Inhibition of B virus (Cercopithecine herpesvirus 1) by Conventional and Experimental Antiviral Compounds

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ABSTRACT

B virus infection of humans results in high morbidity and mortality in up to 80% of identified cases. The main objective of this study was to conduct a comparative analysis of conventional and experimental antiviral drug susceptibilities of B virus isolates from multiple macaque species and zoonotically infected humans. We used a plaque reduction assay to establish the effective inhibitory doses of acyclovir, ganciclovir, and vidarabine, as well as a group of experimental nucleoside analogs with known anti-herpes simplex virus activity. Four of the tested experimental drugs were 10 to 100-fold more potent inhibitors of B virus replication than conventional antiviral agents. Drug efficacy was similar for multiple B virus isolates tested with variations within 2-fold of the median effective concentration ($EC_{50}$) for each drug and was considerably lower than those of B virus TK mutants. We observed no differences in the viral thymidine kinase amino acid sequence between B virus isolates from rhesus monkeys and human zoonoses. Differences in the TK protein sequence of cynomolgus and pigtail macaque B virus isolates did not affect drug sensitivity except in the case of one compound. Taken together, these data suggest that future B virus zoonoses will respond consistently to conventional antiviral treatment. Further, the considerably higher potency of FEAU advocates its compassionate use in advanced human B virus infections.
INTRODUCTION

In its natural host, macaque monkeys, B virus (*Cercopithecine 1 simplexvirus, herpesviridae*) causes lesions on epithelial surfaces (2), and establishes reactivatable latent infection in sensory neurons (30, 37), similar to herpes simplex virus (HSV) in humans. B virus often results in severe pathogenesis, including paralysis, encephalitis, and in many cases, rapid death following infection of humans (reviewed in (22)). Nearly all reported cases of B virus zoonosis have been associated with individuals handling macaques during the course of research or technology development (33). Five fatalities, along with at least 23 surviving cases, have occurred in the past 20 years, underscoring that zoonotic infections remain a problem in the laboratory animal environment (8-10).

The CDC’s B Virus Working Group currently recommends treatment of confirmed zoonotic infections with herpesvirus-specific antiviral drugs, including acyclovir (ACV) and ganciclovir (GCV) (11). Both agents in this class of compounds are phosphorylated to active form by virus-encoded thymidine kinase. The resulting nucleoside triphosphate analog inhibits viral DNA replication by termination of chain elongation and by direct inhibition of herpesvirus DNA polymerase (24). While ACV is effective against B virus both in cell culture and animal models (6), the dose required for 50% plaque reduction is more than tenfold higher for B virus compared to HSV-1 (38). GCV is twice as potent as ACV in B virus plaque reduction, yet the median effective concentration ($EC_{50}$) of GCV for B virus is almost 10 times higher than for HSV-1 (38). In some, but not all cases of zoonotic B virus infection, acyclovir and ganciclovir have proven to be effective at curtailing disease progression (8, 9). Zoonotic infections that have progressed to extensive CNS involvement, including respiratory arrest, appear to be refractory to conventional antiviral intervention (12, 15) J. Hilliard, unpublished data).
Vidarabine (9-ß-D-arabinofuranosyladenine, ara-A, VDB), an antiviral agent effective against HSV, may be useful for treatment of early stages of zoonotic B virus infections, but it has not been used alone in previous cases. Prior to the use of ACV for treatment of HSV, intravenous VDB was used for treatment of encephalitic infections (34). In cell culture, it has been shown to be equally potent against B virus and HSV-1 (5). VDB does not require selective phosphorylation by the viral TK for activity (4), however, since VDB can be converted to its active form by host enzymes it has potential toxicity in humans (29).

Experimental drugs effective in cell culture against HSV include the β-D-2’-fluoro-5-substituted arabinosyl pyrimidines FMAU (2’-fluoro-5-methyl-ara-U) and FEAU (2’-fluoro-5-ethyl-ara-U), both of which require phosphorylation by viral thymidine kinase for activation (19). FMAU and FEAU have similar potency as ACV for inhibition of HSV-1 in cell culture, but tenfold greater potency of FMAU is observed against HSV-2 compared to ACV (19). Unfortunately, FMAU has been reported to be toxic to humans at elevated doses used for cancer chemotherapy (1, 13); FMAU can be incorporated into uninfected cell DNA by host DNA polymerase, suggesting the basis of its toxicity in vivo (7). FEAU has been shown to be a selective inhibitor of HSV DNA synthesis in cell culture (7), but its toxicity in humans has not been investigated. Other β-D-2’-fluoro-5-substituted arabinosyl pyrimidines, such as FMAC (2’-fluoro-5-methyl-ara-C) and FBrAC (2’-fluoro-5-bromyl-ara-C) have been synthesized and tested against HSV (17, 32), R.F. Schinazi, unpublished results). No information on FBrAC toxicity is known.

The goal of this study was to determine the general variability of drug susceptibility and the efficacy of a class of experimental antiviral agents by using a panel of B virus isolates from multiple macaque species and humans. The results presented here suggest that B virus isolates
Running title: B Virus Antivirals

in the wild are susceptible to antiviral agents that require the viral TK for activity. Further, our findings that specific experimental nucleoside analogs are appreciably more effective than conventionally used antiviral agents at blocking B virus replication suggest that these drugs may benefit the treatment of high morbidity human cases.

MATERIALS AND METHODS

Cells and Viruses. African green monkey kidney (Vero) cells (ATCC CCL-81) were obtained from the American Type Culture Collection (Manassas, VA) and propagated in Dulbecco's Modified Eagle's Medium (Mediatech) supplemented with 7% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Infections were done using DMEM supplemented with 1% FBS. All B virus plaque reduction assays were performed under biosafety level 3 conditions. B virus infections for viral DNA purification and virus stock production were done under biosafety level 4 conditions as mandated by the 5th Edition of the Biosafety in Microbiological and Biomedical Laboratories manual (National Institutes of Health) for propagating and handling large volumes and titers of select agent.

The E2490 reference strain of rhesus macaque B virus was originally obtained from Dr. R. N. Hull, Eli Lilly Research Laboratories (Indianapolis, IN). All other wild-type viruses used in this study were diagnostic isolates obtained from the NIH's NCRR-supported National B Virus Resource Center, Georgia State University, Atlanta, GA. Restriction endonuclease digestions of these isolates' viral genomes are shown in Figure 1. MR, rhesus macaque isolate; E2490, prototype laboratory strain; A, human isolate; MJ, Japanese macaque isolate; MC, cynomolgous macaque isolate; MP, pigtail macaque isolate.
The TK mutants were generated by co-transfection of NaI-gradient purified E2490 viral DNA with plasmids containing the TK gene with either a deletion (pBVΔTK) or a premature stop codon (pBVTKstop) into rabbit skin cells (ATCC CCL-68) (Figure 2a, technique described in reference 27). For pBVTKstop, a SpeI linker containing stop codons in all frames (#1061, New England Biolabs) was inserted into the BglII site of pBVTK. Dilutions of the subsequent transfection stocks were plated on Vero cells and individual plaques screened by PCR and Southern Hybridization. After 3 (BVΔTK) or 4 (BVTKstop) rounds of plaque purification, one plaque with no evidence of wild-type virus was chosen as the representative mutant of each virus for preparation of stocks.

The DNA sequence of both viral mutants was confirmed by sequencing. Confirmation of the deletion and stop codon insertion using PCR is shown in Figure 2b. The parent virus PCR amplimer is 1143 bp (lane 1). The deletion from ApaI to BspEI removes a 759 bp fragment, resulting in a 384 bp amplimer (lane 2). The insertion of the stop codon linker into the BglII site results in an 1157 bp amplimer (lane 4) that when cut with SpeI yields 856 bp and 301 bp fragments (lane 3). Manipulation of these drug-resistant TKnull viruses is restricted to a biosafety level 4 laboratory in accordance with the Georgia State University Institutional Biosafety Committee and the Office of Biotechnology Activities at the National Institutes of Health.

**Antiviral Compounds.** Acyclovir (ACV), Ganciclovir (GCV) and Vidarabine (VDB) were purchased from Sigma (St. Louis, MO). The compounds 1-(2-fluoro-5-methyl-beta-D-arabinofuranosyl)uracil (FMAU), 1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-ethyluracil (FEAU), 1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-bromylcytosine (FBrAC) and 1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-methylcytosine (FMAC) were synthesized in one of our laboratories (R.F.S.).
Plaque Reduction Assay. Confluent monolayers of Vero cells in 12-well microplates were infected with approximately 200 plaque forming units of virus and incubated at 37°C with twofold serial dilutions of antiviral drug. After one hr, the inoculum was removed and replaced with media containing 1% methycellulose and antiviral drug. Control wells did not contain antiviral drug. The cells were fixed in 100% methanol 40 hr after infection and stained with Giemsa. Plaques were visually inspected and counted using a dissecting microscope. The number of plaques at each drug concentration was plotted versus log of the drug concentration, and the slope of the regression line in the linear range was determined. The amount of drug required to reduce the number of plaques by 50% of those in the control wells (EC\(_{50}\)) was calculated from the equation of the regression line. Each drug was tested against each isolate at least twice in replicate wells and the EC\(_{50}\) was calculated as an average value. Cytotoxicity in Vero cells for all the compounds was determined previously using an MTT assay. None of the compounds were cytotoxic when evaluated up to 10 μM (data not shown).

Viral DNA analysis. Viral DNA was isolated from infected cells by the method of Roizman and colleagues (23). Restriction digests were separated on 0.8% agarose gels. Each PCR amplification of the TK gene from rhesus, Japanese, cynomolgus and human B virus isolates was performed with the primers 5'CCAACGCTCCGTAACACCAG3' and 5'ACCATCTTTATTGCAGCCAGGG3'. Amplimers were purified after extraction from 1% agarose gels and both strands were sequenced using an automated sequencer 377 (Applied Biosystems), with the above primers and three internal primers 5'AAGATCGTGTCCTCGATCTC3', 5'GAGATCGAGGACACGATCTT3', and 5'CACCTGAGCGCTGGCCATTG3'. Because PCR amplification of the pigtail macaque B virus TK gene was not possible with the rhesus macaque-based PCR primers, restriction
fragments containing the pigtail B virus TK gene were cloned. These plasmids were subcloned and the pigtail B virus TK gene was then sequenced using vector-specific primers and two internal gene-specific primers 5’AAGATCGTGTCCTCGATCTC3’ and 5’GAGATCGAGGACACGATCTT3’. Sequence alignment was performed using MegAlign 4.03 (DNAStar, Madison, WI) using the Joten-Hein algorithm.

RESULTS

Characterization of the Virus Panel. One of the goals of this work was to examine the variability of drug potency and susceptibility against multiple B virus isolates from macaque and humans. We chose a large panel of B virus isolates from various species of macaques and human isolates; many of the viruses were isolated recently and have been maintained at three or fewer passages. To verify the genotype and observe the genetic variability of the B virus isolates, we performed restriction fragment length polymorphism (RFLP) analysis with Sal I restriction endonuclease (Figure 1). Numerous polymorphisms were observed, similar to those noted in a previous study of multiple isolates from cynomolgus macaques (31). It is also noted that the seven B virus isolates from human zoonoses were genotyped as rhesus B virus. B virus isolates from cynomolgus and pigtail macaques had restriction profiles distinct from those of rhesus macaques as previously reported by others (25). Recently, sequencing and RFLP analysis of PCR amplimers demonstrated specific differences that distinguish B virus from Japanese and rhesus macaques (21). Our results show that Japanese macaque B virus RFLPs were very similar to those from rhesus macaque virus isolates, yet these isolates had distinct RFLPs generated by digestion with Sal I (Figure 1) and other restriction endonucleases (data not shown).
In order to determine if a B virus isolate was resistant to ACV or GCV, we created two Thymidine Kinase defective B viruses as controls (Figure 2). These viruses were 6-fold more resistant to ACV and 16-fold more resistant to GCV than the wild-type parental strain.

**Sensitivity of B virus Isolates to Vidarabine.** As previously reported (5), B virus and HSV-1 have similar sensitivity to VDB in cell culture, suggesting the hypothesis that the reason B virus is less sensitive to ACV and GCV than HSV is due to variation in the viral TKs, not the viral DNA polymerases. To detect differences in the B virus DNA polymerases, the sensitivity of the B virus isolates to VDB was determined by plaque reduction assay. The rationale for the use of VDB was to determine whether mutations affecting the viral DNA polymerase might be present, since these mutations could potentially result in an observed resistance to drugs that require phosphorylation by the viral TK and may be mistakenly attributed to a partial or total loss of TK function.

We found that each rhesus isolate had very similar sensitivity to VDB (mean EC$_{50}$ 34.3 µM, range 26.5 – 40.4 µM, Figure 3). As expected, the mean VDB sensitivities of the BVΔTK and the BVTKstop mutants were similar to the rhesus isolates (37.8 µM and 46.1 µM, respectively, Figure 2C). While it cannot be completely ruled out that mutations in the viral DNA pol might exist that do not effect the sensitivity of VDB, these results indicate that the DNA polymerase genes in these isolates are similar, suggesting that large differences observed with TK-activated antiviral agents would likely be due to variation in the viral TK.

**Sensitivities of B Virus Isolates to Acyclovir and Ganciclovir.** Fatalities following zoonotic infection from 1987-1997 occurred despite pharmacologic intervention with ACV and/or GCV. To rule out that the fatality-associated viruses were strain variants that were “naturally” resistant to these antiviral agents, we determined the sensitivities of our panel of B virus isolates to these
antivirals using plaque reduction assays. As controls, the TK
null mutants were used to determine a
standard for completely resistant virus.

Our results for ACV and GCV (Figure 4) were similar to that of previously published
studies for rhesus B virus (38). The EC\textsubscript{50} for each drug against each isolate was within a two-
fold difference when compared to the mean sensitivity of the isolates from rhesus monkeys and
humans (33.2 µM, range 19.3 - 45.7 µM). The sensitivity of Japanese, cynomolgus and pigtail B
virus isolates to ACV and GCV was very similar to that of the human and rhesus B virus
isolates, suggesting that future human infections with these B virus genotypes could be treated
with these antivirals. Because the drug sensitivity of the isolates did not approach the levels of
the TK\textsubscript{null} mutants (Figure 2C), our results indicate that the isolates in this panel have a wild-type
antiviral drug sensitivity, however, it is possible that the variability observed in this assay could
be due to minor differences in the TK genes.

**Experimental Drugs.** While ACV and GCV have been shown to be effective in some cases of
B virus zoonosis, the prognosis for patients is bleak once clinical signs indicate entry of B virus
into the CNS. To the end of discovering more efficacious B virus antiviral agents, we tested a
panel of experimental compounds with known activity against HSV. FMAU and FEAU were
found to be approximately 100 times more potent than conventional drugs (Figure 5, upper
panel). FMAC and FBrAC had approximately 10 times greater potency than ACV (Figure 5, lower panel). Each of these agents requires the viral TK for activation, indicating that either they
are activated with higher efficiency by TK or their phosphorylated forms are recognized by the
viral DNA polymerase with higher affinity than the triphosphate forms of ACV or GCV. These
data demonstrate a more efficacious panel of B virus antiviral agents exists within the class of 2'-
fluoro-5-substituted arabinosyl pyrimidines.
**Thymidine Kinase Heterogeneity.** Most of the B virus isolates had similar sensitivities to each drug with a range less than twofold from the mean. It is possible that the observed variation between these isolates is due to minor differences at the amino acid level in the viral proteins that can confer resistance to nucleoside analogs (TK or DNA polymerase). To determine whether apparent functional mutations existed in TK, the TK ORF was sequenced from rhesus, Japanese, cynomolgus, pigtail and human B virus isolates.

Striking conservation between B virus TK ORFs from rhesus macaque, Japanese macaque, and human isolates was observed; one nucleic acid substitution was present in 8 of 22 of these isolates, but the difference did not affect the amino acid sequence. Cynomolgus-derived B virus isolates have 7 unique TK amino acid differences compared to rhesus and pigtail B virus TK (Figure 6), and these differences may underlie the increased sensitivity of the cynomolgus isolates to FBrAC (Figure 5, lower panel). While 27 unique amino acid differences were present in the pigtail B virus isolate relative to rhesus and cynomolgus B virus TK (Figure 6), the drug sensitivity of the pigtail B virus fell into the range of the rhesus B virus isolates with all tested antiviral agents. We hypothesize that the observed amino acid differences are likely in domains that are not functional with respect to drug interaction, or alternatively in functional domains that are conserved sufficiently to retain function.

Included in these studies were isolates from three humans and two rhesus macaques sampled during a cluster of zoonotic infections in Pensacola (9). The three human isolates and two monkey isolates are designated A1, A2, A3, MR1, and MR2, respectively. While there were minor differences in drug sensitivities among these isolates, the DNA sequences of the TK ORF were identical (data not shown). Taken together, the similar drug sensitivity and identical
DISCUSSION

These data provide support for the value of and choices for antiviral intervention against zoonotic B virus infections, extending data originally demonstrating the efficacy of conventional drugs that target viral DNA replication. The potential of 2’-fluoroarabinofuranosyl 5-substituted pyrimidine nucleosides is demonstrated for the first time; these data may serve as the basis of compassionate use when the onset of severe morbidity signals unlikely success with conventional therapeutic approaches, as learned from the five fatal cases in recent years. Our data further suggests there is no immediate host pressure that induces drug susceptibility-related changes in B virus during human infection. Each isolate, whether isolated from a macaque or a human, was similarly sensitive to the antiviral agents used in these studies, suggesting that time to treatment is a major factor in human survival.

The TKnull mutants had similar VDB sensitivity to the wild-type isolate panel, supporting the hypothesis that the increased requirements of ACV and GCV for B virus plaque reduction is due to the differences between B virus and HSV TKs. While most of the known functional domains of TK are identical between the rhesus B virus and HSV, it is tempting to speculate that the substitution of Pro150 in the B virus TK would place a kink in the nucleoside-binding domain not present in the same position in HSV TK (Ala168). Indeed, a change in the homologous amino acid in HSV-2 (Ser169) is responsible for the resistance of that virus to brivudin (BVdU) (35) and this same amino acid variation in the binding pocket is present in cynomolgus B virus TK (Ser150). Zwartouw and colleagues (38) demonstrated the increased resistance of
cynomolgus B virus to BVdU almost 20 years ago. Site-directed mutagenesis and a structure-activity relationship analysis of B virus TK would be required to determine the role of individual amino acids in the relative resistance of B virus to ACV and GCV.

B virus isolates from recent zoonotic infections demonstrated no variation in TK genes relative to isolates from rhesus macaques and all appear similar to those from rhesus monkeys by restriction endonuclease profiles. These data validate a rhesus origin of B virus associated with these specific zoonoses. However, this is a small sample set and it was noted that affected individuals had been working specifically with rhesus macaques. Thus, our data does not rule out the possibility that B virus from macaque species other than *macaca mullata* may share its pathogenic potential (25). Even so, B virus can be transmitted to non-macaque monkeys (18, 36) and other macaques can harbor B virus acquired from rhesus monkeys (21, 25). Thus, regardless of the monkey species, if a monkey has been in contact with macaques, B virus should be presumed to be a hazard. Few cases previously reported in the literature have been associated with specific macaques (22), in fact, many affected individuals had histories of contact with multiple macaque species.

In each of the recent symptomatic cases of B virus zoonosis, treatment has consisted of acyclovir and/or ganciclovir (3, 10, 12, 16, 20). Of eleven infected individuals, six survived. Data presented here suggests that variable infection outcomes regarding antiviral therapy were not associated with virus mutations. It is possible that each individual had different host-dependent susceptibilities to B virus infection. Alternatively, since four of the five fatally infected individuals were not treated until severe neurological symptoms were detected, whereas three of the six survivors were hospitalized prior to the onset of acute neurological symptoms, time for effective intervention may have been limited. This would underscore the importance of
293 early identification of zoonotic disease, which can be generally accomplished early after
294 infection for less than US$300 of laboratory evaluations.
295 Zwartouw and colleagues (38) compared ACV and GCV against B virus in
296 experimentally infected rabbits, observing 100% survival in the GCV treated group for 5 months,
297 compared to 33% survival in those treated with ACV. Interestingly, one of the patients from the
298 Michigan cluster of human infections with B virus was given ganciclovir 5 mg/kg i.v. twice
299 daily, following a lack of success with ACV used at 15 mg/kg for three days, resulting in
300 abatement of neurological symptoms and stabilization of CSF antibody levels (12). In four other
301 human cases, however, GCV was unable to reverse the fatal progression of disease (10, 17, 20),
302 substantiating the need for more efficacious antivirals for late stage infection.
303 During the preparation of this manuscript, a biochemical analysis of B virus TK was
304 published, including some overlap with the present study (14). Our somewhat different approach
305 was to determine if variability existed among wild isolates of B virus, hence our inclusion of
306 many more virus strains, instead of just 3 strains. Instead of characterizing the biochemical
307 properties of B virus TK, we sought out compounds that had significantly greater efficacy in cell
308 culture against a wide scope of B virus isolates, so that infectious disease physicians would have
309 more powerful choices to treat cases of advanced human B virus infection. The creation of the
310 TK
null
mutant viruses as opposed to the production of recombinant protein allowed us to examine
311 TK-mediated drug resistance in the context of viral infection. The authors of the previous study
312 could not detect the phosphorylation of ACV or GCV with recombinant B virus TK; however,
313 the increased resistance of the TK mutants to these drugs suggests that in cell culture infections,
314 B virus TK does activate ACV and GCV.
Our studies indicate that a new, improved group of antiviral agents, 2'-fluoroarabinofuranosyl 5-substituted pyrimidine nucleosides, should be considered for use therapeutically to combat zoonotic B virus infection. Comprehensive analyses examining the cellular toxicity and effectiveness of a number of these compounds against HSV have been reported. FEAU has been shown to be the most selective anti-herpes simplex virus antiviral in this class; it was not incorporated into uninfected cell DNA (17) and was tenfold less toxic than ACV in cell culture (19). Other laboratories have demonstrated the in vivo safety of FEAU; no negative effects were reported in a monkey following 6 intravenous doses for FEAU at 30 mg/kg (7). Further work has demonstrated the in vivo safety and effectiveness of FEAU against Simian Varicella Virus in African Green Monkeys (26), as did analysis of FEAU in both a mouse model of HSV-2 (19) and a rabbit model of HSV-1 (28). In view of the impressively low doses required to inhibit B virus replication, FEAU should be considered as a compassionate use treatment for human cases in which there is progressive or severe morbidity.

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Figure 1. **Confirmation of B virus isolate genotype by RFLP.** Viral DNA was digested with Sal I restriction enzyme and separated on a 0.8% agarose gel. MR, rhesus macaque isolate; E2490, prototype laboratory strain; A, human isolate; MJ, Japanese macaque isolate; MC, cynomolgous macaque isolate; MP, pigtail macaque isolate. Size markers are indicated in kilobase pairs and reflect the positions of BstEII-digested lambda DNA fragments.

Figure 2. **Design and confirmation of recombinant B virus harboring mutations in thymidine kinase.** (A) Overview of TK mutant construction. 1, Orientation of B virus genome. 2, BamH I – Sal I fragment containing the complete TK open reading frame. 3, Apa I – BspE I collapse of pBVTK, deleting 754 amino acids from the center of TK. 4, Stop codon linker insertion into the Bgl II site to prematurely stop TK translation. (B) Confirmation of mutants by PCR. M, ΦX174/HaeIII marker; 1, E2490; 2, BVΔTK; 3, BVTKstop PCR product cut with SpeI; 4, BVTKstop (uncut). (C) Antiviral sensitivities of E2490, BVΔTK and BVTKstop versus Acyclovir, Ganciclovir and Vidarabine. Error bars indicate the standard deviation.

Figure 3. **Vidarabine sensitivity of B virus isolates.** Plaque reduction assays were performed and the 50% effective concentration (EC₅₀) of each drug against each isolate was determined. Each bar corresponds to the mean 50% effective concentration; Error bars reflect the standard deviation. Each isolate was tested against each drug in at least two independent experiments.

Figure 4. **Sensitivities of B virus isolates to conventional antivirals.** Plaque reduction assays were performed and the 50% effective concentration (EC₅₀) of each drug against each isolate was determined. Each bar corresponds to the mean 50% effective concentration; Error bars reflect the standard deviation. Each isolate was tested against each drug in at least two independent experiments. N.D., not done.
Figure 5. Sensitivities of B virus isolates to experimental antivirals. Plaque reduction assays were performed and the 50% effective concentration (EC$_{50}$) of each drug against each isolate was determined. Each bar corresponds to the mean 50% effective concentration; Error bars reflect the standard deviation. Each isolate was tested against each drug in at least two independent experiments. N.D., not done.

Figure 6. Amino acid alignment of thymidine kinase from three B virus genotypes, HSV-1 and HSV-2. Thymidine kinase open reading frames from the B virus strains E2490 (Rhesus BV), MC1 (Cynomolgus BV), and MP1 (Pigtail BV) were aligned using the Juton-Hein method. The thymidine kinase open reading frame from HSV-1 strain F and HSV-2 strain 333 were included for comparison. Shaded amino acids indicate identity with known functional domains.
Rhesus BV  MASHAGQOCAPALDRVAGPAGHDNRPSAL ------------ LRIYVDGPHGLGKTTTAAALAAALGRRDEIEYVPEPMAYWQTLGGPQTITR  79
Cynomolgus BV  MASHAGQOCAPALDRVAGPAGHDNRPSAL ------------ LRIYVDGPHGLGKTTTAAALAAALGRRDEIEYVPEPMAYWQTLGGPQTITR  79
Pigtail BV  MASHAGQOCAPALDRVAGPAGHDNRPSAL ------------ LRIYVDGPHGLGKTTTAAALAAALGRRDEIEYVPEPMAYWQTLGGPQTITR  79

HSV-1  MASHAGHQDAPALDRVAGPAGHDNRPSAL ------------ LRIYVDGPHGLGKTTTAAALAAALGRRDEIEYVPEPMAYWQTLGGPQTITR  79
HSV-2  MASHAGHQDAPALDRVAGPAGHDNRPSAL ------------ LRIYVDGPHGLGKTTTAAALAAALGRRDEIEYVPEPMAYWQTLGGPQTITR  79

P-Loop Motif

Rhesus BV  IFDAQHRLDRGEISASEAAMAMASAQVTMSTPYAYOTESAVAPHIGAELPPGHGPHPNIDLTLVFDRHPVA  179
Cynomolgus BV  IFDAQHRLDRGEISASEAAMAMASAQVTMSTPYAYOTESAVAPHIGAELPPGHGPHPNIDLTLVFDRHPVA  179
Pigtail BV  IFDAQHRLDRGEISASEAAMAMASAQVTMSTPYAYOTESAVAPHIGAELPPGHGPHPNIDLTLVFDRHPVA  179

HSV-1  IYTTQHRLDQGEISAGDAAVVMTSAQITMGMPYATDAVLAPHIGGEAVGPQAPPPA--LTLVFDRHPIA  197
HSV-2  IYNTQHRLDRGEISAGDAAVVMTSAQITMSTPYAATDAVLAPHIGGEAVGPQAPPPA--LTLVFDRHPIA  198

Mg$^2+$ Binding  Nucleoside Binding

Rhesus BV  TPGTNLVLGALPEAVHAERLAQRQRPGERLDLAMLSAIRRVYDMLGNAIVYLQQGGSWRADWRRLSPARPAAA-SGRPARILPRPEIEDTIFALFCAPEL  279
Cynomolgus BV  TPGTNLVLGALPEAVHAERLAQRQRPGERLDLAMLSAIRRVYDMLGNAIVYLQRGGSWRADWRRLSPARSAAA-SGRPARILPRPEIEDTIFALFCAPEL  279
Pigtail BV  SPGTNIVLGALPETVHAERLAQRQRPGERLDLAMLSAIRRVYDMLGNAITYLQRGGSWRADWRRLSPARPAAA-SGRPARILPRPEIEDTIFALFCAPEL  279

HSV-1  LPGTNIVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGLLANTVRYLQGGGRWREDWGQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPEL  297
HSV-2  APGTNLVLGVLPEAEHADRLARRQRPGERLDLAMLSAIRRVYDLLANTVRYLQRGGRWREDWGRLTGVAAATR-PDPEDGAGSLPRIEDTLFALFRVPEL  298

Lid Motif

Rhesus BV  LDGTGEPYRVFAWTLDLLAERLRPMHLLVLDYNQAPHHCWMDLMEMIPEMTPTLPATPGSMLTLQLLAREFAREMTPARGGDAGGEGSETQ. 371
Cynomolgus BV  LDETGEPYRVFAWTLDLLAERLRPMHLLVLDYNQAPHHCWMDLMEMIPEMTPTLPATPGSMLTLQLLAREFAREMTPARGGDAGGEGRETQ. 371
Pigtail BV  LDETGEPYRVFAWTLDLLAERLKAMHLFVLDYHQAPHHCWMDLMELIPEMTPTLPATSGSMMTLQILAREFAREMTPARGGDAGGEGPETR. 371

HSV-1  LAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMIQTHVTTPGSIPTICDLARTFAREM------------GEAN. 376
HSV-2  LAPNGDLYTFALALDVLADRLLPMHLFVLDYDQSPVGCRDALLQLTAGMIPTRVTTAGSIAEIRDLARTFAREV------------G-GV. 376