (Lawetz and Liuzzi, 1998). In all cases, compounds were tested in three-fold serial dilutions and effective concentrations to achieve 50% inhibition of virus replication (i.e., EC_{50}) were determined from dose–response curves using SAS software (SAS Institute, Cary, NC). A non-linear regression analysis based on the Hill equation was applied to the percent inhibition versus concentration data. The cytotoxicity of compounds for BHK cells was determined with the modified tetrazolium MTT assay described previously (Lawetz and Liuzzi, 1998) under the same cell culture conditions but in the absence of infection.

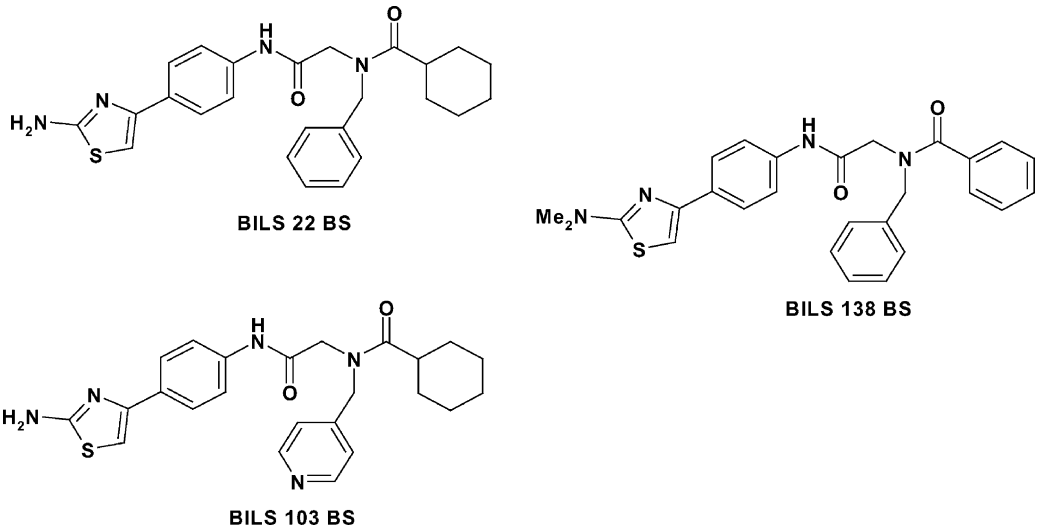

Fig. 1. Molecular structures of aminothiazolylphenyl-based inhibitors of HSV helicase-primase. In BILS 138 BS, Me indicates methylation of the exocyclic nitrogen.

2.4. Selection of resistant viruses

BHK-21/C13 cells were seeded in 150 cm^2 culture plates (Corning, Cambridge, MA) and infected with HSV-1 KOS at an MOI of 1. The infections were allowed to proceed in the presence of inhibitors until the infected cells exhibited 90% cytopathic effect (approximately 48 h). The viral pools from each passage were harvested, titered and used to infect cells for the next passage. This process was repeated eight times in the absence of drug and in the presence of 1 μM BILS 22 BS or 4 μM ACV. From passage eight viral pools, two HSV-1 isolates resistant to BILS 22 BS ($K22^r1$ and $K22^r5$) were plaque purified three times on Vero cells in the presence of inhibitor. Alternatively, 10^6 plaque forming units (pfu) of HSV-1 KOS were plated directly on Vero cells in the presence of 1 μM BILS 138 BS. Resistant plaques were picked and plaque purified as described above. This yielded the resistant HSV-1 clone $K138^r3$.

2.5. Marker transfer experiments and DNA sequencing

Infectious HSV DNA was isolated from Vero cells infected with HSV-1 KOS, $K22^r1$, and $K22^r5$ as described previously (Bonneau et al., 1996). The purified DNA from resistant HSV-1 isolates was used to PCR amplify the UL5, UL52, and UL8 genes by employing the following pairs of oligonucleotides: UL5, CGGGTTCGGTGGAACTGT and GATCGTCATCGACGAGGCCG; UL8, CAACCCCAACGCTGACATCATCCT and ACCCCGCCGGGATCATGGGGAGCTG; UL52, CGCATCTTGTCCTGATGGGCCGCCA and CGTGTGTACCATAGGGTCTCCAGA. The PCR DNA fragments were run on 1.5% agarose gel and purified using a QIAEX gel extraction kit (Qiagen, Valencia, CA). The purified DNA fragments were then co-transfected into Vero cells along with infectious HSV-1 KOS DNA by $CaPO_4$ co-precipitation (Bonneau et al., 1996). Transfected cells were incubated for 5–7 days in the absence of inhibitor until the maximum cytopathic effect was achieved. Then, the progeny virus was harvested and plated on Vero cells in the presence or absence of 1 μM BILS 22 BS to determine the number and the frequency of resistant plaques. Subsequent experiments followed the same protocol as above but used PCR-amplified DNA corresponding to the following regions of the HSV-1 genome: 13680–14399 and 14400–15458. These correspond to the N-terminal and C-terminal halves of the UL5 gene, respectively. The PCR-amplified DNA fragments from the genetic mapping experiments corresponding to nucleotides 13680–14399 of the HSV-1 genome (N-terminus of the UL5 gene) were sequenced and nucleotide changes were identified by comparison with wild-type HSV-1 sequences.

2.6. Single-step growth kinetics

Confluent BHK cells in 24-well culture plates were mock-infected or infected with wild-type HSV-1 KOS or with the resistant HSV-1 clones $K22^r1$ and $K22^r5$ at a MOI of 10 in α-MEM containing 2% heat inactivated dialyzed FBS. After 1 h of virus adsorption, the infected cells were washed once with fresh medium and then incubated at 37 °C for various time periods. Viral yields at each time point were measured by a plaque assay on Vero cells as described above.

2.7. Neurovirulence studies in mice

Balb/c mice were anesthetized with halothane and inoculated by direct injection of 20 μl of viral suspension in the right cerebral hemisphere using a 30-gauge needle inserted at a depth of 1–2 mm. Mortality was monitored daily over a 14-day period. For each virus, at least four different viral titers were studied. In each experimental protocol, groups of eight mice were inoculated at each titer. In these experiments, the HSV-1 KOS mutant ICP6Δ was used as a control for highly attenuated neurovirulence (Jacobson et al., 1989). The results shown represent the average of two different experimental protocols except for ICP6Δ. Fifty percent lethal dose (LD_{50}) values were determined from mortality versus inoculum plots.

2.8. Pathogenicity in the mouse eye model and reactivation from latent infection

Three- to four-week-old female Balb/c mice were used for these experiments. All animals were inspected microscopically prior to infection and only those animals showing no corneal defects were selected for the study. Mice were anesthetized by inhalation of 2.5% halothane. While under anesthesia, the right cornea was scratched three times vertically and three times horizontally using a sterile 30-gauge needle. A 5-μl drop of α-MEM with 2% FBS containing 10^6 pfu of virus was placed on the cornea and the eyes were massaged twice. Thirty seconds later, excess inoculum was removed with a cotton swab. In these experiments, 19 mice per group were infected with wild-type HSV-1 KOS or with the resistant isolates $K22^r1$ or $K22^r5$. As a negative control, mice were infected with the HSV-1 KOS-derived mutant ICP6Δ since it has been demonstrated not to reactivate from latency (Jacobson et al., 1989). All animals were examined for stromal keratitis 33 days post-inoculation using a previously described scoring system (Brandt et al., 1996). Subsequently, animals were sacrificed by cervical dislocation and the right trigeminal ganglia were removed and placed in ice-cold α-MEM. One half of the samples were homogenized, frozen and thawed three times, and titrated for infectious virus using a plaque assay on Vero cells. The remaining samples were minced and plated on Vero cells in α-MEM with 2% FBS and incubated at 37 °C. Every 2–3 days for 2 weeks, the cells were inspected for signs of virus-induced cytopathic effects indicative of reactivation from latency.

2.9. Pathogenicity studies in the cutaneous mouse model of HSV-1 infection

Four- to six-week-old female SKH-1 hairless mice were anesthetized with halothane and the stratum corneum of the dorsal flank on either side of the spinal column and midway in the thoracic region was punched with a twelve-needle device. The puncture sites were inoculated with 7.3×10^7 pfu of HSV-1 KOS or 3.4×10^7 pfu of the resistant isolate K22^{r}1. Virus-mediated pathology was quantitated daily as described elsewhere (Duan et al., 2003). The course of disease was measured for a period of 14 days with peak disease represented by the peak mean disease score and the overall extent of disease represented by the area under the curve (AUC) of the mean daily disease score versus days post-inoculation with virus. Eight animals were employed in each experimental group and the observer was not blinded to the treatment. Oral BILS 45 BS was evaluated using a 5-day treatment period commencing 3 h post-inoculation. The total daily dose of 100 mg/kg was administered in three equal doses per day by gavage and the vehicle for drug administration was 0.03N HCl.

2.10. Isolation of HSV helicase-primase from infected cells

Confluent BHK cells were infected with wild-type HSV-1 KOS or resistant HSV-1 strains at a MOI of 5 for 12 h. HSV-infected cells from 50 roller bottles were resuspended and lysed as described for the extraction of recombinant helicase-primase (Dracheva et al., 1995). The nuclear fraction was retained and resuspended in an equal volume of Buffer A (20 mM HEPES pH 7.5, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM Pefabloc SC, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol) containing 1 M NaCl. The highly viscous preparation was ultracentrifuged and the supernatant was dialyzed overnight at 4 °C against Buffer A containing 50 mM NaCl. For purification, the nuclear extract was injected onto a 6-ml Resource Q column and the proteins were eluted with a linear gradient from 0.05 to 0.8 M NaCl in Buffer A. Fractions at ∼400 mM NaCl containing helicase-primase were pooled, diluted to 100 mM NaCl with Buffer A and injected into a 5-ml HiTrap Heparin column. The column was eluted with a linear gradient from 0.1 to 1.0 M NaCl in Buffer A. Fractions at ∼300 mM NaCl containing helicase-primase were pooled and injected into a 124-ml Superdex S200 gel filtration column pre-equilibrated with Buffer A containing 300 mM NaCl. Fractions were assayed for DNA-dependent ATPase and peak fractions containing purified helicase-primase were pooled, aliquoted and stored at −80 °C.

2.11. Production of a baculovirus with the K356N mutation in the UL5 gene and isolation of recombinant helicase-primases

A background, wild-type UL5-containing baculovirus was constructed by PCR amplifying the UL5 gene and cloning it into the pBlue Bac 4 vector (InVitrogen, Groningen, The Netherlands). The N-terminus of the UL5 gene from the K22^{r}1 virus was amplified by PCR (HSV-1 genome positions 12861–14380) as described above. This PCR fragment and wild-type UL5 containing pBlue Bac 4 were digested with the restriction enzymes Pvu II and Sal 1. The resulting digests were purified on a 1.5% agarose gel and eluted using the QIAEX gel extraction kit. The K22^{r}1 fragment was then ligated into the digested pBlue Bac 4. The resulting shuttle vector was used to generate a recombinant baculovirus by homologous recombination as previously described (Summers and Smith, 1987). The recombinant baculovirus expressing the UL5 with a K356N mutation was referred to as BacUL5-K356N. The wild-type and mutated HSV-1 helicase-primase holoenzymes were expressed in Sf21 insect cells and purified to homogeneity as described elsewhere (Dracheva et al., 1995).

2.12. Helicase-primase biochemical assays

DNA helicase and DNA-dependent ATPase activities were measured as described previously (Crute et al., 2002). The reaction mixtures (80 μl) contained 40 mM HEPES pH 7.5, 10% glycerol, 5.5 mM $MgCl_2$, 1 mM DTT, 50 μg/ml acetylated BSA, 3.3% DMSO, 4 mM ATP, 100 μg/ml calf thymus DNA and enzyme. Primase activity was measured essentially as described before (Crute et al., 2002) except that the reaction mixtures contained 10 μM GTP and 10 μCi [α-^{32}P]GTP (specific activity 3000 Ci/mmol) and that the reactions were quenched by the addition of 10 units of calf intestinal phosphatase (Roche Diagnostics, Laval, PQ). Products of the primase reaction were separated by electrophoresis through 20% polyacrylamide gels containing 6 M urea and the primers were visualized by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). When assessing the effect of inhibitors, reactions were run in the presence of increasing concentrations of inhibitors and IC_{50} values were derived as described for the ELISA antiviral assay.

2.13. Statistical analyses

The statistical significance of differences in the AUC of disease curves and mean day of death was determined using ANOVA followed by a S.N.K. post hoc analysis. Survival curve data was analyzed using a Fisher Exact test employing the Bonferoni correction. The critical *P*-value in either case was 0.05.

3. Results

3.1. Isolation of HSV-1 mutants resistant to helicase-primase inhibitors

Two different protocols were adopted to select for HSV-1 mutants resistant to helicase-primase inhibitors. In the first

protocol HSV-1 KOS was passaged at a MOI of 1 on BHK cells in the presence of 1 μM BILS 22 BS. Viral pools from each passage were harvested, titered and tested for resistance by using the ELISA antiviral assay. These experiments indicated that the viral pools from passages 2 to 8 were over 1000-fold resistant to BILS 22 BS as compared to wild-type HSV-1 KOS. In comparison, viral pools from infected cells grown in the presence of ACV for eight consecutive passages exhibited a 35-fold resistance to ACV (data not shown). After the eighth passage, two resistant isolates termed K22^{r}1 and K22^{r}5 were plaque purified for detailed characterization. In the second strategy, a resistant HSV-1 isolate K138^{r}3 was selected by using a single-step protocol by plating HSV-1 KOS directly on Vero cells in the presence of 1 μM BILS 138 BS. This latter compound was not active against HSV helicase-primase, but was metabolized inside cells to the corresponding free aminithiazole with potent antiviral activity.

3.2. Sensitivity of resistant mutants to antiviral compounds in vitro

The phenotype of the helicase-primase-resistant HSV-1 strains was assessed in the ELISA-based antiviral assay. The results from these experiments are summarized in Table 1. Isolates K22^{r}1, K22^{r}5, and K138^{r}3 were approximately 2500-, 316-, and 38-fold resistant to BILS 22 BS. Similar results were also obtained with a plaque reduction assay, although the degree of resistance was generally lower (data not shown). All three isolates were cross-resistant to all tested aminothiazolylphenyl-based inhibitors of helicase-primase (see Table 1 and data not shown), including BILS 45 BS which has been shown to have antiviral activity in vivo (Duan et al., 2003 and see below). However, all three isolates were sensitive to ACV, indicating that a different antiviral mechanism was involved. Furthermore, the resistance phenotype was maintained after five passages of the resistant isolates in the absence of inhibitor (data not shown), indicating that the resistance phenotype was stable.

Table 1
Effect of helicase-primase inhibitors or ACV on the growth of wild-type and resistant HSV-1 in cell culture

Compound	EC_{50} (μM)[a]			
	KOS	K22^{r}1	K22^{r}5	K138^{r}3
ACV	0.65 (1)	0.38 (0.6)	0.49 (0.8)	0.33 (0.5)
BILS 22 BS	0.006 (1)	15.0[b] (2500)	1.9 (316)	0.23 (38)
BILS 103 BS	0.010 (1)	44[b] (4400)	4.6 (460)	0.48 (48)
BILS 138 BS	0.005 (1)	>81 (>16000)	>81 (>16000)	>81 (>16000)

[a] Antiviral activity was measured by ELISA as described in Section 2. Values shown represent the mean from at least three independent determinations. The 50% cytotoxic concentrations, determined using a modified MTT assay under the conditions outlined for the antiviral ELISA assay were >200, 47, 74, and 81 μM for ACV, BILS 22 BS, BILS 103 BS, and BILS 138 BS, respectively.

[b] Antiviral effect may be due in part to the cytotoxic effect of the compound. Values in parenthesis represent the fold resistance of each isolate as compared to wild-type HSV-1 KOS.

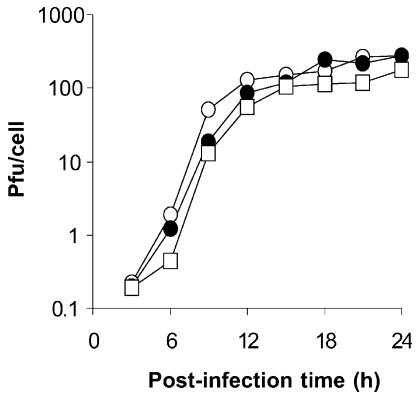

Fig. 2. Growth properties of helicase-primase-resistant HSV isolates in cell culture. Confluent BHK-21 cells were infected at a MOI of 10 with wild-type HSV-1 KOS (○) or the resistant isolates K22^{r}1 (●) and K22^{r}5 (□) and virus yields were measured at various time intervals post-infection as described in Section 2.

3.3. Characterization of the resistant viruses in vitro

Single-cycle growth curves were performed with the isolates K22^{r}1 and K22^{r}5 in order to determine whether any of the resistant viruses were defective for replication in cell culture. Fig. 2 shows that there were no significant differences in the yields and kinetics of infectious virus production for the two isolates. Thus, both mutant viruses were able to replicate at near normal levels. The same conclusion was reached when monitoring overall protein synthesis or DNA synthesis (data not shown). Taken together, the data presented suggested that the resistant isolates possessed in vitro growth properties very similar to those of the parental wild-type strain KOS.

3.4. Pathogenicity of resistant viruses

To determine the neurovirulence of the HSV isolates resistant to BILS 22 BS, Balb/c mice were inoculated intra-cranially with HSV-1 KOS, K22^{r}1, and K22^{r}5. Inoculation with HSV-1 KOS resulted in titer-dependent mortality with an estimated LD_{50} of 200 pfu (see Fig. 3A). When compared to its parental strain, the K22^{r}5 was not significantly attenuated for neurovirulence with a LD_{50} of 500 pfu. The K22^{r}1 strain was significantly attenuated for neurovirulence with a LD_{50} value of 2000 pfu (Fig. 3A). As expected from published reports (15, 16), the HSV-1 KOS ICP6Δ showed marked reduction in neurovirulence with a LD_{50} of $\sim 10^7$ pfu. These results clearly demonstrate that the neurovirulence of the resistant isolates K22^{r}1 and K22^{r}5 was similar to that of wild-type KOS.

To assess the replication competence of the resistant viruses at a peripheral site as opposed to the central nervous system, we studied the pathogenicity of the isolate

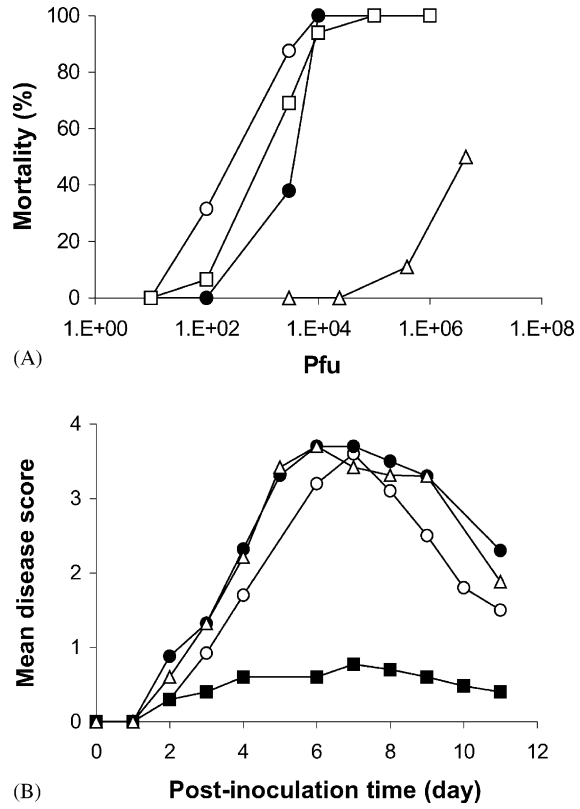

Fig. 3. Pathogenicity of resistant isolates in vivo. (A) Balb/c mice were inoculated intra-cranially with increasing amounts of HSV-1 KOS (○), K22^{r}1 (●), K22^{r}5 (□), and ICP6Δ (△), and mortality was recorded over a period of 14 days. Each data point is the mean of 8–16 mice. Significant differences in mortality from HSV-1 KOS were observed only for the K22^{r}1 strain where mortality at inoculums of 10^3 and 10^2 pfu were significantly lower. There were no significant differences in mean day of death at inoculums of 10^4 and 10^3 pfu. (B) Female SKH-1 hairless mice were infected by cutaneous inoculation with HSV-1 KOS or the resistant isolate K22^{r}1, and the effect of BILS 45 BS was evaluated as described in Section 2. (○) KOS; (●) K22^{r}1; (■) KOS plus BILS 45 BS; (△) K22^{r}1 plus BILS 45 BS. Each data point is the mean of 8–16 mice. The only group for which the peak disease score and AUC for disease was significantly different from HSV-1 KOS was that which received BILS 45 BS.

K22^{r}1 in a mouse model of cutaneous infection (Crute et al., 2002). Inoculation of immunocompetent SKH-1 hairless mice with HSV-1 KOS leads to the development of cutaneous disease pathology characterized by a progression phase and a regression phase with no mortality. In this model, the maximum mean composite disease score approaches a value of 3–4 after 6–7 days post-inoculation. As shown in Fig. 3B, orally administered BILS 45 BS at a dose of 100 mg/kg per day produced an 85% reduction of peak and overall cutaneous disease pathology as measured from the area under the curve. Inoculation of SKH-1 mice with the resistant isolate K22^{r}1 lead to the development of cutaneous disease indistinguishable from that of wild-type HSV-1 KOS. Fig. 3B also clearly shows that BILS 45 BS was ineffective at preventing pathology mediated by the resistant HSV-1 isolate, totally consistent with the increased

Table 2
Incidence of ocular disease and reactivation following corneal infection of Balb/c mice with wild-type or helicase-primase inhibitor-resistant HSV-1

HSV-1 strain	Ocular disease incidence (%)[a]	Reactivation rate (%)[b]
KOS	96	100 (15/15)
K22^{r}1	100	100 (16/16)
K22^{r}5	84	94 (15/16)
ICP6Δ	0	0 (0/16)

Balb/c mice were infected with the respective HSV strains by corneal inoculation as outlined in Section 2.

[a] Ocular disease was assessed 33 days post-inoculation and is reported as percent of infected animals showing signs of disease.

[b] Reactivation rates were scored from the recovery of infectious virus upon co-cultivation of explanted trigeminal ganglia with Vero cells as specified in Section 2. Values in parenthesis represent the number of mice with reactivation vs. the total number of mice infected.

EC_{50} of BILS 45 BS from 0.021 μM against HSV-1 KOS to 15.8 μM against the K22^{r}1. Combined, the results demonstrate that the pathogenicity of K22^{r}1 was not attenuated in a cutaneous model of HSV-1 disease. Moreover, the ensuing pathology was totally refractory to therapy with helicase-primase inhibitors.

The mouse eye model was used to assess the pathogenicity of the resistant mutants at a peripheral site distinct from the skin. Inoculation of mice with either of the two mutants K22^{r}1 and K22^{r}5 or wild-type KOS at a dose of 10^6 pfu per eye led to comparable induction and severity of keratitis (data not shown). When mice were observed for ocular disease at the end of the 33-day period, the incidence of ocular disease in mice inoculated with KOS, K22^{r}1, or K22^{r}5 was greater than 84% (see Table 2). In contrast, and as shown previously, mice inoculated with HSV-1 ICP6Δ showed no sign of ophthalmic disease.

3.5. Establishment and reactivation from latency

After corneal inoculation of Balb/c mice, HSV establishes reactivatable latent infection in the trigeminal ganglia (Jacobson et al., 1989; Coen et al., 1989). This model can therefore be used to evaluate the establishment and reactivation from latency. To examine the ability of the mutant viruses to reactivate from latency, we cultured homogenized explanted ganglia in the absence and in the presence of Vero cells. None of the trigeminal ganglia showed any infectious virus at the time of explant. However, 100% of the mice infected 33 days previously with the isolate K22^{r}1 (i.e., 16/16) and 94% of the mice infected with the isolate K22^{r}5 (i.e., 15/16) yielded infectious virus upon co-cultivation with Vero cells. Likewise, all the animals infected with the wild-type KOS (i.e., 15/15) yielded infectious virus. By contrast, none of the ganglia from mice infected with ICP6Δ yielded infectious virus upon co-cultivation with Vero cells, in agreement with previous reports (Jacobson et al., 1989). Thus, both isolates K22^{r}1 and K22^{r}5 were able to replicate in the eye and reactivate from latent infection.

Table 3
Effect of helicase-primase inhibitors on the DNA-dependent ATPase activity of helicase-primases isolated from cells infected with wild-type or resistant HSV strains

Compound	IC_{50} (μM)[a]				
	Recombinant[b]	KOS[c]	$K22^r1$	$K22^r5$	$K138^r3$
BILS 22 BS	0.023	0.013 (1)	>50 (>3800)	16 (1230)	5 (385)
BILS 103 BS	0.038	0.031 (1)	>50 (>1600)	50 (1600)	20 (645)

[a] DNA-dependent ATPase activity and IC_{50} values were determined from dose–response curves as described in Section 2. Values shown are representative of at least three independent determinations.
[b] Recombinant helicase-primase holoenzyme was isolated from insect cells as outlined in Section 2.
[c] Wild-type and helicase-primase inhibitor-resistant enzymes were purified from BHK cells infected with HSV-1 KOS, $K22^r1$, $K22^r5$, or $K138^r3$ as described in Section 2. Values in parenthesis represent the fold increase in IC_{50} as compared to wild-type HSV-1 KOS.

3.6. Effect of inhibitors on helicase-primase holoenzyme isolated from cells infected with resistant HSV-1 mutants

To determine whether resistance of HSV-1 was due to reduced sensitivity of the viral helicase-primase, holoenzymes from cells infected with wild-type or with the respective resistant viruses were isolated and the partially purified enzymes were tested against BILS 22 BS and BILS 103 BS. As expected, the IC_{50} of BILS 22 BS against recombinant and partially purified helicase-primases was comparable (see Table 3). However, a marked increase in IC_{50} was seen when BILS 22 BS was tested against helicase-primases isolated from cells infected with the resistant viruses. The DNA-dependent ATPase isolated from cells infected with the most resistant isolate $K22^r1$ was not inhibited at all. This translated into a greater than 3800-fold increase in the IC_{50} value. On the other hand, the DNA-dependent ATPase isolated from cells infected with the resistant isolates $K22^r5$ or $K138^r3$ were inhibited by 50% at a concentration of BILS 22 BS of 16 and 5 μM, respectively. This corresponded to a 1230- and 385-fold loss of sensitivity as compared to wild-type helicase-primase. Similar increases of IC_{50} values were observed for BILS 103 BS (see Table 3). Overall, these results correlated well with the fold resistance of the respective viruses in cell culture and therefore these results implied that antiviral resistance was most likely due to reduced sensitivity of the helicase-primase to the inhibitors.

3.7. Genetic mapping of the gene(s) conferring resistance to helicase-primase inhibitors

In order to identify the viral gene(s) responsible for the BILS 22 BS resistance the UL5, UL8, and UL52 genes were PCR-amplified from the $K22^r1$ and $K22^r5$ isolates and used for marker transfer experiments. Initially, each of the three genes was used separately for recombination into wild-type HSV-1 KOS. Only the DNA fragments containing the UL5 gene from each of the isolates were able to produce large numbers of plaques resistant to BILS 22 BS (see Table 4). Subsequent marker transfer experiments were performed using two fragments of the UL5 gene comprising the N-terminal and C-terminal halves of the UL5 ORF. In these experiments only the N-terminal portion of the UL5 gene was able to produce resistant virus. Therefore, both UL5 gene fragments derived from the resistant isolates $K22^r1$ and $K22^r5$ contained mutations in the N-terminal portions of the gene that conferred resistance to BILS 22 BS. These experiments therefore defined the resistance locus within the N-terminal half of the UL5 gene.

3.8. Sequencing of the resistance-conferring region of UL5

Since the resistance mutations mapped to the N-terminus of the UL5 ORF, these DNA fragments were sequenced to determine whether any mutations were present. As shown in Fig. 4, the isolate $K22^r1$ contained a single base pair change at nucleotide 1068 of the UL5 ORF. This would correspond to a K356N (lysine to asparagine change at amino acid 356) change in the amino acid sequence. The $K22^r5$ isolate also contained a single base pair change at nucleotide 1055 of the UL5 ORF. This would create a G352V (glycine

Table 4
Marker transfer of the resistance-associated mutations to wild-type HSV-1 KOS

ORF	Resistant plaques	Frequency
Virus: $K22^r1$		
UL8	1	1.2×10^{-6}
UL52	1	5.7×10^{-7}
UL5	~3150	8.4×10^{-3}
UL5 (15458–14400)	0	$<1 \times 10^{-6}$
UL5 (14399–13680)	216	1×10^{-4}
Virus: $K22^r5$		
UL8	37	7×10^{-5}
UL52	0	$<1 \times 10^{-6}$
UL5	~3000	5.7×10^{-3}
UL5 (15458–14400)	0	$<1 \times 10^{-6}$
UL5 (14399–13680)	216	1.5×10^{-4}

Infectious DNA isolated from wild-type HSV-1 KOS was co-transfected with the PCR-amplified UL8, UL52, or UL5 genes derived from the resistant isolates $K22^r1$ or $K22^r5$ to locate the resistance-conferring helicase-primase gene. Subsequent co-transfection experiments utilized PCR-amplified DNA fragments corresponding to the HSV genome 13680–14399 and 14400–15458 that represent the N-terminal and the C-terminal halves of the UL5 gene. Transfection yields were tested for their ability to form plaques in the presence of 1 μM BILS 22 BS and the frequency of resistant virus was calculated as the ratio of resistant plaques compared to virus yields obtained in the absence of inhibitor.

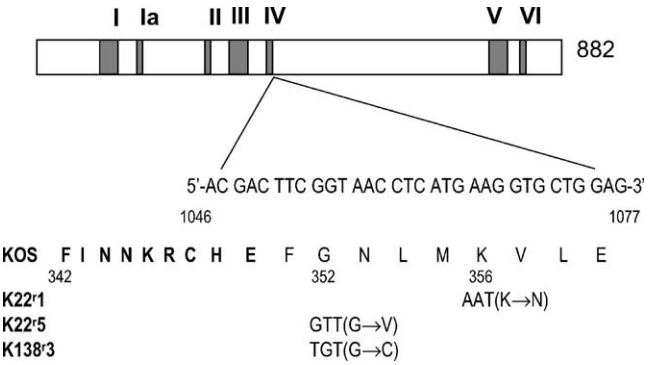

Fig. 4. Mutational changes in the UL5 gene of resistant HSV isolates. The HSV-1 UL5 protein composed of 882 amino acids and the seven conserved helicase motifs numbered I and Ia–VI are shown. The predicted amino acid sequence for HSV-1 KOS is shown underneath the nucleotide sequence surrounding the resistance mutations and the amino acids corresponding to the conserved motif IV are highlighted in bold. Numbering of the amino acids and nucleotides represent the numbering of the UL5 ORF.

to valine change) change in the amino acid sequence of the UL5 ORF. DNA sequence analysis of the isolate K138^{r}3 indicated a third mutation that caused resistance to BILS 22 BS. This isolate contained a new mutation at nucleotide 1054 of the UL5 ORF. This would create a mutation at the same amino acid as in the K22^{r}5 virus but instead made a G352C (glycine to cysteine change) mutation. These results indicated that mutations clustered at the N-terminus of UL5 are both necessary and sufficient to confer resistance to helicase-primase inhibitors.

3.9. Isolation of recombinant helicase-primase containing the UL5 (K356N) mutation and effect of inhibitors

To examine the effect of the K356N mutation on the biochemical activities of the helicase-primase in vitro, a recombinant baculovirus containing the UL5 (K356N) gene was constructed and mutant recombinant helicase-primase was purified. Biochemical assays that assessed the helicase, primase and DNA-dependent ATPase activities of the enzyme indicated that the enzymatic activities of the helicase-primase holoenzyme were not significantly altered by the presence of the K356N mutation (data not shown). To determine whether the K356N amino acid change in the UL5 subunit affected the ability of this class of inhibitors to inhibit any of the enzymatic activities of the mutated holoenzyme, we measured the IC_{50} of BILS 22 BS against the various activities of the mutated helicase-primase and compared them to those of wild-type enzyme. The results showed that none of the activities of the mutated holoenzyme was inhibited by BILS 22 BS up to highest concentration tested of 50 μM. By contrast, all three enzymatic activities of the wild-type helicase-primase were inhibited by the BILS 22 BS with IC_{50} values of 0.023, 0.036, and 0.059 μM in the DNA-dependent ATPase, DNA helicase and RNA primase assays, respectively. Thus, these results indicate that a single point mutation in the UL5 gene can abolish the ability of helicase-primase inhibitors to inhibit all three activities of the helicase-primase holoenzyme.

4. Discussion

We reported previously on the discovery of specific aminothiazolylphenyl-based inhibitors of the HSV type 1 helicase-primase (Crute et al., 2002; Duan et al., 2003). Compounds in this class exhibited potent antiviral activity against wild-type and ACV-resistant HSV-1 and HSV-2 in cell culture assays. The most optimized inhibitors also showed antiviral activity in murine models of wild-type and ACV-resistant HSV disease after oral administration. To confirm the mechanism of antiviral action and to determine the propensity for the development of resistance to this novel class of antiherpetic agents, we now report the isolation and characterization of resistant HSV-1 isolates. Viruses resistant to aminothiazolylphenyl-based inhibitors emerged quite rapidly. During these experiments it was observed that viral pools from passage 2 were already resistant to the helicase-primase inhibitor, suggesting that the resistant isolates pre-existed in the laboratory stocks of HSV-1 KOS. This was verified experimentally by plating large amounts of virus directly in the presence of inhibitor. From these experiments, the rate of appearance of resistant virus was estimated at about 10^{-6}, which is significantly lower than that of ACV-resistant mutants (Shin et al., 2001; Coen, 1991). The low rate of resistance mutation would be consistent with the rate of appearance of single point mutations in HSV-1 (Hall et al., 1984; Hwang et al., 1999).

The emergence of HSV mutants that are resistant to antiviral drugs is an important concern for any new antiviral agent. Although the frequency of ACV-resistant mutants has been reported to be as high as 7.5×10^{-4} (Shin et al., 2001; Coen, 1991), disease due to acyclovir-resistant virus is limited mainly to patients with AIDS (Coen, 1991; Safrin, 1996; Kimberlin et al., 1995) and remains rare in immunocompetent patients. In these instances, resistant HSV disease is due to the less frequent (∼5% of ACV-resistant HSV) but more pathogenic mutant viruses that have partial or altered thymidine kinase activities or altered DNA polymerase activity. Thus, it would appear that HSV-1 mutants with resistance to helicase-primase inhibitors are not more frequent than pathogenic ACV-resistant isolates. Therefore, if the assays in mice are predictive of pathogenicity in humans, it is quite possible that the incidence of helicase-primase inhibitor-resistant disease might remain low.

The present study also provides a detailed examination of the virulence of the HSV-1 isolates resistant to helicase-primase inhibitors that may indicate the potential for pathogenicity of drug-resistant mutants in the clinic. Single-step growth curves showed that the helicase-primase-resistant viruses were not impaired for growth in cell culture. Furthermore, when grown for five

passages in the absence of selective pressure, the two resistant isolates K22^{r}1 and K22^{r}5 maintained their full resistance phenotype, suggesting that the identified amino acid changes did not affect virus replication. These results concord well with the observation that recombinant helicase-primase with a K356N mutation in the UL5 subunit retained essentially wild-type DNA-dependent ATPase, DNA helicase and RNA primase activities. In neurovirulence studies, the two resistant isolates K22^{r}1 or K22^{r}5 were not significantly attenuated as compared to wild-type HSV-1 KOS following intra-cranial inoculation in Balb/c mice. Both resistant isolates were also fully competent for replication and reactivation from latency following corneal inoculation of mice. Inoculation of mice with K22^{r}1, the only mutant virus examined for pathogenicity in the skin of SKH-1 mice, resulted in cutaneous disease that was essentially indistinguishable from that produced by HSV-1 KOS. As expected, however, the K22^{r}1-mediated pathology was refractory to treatment with the orally active inhibitor BILS 45 BS. Our results independently confirm a previous report (Betz et al., 2002) that the resistant virus with a K356N mutation in the helicase gene exhibited essentially wild-type replication and pathogenicity. We have not investigated the properties of the resistant mutant K138^{r}3 that has a G352C substitution in the UL5 gene. Most probably, this mutant virus is not attenuated for virulence, although an attenuated virus resistant to helicase-primase inhibitors has been reported (Kleymann et al., 2002).

Our study shows that aminothiazolylphenyl-based compounds inhibit HSV-1 growth by targeting the UL5 subunit of the HSV-1 helicase-primase, fully in agreement with their mechanism of action in vitro. Marker rescue experiments and DNA sequence analysis demonstrated that single amino acid substitutions in the UL5 protein conferred resistance to helicase-primase inhibitors. Of the three resistant HSV-1 isolates selected, K22^{r}1 had a lysine mutated to an asparagine at amino acid residue 356 of the UL5 protein (i.e., K356N). This mutation is equivalent to that reported by others who found the same change in the UL5 protein of HSV-2 after selection with a distinct aminothiazolylphenyl inhibitor (Spector et al., 1998). The other two resistant isolates K22^{r}5 and K138^{r}1 had mutations at glycine residue 352 in the UL5 protein. In K22^{r}5 the glycine was changed to valine (G352V) and in K138^{r}1 the glycine was changed to cysteine (G352C). Combined with the resistance mutations identified by Spector and co-workers in the HSV-2 UL5 protein, the additional mutations identified here thus further help to define the binding site of aminothiazolylphenyl-based inhibitors. Recently, a chemically distinct class of HSV antivirals that target the viral helicase-primase has been described (Kleymann et al., 2002; Betz et al., 2002). Interestingly, several resistant mutants to thiazolylsulfonamide-based inhibitors exhibited single amino acid substitutions in the viral UL5 and one mutant had a substitution in the UL52 gene. Almost all the mutations in the UL5 gene were located between amino acids 352 and 356 and included the amino acid substitutions selected by aminothiazolylphenyl-based inhibitors described in this report. These results strongly suggest that both classes of HSV helicase-primase inhibitors share a common or closely overlapping binding site. It appears therefore likely that both classes of compounds inhibit the enzyme by the same mechanism of action. Interestingly, Kleymann and co-workers also identified a novel resistance mutation in the UL52 gene, suggesting that the binding site for both classes of inhibitors may be located at the interface between helicase and primase subunits.

In conclusion, we have demonstrated that several point mutations localized in the HSV-1 UL5 subunit are responsible for conferring high level of resistance to aminothyazolylphenyl-containing inhibitors of the HSV-1 helicase-primase. These results confirm that helicase-primase is the target of antiviral action of this class of antiherpetic agents. Moreover, our results indicate that the resistant HSV-1 mutants are not attenuated in terms of replication in cell culture and pathogenicity in several murine models. It remains, however, to be determined whether the genotypic changes identified for HSV mutants selected in the presence of helicase-primase inhibitors would be isolated from patients treated with these new agents. Obviously, the significance of the work described here and the potential for the development of resistant HSV disease to this novel class of antiherpetic agents must await clinical trials in humans.

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