VALIDATION OF BABOON PAPIINE HERPESVIRUS 2 AS A MODEL FOR MACAQUE MACACINE HERPESVIRUS 1 DRUG SENSITIVITY

By

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This is dedicated to Butters

Jamie, Frank, Butters, Margarine, Suga, Flour, Beth, Bubby, Yeller, Corky, Onkers

For all that you taught me, everything I do in my research is to help your kind.

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ABSTRACT

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Title of Study: VALIDATION OF BABOON PAPIINE HERPESVIRUS 2 AS A MODEL FOR MACAQUE MACACINE HERPESVIRUS 1 DRUG SENSITIVITY

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Macacine herpesvirus 1 (Monkey B virus; BV) is a macaque alpha-herpesvirus that is usually fatal when transmitted to humans, with a mortality rate close to 80%. Current antiviral treatments for BV infection are not fully effective and complete recovery is rare. Consequently, BV is a Risk Group 4 virus making work with BV both dangerous and expensive. These limitations have led to little BV research being accomplished. A related Risk Group 2 baboon virus (*Papiine herpesvirus 2*; HVP2), represents a potential model for BV that does not have these limitations.

The ability of the HVP2 model to predict sensitivity of BV to antiviral drugs was tested by comparing the drug sensitivity *in vitro* (cell culture) and *in vivo* (BALB/c mice). Five commonly used nucleoside analogs were tested: acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV), cidofovir (CDV), and 5-ethyl-2-deoxyuridine (EDU) were tested *in vitro* and *in vivo.* Additionally, seven other antivirals were tested *in vitro*: iododeoxyuridine (IDU), trisodium phosphonoformate (PFA), trifluorothymidine (TFT), brivudin (BVDU), adenine arabinoside (AraA), 5-bromo-deoxyuridine (BUdR) and 9-(4-hydroxybutyl)-N2-phenylguanine (HBPG). In plaque assays the drugs CDV, EDU, GCV and PCV were most effective. In mice, GCV and CDV showed efficacy, while PCV, ACV and EDU were not effective. For all but one of the twenty-four HVP2 *in vitro* tests, drug efficacy *in vitro* and *in vivo* against HVP2 paralleled that of BV. These results indicate that drug sensitivity of HVP2 is predictive of BV drug sensitivity.

Delaying initiation of drug therapy, which mimics a human exposure to BV where seeking of medical attention can often be delayed by days, further tested the predictive nature of GCV and CDV treatment. In both drug therapy onset studies, as the time treatment delay increased so did the neurological involvement. There was however a temporally abrupt break in protection from death, with HVP2 paralleling that of BV. With all testing done, results for HVP2 paralleled those of BV. This work serves to validate the accuracy of HVP2 as a predictive model for BV drug sensitivity. This validation of the HVP2 model represents a unique and safe alternative to screen or discover anti-BV drugs.

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LITERATURE REVIEW

Viruses are some of the smallest of all biological entities, and have historically been characterized by their ability to pass through filters that retain bacteria. In their most basic form viruses are small segments of nucleic acid encased in a simple protein shell. They have no metabolism of their own but rather invade cells and manipulate the host cell machinery to their own end. They can be compared to Von Neumann machines, but I see them more as brute chemical zombies.

The Herpesviruses

Few virus families exhibit as much variation as that of the family *Herpesviridae*. Family *Herpesviridae* is defined by the Baltimore classification as Group I: dsDNA virus, and by the International Committee on Taxonomy of Viruses (ICTV) as Order: *Herpesvirales*, Family: *Herpesviridae*. The Herpesviruses draw their name from the Greek herpes, ερπειν, which means "to creep" and probably refers to the recurrent skin lesions some of these viruses cause. Of the approximately 130 identified herpesviruses, 8 are currently recognized as a human herpesviruses (Field, 2381). In Shakespear's Romeo and Juliet, Mercutio speaks to Romeo about the lures of the women saying "O'er ladies lips, who strait on kisses dream, which oft the angry Mab with blisters plagues." (Romeo & Juliet. 1.4.74-75) Never has a venereal disease been so eloquently defined.

Membership in the family *Herpesviridae* is based on the structure of the virion, which has four distinct architectural elements. First, the viruses contain a dense fibrillar

core onto which DNA is wrapped in the form of a torus; the exact arrangement of this DNA nucleoid is not known. Second, herpesviruses have linear, double-stranded DNA genome with a G+C content ranging from 32 to 75%. Herpesvirus genomes vary in size from 85 kbp for varicella zoster to 250 kbp for the cytomegaloviruses. Third, the nucleoid, or core, is encapsulated in an icosadeltahedral capsid. The capsid is 100-110 nm in diameter made up of 12 pentameric capsomeres and 150 hexameric capsomeres. A tightly adhered asymmetrically arranged tegument then covers the capsid. Fourth, surrounding the capsid-tegument structure is a typical lipid bilayer envelope. The viral envelope element of the virion is 200-300 nanometers in diameter and consists of polyamines, lipids, and glycoproteins. Some virion glycoproteins are derived from host cell membranes but most are encoded by the virus. Herpesvirus envelopes contain glycoproteins that are both more numerous and shorter than those of other enveloped viruses. (Fields, 2387)

When viewing herpesviruses by electron microscopy it is not possible to distinguish the specific type of herpesvirus based upon virion structure. Also, the outer layers of tegument and envelope may be missing from some viral particles due to a lack of viron structural rigidity. Different herpesviruses are distinguished based upon biological properties, the immunological specificity of their virion proteins, and the size and base composition of the genome. Based on these properties, herpesviruses are divided into four subfamilies designated alpha (α), beta (β), gamma (γ), and delta (δ). All of the mammalian herpesviruses fall in the alpha, beta, and gamma subfamilies; the delta subfamily is found in reptiles, amphibians, and fish.

The human herpesviruses known most to the general public are those within the alpha subfamily. This subfamily includes the herpesviruses responsible for cold sores and genital herpes (herpes simplex 1 and 2; HSV1 and HSV2, or HHV-1 and HHV-2), and the chickenpox virus (varicella zoster; VZV or HHV-3). In general alpha-herpesviruses have a short replicative cycle of several hours. Alpha-herpesviruses cause prompt destruction of the host cell, which is the cause of the cytopathic effect visible in cell culture monolayer. They also generally have a broad host range and characteristically establish latency in sensory nerve ganglia.

The beta subfamily includes human cytomegalovirus (CMV, HHV-5) and the relatively new viruses HHV-6 (variants A and B) and HHV-7. Beta-herpesviruses typically have a long replicative cycle of several days, with infections progressing slowly in cell culture. Beta-herpesviruses also have a fairly restricted host range and establish latent infections in secretory glands, cells of the mononuclear phagocyte system, or the kidneys. A characteristic of these viruses is the formation of enlarged or giant cells, from which the cytomegaloviruses get their name.

Gamma-herpesviruses include Epstein-Barr virus (EBV, HHV-4) and Kaposi's sarcoma virus (KSHV, HHV-8). Gamma-herpesviruses have a very restricted host range and primarily replicate in lymphoblastoid cells in which they may cause lytic infections. Latency has been shown to occur in lymphoid tissue.

Herpesvirus Infection

HSV1 is the prototypic herpesvirus, and consequently much of the research done on herpesviruses has been done on HSV1. As a result, much of what is known of

herpesvirus replication and latency is from HSV1 studies. It is assumed herpesviruses replication has a large amount of similarity across the subfamilies.

Herpesvirus infection and subsequent replication begins with the virus entering a host cell. Viral envelope glycoproteins bind to cell surface proteins, bringing the viral envelope and cell plasma membrane into close proximity. Cell tropism of the virus is determined by the availability of the correct receptor on the surface of the host cell. In HSV1, envelope glycoprotein C (gC) binds to host cell surface receptor heparan sulfate. A second viral glycoprotein, gD, then acts as a coreceptor to bind one of three possible host receptors. One of these is a member of the tumor necrosis factor (TNF) family called HveA, which itself a member of the immunoglobin superfamily nectin-1. The other two possibilities are members of the family of 3-O-sulfated heparan sulfate.

The last step of entry is fusion of the viral envelope with the plasma membrane of the host cell. Once bound, gD changes conformation and interacts with viral envelope proteins gH and gL. This interaction forms a hemifusion state complex. Viral glycoprotein gB then interacts with the gH/gL heterodimer and host surface glycosaminoglycans to create an entry pore through which the viral capsid-tegument structure to transit into the host cytoplasm.

Once the tegument-surrounded nucleocapsid enters the cytoplasm it is transported via the microtubular network to nuclear membrane pores. The viral DNA is injected into the host nucleus through an axial channel portal on the vertex of the capsid. Twelve copies of the portal protein UL6, which contains a leucine zipper motif that allows the copies to bind together to arrange to a ring, form the portal (Newcomb, et al 2003). Each capsid contains a single vertex-located portal.

Tegument proteins are collectively introduced into the cytoplasm along with the capsid. Some tegument proteins play a key role in creating an intracellular environment that is conducive to viral replication. One important tegument protein of note is a product of viral gene UL41, the virion host shutoff protein (vhs). The vhs protein is released directly into the cytosol where it immediately impacts the host cell. The vhs protein is an endonuclease that degrades host mRNAs, leading to a decrease in host protein synthesis and rapid turnover of viral mRNA which promotes the ordered expression of the three kinetic classes of viral genes. In addition, vhs is also an important determinant of virulence because of its role in hampering the host cell interferon- mediated host immune response and by down regulating host major histocompatibility complexes I and II.

Herpesvirus proteins are grouped into three kinetic classes; immediate-early (IE), early (E), and late (L). The immediate-early, early, and late classes are also referred to as α, β, and γ. There is also the possibility of an additional stage, a lytic stage called delayed-late. The IE proteins are the first viral genes expressed following infection. Transcription of IE genes requires no prior viral protein synthesis and instead is initiated by the viral tegument αTIF (or VP16) protein. VP16 stimulates the transcription of several IE genes, one of which is ICP4. Viral genes are also regulated post-transcriptionally, but the major control of gene expression is through transcriptional regulation. IE proteins are involved in transcriptional regulation of viral E genes and are generally not found in the mature virion.

The IE protein ICP4 aids in the shift from IE to E gene expression by repressing its own transcription. Some E gene products also down regulate IE gene expression. An example of this is the E protein ICP8 which decreases expression of the ICP4 gene. E

proteins are generally involved in DNA replication and include the DNA polymerase and other proteins involved in DNA replication.

During the final replicative stage, the late (L) or gamma genes are expressed. L protein synthesis is initiated after the start of DNA synthesis. The L proteins are synthesized in large amounts and are primarily structural components of the virion. L genes are translated in the cytoplasm and many of the proteins are then transported back to the nucleus for capsid assembly. Glycoproteins that are components of the viral envelope are made in the rough ER and move to the nuclear membrane by diffusion. Viral surface proteins also concentrate in areas of the outer plasma membrane where viral capsids eventually bud.

Assembled nucleocapsids have several possible pathways for exiting the host cell. The classic route is budding through the double nuclear membrane in a process called primary and secondary envelopment. The primary envelope is acquired by budding through the inner nuclear membrane. This envelope then fuses with the outer nuclear membrane releasing the naked capsid into the cytoplasm. The virion then acquires its final envelope by budding into cytoplasmic vesicles and leaving the cell by means of exocytosis pathways. Alternatively, the nucleocapsid may acquire its secondary envelope from the outer nuclear membrane. This envelope is kept intact as the virion enters the cytoplasmic space, and the enveloped virion is released from the infected host cells by cell lysis.

Herpesviruses encode their own DNA-dependent DNA polymerase for replication of the viral genome. However, the host RNA polymerase II is responsible for transcription of all viral gene products. The virus also encodes additional “non-essential”

enzymes that are not required for viral replication in tissue culture. These proteins allow the virus to replicate in non-dividing cells that lack certain precursors of DNA synthesis. One such example of non-dividing cells are neurons, where herpesviruses establish latent infections. Examples of these nonessential gene products include the thymidine kinase (TK), deoxyuridine triphosphatase, and uracil N-glycosylase enzymes.

Viral DNA Replication

The HSV1 linear genome is 152-kbp. It contains three origins of replication (ori) and 75 open-reading frames (Figure 1). The genome has two unique regions, U_L and U_s (unique long and short), that are flanked by repeat regions 'a' and 'b' or 'c'. The repeat regions are present at both ends of the genome as terminal repeats (TR) and as an internal repeat region (IR).

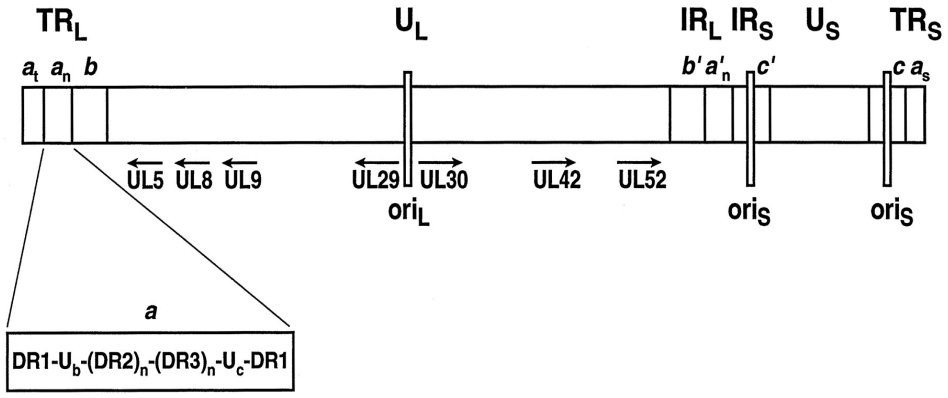

Figure 1. Structure of the Herpes Simplex Virus Genome.
A representation of the HSV1 genome. Terminal repeats (TR_L and TR_S) and internal repeats (IR_L and IR_S) are shown, as well as the positions of the 'a', 'b', and 'c' repeats within them. The origins of DNA replication (ori_L and ori_S) are shown, and the positions and direction of transcription of the seven essential DNA replication genes are indicated by the arrows. The enlarged area shows the composition of a unit length 'a' sequence. It consists of direct repeats (DR) 1, 2, and 3 and unique (U) domains 'b' and 'c'. Source: Boehmer 1997

For HSV1, (and most other alpha-herpesvirus) DNA replication requires 7 viral proteins, all of which are encoded by genes located within the U_L domain: the

hetrodimeric DNA polymerase (UL30) and accessory protein (UL42), origin-binding protein (UL9), the ICP8 ssDNA binding protein (UL29), and the hetrotrimeric helicase-primase composed of the UL5/UL8/UL52 proteins. Before replication begins prereplicative sites are needed on the parent DNA. These sites are formed by several early proteins that are transported to the nucleus and assemble on the parental viral DNA near ND10 structures (Field, 2421). Genome replication has two phases; the first being bidirectional theta replication, and the second rolling-circle replication. The rolling-circle replication mode is the predominant form of HSV genome replication.

Viral DNA replication begins at the palidromic A-T rich oriL and oriS sites. The origin binding protein UL9 is a homodimer with 3' to 5' helicase activity that specifically binds to the sequence CGTTCGCACTT. Binding of UL9 causes the DNA to bend, inducing the formation of a single-stranded stem-loop. UL9 then begins to unwind the DNA and aids in synthesis of an initiating primer. The ssDNA binding protein ICP8 associates with ssDNA in the unwound areas of DNA which recruits the helicase-primase proteins UL5, UL8 and UL52. The helicase domain then assembles at each replication fork and initiates theta replication. The helicase activity unwinds the ICP8 coated ssDNA strands, while the primase activity produces oligoribonucleotides 6 to 13 bases in length that serve as primers (Field, 2423).

The heterodimeric DNA polymerase UL30 and processivity factor UL42 associate with the replication structure to begin DNA synthesis on the leading strand. UL30 also has an intrinsic 3' to 5' exonuclease that acts as a proofreader. While the accessory protein UL42 appears to only increase the affinity of UL30, it is essential for DNA replication *in vivo*. An unknown mechanism causes the theta mode of replication to

switch to a rolling-circle replication style. The circular DNA structure is nicked to produce the rolling-circle style, thereby making the UL9 protein unnecessary. The UL9 protein disassociates from the replication complex at this point. During the rolling-circle replication phase, long concatemeric strands of DNA are produced. While genome replication is starting, additional viral proteins involved in genome replication are synthesized. Some of these are "non-essential" enzymes. While not required for replication of the virus *in vitro*, disruption or deletion of these genes often has a profound effect on the *in vivo* virulence of HSV, suggesting they are important *in vivo*.

More than 30 viral gene products are required for HSV capsid formation. Viral genomes are encapsidated with the aid of cleavage/packing proteins. Empty capsids assemble in the nucleus and contain scaffolding proteins that are involved in DNA packaging. Initial stages of capsid assembly begin in the cytoplasm with synthesis of the major capsid protein VP5 (UL19) and the minor capsid proteins VP26 and VP23. The VP5 capsid protein is transported to the nucleus, where final capsid assembly occurs. Once in the nucleus VP5 associates with UL26 and UL26.5 to complete the capsid scaffolding. UL18 and UL38 then assemble around the scaffolding, creating a procapsid. As DNA is packaged into the capsid, autoproteolytic cleavage occurs and the internal scaffolding proteins are removed.

Viral DNA encapsidation is directed by motifs within the Uc and Ub domains (adjacent to the L and S termini; see Figure 1) of the 'a' sequence named Pac1 and Pac2. Within the viral genome there are 17 to 20 bp direct repeats called DR1 that flank the 'a' sequences. Cleavage events cause genomic termini to be created near the end of 'a' DR1 sequence. When fusion of genomic ends occurs, a 200 bp fragment is made, Uc-DR1-Ub,

that spans the junction between tandem a sequences (Tong and Stow, 2010). The cleavage/packing motifs contain all the essential cis-acting sequences necessary for DNA packing. The ‘a’ sequences of the motifs Pac1 and Pac2 are recognized and bound to by viral proteins. The Pac2 sequences are cleaved, which initiates DNA packaging. Genome packaging then proceeds from Pac2 toward the Pac1 terminus.

The concatemeric DNA is fed into the capsid until a “headful” of DNA is reached; this appears to be determined when another ‘a’ sequence is reached, indicating one genome equivalent has been packaged. An asymmetric cut within the Uc domain of the ‘a’ sequence is introduced on both strands of DNA. The two nicked ends are then joined. Once the genome has been encapsidated, the nucleocapsid is ready to bud.

Latency

A latent infection is one in which the viral DNA remains in the host cell but no infectious virus is detectable. During latency very few viral proteins are expressed, allowing the virus to evade host defenses. HSV establish latent infections in sensory neurons innervating the mucosal tissue of the site of primary infection. During primary infection the virus replicates in columnar epithelium of mucosal tissue. The virus then enters unmyelinated sensory nerve endings and undergoes retrograde transport via microtubules to the nucleus of the neuron. HSV1 tends to establish latency in trigeminal ganglia which innervate the oral cavity, while HSV2 establishes latency in sacral ganglia which innervate the genital tract. However, latent infections have also been found in the vagus nerve, which innervates the lungs and stomach (Roizman, 1985, pg 12).

Latency is a complex process that involves many host cell factors, cellular repressors and activators, and viral functions. During latency the majority of gene

expression is limited to the Latency-Associated Transcripts (LAT). LAT RNAs are known to regulate host cell genomes and to protect neurons from apoptosis through down-regulation of viral lytic gene expression. HSV1 LAT RNAs have two ORFs (O and P) that are completely repressed by ICP4 during productive infection (Fields, 2469). These ORFs overlap and are antisense to the ICP 34.5 gene. The ICP 34.5 protein plays a large role in neurovirulence, as deletion of the ICP 34.5 gene removes the ability of HSV to both invade and replicate in the central nervous system (CNS).

The virus can remain latent for long periods of time, and the exact process of reactivation is not well understood. It does appear that reactivation is dependent on an intact anterior nerve route and peripheral nerve pathways. Possible triggers for reactivation are stress and stressors such as hyperthermia, UV light, illness and menstruation. When HSV reactivates it is transported anteriograde to the mucosal epithelial layers where it is released from the neuron to infect squamous epithelial cells. Replication in epithelial cells can result in either lesion formation and shedding of virus or asymptomatic shedding.

Monkey B Virus

Macacine herpesvirus 1 (Monkey B virus; BV) is an alpha-herpesvirus closely related to HSV1 and HSV2. BV occurs naturally in macaque monkeys of the genus *Macaca*, such as rhesus and cynomolgus monkeys. BV was first isolated in 1932, and prior to 2008 was known as *Cercopithecine herpesvirus 1* or *Herpesvirus simiae*. Of the 35 known nonhuman primate herpesviruses, BV is the only one that is highly pathogenic in humans. Serious disease caused by BV is rare in macaques, but when BV is

transmitted to humans it is often lethal. Consequently BV is a Risk Group 4 virus and until December 2012 was listed as a Select Agent by the CDC.

BV has a typical herpesvirus virion structure: an icosahedral capsid and tegument covered by a lipid envelope. The complete genome sequence was determined for strain E2490 which is an attenuated vaccine strain that was originally isolated from a rhesus macaque (Hull, 1971 and Perelygina et al, 2003). The total genome length is 156,789 bp, with an overall G+C content of 74.5%. The genome of BV is thus slightly larger than the HSV1 and HSV2 genomes (152kb and 155kb respectively). Similar to HSV, the U_L and U_s regions are flanked by inverted repeated regions. BV has 74 genes, all but one being homologous to HSV genes. Surprisingly, the one gene that BV lacks is a homolog of the HSV ICP 34.5 gene, which is a known neurovirulence factor in HSV1 and HSV2. This suggests that replication of BV in neuronal cells involves mechanisms somewhat different from those of HSV.

There have been about 50 reported cases of human BV infection, with 26 of these being well documented (as of 2002, Cohen et al, 2002). Most human infections have resulted from bites or scratches by macaques. However, some cases have resulted from needlesticks, splash of urine into the eye, and contamination of a cut with media from primary macaque cells. Incubation time, site of symptoms, and progression of clinical symptoms varies from case to case. The incubation from initial exposure to development of symptoms can vary from 2 days to 10 years (Weigler, 1992). For well documented cases, initial symptoms usually present 1-3 weeks after exposure, with some cases developing as late as 5 weeks. Symptoms may present near the site of exposure and then progress, or may be limited to either the peripheral nervous system (PNS) or CNS.

Initially flu-like symptoms may present as fever, chills, and myalgia. A vesicular rash may develop at the site of exposure that can tingle, itch, be painful, or numb. The virus usually spreads along nerves to the spinal cord, and then ascends to the brain. Once the infection reaches the brainstem the chances of survival fall dramatically, with encephalomyelitis being a sign of the terminal stages of the disease. Patients who have survived BV infection tend to have residual neurological problems or experience progressive neurological deterioration.

Baboon Herpesvirus 2

Papiine herpesvirus 2 (Herpesvirus papio 2; HVP2), formally *Cercopithecine herpesvirus 16,* is a simian alpha-herpesvirus that is closely related to HSV and especially to BV. When HVP2 was originally isolated from a babboon in 1969, it was identified as SA8 (*Cercopithecine herpesvirus 2*). However, based upon differences in DNA sequence and antigenicity of the viral glycoproteins, HVP2 was later recognized as a distinct virus (Eberle, 95). Over 90% of adult baboons carry HVP2. The virus causes orogenital infections similar to those of HSV in humans, and the virus also establishes latent infections in sensory ganglia. HVP2 is seldom virulent in primates other than its natural host. However, both HVP2 and BV are neurovirulent in mice.

The genome of HVP2 strain X313 has been sequenced and is 156,487 bp in length with an overall G+C content of 76.5% (Tyler and Severini, 2006). Similar to other simplexviruses, HVP2 has U_L and U_S regions bordered by inverted repeats that end with an ‘a’ sequence. All ORFs of HVP2 are homologous with those of SA8 and BV. HVP2, like SA8 and BV, is also missing a homolog of the HSV neurovirulence gene ICP 34.5. While HVP2 is 85% homologous to its closest relative SA8, there are two regions in

which HVP2 shows greater homology to BV. The first of these regions is the UL41 - UL44 genes, and the second is within the large UL36 gene; the significance of this is unknown.

Antiviral Therapy- TK Class Drugs

Nucleoside analogs are the largest and most widely used class of antiviral drugs used to treat HSV infections. These nucleoside analogs are also called the "TK class" because they interact with and are first phosphorylated by the viral thymidine kinase (TK), which is a product of the UL23 gene. This drug class was first introduced in the 1960s with adenine arabinoside (AraA) being one of the first. AraA was later replaced in popularity by the less toxic acyclovir (ACV), a drug which earned its developer Gertrude Elion the 1988 Nobel Prize in Medicine. For the treatment of HSV, the TK drug class currently has three major members; acyclovir, penciclovir, ganciclovir (and their respective prodrug forms).

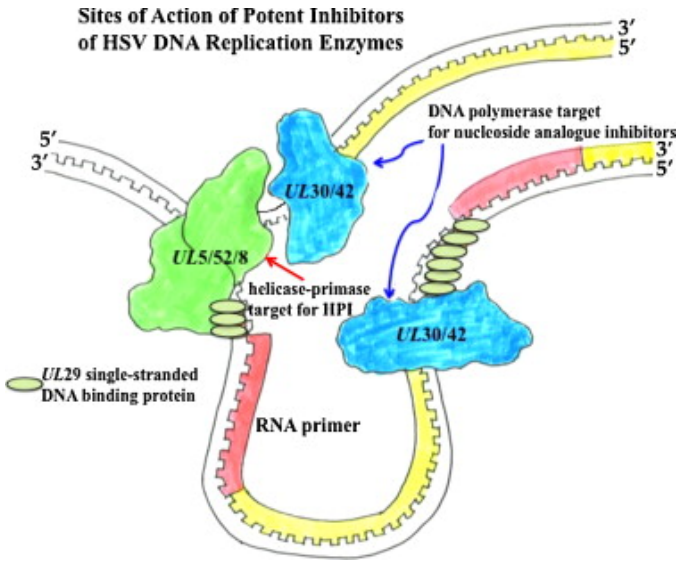

Figure 2. Sites of Action of Potential Antiviral Drug Classes.
Diagram showing the site of action of nucleoside analogs (e.g. ACV or GCV) and helicase-primase inhibitors (e.g. BAY 57-1293 or BILS 22). Source: Field 2011.

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Acyclovir

Acyclovir (ACV) is the most widely used and prototypic antiviral agent. ACV is most active, and highly documented, against HSV. ACV is a deoxyguanosine analogue with an acyclic side chain that lacks a 3'-hydroxyl group, as seen in the structure shown in Figure 3. ACV interferes with viral DNA synthesis by terminating DNA chain elongation. The mechanism of activity is demonstrated in Figure 3. ACV is monophosphorylated by the viral TK which gives ACV specificity for virus infected cells as compared to uninfected host cells. The ACV-mp is then diphosphorylated and triphosphorylated by host cell kinases. The triphosphate acyclideoxyguanosine analogue form competes with the natural nucleoside deoxyguanosine as a substrate for the viral DNA polymerase. Once incorporated into the viral DNA, the lack of a 3'-hydoxyl group (which is required for addition of the next nucleoside) results in the growing DNA chain being terminated. ACV triphosphate is a better substrate for the viral polymerase as

compared to the host DNA polymerase, so there is little incorporation of ACV into host DNA, and consequently ACV has a low toxicity.

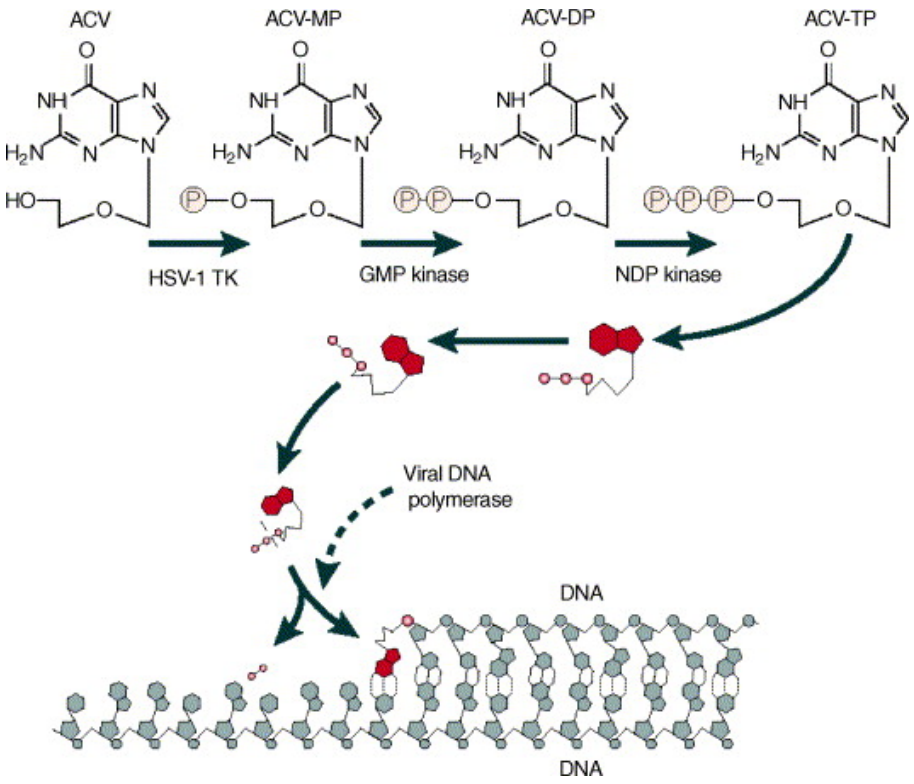

Figure 3. Mechanism of Antiviral Action of ACV.
The figure shows the triphosphorylation ACV must undergo before it can interact with the DNA polymerase. The first phosphorylation is preformed by the viral thymidine kinase, and the following two by host cellular kinases. Source: De Clercq 2004

In humans the oral bioavailability of ACV is poor, with only 15%-20% of the drug being absorbed, making frequent dosing a requirement (Van der Plas et al, 2011). An oral 200 mg dose gives a peak serum concentration of 0.5µg/ml (Wagstaff et al, 1994). IV dose rates of 2.5 to 15 mg/kg/day give a steady-state serum concentration of 6.7 to 20.6 µg/ml. In a healthy adult, ACV has a half-life of 2 to 3 hrs, with 60% of administered drug being excreted in urine (Snoeck).

For treatment of genital HSV, dosing options include ACV orally at 200 mg five times a day or 800 mg orally two times per day, for 5 days (De Clerq, 2004). A typical treatment for oral HSV is 400 mg orally twice daily for 5 days. For treatment of HSV encephalitis, IV ACV is administered at 30 mg/kg/day for 14-21 days (De Clerq, 2004). Higher dose rates of 45-60 mg/kg may be needed, with toxicity being the limiting factor (Van der Plas).

Valacyclovir (VACV) is the prodrug form of ACV, which means it is converted to the active compound during first-pass hepatic metabolism and has a greater bioavailability than the parent molecule. The bioavailability of VACV is 54% as compared to the 15%-20% for ACV (Snoeck). VACV is better known by its trade name, Valtrex. While VACV can be used to treat herpes zoster, it's primarily used in treatment of genital HSV. Dosing is similar to ACV, VACV being prescribed orally at 500 mg twice daily for 5 days (De Clerq, 2004).

ACV is a relatively safe drug that is generally well tolerated with minimal side effects. Oral ACV can cause mild gastrointestinal upset, rash, and headache. IV ACV occasionally causes rash, sweating, nausea, headache, hematuria, and hypotension. If IV ACV is given too rapidly or to a dehydrated patient, ACV crystals can form in the renal tubules causing an obstructive nephropathy leading to reversible nephrotoxicity. The most serious potential side effect of ACV is neurotoxicity, which usually only occurs in patients with compromised renal function whose drug serum peak concentrations result in toxic levels. ACV neurotoxicity results in lethargy, confusion, hallucinations, tremors, and seizures.

Penciclovir

Penciclovir (PCV) is another acyclic nucleoside analogue of guanosine, and like ACV, prevents viral DNA synthesis through interaction with the viral DNA polymerase. PCV becomes triphosphorylated by the same process of viral TK and host cell enzymes. However, the affinity of ACV for viral TK is 100-fold less than that of PCV (Vera). This results in an increase of phosphorylation of PCV compared with that of ACV. Triphosphatorylated PCV competes with the natural nucleoside deoxyguanosine as a substrate for the viral DNA polymerase. Unlike ACV, PCV does not cause DNA chain termination but rather retards the rate of subsequent nucleotide incorporation. PCV has lower potency against HSV than ACV, as seen by the EC_{50} in Table 1 (Crumpacker). However, it has a longer half-life leading to higher intracellular concentrations that make it an effective antiviral.

Virus	EC_{50} (μg/ml)	
	PCV	ACV
HSV1	0.4 ±0.1	0.2 ± 0.2
HSV2	1.5 ± 0.4	0.6 ± 0.2
VZV	3.1 ± 0.8	3.8 ± 0.7

Table 1. EC_{50} Comparison Between PCV and ACV
Shown is the activity of ACV and PCV against herpesviruses in MRC-5 cells. There were several isolates tested for each virus: 17 of HSV1, 13 of HSV2, and 5 of VZV.

PCV itself has a low bioavailability, so the prodrug famcyclovir is often administered instead. Famcyclovir (FCV) is the inactive diacetyl ester form of PCV that, once metabolized, has a bioavailability of 70%. PCV has an intracellular half-life of 7 to 20 hrs and a serum half-life of about 2.5 hrs, with 75% being excreted in urine.

PCV is principally used for treating mucocutaneous HSV infections, particularly recurrent herpes labialis. PCV treatment of cold sores is through topical ointment with application every 2-4 waking hours (De Clerq, 2004). FCV is favored over PCV for

treatment of HSV using an oral dose. For genital herpes therapy FCV is prescribed 750 mg/day orally for 5 days (De Clerq, 2004). For oral herpes, FCV is given at 250 mg orally twice daily for 5 days. IV PCV treatment for HSV encephalitis is not typical. The side effects of PCV are similar to ACV, but there is less chance of toxicity.

Ganciclovir

Ganciclovir (GCV) is a nucleoside analog that is structurally similar to ACV, but differs by having an extra hydroxymethyl group on the acyclic side chain. Unlike ACV, GCV is phosphorylated by other enzymes in addition to the viral TK and so it has less virus-specificity and a greater potential for toxicity. The bright side of the ease of phosphorylation is that GCV has efficacy against herpesviruses that lack a TK enzyme, like CMV. GCV can be incorporated by the cellular DNA polymerase as well as by the viral polymerase, which gives GCV significant myelotoxicity. GCV is not recommended for the treatment of HSV infections unless treatment or suppression of both HSV and CMV is required (Van der Plas).

GCV has a poor bioavailability with less than 10% of drug being absorbed following oral administration (Frenkel). The prodrug valganciclovir (VGCV) is an L-valine ester form that once quickly metabolized, has a bioavailability of greater than 60% with a plasma half-life of about 30 mins (Jung). VGCV is not typically used to treat HSV, but instead is usually used to treat CMV. Oral VGCV therapy dosage is 900 mg twice daily for 2 weeks (De Clerq, 2004). IV administration of GCV as a 1 hr constant rate infusion at a dose of 5 mg/kg gives peak serum levels of 8 to 11 μg/ml. Typical dose rates of IV GCV are 10 mg/kg once to twice a day for 2 to 3 weeks (De Clerq, 2004).

GCV has the highest chance of adverse side effects of all the analog nucleosides. Bone marrow suppression from IV therapy is the most common serious side effect. About 40% of GCV patients experience dose-related neutropenia of < 1000 WBC/μl (Markham). Unlike ACV, GCV is mutagenic, carcinogenic, and can cause irreversible toxicity. Because of the high incidence of adverse side effects and long-term effects of mutagenicity and toxicity, GCV use in pediatric patients is highly discouraged (Frenkel). Oral VGCV is much better tolerated than IV GCV, with milder side effects (usually including diarrhea, nausea, neutropenia, and headache).

Cidofovir

Cidofovir (CDV) is an acyclic nucleoside phosphonate drug that is active against virtually all DNA viruses, including all eight human herpesviruses (De Clercq, 2007). A lot of attention has also been paid to the fact that CDV is active against smallpox. This is important in the light of the potential use of variola major as a biological weapon. In a mouse model, mice exposed to mousepox virus (the murine equivalent of smallpox) a single treatment of CDV was able to fully protect mice from death, even if treatment was delayed by 6 days (Israly). CDV is a prodrug that must undergo a two-stage phosphorylation that converts it into its active DNA nucleoside form (Donne). Intracellurally, CDV is diphosphorylated by cellular kinases, and then two units must be incorporated into the growing DNA to cause chain termination. It is a competitive substrate for the viral DNA polymerase.

When CDV is used in humans in the treatment of HSV1, it is given intravenous at a dose rate of 100 or 250 mg/kg/day. In mice, one dose even as late as 4 days post exposure was able to provide protection from death (De Clercq, 2007). In a case of a

human with severe combined immunodeficiency, the problem of a severe full-body HPV infection called generalized verrucosis occurred. Intraveneous CDV was used at the rate of 5 mg/kg weekly for 2 weeks, then every other week for a total of 3 months. At the end of therapy there was no sign of lesions (Potthoff).

5-Ethyl-2-Deoxyuridine

The antiviral 5-ethyl-2-deoxyuridine is referred to by the name EDU. It is a nucleoside analog like ACV, PCV, and GCV. Unlike the other drugs which are acyclic purine analogs, EDU is a cyclic pyrimidine nucleoside analog. Like similar nucleoside analogs, the active form of EDU is triphosphorylated. The kinases can be host or viral. DNA with incorporated EDU is less efficient as a template for further DNA replication, and so with more EDU incorporation the greater the likelihood of viral replication termination.

Foscarnet

Trisodium phosphonoformate (PFA) was originally constructed in 1924 by Nylen and is a salt of phosphonoacetic acid (PAA) (Helgstrand). PFA is an antiviral most commonly used clinically in AIDS chemotherapy under the names Foscarnet and Foscavir. Although therapy of HIV and CMV infections are its primary indications, PFA is also effective against HSV. PFA is used as a salvage therapy for HSV infections that do not responsed to ACV (Stranska).

PFA does not depend on conversion to an active from by viral TK or any other viral or cellular enzyme (Snoeck, Gilbert). PFA acts by inhibiting reverse transcriptase in HIV and inhibiting DNA polymerase in CMV and HSV. Action is inhibited by blocking

the pyrophosphate binding sites as it is an analogue of ubiquitous pyrophosphate. (Ferguson)

PFA has a relatively high therapeutic index. In Vero cells infected with HSV1, 100 uM PFA reduces viral DNA synthesis by 85% and cellular DNA synthesis by 15% (Helgstrand). This shows PFA selectivity on viral DNA synthesis. However, PFA has poor oral bioavailability due to its polyanionic nature at physiological pH. When prescribed to treat ACV-resistant HSV or VZV in immunocompromised patients, PFA is administered IV at 120 g/kg/day (De Clercq, 2004).

TK Drug Resistance

Most cases of viral TK drug resistance arise in severely immunocompromised patients receiving prolonged antiviral therapy, with 1% of PFU isolated having drug resistance (Field, 1986). HSV resistance to TK antivirals, and in particular ACV, is through one of three mechanisms: complete TK deficiency, decreased TK production, or TK with altered substrate specificity (Gilbert). Mutations of the TK gene are usually due to an addition or deletion of a nucleotide in long homopolymer runs of G or C. Two studies (Morfin, Gaudreau) have shown that 50% of ACV-resistant HSV contained such a mutation. The resulting framshift mutation causes a truncated or absent TK polypeptide.

Another way for HSV to become resistant to ACV is through a mutation in the HSV DNA polymerase. Unlike TK, the DNA polymerase is essential for virus replication under all conditions. Therefore, only mutations in the polymerase leading to an altered affinity for ACV are possible. These types of drug resistance mutations are rarer than TK mutations, with cell culture plaque-purified isolates being present at $<1/10^6$ PFU (Field, 2011).

When tested in animal models, TK-deficient (ACV resistant) HSV showed dramatically reduced pathogenicity, and these strains were generally impaired in their ability to reactivate from latency (Field, 2001). This can be explained by the TK being unnecessary to establish latency, but being necessary for reactivation and/or viral replication in neurons following reactivation (Efstathiou).

Antiviral Therapy- Helicase-Primase Inhibitors

Before the herpesvirus DNA polymerase can incorporate nucleotides into a growing DNA strand, the double-stranded viral DNA must first be unwound and primed. This is accomplished by the hetrotrimeric helicase-primase composed of the UL5/UL8/UL52 proteins. A new class of drugs, the helicase-primase inhibitors (HPI), use non-nucleoside thiazoylphenyl compounds that target the three proteins of the HSV helicase-primase enzyme to stop viral DNA replication. These compounds appear to work by enhancing the affinity of the helicase-primase complex for HSV DNA, thereby preventing normal progression through helicase or primase catalytic cycles (Biswas, 2011).

The two most notable HPI are the thiazoylphenyl derivatives BILS 179-BS and BAY 57-1293. BILS 179-BS is orally effective against HSV1 and HSV2 in animal models, with a dose rate of 15 mg/kg/day preventing mortality, lessening clinical signs, and reducing infectious virus in tissues below the level of detection within a few days (Biswas, 2007). Most importantly, BILS 179-BS was more effective than ACV when the treatment was reduced or when initiation of treatment was delayed up to 65 hrs after infection (Crute). Antiviral activity was also demonstrated for multiple strains of HSV, including strains resistant to nucleoside analogs. The EC_{50} for HSV1 strain KOS (WT

strain) was 0.08 uM for BILS 179-BS and 0.97 uM for ACV. For HSV1 strain 615.9 (nucleoside resistant strain) the EC_{50} was 0.13 uM for BILS 179-BS and 29.8 uM for ACV.

BAY 57-1293 was shown to be much more potent than VACV, while still having the same level of safety and low side effects. BAY 57-1293 allowed viral expression of IE proteins but budding capsids did not contain viral DNA, presumably as result of reduction in E and L gene expression (Blaustein).

While not as common as resistance to nucleoside analogs, drug resistance to HPI has been documented. Point mutations have been found in the HSV1 UL5 helicase (Sukla). Sukla showed that while HSV1 TK mutations have a frequency of $<1/10^{-4}$ PFU, the HSV1 UL5 mutation had a lower frequency of $<1/10^{-6}$ PFU. This is the same likelihood of a DNA polymerase mutation. Interestingly, some clinical isolates of HSV1 were shown to contain HPI drug resistant mutants at high frequency, even in samples of patients who had not previously been administered HPI. Individuals having pre-existing HPI resistance mutations could explain this finding. This theory was supported by the findings of a study where 5/30 HSV1 clinical isolates were shown to have pre-existing mutations (Field, 2011). This finding may have an impact on the use of HPI as an antiviral in general population use.

Animal Model Drug Sensitivity of BV

The choice of antiviral drugs and dosing usually comes from studies done in animal models. However, these studies are not completely predictive of drug efficacy in humans for several reasons (Cohen). The first is that the animals are usually experimentally infected with a virus dose that is much larger than any exposure a human

would reasonably receive, often many times the lethal dose. Next, the animal model typically used for BV, the rabbit, is much more sensitive to BV than humans are; the virus invades the CNS and causes death much quicker than in humans. Attached is a concern regarding the drug most commonly used to treat BV infections, ACV. ACV has a shorter plasma half-life in rabbits than humans. Another concern not widely addressed yet is that studies using the rabbit model infect the animals by injection (intramuscular, subcutaneous or intracerebral) rather than a scarification model more typical of exposure from bites and scratches. BV may not be able to infect as readily via a needle stick exposure as compared to a scratch exposure because herpesviruses generally replicate in the dermis where they can readily invade unmyelinated nerve endings.

ACV was evaluated for efficacy in rabbits infected by subcutaneous injection with a lethal dose of BV and then giving IV ACV for 5-14 days (Boulter). It was found that rabbits that received antiviral therapy for 2 weeks started within 24 hrs of infection were completely protected from death. This compares to subjects that had treatment stopped at 10 DPI and then died. It was therefore suggested that a 14 day regime is needed because ACV does not eliminate BV in less than 12 days, instead just suppressing replication so that if therapy is removed a fatal infection still develops. However, the survival rates relied heavily upon the rabbits keeping a constantly high plasma ACV concentration, leading to the animals receiving the IV ACV at very short intervals. The rate found to be the most effective was 200 mg/kg every 8 hrs. One surprising finding was that about half of the rabbits that were delayed drug therapy by 5 days were still able to survive as long as they received a high dose of drug at short intervals for the 14 days of therapy.

Later rabbit model testing compared the efficacy of ACV to GCV. Infection was similar and therapy was again started the day after infection, but the duration of drug therapy was varied from 14 to 21 days. For ACV to protect from death a dose rate of 500 mg/kg/day for 21 days was needed, but to prevent the development of any clinical symptoms a rate of 700 mg/kg/day for 21 days was needed (Zwartouw). GCV proved to be much more effective, with only 100 mg/kg/day for 14 days being needed to prevent the development of clinical signs of infection.

Treatment of BV in Humans

The CDC guidelines for a possible BV exposure recommend that first aid should be started immediately: cleaning the exposed area with soap and then irrigating with running water for 15-20 min. Collection of a sample for PCR is not advised, as it may force virus deeper into the wound. The CDC advises that decisions about whether to implement antiviral therapy or not should take into account four criteria. The first is the type and physical condition of the infecting animal. Only macaque monkeys can carry BV, non-macaque monkeys cannot shed virus in an asymptomatic infection. The physical state of the animal is also important: presence of herpetic lesions, immunecompromised, or stressed. The second criterion is the thoroughness and timelines of wound cleaning; there is less chance of infection if the wound was cleansed within 5 min of exposure and was cleansed for the recommended 15 mins. The third criterion is the nature of the wound. Smaller abrasions on extremities are considered lower risk than bites and scratches that can present as deeper wounds. The last criterion is if the exposure came from material that had contact with macaques. Sticks from needles that were used with macaque CNS, eyelids, or mucosa have a high risk potential as compared to the lower

risk of a needlestick from a needle that had contact with peripheral blood since viremia is not part of BV pathology. Also, scratches from possible contaminated objects, such as animal cages, carries a lower potential.

If the exposure criteria are considered high risk, antiviral therapy is started. Recommended prophylactic therapy is oral with ACV or VACV. VACV may be selected more often due to the prodrug giving higher serum levels than administering oral ACV (Cohen). ACV is administered at 800mg 5 times a day for 14 days, and VACV at 1g every 8 hours for 14 days (CDC). For patients showing any signs indicative of infection such as lesions at the site of infection or if infection is confirmed by PCR, anti-viral drugs are given IV rather than orally so as to obtain higher effective doses. For cases without clinical signs of CNS disease, both ACV and GCV have been recommended. IV ACV is administered at a rate of 12.5-15 mg/kg every 8 hrs, and IV GCV at 5 mg/kg every 12 hrs. However, once CNS symptoms have appeared IV GCV is strongly advised, and is administered at the same rate of 5 mg/kg every 12 hours. Even in cases of where no CNS symptoms are apparent, GCV is often preferred over ACV because both a higher dose of ACV is required due to the lower sensitivity of BV to ACV, and since BV infection can rapidly develop into encephalomyelitis for which GCV is more effective than ACV (Cohen).

Administration of BV-specific immunoglobulin may also be part of the treatment regimen. The proposed use to the of BV-specific immunoglobulin is based solely on the effectiveness of IV immunoglobulin as a prophylactic against other alpha-herpesviruses, most notably varicella zoster (CDC).

There is little data to assess the effectiveness of post exposure prophylactic treatment for BV infection. There are no cases known in which humans who received treatment within 72 hours of exposure developed an infection (Cohen). It should be noted that the number of people with potential exposure is rather large, but the number of well documented cases of infection is quite small. This leads to not knowing the rate of infection as compared to exposure.

The rate of mortality from BV infection seems to vary based upon the means and location of exposure. A superficial scratch that can be easily cleaned has a mortality of only 0.5%-5%. Deep bites on fingers and hands only have an expected mortality of 15%-20%, whereas bites to head and neck have an expected mortality of 30% all the way up to 100% (Cohen). Regardless of the site of infection, once the virus reaches the CNS mortality is almost certain.

HVP2 as a Model for BV

As previously mentioned, serious disease from BV is rare in healthy macaques, but when BV is transmitted to humans it is often lethal. Consequently, BV is a Risk Group 4 virus and is listed as a Select Agent by the CDC. Working with BV is dangerous and expensive, and must be performed under strict BSL-4 containment. These limitations have led to little BV research being accomplished. Another historical difficulty with BV research has been the lack of a reliable small animal model. Rabbits are very sensitive to BV infection, but their size, cost, and limited analytical reagents make this a less than ideal model. This problem was lessened by the development of a BALB/c mouse model for BV (Ritchey, 2005).

The limitations in working with BV led to HVP2 being proposed as a potential model for BV (Rogers, 2006). HVP2 is antigenically and genetically very similar to BV, so the use of HVP2 to study BV is a possibility. Another similarity that HVP2 has to BV is that HVP2 has varying levels of virulence between strains. In mice different strains of BV vary in virulence ranging from completely apathogenic to extremely neurovirulent. Similarly, HVP2 varied in range of virulence. However, HVP2 virulence in mice was shown to fall into two distinct phenotyes: high neurovirulence (HVP2nv strains which all produced fatal CNS infections) and apathogenic (HVP2ap which show little to no signs of clinical infection) (Rogers, 2003). In mice HVP2nv infection exhibits the same pathogenicity as BV does in human infections. Thus, the HVP2nv mouse models hold great promise as a model for human BV infection.

INTRODUCTION

Macacine herpesvirus 1 (Monkey B virus; BV) is an α-herpesvirus naturally occurring in macaque monkeys, such as the rhesus macaque and Japanese snow monkey. Biologically and genetically BV is similar to the human herpesviruses HSV1 and HSV2. In the native host, BV causes recurrent oral or genital lesions throughout the life of the host. Infection primarily occurs through mucosal tissues, such as the mouth and genitals. Macaques are primarily infected before reaching sexual maturity through oral infection, but after sexual maturity infections usually occur genitally. Adult populations have up to 90% infection.

While serious disease is rare in the natural host, when transmitted to non-macaque host species BV causes severe infection. Of the 35 known nonhuman primate herpesviruses, BV is the only one that is highly pathogenic in humans. There have been about 50 reported cases of human BV infections, with 26 of these being well documented (as of 2002, Cohen). Most zoonotic infections have resulted from bites or scratches by macaques. However, some cases have resulted from needlesticks, splash of urine into the eye, and contamination of a cut with primary macaque cells. By analogy to HSV, BV replicates in epithelial tissue at the site of infection, where it gains access to the unmyelinated ends of sensory nerves. The virus then moves up the sensory nerve axon and, in the native host, establishes latency in the sensory ganglia. In non-macaque hosts the virus continues up the nerve and into the spinal cord, and then ascends to the brain.

Once the infection reaches the brainstem the chances of survival fall dramatically, with encephalomyelitis being a sign of the terminal stages of the disease. Patients who have survived BV infection tend to have residual neurological problems or progressive neurological deterioration (Cohen).

Treatment of BV infection is best started immediately after exposure. Drug therapy use drugs initially developed for treatment of HSV. However, BV can be 10 times or more less sensitive to these drugs. The current recommended post exposure prophylactic treatment for BV infection is oral valacyclovir (Cohen, CDC). This prodrug of acyclovir (ACV) gives higher serum drug levels than administering oral ACV (Cohen). However, once CNS symptoms have appeared, IV ganciclovir (GCV) is strongly advised. For patients showing clinical signs, anti-viral drugs are given IV rather than orally so as to obtain higher systemic concentrations. Even in cases where no CNS symptoms are apparent, GCV is often preferred in use over ACV. This is because higher doses of ACV are required due to the lower sensitivity of BV to ACV, and because BV infections can rapidly develop into encephalomyelitis for which GCV is more effective than ACV.

BV is a BSL-4 level virus, and because of its potential to be used as a biological weapon was until recently listed as a Select Agent by the CDC. Working with BV is dangerous and expensive. Also, due to the infrequency of BV infections, pharmaceutical companies have not seen investigating new treatments of BV as fiscally beneficial. Together, these limitations have led to little BV research being accomplished.

There are areas of concern regarding the BV mouse model. The first is that traditionally, there has not been a reliable small animal model; only a rabbit model was

available. This limitation was resolved in the introduction of a mouse model. However, different strains of BV vary in their virulence in mice, ranging from completely apathogenic to extremely neurovirulent (Rogers, 2003). Also, route of inoculation with BV can vary the level of virulence. Lastly, BV infections in mice are not strictly dose dependent, so calculating lethal dose levels can be difficult (Ritchey, 2005).

In the search to overcome these limitations, another α-herpesvirus was studied as a potential model for BV. *Papiine herpesvirus 2* (HVP2) is naturally occurring in baboons, but unlike BV, there have been no known zoonotic transmissions. HVP2 is antigenically and genetically very similar to BV, so the use of HVP2 to study BV is a possibility. The thymidine kinase enzyme, which is the target of current antiviral drugs, is highly conserved between the two viruses. Therefore, we hypothesize that drug sensitivity of HVP2 should be similar to that of BV. Also, most HVP2 strains exhibit the same pathogenicity in mice as BV does in human infections (Rogers, 2006). Thus, the HVP2 mouse model may be predictive of human BV infection.

The aim of this research project is to validate the proposed model of HVP2 for BV drug sensitivity. Here we describe further *in vitro* and *in vivo* testing of HVP2 drug sensitivity as compared to BV drug sensitivity. We used drugs recommended for treatment of BV, as well as others that are not currently used in treatment of BV but are licensed for treatment of other herpes infections in humans. Validating the use of the lower zoonotic potential virus HVP2 as a model for BV could provide a safe alternative or complement to anti-BV drug discovery.

METHODS

Viruses and Cells

Vero (African green monkey kidney) cells were obtained from the Oklahoma Animal Disease Diagnostic Laboratory (Stillwater, OK). Cells were grown in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine. After infection cells were maintained in the same media but containing 2% FBS plus 100 U/ml penicillin and 100 µg/ml streptomycin.

HVP2 strain OU1-76 was originally isolated from a throat swab of an infant olive baboon (*Papio anubis*) at the University of Oklahoma Health Sciences Center (Eberle, 1997). HVP2 strain X313 was isolated from a yellow baboon (*Papio cynocephalus*) during an outbreak at the Southwest Foundation for Biomedical Research (Eberle, 1995). BV strain E90-136 was isolated from a fatal infection in a young cynomolgous macaque (Simon).

Drugs

Antiviral drugs used in the study included acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV), cidofovir (CDV), 5-ethyl-2-deoxyuridine (EDU), iododeoxyuridine (IDU), trisodium phosphonoformate (PFA), trifluorothymidine (TFT), adenine arabinoside (AraA), brivudin (BVDU), 5-bromo-deoxyuridine (BUdR), and 9-(4-hydroxybutyl)-N2-phenylguanine (HBPG). AraA, TFT, HBPG, CDV, EDU, and HBPG were obtained from Dr George Wright (GLSynthesis, Worchester, MA). GCV, PCV, and

ACV were obtained from LKT Laboratories (St. Paul, MN). GCV, EDU, IDU, PFA, BVDU, and BUdR were obtained from Sigma-Aldrich (St. Louis, MO).

Plaque Assays

Drug sensitivity testing was performed using confluent Vero cells in 6-well trays. Cells were infected with 100 PFU of virus (200 μl/well). Virus was absorbed at 37°C for 30 min, with rocking of the plates every 10 min to prevent drying of the cell monolayer. Cells were then overlaid with a solution of 1% FBS-DMEM with 0.5% methylcellulose that contained the test drug. Cells were incubated at 37°C and plaques counted at 2 days PI. Several concentrations (3-6) were tested for each drug, and each concentration was tested in duplicate. Control wells had no drug in the overlay medium. All assays were repeated at least three times to confirm results.

The efficiency of each drug was expressed as the percent plaque reduction. This was calculated as follows: 100- [(Drug Well Avg / Control Well Avg) x 100]. The average plaque count of each set of duplicate wells was determined, and the average plaque count for each concentration of drug was divided by the control wells average. This number was then multiplied by 100, and the resulting value was subtracted from 100. The resulting number is the percent plaque reduction. This value is useful because it normalizes variation between individual experiments so that multiple assays preformed at different times can be compared with each other.

Calculation of EC_{50} Values

Drug sensitivity was expressed as an EC_{50} value, which is the concentration of drug at which the average plaque count is reduced by 50%. The more potent a drug, the lower the EC_{50} value will be. Along with reducing the plaque count, drugs can also

reduce plaque size. As drug concentrations increase, the size of plaques can be reduced to a fraction of the no-drug control plaque size. The presence of a plaque indicates a PFU was able to cause destruction of numerous host cells, and that the drug was not able to stop viral replication. Therefore, while plaque size reduction was noted, only actual plaque numbers were used to determine EC_{50} values.

EC_{50} values were found by plotting the linear function of the percent reduction versus log concentration. The mean EC_{50} values were then analyzed to determine significance using a 2-factor factorial completely randomized ANOVA.

Mouse Husbandry

Female 10–12 g BALB/c mice were obtained from Charles River Laboratories (Wilmington, Mass.). In all experiments test groups of 8 mice were used. Mice were given commercial food and water *ad lib*. The course of infection in unmedicated control mice is usually 6-8 days until death, but due to the nature of the drug therapy, some mice developed neurological signs of infection but did not progress to death in the normal 6-8 day time period. For this reason all mice used in drug therapy experiments received acetaminophen in their drinking water at a concentration of 2 mg/ml.

Mouse Inoculations

Infection via skin scarification mimics human BV infections resulting from monkey scratches. Disrupting the outer epithelial layer of skin also exposes the dermis, where the unmyelinated ends of sensory nerves are located. These unmyelinated nerve endings are where the virus can effectively invade neural tissue. Skin scarification has been shown to be a more reliable route of infection than the alternative intramuscular infection (Rogers, 2006), and so it was used as the route of infection of all experiments.

The left hind flank was shaved and the skin superficially scarified with a 22 gauge needle to expose fresh epithelial cells for infection. Virus (10^5 PFU in 10ul) was applied to the scarification site using a micropipettor and 'rubbed in' in with the side of the pipette tip.

***In vivo* Drug Administration**

Three to eight doses of each drug were employed in *in vivo* tests to determine the efficacy of nucleoside analogs. Drugs were administered by intraperitoneal injection twice-daily for 7 days using an insulin syringe with a 28 gauge needle. To allow maximal antiviral drug activity, the drug regimen was initiated starting one day prior to infection (-1 DPI) to allow steady state plasma concentrations of drug to be established before infection.

In experiments designed to assess the effects of delayed onset of drug therapy, only one drug dose rate was used. Start of therapy varied by 24 hr for each group, the first group beginning therapy on -1 DPI. For BV drug therapy groups extended to 4 DPI, and for HVP2 to 5 DPI. For all groups, drugs were given twice daily for 7 days by intraperitoneal injection. In the initial *in vivo* experiments the day of start of therapy remained the same, where the dose of drug changed for each test group. In this experiment the dose remained the same, and the day of start of drug therapy varied. The GCV delayed study used a dose of 100 mg/kg/day, and the CDV delayed study used a dose of 25 mg/kg/day. The same strain of BV (E90-136) was used for both delayed experiments, whereas only HVP2 strain OU1-76 was used in these experiments.

Neurological Scoring

Progression of symptoms and characterization of disease was assessed using a scoring method based on neurological signs. While ulcerative skin lesions are often the

first sign of disease, they were not factored into scoring due to the difficulty of detecting them in re-grown hair and the successive outbreaks of lesions that can occur over the course of an experiment. Mice were observed twice daily, and scored based on signs of neurological impairment. The scoring of signs was on a scale of 0 to 5 as shown in Figure 4 and summarized in Table 3. Mice were euthanized when they developed a severe CNS infection; signs of this included sunken flank, rapid breathing, tremors, immobility, lack of balance and/or lack of alertness. Mice were also euthanized if they self-amputated limbs, regardless of the health status of the total mouse. Mice that survived to the end of study were bled by cardiac puncture when euthanized and serum tested by ELISA for antiviral IgG to confirm viral infection. Euthanasia was by overdose of isoflurane followed by cervical dislocation.

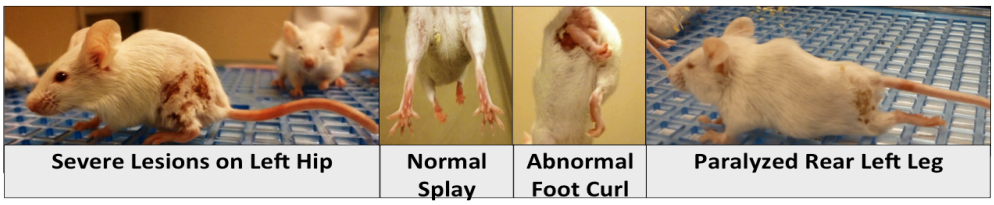

Figure 4. Visual Representation of Disease Progression
Exceptionally severe herpetic vesicles at site of infection (**left panel**). The abnormal splay reflex of the ipsilateral hind limb when the mouse is lifted by the tail (**middle panels**). Paralysis of the limb with rapid neuromuscular atrophy (**right panel**),

Score	Clinical Presentation of Neurological Impairment
0	None; appears normal.
1	Slightly abnormal tail-lift reflex response, adduction of ipsilateral foot/leg.
2	Mild paresis or paralysis of ipsilateral hind leg. Still active and alert.
3	Obvious paresis or paralysis of ipsilateral hind leg. Still active and alert.
4	Paralysis, scruffy coat, inactive, not alert; but not to be euthanized at this time.
5	Dead in cage or euthanized.

Table 2. Neurological Scoring of Infected Mice
The scoring system from 0-5 on which all graphs are based. The scoring follows the natural progressions of neurological infection from herpesvirus.

Tissue Collection

Mice used in experiments to track the progress of infection were euthanized by isoflurane overdose, cervical dislocation was not preformed due to the damage it would cause to neural tissue in question. A thoracic-lumbar laminectomy was performed to reveal the spinal cord (SC) and dorsal root ganglia (DRG) in the lumbar region. The SC and DRG were extracted using disinfected scissors and forceps, tissues placed in a sterile microfuge tube, and stored at -80°C.

Virus Isolation.

Stored tissue samples were thawed, 250 ul of 2% DMEM added to each tube, and tissue samples homogenized using a motorized pestle. Insoluble debris was removed by centrifugation at 14,000 x g for 2 min, and the clarified supernatant transferred to a new sterile tube. Infectious virus was detected by plaque assay on Vero cells (Roger, 2009).

ELISA

The humoral immune response of antiviral IgG antibodies was assessed by ELISA (Ohsawa). Collected blood was allowed to coagulate, spun at 14,000 x g for 5 min, the serum transferred to a new sterile tube, and stored at -20°C. Immulon-2 U-bottomed microtiter plates (Dynax Technologies, Inc., Chantilly, VA) were coated with HVP2 or BV infected cell antigen. A second set of wells were also coated with uninfected cell antigen to serve as controls. Serum samples were diluted 1:100 for testing. Secondary antibody was bioinylated anti-mouse IgG followed by an avidin-biotinylated peroxidase complex and substrate. Plates were read at 490nm. The OD_{490} values on uninfected antigen was subtracted from the OD_{490} values on infected cell antigen. Resulting OD_{490} values of greater than 0.100 were considered positive.

RESULTS

--IN VITRO RESULTS--

	BV E90-136	HVP2 OU1-76	HVP2 X313
ACV	23.31 ± 4.27	39.36 ± 6.51	29.92 ± 5.04
AraA	5.7 ± 1.55	11.43 ± 1.14	4.8 ± 0.32
BUdR	>200	>200	>200
BVDU	>200	>200	>200
CDV	12.41 ± 2.05	3.14 ± ± 0.37	8.36 ± 1.21
EDU	14.21 ± 5.75	13.68 ± 2.18	3.66 ± 1.60
HBPG	>200	>200	>200
GCV	18.35 ± 3.60	10.62 ± 0.97	15.14 ± 3.80
IDU	1.26 b ± 0.46	31.74 a ± 5.46	11.36 ab ± 2.82
PCV	11.32 ± 1.44	13.73 ± 2.15	15.46 ± 1.57
PFA	>100	>100	>100
TFT	1.32 ± 0.18	0.65 ± 0.19	1.27 ± 0.02

Table 3. EC_{50} Values of the Virus Strains Tested.
Shown are EC_{50} means ± standard error. The lower case letters denote a statistical significance among groups, values with the same letter are not statistically different from each other.

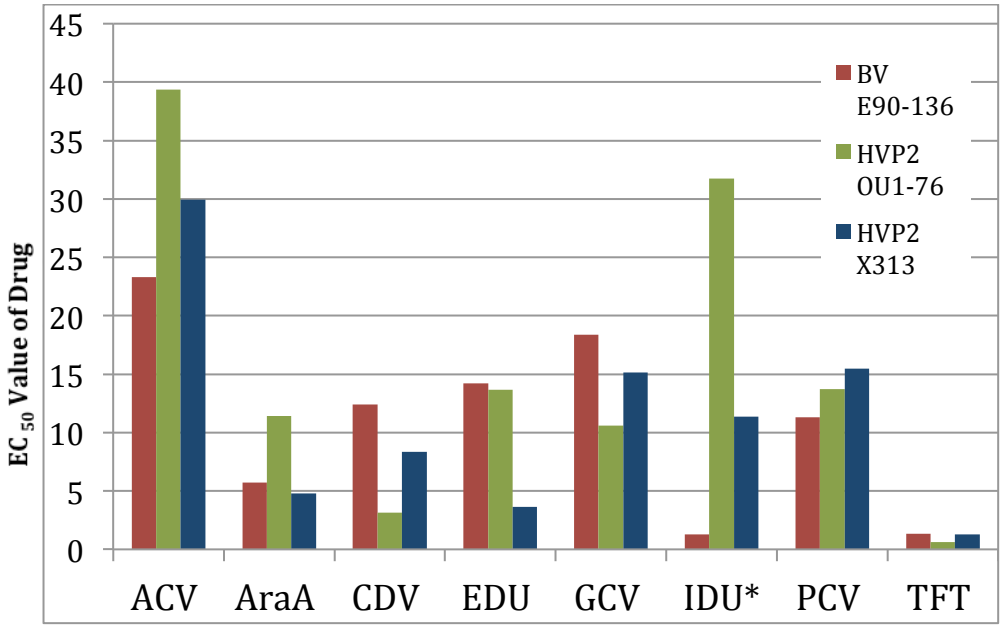

Figure 5. Visual Representation of EC_{50} Values of the Viruses Tested.
Only 8 of the 12 drug examine are shown; the other 4 drugs were not effective. Shown are the mean EC_{50} values. The asterisk denotes statistical significance difference among viruses.

For HVP2 to be validated as a predictive model of BV drug sensitivity, a number of parallels must be demonstrated, among which similar EC_{50} values. Because of potential difference between strains of HVP2 in antiviral drug sensitivity, two HVP2 isolates were tested. A summary of the results for the *in vitro* drug testing is shown in Table 4 and Figure 5. For 11 of the 12 drugs tested *in vitro*, the drug sensitivity of the two HVP2 strains paralleled each other. For IDU the EC_{50} values the two HVP2 strains were significantly different. For all 12 drugs tested the sensitivity of HVP2 X313 paralled BV E90-136. HVP2 OU1-76 drug sensitivity paralleled that of BV for all drugs except IDU.

Drugs that showed efficacy against all three viruses were GCV, PCV, ACV, EDU, CDV, TFT, and AraA. In contrast, PFA, BVDU, BUdR, and HBPG were not effective against any of the three viruses. The EC_{50} for PFA was close to 100 µg/ml, but the EC_{50} values for BVDU, BUdR, and HBPG were all over 200 µg/ml.

The EC_{50} values of the first line choice for drug therapy, ACV, were similar for both HVP2 strains and BV. While PCV is not as widely used in treatment of BV, the EC_{50} value for PCV against all three viruses was lower than the ACV EC_{50} value. The biggest difference between ACV and PCV can be seen for HVP2 OU1-76 where the difference between the ACV and PCV EC_{50} values was 25.63 µg/ml. GCV did prove more potent against BV as compared to ACV with the EC_{50} values being the lowest overall of the three commonly used antivirals. However, the GCV EC_{50} values were very similar to the EC_{50} values for PCV. For BV, the EC_{50} of PCV was lower than that of GCV (PCV at 11.32 µg/ml vs GCV at 18.35 µg/ml). For both HVP2 strains the EC_{50} of GCV was lower than that of PCV, but only slightly. In HVP2-X313 the difference

between the PCV and GCV EC_{50} values was only 0.32µg/ml. For all three drugs the differences between EC_{50} values for HVP2 and BV were not statistically significant.

Surprisingly, the older drug AraA showed greater potency than the newer drugs that replaced the widespread use of AraA. The lowest AraA EC_{50} was that of HVP2-X313 at 4.8µg/ml, and BV E90-136 was close with an EC_{50} value of 5.70 µg/ml.

Several other drugs tested also showed promising efficacy. EDU and CDV both had *in vitro* EC_{50} values just as low as that of GCV. For both drugs, the EC_{50} values for BV were similar. The BV EDU EC_{50} value was 14.21 µg/ml and the CDV EC_{50} value was 12.41 µg/ml. Both drug EC_{50} values were also slightly less than the EC_{50} value of GCV for BV. EDU showed the most potency against HVP2-X313, with an EC_{50} value of 3.66 µg/ml, while CDV was most potent against HVP2-OU1-76 with an EC_{50} value of only 3.14 µg/ml.

Two additional drugs with determinable EC_{50} values were tested *in vitro*: IDU and TFT. Both of these drugs have very low EC_{50} values but are highly toxic when used systemically *in vivo* (Hamuy). TFT had the lowest EC_{50} values, with values for all three being < 2 µg/ml and with very low standard error values. There was no statistical difference between the TFT EC_{50} values for HVP2 and BV. However, with IDU there was a statistical difference between viruses. The difference between EC_{50} values for the two HVP2 strains was not statistically significant. The IDU EC_{50} for HVP2-OU1-76 is statistically significant from the BV EC_{50} value. On the other hand, HVP2 strain X313 has an EC_{50} value lower than that of OU1-76, and the difference between the BV EC_{50} and the X313 EC_{50} value is not statistically significant. To summarize, whereas the two

HVP2 strains had higher EC_{50} values for IDU than BV, only one HVP2 strain had a sensitivity that was significantly different than for BV.

An EC_{50} value of 40 μg/ml seems to be the point that separates the effective and non-effective drugs; drugs that were efficacious had EC_{50} values below 20 μg/ml. Drugs with an EC_{50} of >40 μg/ml were not always effective, with an E_{max} not being reached. PFA showed some degree of efficacy, with an EC_{50} value close to 100 μg/ml. The drugs that were not effective were PFA, BUdR, BVDU, and HBPG.

--IN VIVO RESULTS—

Drug sensitivity

Drug	Dose Rate (mg/kg/day)	Type of Event Curve	Median Time (Days)		
			BV E90-136	HVP2 OU1-76	HVP2 X313
ACV	150	Mortality	7	8	8
PCV	200	Mortality	8	9	13
GCV	100	Paralysis	>14	9	14
	50	Mortality	>14	13	>14
	25	Mortality	13	14	11
EDU	400	Mortality	6	8	7
CDV	25	Paralysis	14	14	9
	12.5	Paralysis	8	8	7
	6	Paralysis	7	8	7
	6	Mortality	7	14	10
	3	Mortality	6	8	7
	1.5	Mortality	6	8	7

Table 4. Median Time of Mice Reaching Paralysis or Death
The data is expressed as the number out of the total group to reach the respective event.

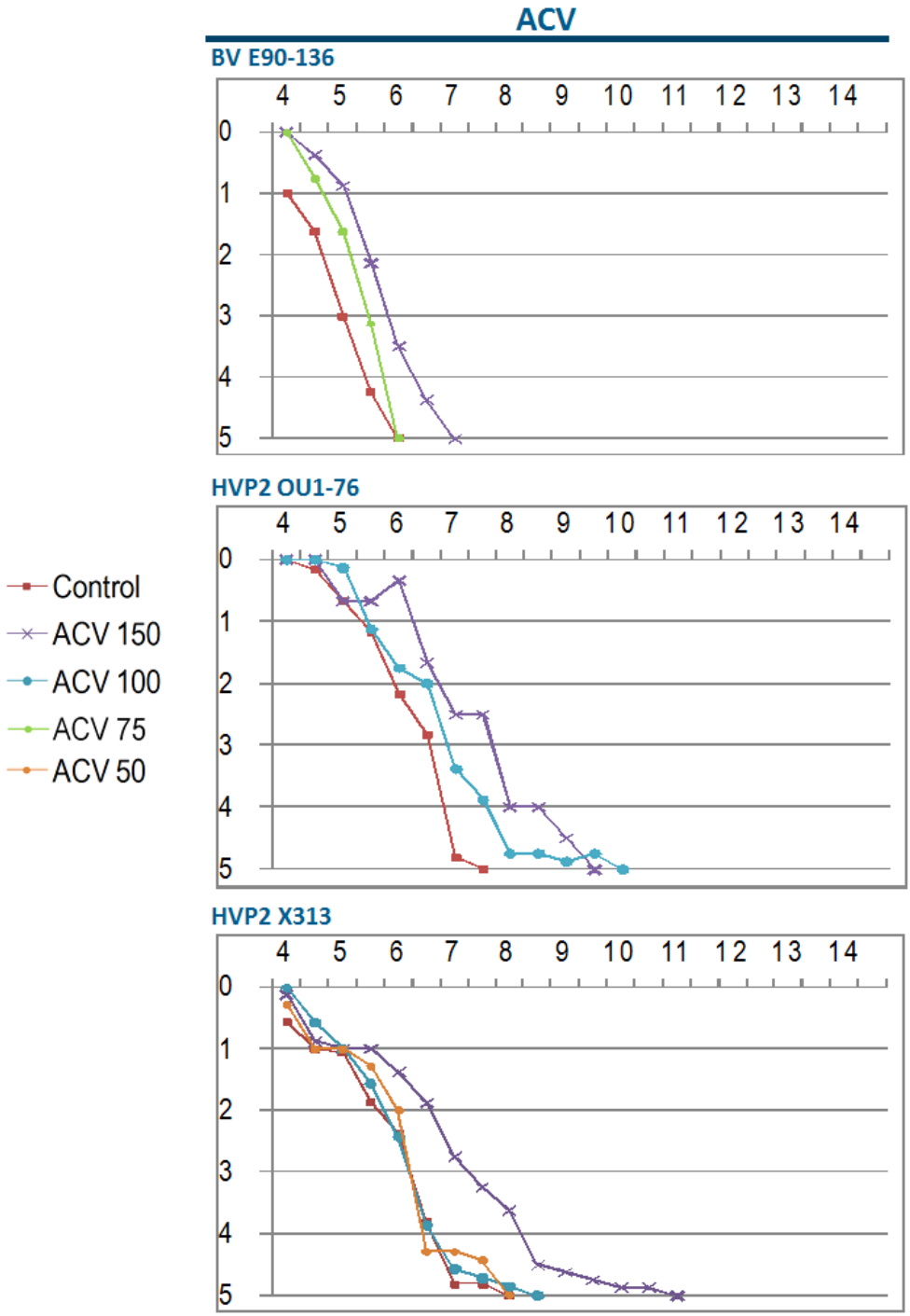

Figure 6. Neurological Scores of Mice Receiving ACV.
Mice were dosed with ACV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Mice were scored twice daily based on progression of neurological scores (Table 3). Average of neurological scores for each group, and each dose, are shown.

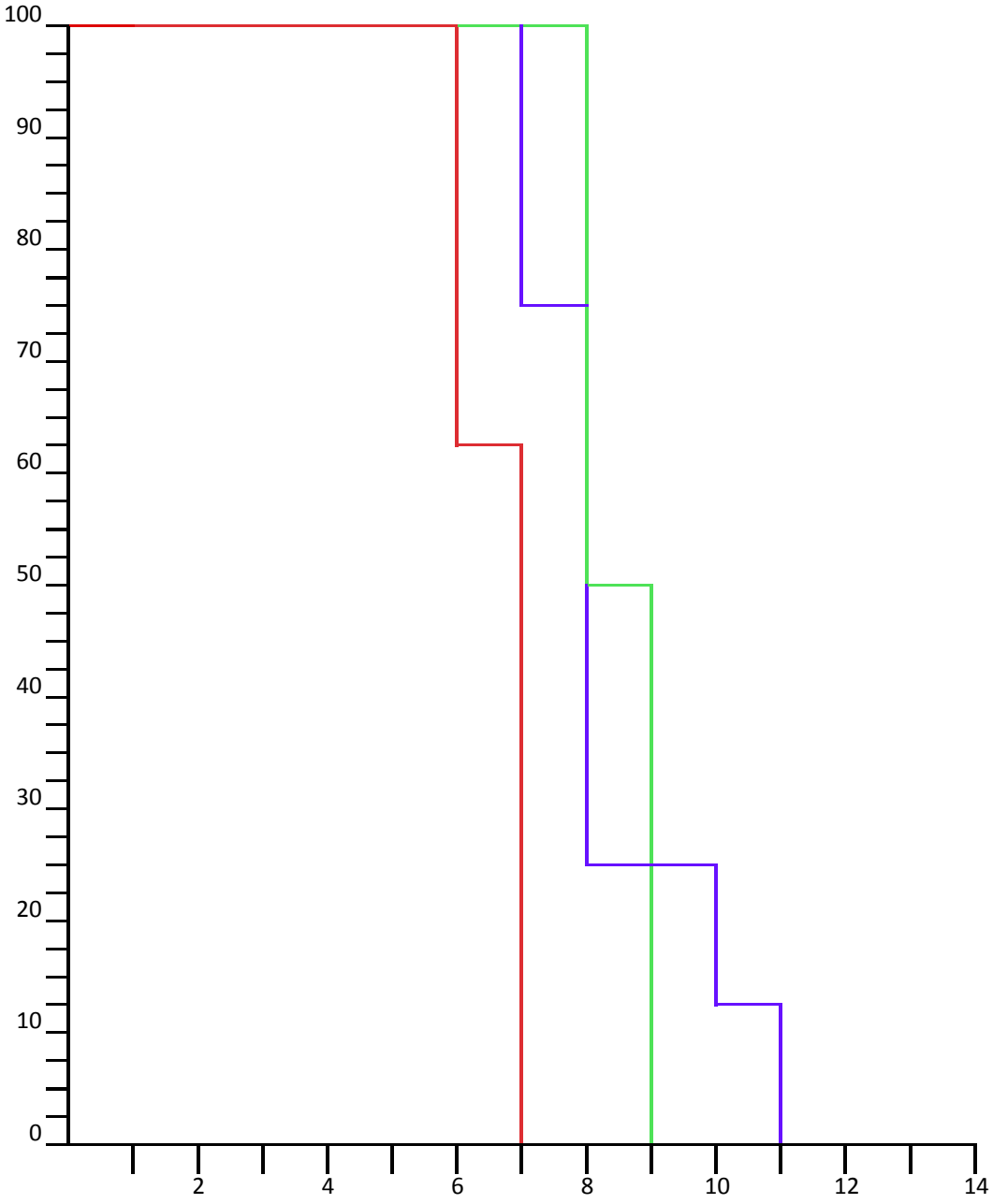

Figure 7. Kaplan-Meier Survival Analysis for ACV 150 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with ACV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

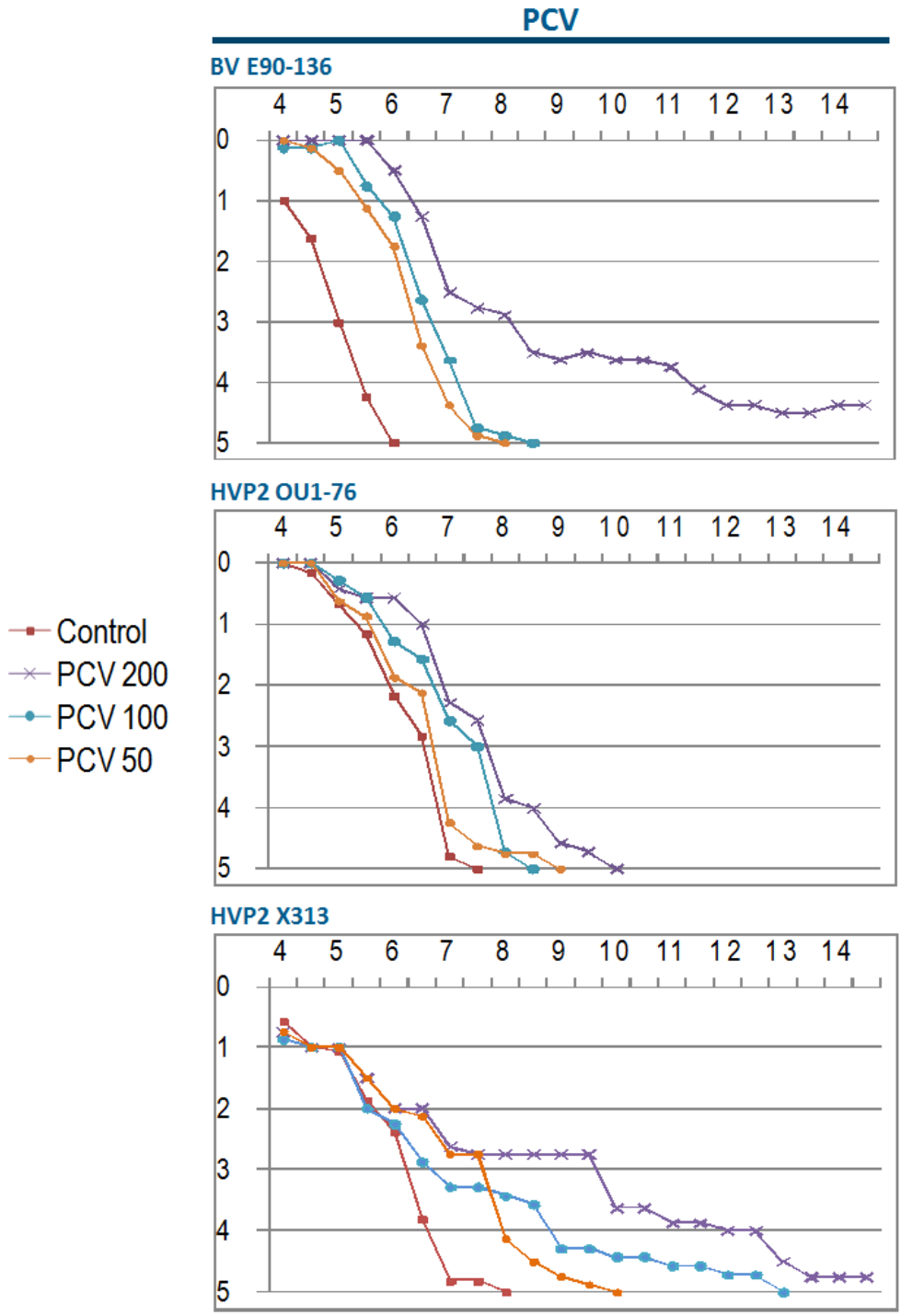

Figure 8. Neurological Scores of Mice Receiving PCV.
Mice were dosed with PCV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Mice were scored twice daily based on progression of neurological scores (Table 3). Average of neurological scores for each group, and each dose, are shown.

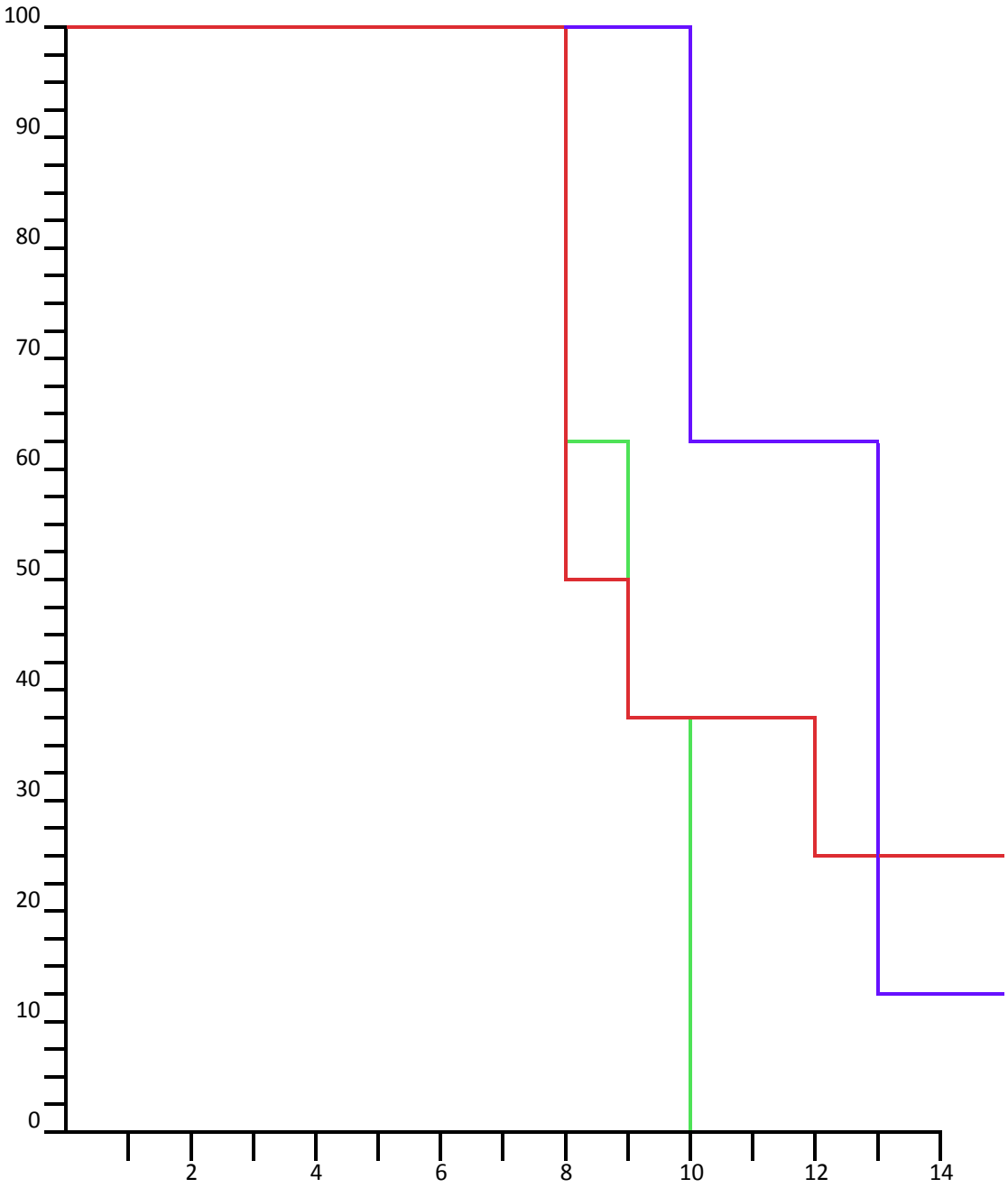

Figure 9. Kaplan-Meier Survival Analysis for PCV 200 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with PCV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

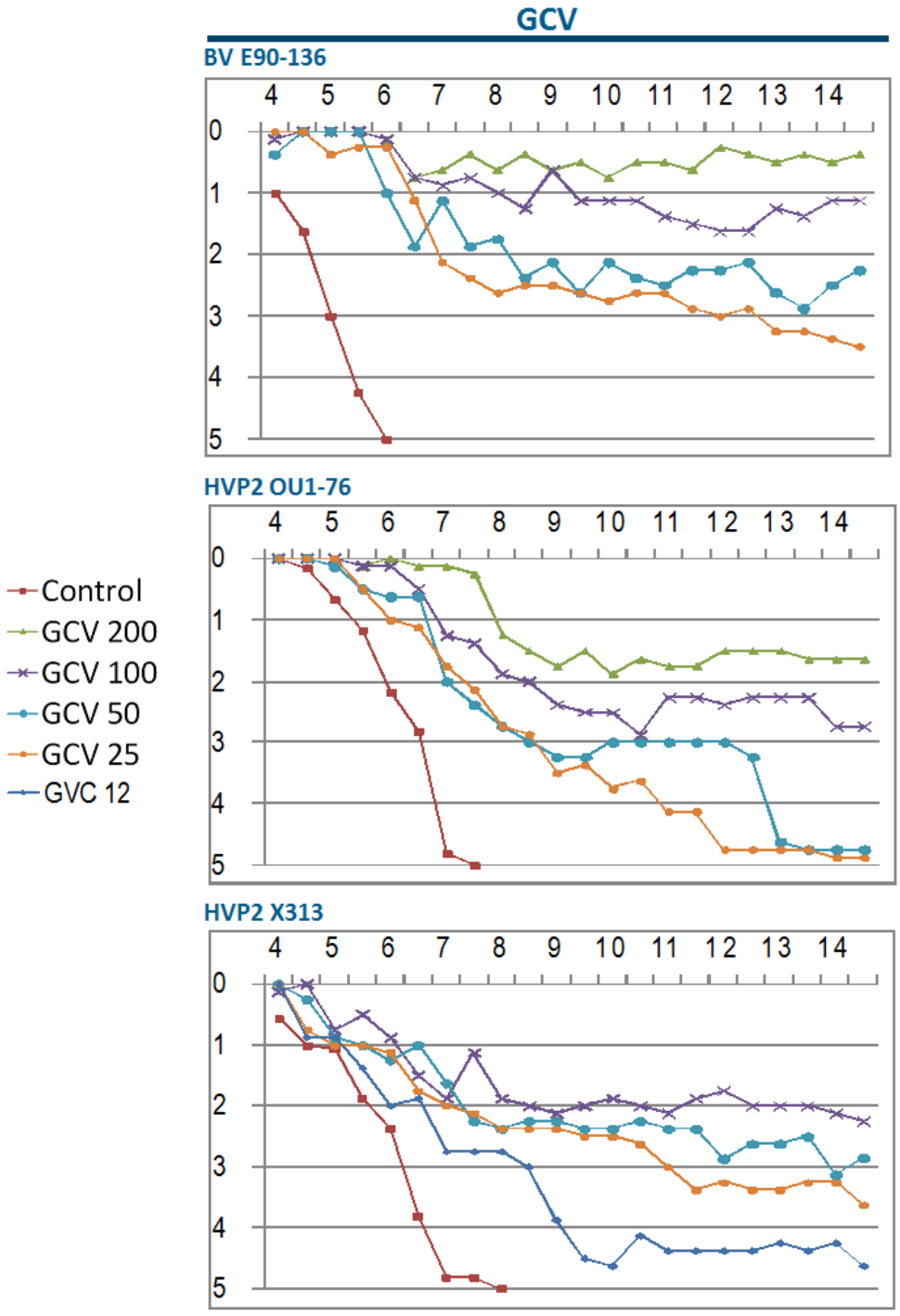

Figure 10. Neurological Scores of Mice Receiving GCV.
Mice were dosed with GCV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Mice were scored twice daily based on progression of neurological scores (Table 3). Average of neurological scores for each group, and each dose, are shown.

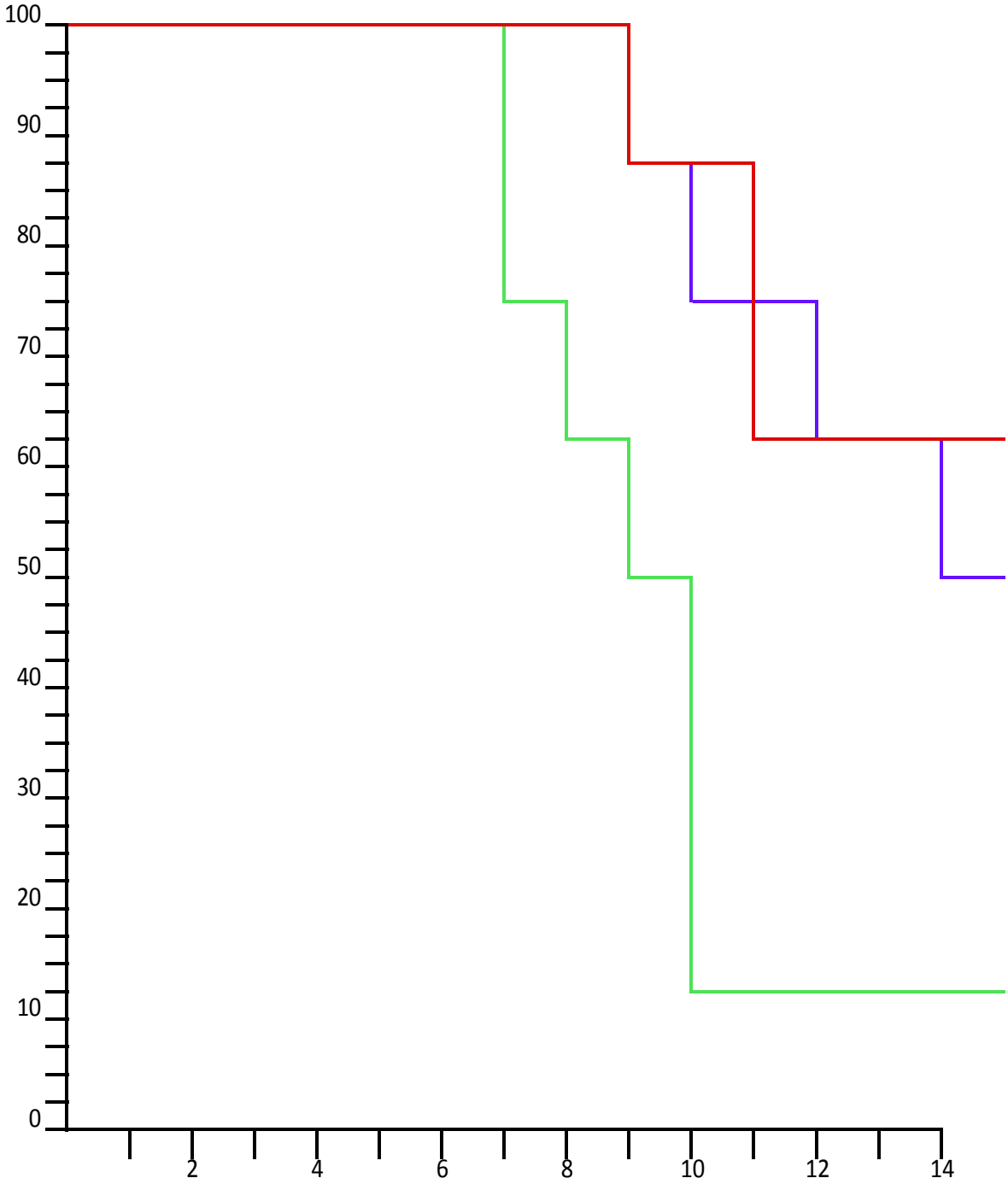

Figure 11. Kaplan-Meier Event Curve of Score of 3 for GCV 100 mg/kg/day. Shown is the percent of mice with an average score of 3, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with GCV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 3, which is representative of full paralysis of one leg (Table 3).

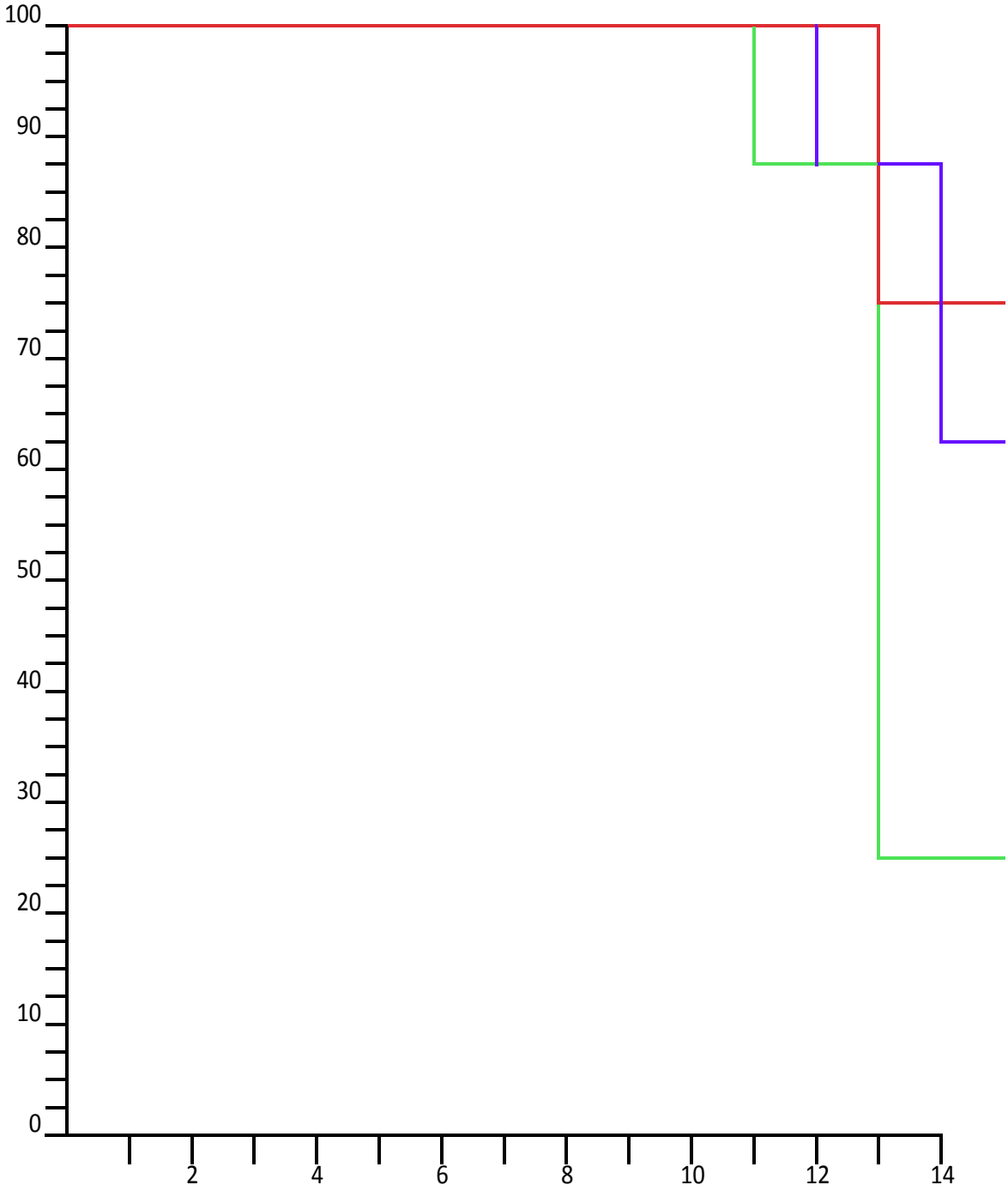

Figure 12. Kaplan-Meier Survival Analysis for GCV 50 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with GCV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

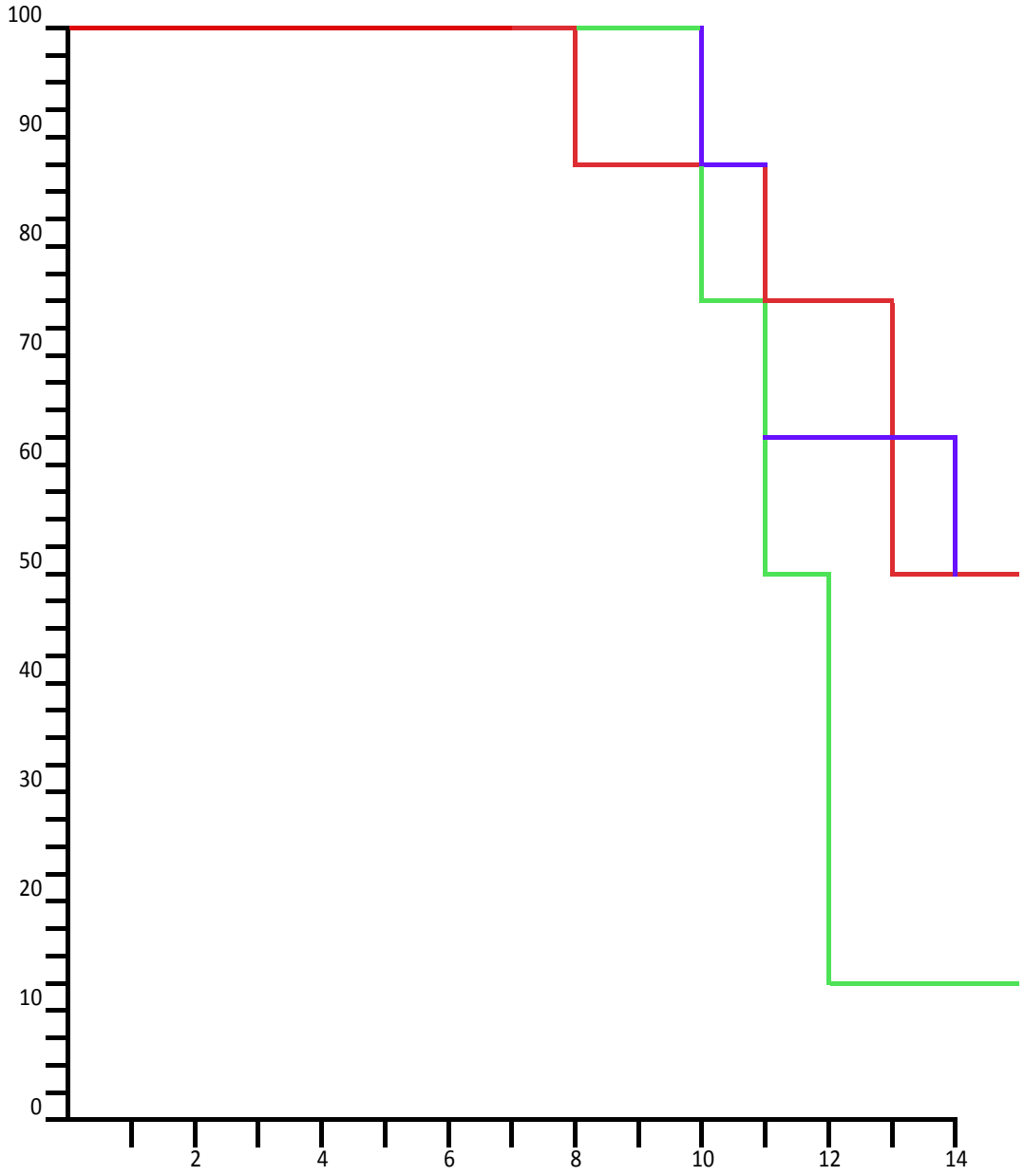

Figure 13. Kaplan-Meier Survival Analysis for GCV 25 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with GCV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

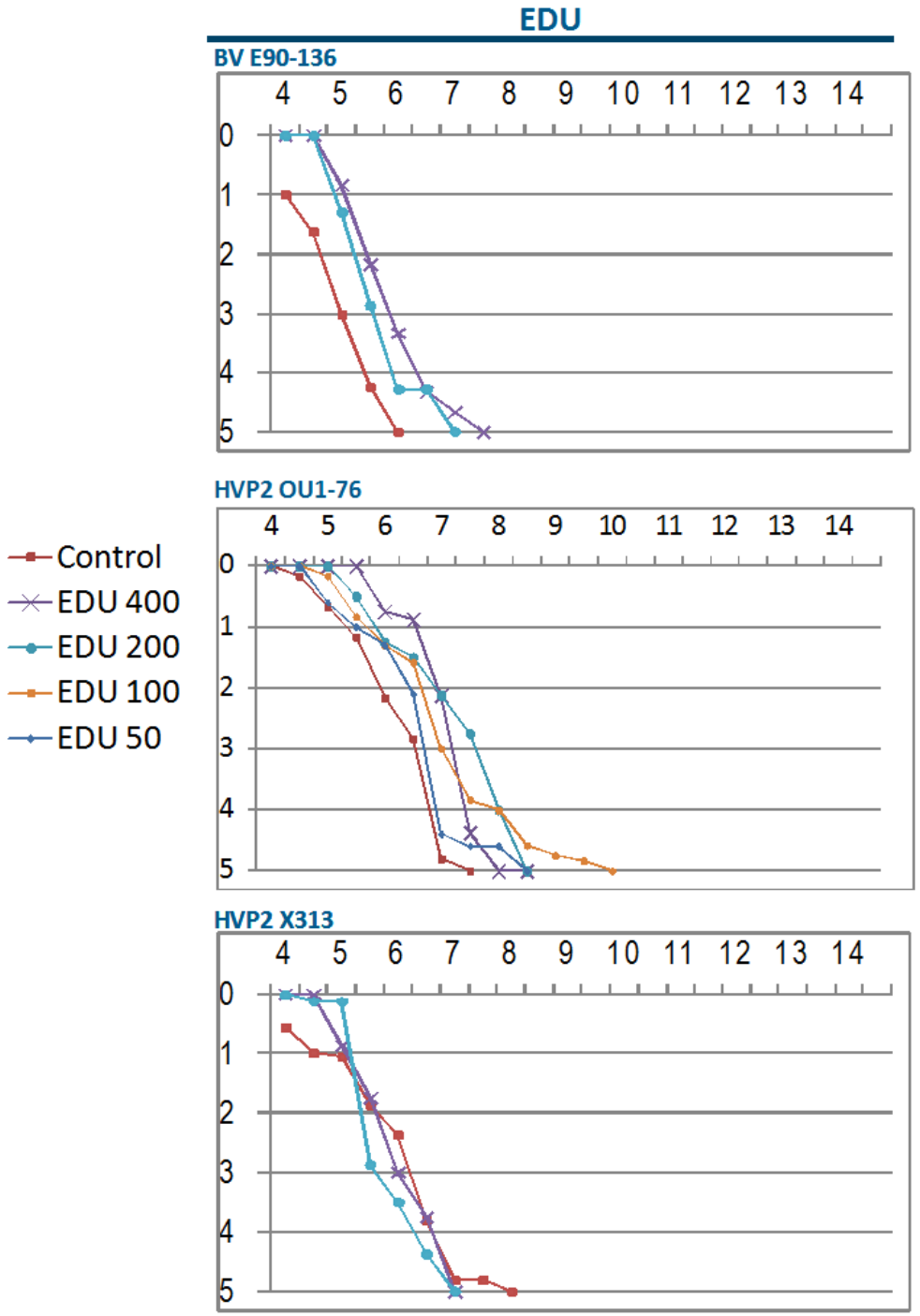

Figure 14. Neurological Scores of Mice Receiving EDU.
Mice were dosed with EDU by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Mice were scored twice daily based on progression of neurological scores (Table 3). Average of neurological scores for each group, and each dose, are shown.

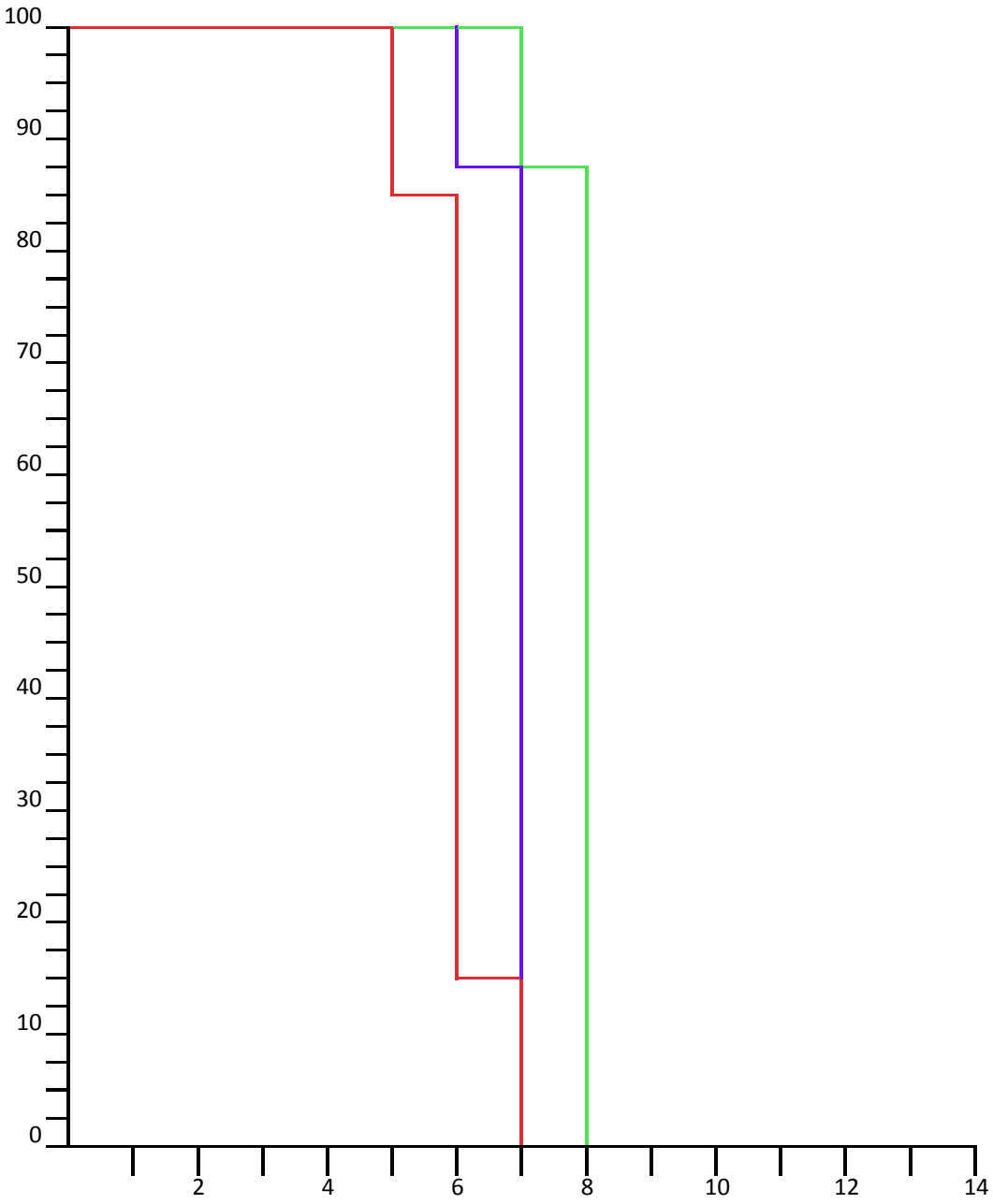

Figure 15. Kaplan-Meier Survival Analysis for EDU 400 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with EDU by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

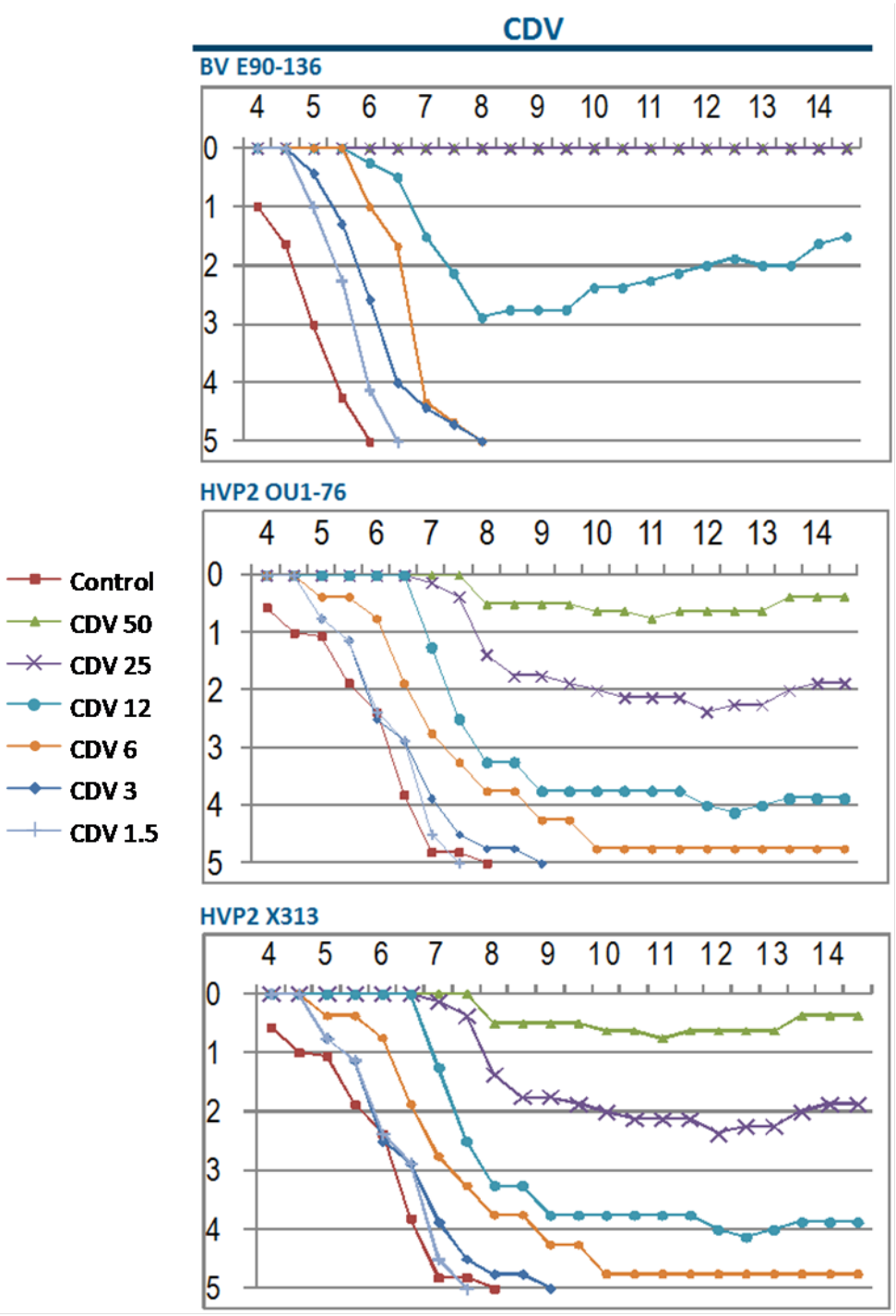

Figure 16. Neurological Scores of Mice Receiving CDV.
Mice were dosed with CDV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Mice were scored twice daily based on progression of neurological scores (Table 3). Average of neurological scores for each group, and each dose, are shown.

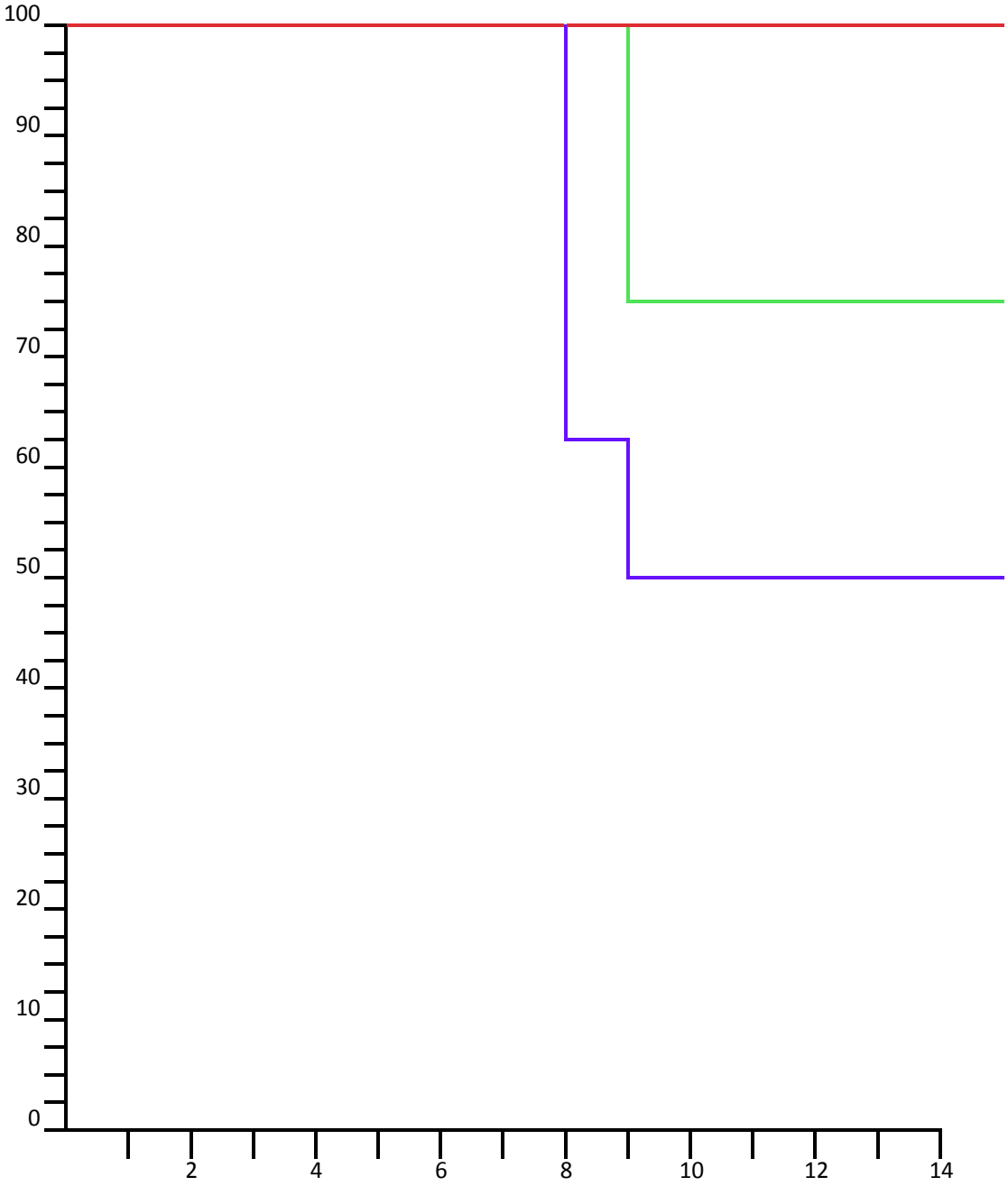

Figure 17. Kaplan-Meier Event Curve of Score of 3 for CDV 25 mg/kg/day. Shown is the percent of mice with an average score of 3, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with CDV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 3, which is representative of full paralysis of one leg (Table 3).

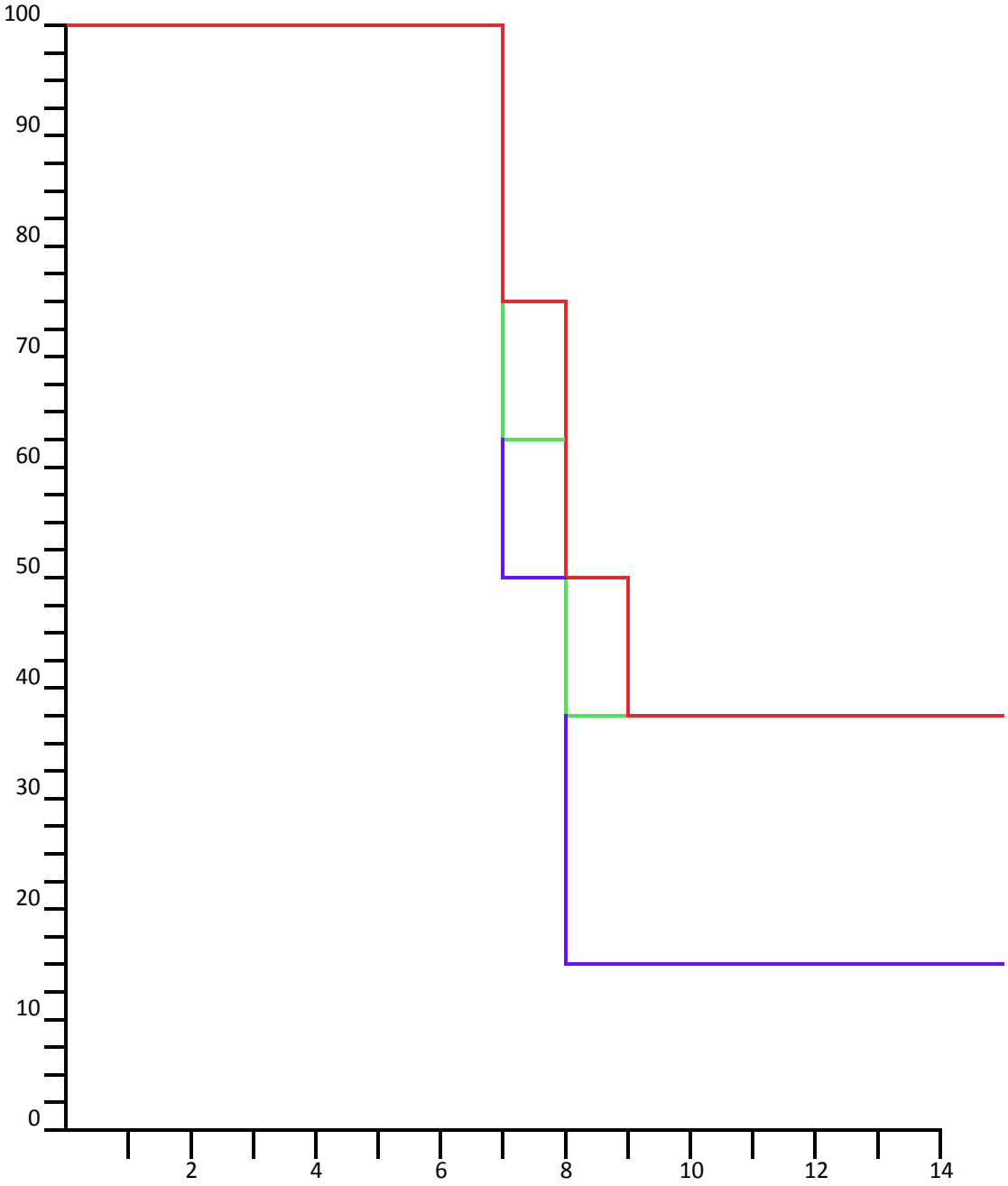

Figure 18. Kaplan-Meier Event Curve of Score of 3 for CDV 12.5 mg/kg/day. Shown is the percent of mice with an average score of 3, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with CDV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 3, which is representative of full paralysis of one leg (Table 3).

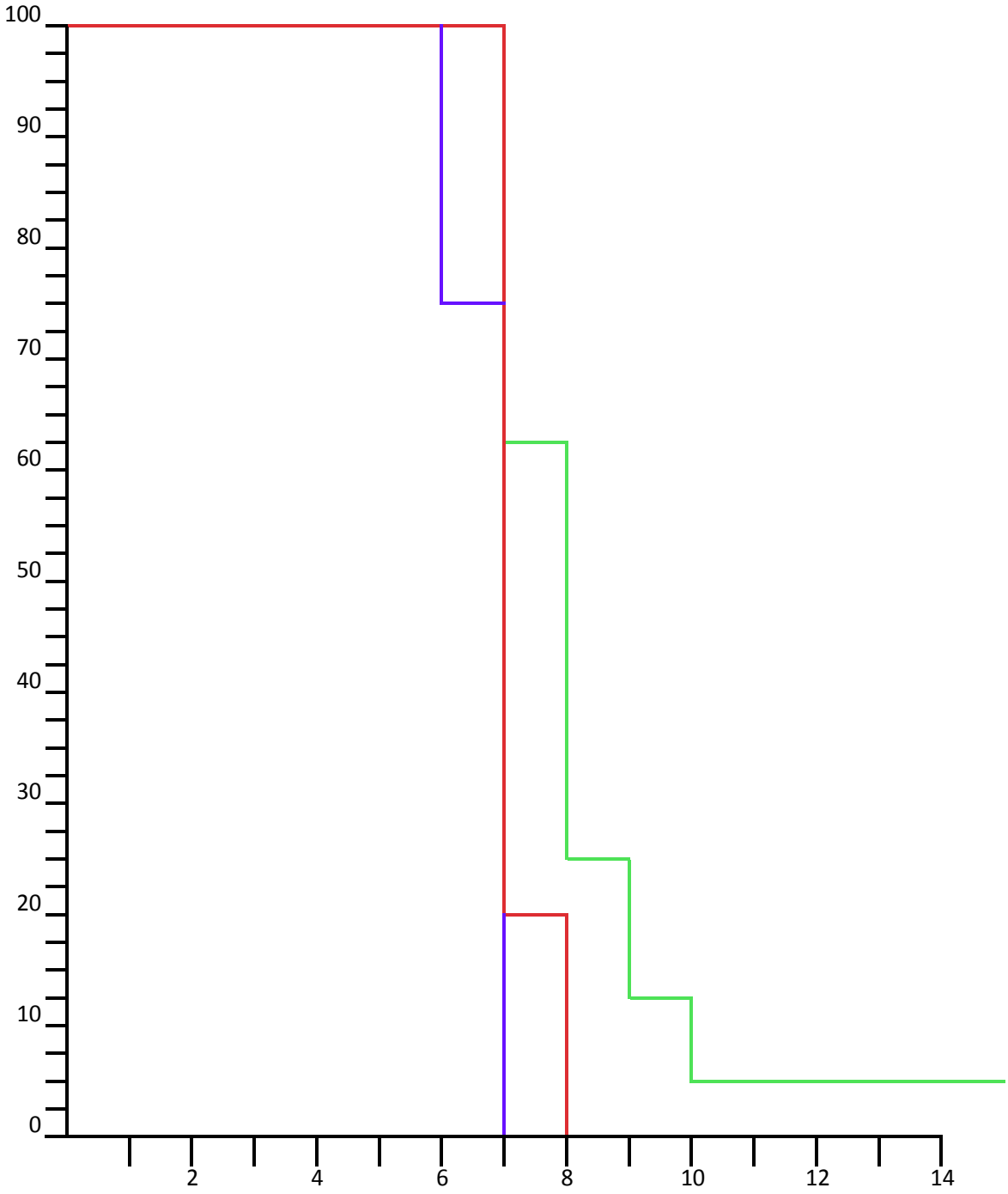

Figure 19. Kaplan-Meier Event Curve of Score of 3 for CDV 6 mg/kg/day.
Shown is the percent of mice with an average score of 3, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with CDV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 3, which is representative of full paralysis of one leg (Table 3).

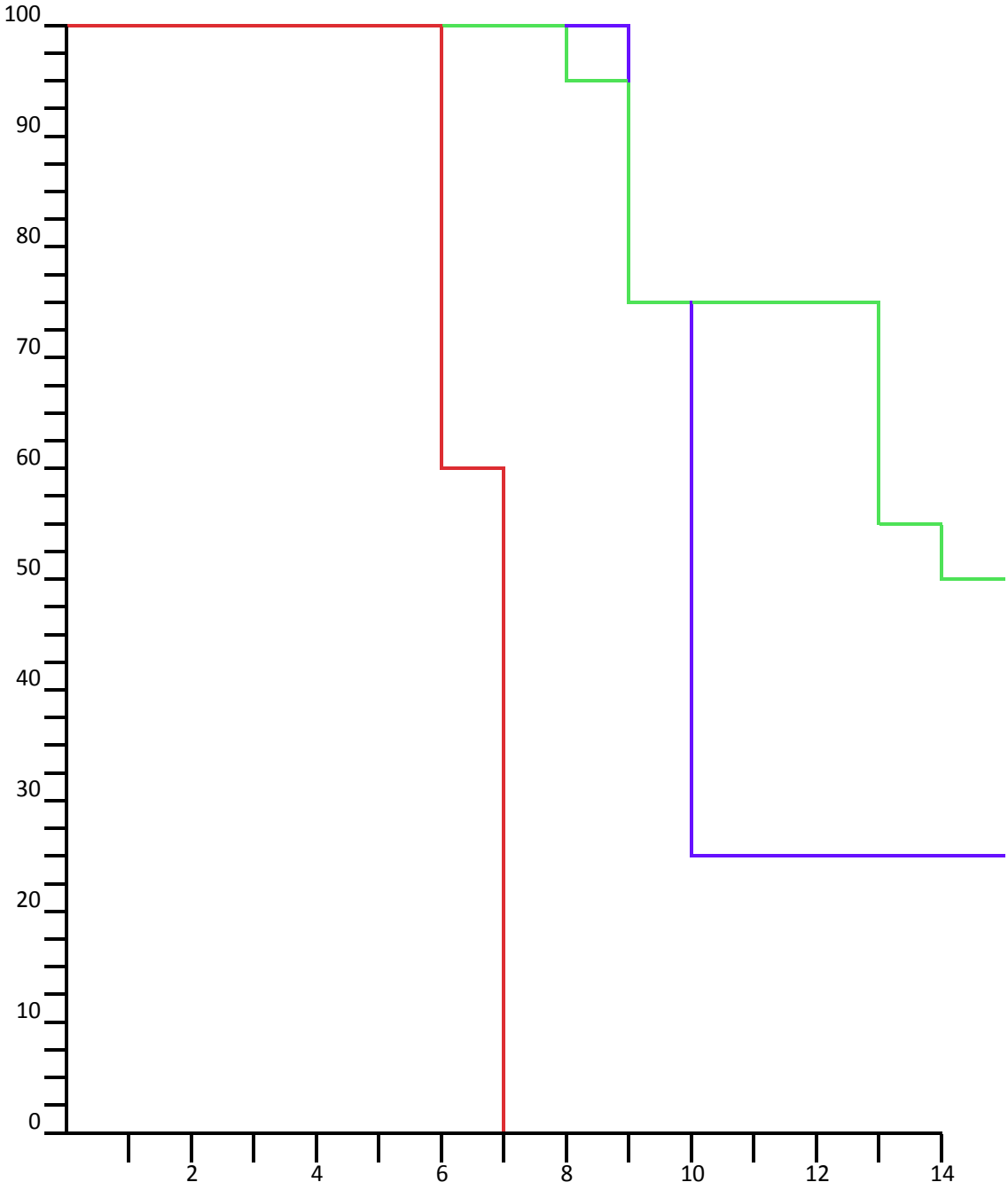

Figure 20. Kaplan-Meier Survival Analysis for CDV 6 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with CDV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

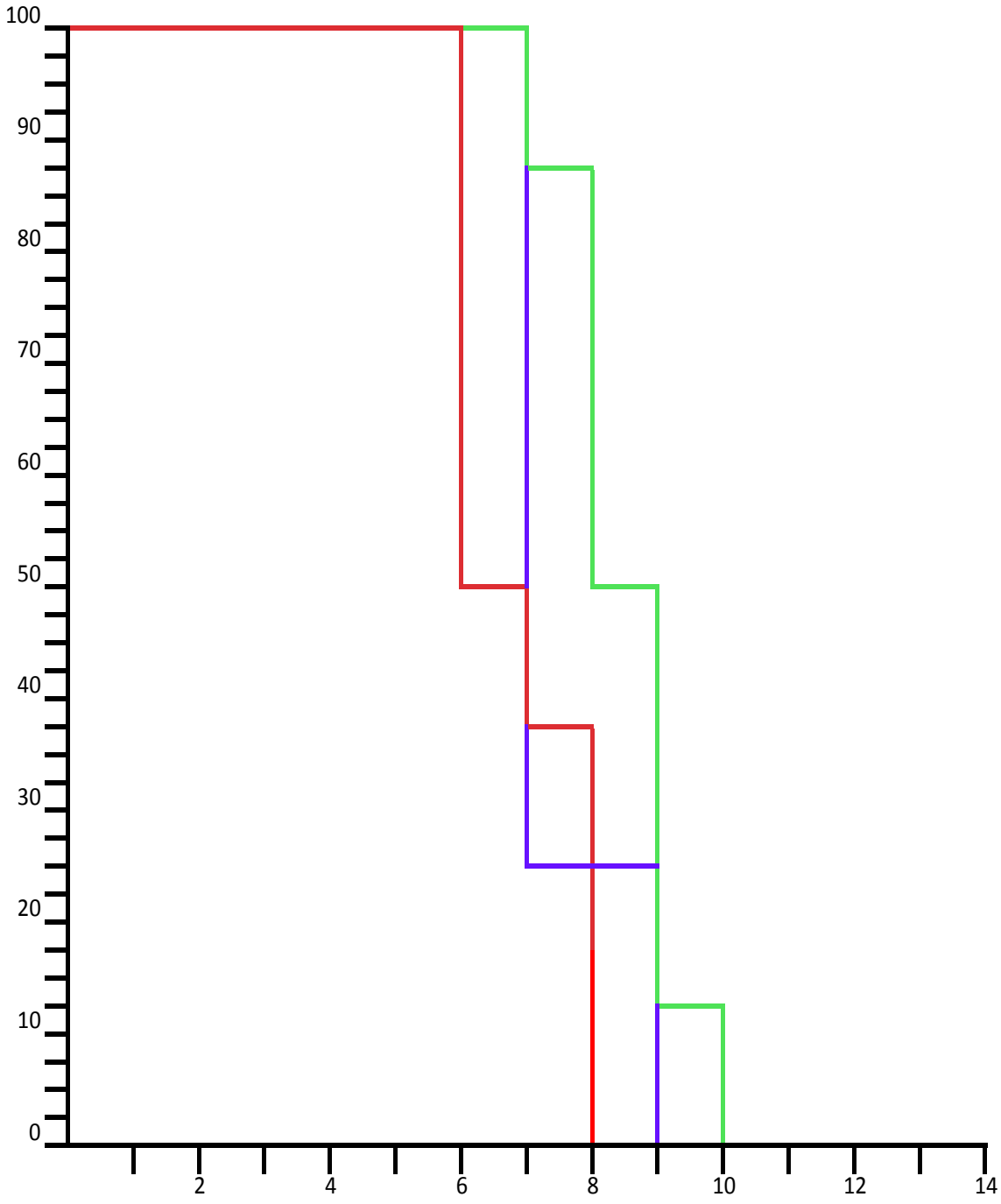

Figure 21. Kaplan-Meier Survival Analysis for CDV 3 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with CDV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

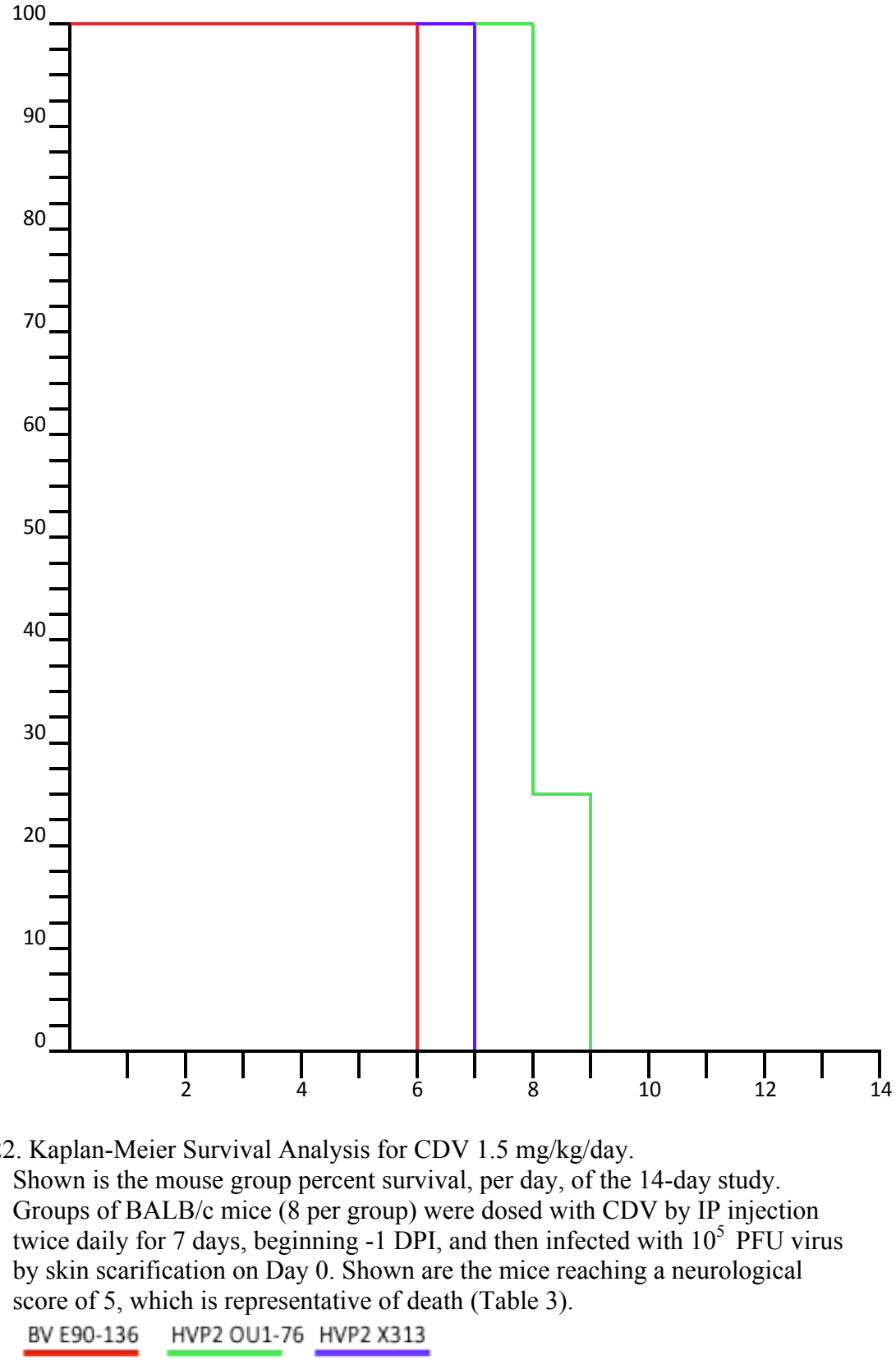

Figure 22. Kaplan-Meier Survival Analysis for CDV 1.5 mg/kg/day.
Shown is the mouse group percent survival, per day, of the 14-day study. Groups of BALB/c mice (8 per group) were dosed with CDV by IP injection twice daily for 7 days, beginning -1 DPI, and then infected with 10^5 PFU virus by skin scarification on Day 0. Shown are the mice reaching a neurological score of 5, which is representative of death (Table 3).

	Treatment in mg/kg/day	Reaching Paralysis			Reaching Death		
		BV	HVP2		BV	HVP2	
		E90-136	OU1-76	X313	E90-136	OU1-76	X313
ACV	75	8/8	-	-	8/8	-	-
	100	-	8/8	7/7	-	8/8	7/7
	150	8/8	6/6	8/8	8/8	6/6	8/8
PCV	50	8/8	8/8	8/8	8/8	8/8	8/8
	100	8/8	8/8	8/8	8/8	8/8	8/8
	200	8/8	8/8	8/8	6/8	8/8	7/8
GCV	25	6/8	8/8	7/8	4/8	7/8	4/8
	50	6/8	8/8	6/8	2/8	6/8	3/8
	100	2/8	8/8	3/8	0/8	0/8	2/8
	200	0/8	4/8	-	0/8	0/8	-
CDV	1.5	8/8	8/8	8/8	8/8	8/8	8/8
	3	7/8	8/8	8/8	7/8	8/8	8/8
	6	5/5	14/14	8/8	5/5	8/14	8/8
	12	6/8	5/8	7/8	0/8	2/8	3/8
	25	0/8	0/8	4/8	0/8	0/8	1/8
	50	0/8	0/8	0/8	0/8	0/8	0/8
	100	0/8	0/8	-	0/8	0/8	-
	200	-	0/8	-	-	0/8	-

Table 5. Number of Mice in Each Test Group Reaching Paralysis or Death
The data is expressed as the number out of the total group to reach the respective event. The dash marks represent doses not tested in that virus.

Drug	EC_{50} (mg/kg/day)		
	BV E90-136	HVP2 OU1-76	HVP2 X313
GCV	26.85 b	52.64 a	28.14 ab
CDV	8.83	7.10	10.68

Table 6. EC_{50} of Drug from the *in vivo* Experiment Which Showed Efficacy.
Shown are the only two drugs that were able to fully protect at least one group of mice from death. The lower case letters denote a statistical significance between groups, groups having the same letter not being significant from one another.

In vivo drug sensitivity models serve to test if drug sensitivity as determined by *in vitro* assays translates to a live animal model. To further examine the predictive nature of HVP2 for BV drug sensitivity, a mouse model was used to compare the *in vivo* efficacy of several drugs. The first drugs tested in the *in vivo* assay were ACV, PCV, and GCV since these drugs represent the current recommended treatment options for zoonotic BV infections. EDU and CDV were also tested because they both showed efficacy against BV and HVP2 *in vitro*. Even though TFT and IDU were effective *in vitro*, their high toxicity made them unable to be used in experiments where drugs were administered systemically.

BV control mice (no drug) began showing neurological signs of infection at or shortly before 4 DPI. Mice developed an abnormal adduction of the ipsilateral hind leg at 4 DPI, followed by ipsilateral hind leg paresis/paralysis at 4-5 DPI, and by 6-7 DPI all mice were dead. Similar to BV, mice infected with HVP2 X313 (no drug controls) developed an abnormal adduction of the ipsilateral hind leg at 4 DPI, this progressed to paresis/paralysis of the ipsilateral hind leg at 5-6 DPI. At 7-8 DPI the infection progressed to the point that mice either died or were humanely euthanized. In the HVP2 OU1-76 control infected mice presented neurological signs 1 day later than X313, then followed the same progression to death. While there was some variation in the timing of the progression of the infection between the three viruses, the progression of neurological disease development was consistent.

ACV was somewhat effective in *in vitro* assays, however the results of *in vivo* testing were not consistent with this (Figures 6 and 7). ACV was not effective against BV or either strain of HVP2; there was no protection from death at any dose for any of the

viruses tested. Even at the highest non-toxic dose tested, mice developed disease and died or were euthanized. The survival time was not significantly different than that of control groups.

PCV was more effective than ACV against BV and both HVP2 strains *in vitro*, but like ACV, was not protective *in vivo.* As seen in Figure 8, even at the highest non-toxic doses tested all mice developed severe disease. PCV did not provide protection against death; instead it only delayed death/euthanasia by 1-2 days (Figure 9).

The *in vitro* EC_{50} of GCV was similar to that of PCV for all viruses. However, while PCV was not able to protect against death for any of the three viruses, GCV did provide protection from death against both HVP2 and BV. Figure 10 shows the neurological scores for all the dose rates of GCV tested against HVP2 and BV. The highest two dose rates (200 & 100 mg/kg/day) both provided full protection from death in all three viruses. Although a GCV dose of 100 mg/kg/day protected fully from death, mice did develop neurological symptoms. Protection against development of a neurological score of $\geq$3 (paralysis) was instead examined (Figure 11). This is an important point for zoonotic of BV infections, since if symptoms progress past this point is it usually indicative of a lethal infection. As doses decreased the neurological scores and the number of mice dying both increased. Figures 12 and 13 show the survival curve of the lower concentrations of GCV 50 mg/kg/day and 25 mg/kg/day.

The data shows that GCV is the most effective of the current recommended therapies. In these experiments GCV appears to be somewhat more protective against BV than against HVP2 with the *in vivo* EC_{50} value for BV being the lowest of the three viruses at 26.85 mg/kg/day. All BV control mice were dead at the time when mice

infected with either HVP2 strain had only progressed showing abnormal leg adduction/paresis. However, in BV infected mice both of the two lowest GCV dose rates still provided full protection from death; the 50 mg/kg/day group average score did not progress past ipsilateral paralysis. In the 25 mg/kg/day group the average score was only slightly higher, there being only one mouse with bilateral paralysis. HVP2 X313 infected mice receiving 25 mg/kg/day GCV were similar to BV infected mice in that symptoms did not progress past ipsilateral paralysis with few mice developing bilateral paralysis. In contrast, HVP2 OU1-76 groups receiving 50 and 25 mg/kg/day GCV had a large number of mice progressing to death, with the group average almost reaching a neurological score of 5 (death).

Based on these *in vivo* experiments EC_{50} values were determined for GCV (Table 6). The BV EC_{50} value was 26.85 mg/kg/day, with HVP2 X313 being close with an EC_{50} value of 28.14 mg/kg/day. HVP2 OU1-76 had an EC_{50} value of 52.64 mg/kg/day; this high value made the OU1-76 EC_{50} significantly different from that of BV, but not from HVP2 X313.

EDU showed efficacy *in vitro*, having EC_{50} values close to or even lower than GCV. But similar to PCV, EDU was not effective *in vivo* (Figure 14). EDU was tested at dose rates as high as 400 mg/kg/day, but for all three viruses EDU provided no protection from death (Figure 15). These results were very similar to those of ACV, with the highest dose rate groups dying only 1-2 days after the controls.

The most unexpected results came with CDV (Figure16). Several of the higher dose groups had no mortality and did not develop any clinical signs of neurological disease. The first experiment with HVP2 OU1-76 used a highest dose rate of CDV 200

mg/kg/day. Not only did this dose group show no signs of neurological involvement, but the100 and 50 mg/kg/day groups also showed no signs of infection. In the group receiving 25 mg/kg/day, only a few mice developed mild symptoms of paresis with a smaller number of mice experiencing paralysis. While some mice did experience paralysis during the 14 day experiment, all mice had recovered to paresis by 14 DPI. Figure 17 shows the event of mice reaching paralysis for the 25 mg/kg/day group, but it does not show the eventual recovery of mice. The efficacy of CDV was even more evident in the BV experiment. BV infected mice showed no signs when given 100, 50 or 25 mg/kg/day; and it was only at doses $\leq$12.5 mg/kg/day that any signs of neurological infection were seen. CDV showed the least efficacy in HVP2 X313 infected mice. Mice receiving a dose of 50 mg/kg/day developed signs of neurological involvement by 8DPI.

In all viruses the efficacy of CDV was severely diminished at doses of $\leq$6 mg/kg/day. All mice were either dead or had a score of 4 and progressed to death after the 14 day experiment cutoff. Figures 20, 21, and 22 show the survival curves for the lowest doses CDV (6, 3, and 1.5 mg/kg/day).

EC_{50} values were determined for CDV (Table 6). All EC_{50} values were similar, with there being no statistically significant difference between any of the tested viruses.

Delayed Drug Administration

The delayed drug experiment was performed to mimic an exposure and treatment regime of a human BV infection. The drug sensitivity assays started drug therapy at one day prior to infection, which allowed the drug concentrations to accumulate before infection. In a human infection, drug therapy would start at the earliest a few hours after infection. However, a treatment regime often begins a few days after infection. This

experiment delays the initiation of drug therapy to examine the length of time that therapy can be delayed while still protecting from death. Two drugs used in the drug sensitivity assays were used in this experiment: GCV at 100 mg/kg/day and CDV 25 mg/kg/day (the lowest dose of each drug that fully protected from death).

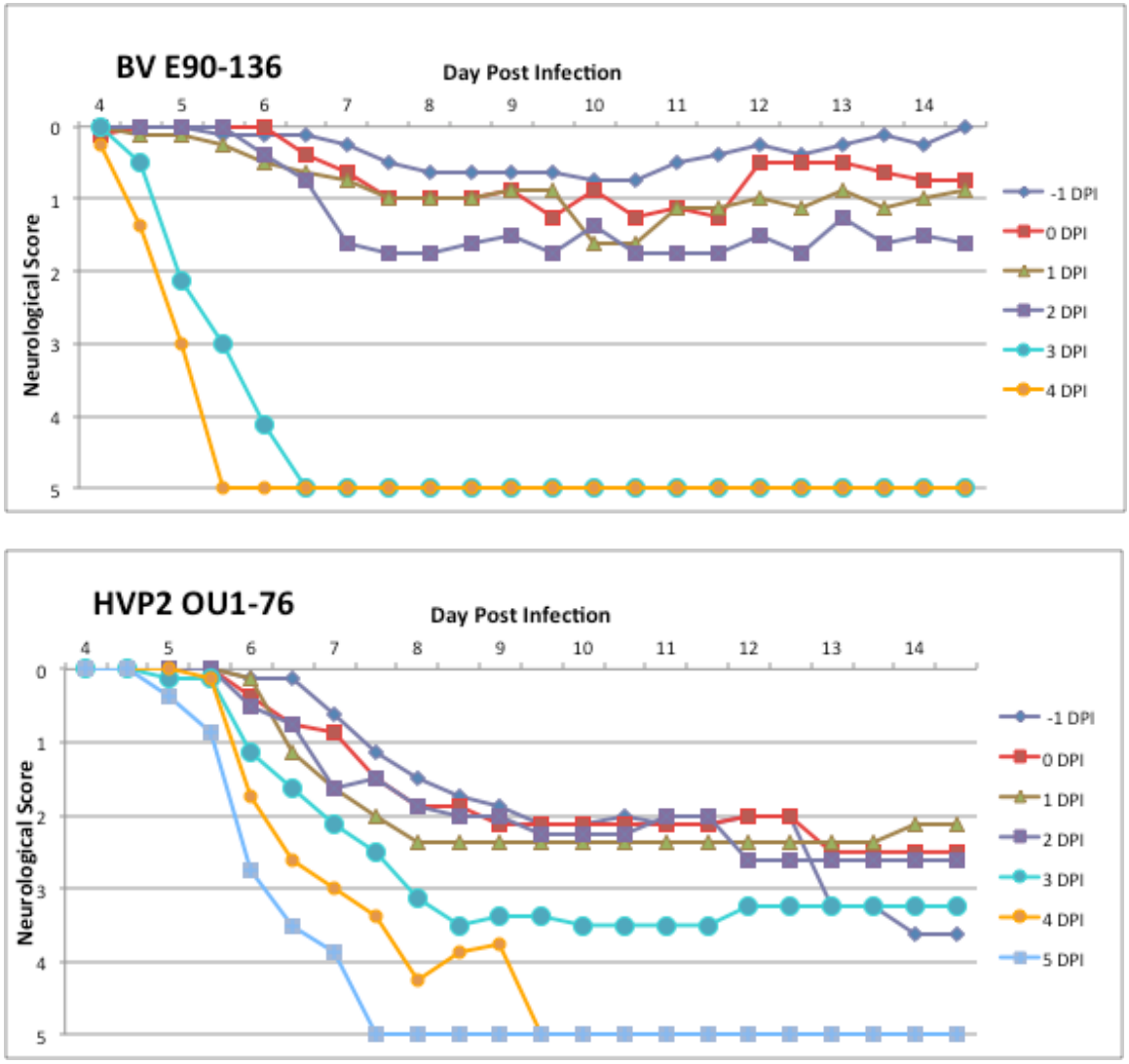

Figure 23. Neurological Scores Following Delayed GCV Administration
Mice were dosed with GCV (100 mg/kg/day) by IP injection twice daily for 7 days, with time of initiation between -1 to 5 DPI. All mice were infected with 10^5 PFU virus by skin scarification on Day 0. Mice were scored twice daily based on progression of neurological scores (Table 3). Average neurological scores for each group are shown.

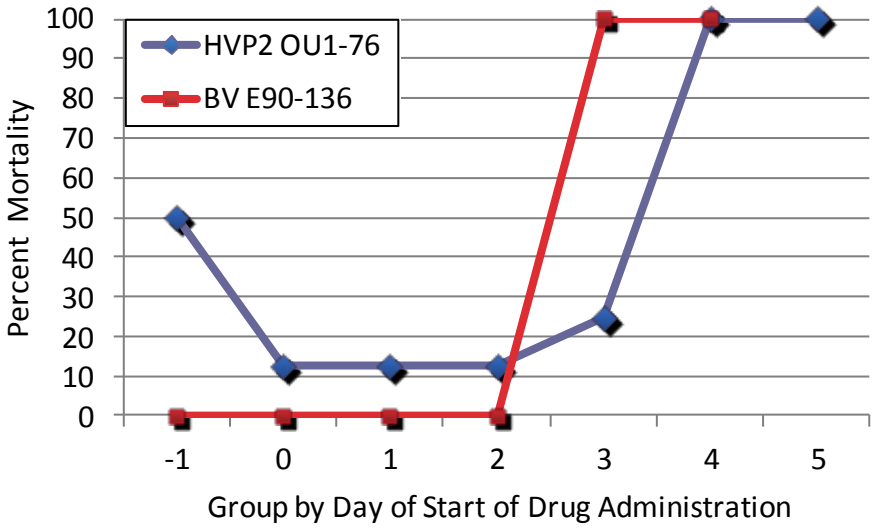

Figure 24. Mortality for Delayed Administration GCV at 100 mg/kg/day.
Shown is the percent mortality of each group of mice receiving GCV 100 mg/kg/day beginning on the day indicated. The start of therapy for BV was only tested out to 4 DPI, while HVP2 was tested to 5 DPI.

The GCV delayed drug trial used the prophylactic -1 DPI start group as controls. The group average scores of BV infected mice starting on prophylactic -1 DPI were similar to those of the GCV 100 mg/kg/day group in the initial drug sensitivity testing experiment (Figure 23, top). All -1 DPI mice lived with little to no neurological involvement, the group average neurological score always being less than 1 (abnormal leg adduction). In the BV experiment, if treatment was started on or before 2 DPI, all mice survived to the end of the study with most showing very mild neurological symptoms such as abnormal leg adduction or paresis. However, when drug administration began on 3 or 4 DPI, all mice quickly progressed to death on a time course comparable to the no drug control groups in earlier experiments (Figure 10 and Figure 23). Clearly, beginning drug therapy on or before 2 DPI is essential for survival.

The break of treatment initiation efficacy (protection from death) was not as clear-cut in HVP2 OU1-76 infected mice (Figure 23, bottom). Due to the slower progression of HVP2 infection and the corresponding later start of development of neurological symptoms, delaying onset of drug therapy until 5 DPI was tested. Mice starting drug therapy on or before 3 DPI led to survival for almost all mice, while all mice starting therapy on 4 or 5 DPI died. Mice in groups 1 DPI and 2 DPI showed the least neurological signs, with mice only showing abnormal adduction of the ipsilateral hind leg which often disappeared by the end of the study. Mice in the -1, 0, and 3 DPI groups were similar in having symptoms develop in some mice that were between ipsilateral hind leg paresis and paralysis. For HVP2, the day that drug therapy was initiated did not appear to be as important as the length of drug treatments.

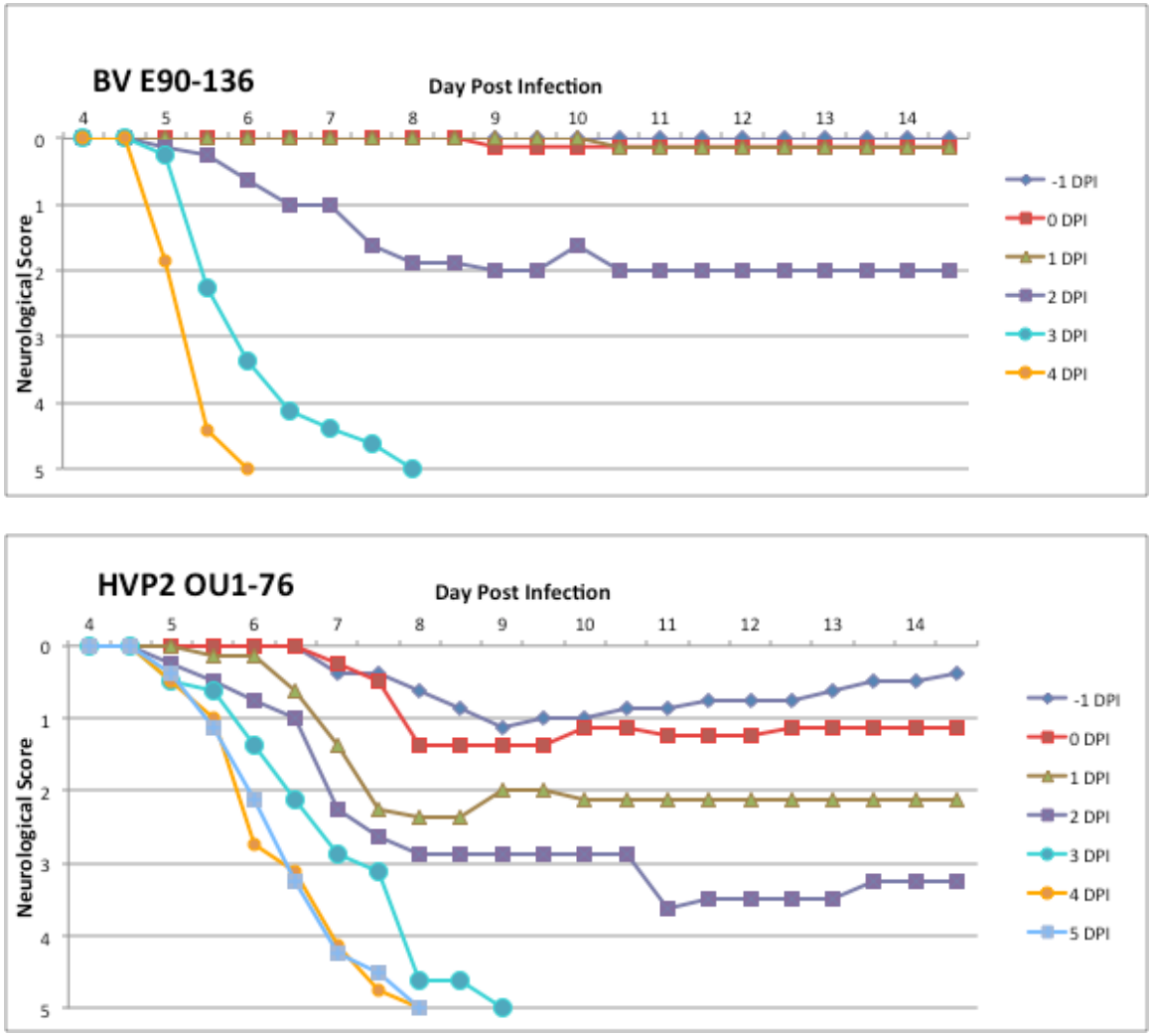

Figure 25. Neurological Scores Following Delayed CDV Administration
Mice were dosed with CDV (25 mg/kg/day) by IP injection twice daily for 7 days, with time of initiation between -1 to 5 DPI. All mice were infected with 10^5 PFU virus by skin scarification on Day 0. Mice were scored twice daily based on progression of neurological scores (Table 3). Average neurological scores for each group are shown.

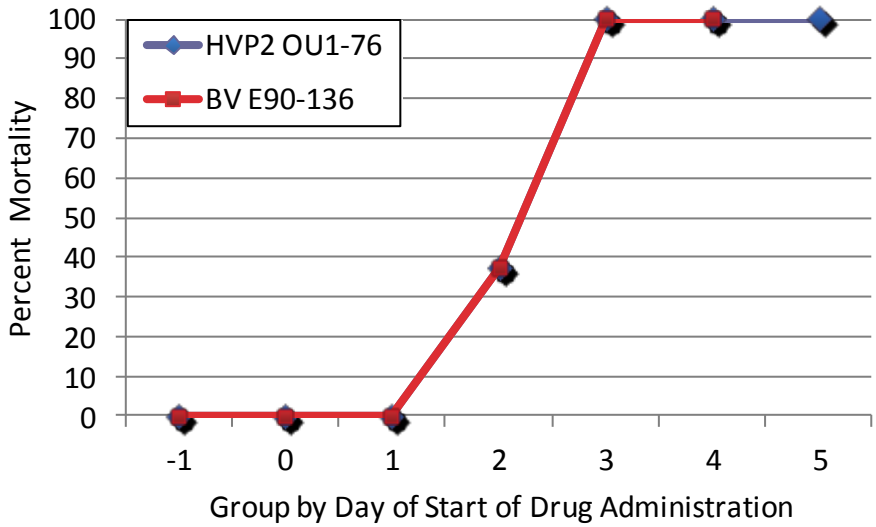

Figure 26. Mortality for Delayed Administration CDV at 25 mg/kg/day.
Shown is the percent mortality of each group of mice receiving CDV 25 mg/kg/day beginning on the day indicated. The start of therapy for BV was only tested out to 4 DPI, while HVP2 was tested to 5 DPI.

Since CDV was effective *in vivo*, CDV was also tested in the delayed therapy model. The dose rate of 12.5 mg/kg/day CDV was initially chosen and tested in HVP2 OU1-76, but there was a large number of deaths in the DPI groups of 0 and 1 (but not DPI group -1) made the use of a higher dose of CDV (25 mg/kg/day) necessary. The CDV delayed therapy experiment results resembled those of the GCV therapy delayed studies. Again, BV had a clear break in efficacy, with the HVP2 OU1-76 having an increasing range of neurological development.

Delayed onset of CDV therapy out to 1 DPI had no effect on efficacy against BV. There were no symptoms of neurological disease in mice starting therapy on or before 1DPI (Figure 25, top). The 2 DPI start of therapy group did develop a group average of paresis, but did not progress further. Again, starting drug therapy on 3 or 4 DPI resulted in all mice processing to death on a time course similar to that of the no drug control groups of initial drug sensitivity (Figure 25 and Figure 16).

The HVP2 infected mice receiving CDV delay therapy did not have the clear-cut break in efficacy as seen BV mice (Figure 25). The 0-2 DPI mice all survived and had at most only paresis. The 3 DPI group had an average neurological score of slightly past paralysis (>3). The 3-5 DPI therapy start groups all died in a time course consistent with the no drug controls.

In the GCV delayed therapy BV infected mice had a sharp rise in mortality between 2 and 3 DPI (Figure 24). Mortality associated with BV increased from 0% to 100% mortality with the delay of drug therapy by one additional day, between 2 DPI and 3 DPI. HVP2 infected mice had a similar rise in mortality between 3 and 4 DPI start of therapy groups. The HVP2 one-day delay in death was expected, as slower onset of

HVP2 neurological involvement was seen throughout the drug sensitivity assays. Interestingly, in the CDV delayed onset of therapy experiment BV and HVP2 had similar courses of mortality (Figure 26). Whereas GCV had a sharp rise in mortality, the mortality of CDV treated mice mortality rose with the groups 2-3 DPI. No mortality was seen in the 1 DPI group, a 37.5% mortality in the 2 DPI group, and 100% mouse death in the 3 DPI group.

Length of viral CNS invasion

Neural Tissue	Day of Tissue Collection						
	0	1	2	3	4	5	6
DRG	0/4	0/4	0/4	4/4	4/4	4/4	4/4
Spinal Cord	0/4	0/4	0/4	0/4	4/4	4/4	4/4

Table 7. Virus Isolation from Neural Tissue.
The table illustrates the number of mice in which the neural tissue tested positive for presence of virus through the process of virus isolation.

Results of the HVP2 delayed experiments showed that the time around 2-4 DPI was very important in determining mortality vs survival. It was hypothesized that once the virus was present in a specific neurological tissue, initiation of drug therapy would be ineffective and result in mortality. The two points along the neuropathogenic pathway that could indicate a point of no recovery are virus in the dorsal root ganglia (DRG) and virus in the spinal cord (SC).

Mice were infected with HVP2 OU1-76 and sacrificed on 24 hour intervals. The DRG and SC were collected and virus isolation preformed on each tissue separately. The 0 DPI group served as controls, since no virus would be present in DRG or SC at the time of inoculation. It was not until 3 DPI that virus was found in DRG, and next day (4 DPI) virus was present in both DRG and SC (Table 7). Virus was also present in both the DRG and spinal cord at 5 and 6 DPI. These results suggest that initiation of drug therapy after virus has reached the DRG will not be effective.

DISCUSSION

Zoonotic BV infection is usually fatal, and even with immediate drug treatment survivors are often left with long term deteriorative neurological symptoms. Also, due to the establishment of latency by herpesviruses, survivors may have latent BV reactivate and cause another active infection. BV has the potential to be used as a biological weapon, and has been classified as a Select Agent, and due to its high lethality needs to be worked with under strict BSL-4 containment. These factors together make studying BV dangerous and expensive. Also, there have only been around 50 documented cases, and most have been veterinarians and research support staff. Pharmaceutical companies have not seen developing BV drug therapy as fiscally beneficial due to the rarity of cases. These factors have led to paucity research on possible new drug therapies for treatment of BV.

The validation of a BSL-2 model to preform preliminary testing of new drug therapies for BV would allow more testing to be done with both reduced cost and danger. We have proposed use of the non-zoonotic baboon virus HVP2 as such a model. HVP2 has been shown to have pathogenicity in mice similar to BV, both of which mimic human infection (Ritchey, 2001, Rogers, 2006). The first step in validation of the HVP2 model is to show parallel drug sensitivity between HVP2 and BV.

Twelve anti-herpetic drugs licensed for use in humans were selected and tested *in vitro* to determine EC_{50} values against BV and HVP2. Some drugs were selected even

though their lack of efficiency in BV was known; our aim was hoped to show parallel sensitivity to drugs that were efficacious and not effective.

For all drugs tested, the sensitivity of HVP2 paralleled that of BV. In the *in vitro* experiments, the drugs ACV, PCV, GCV, EDU, CDV, IDU and TFT were the most efficacious. PFA, BVDU, BUdR, and HBPG were not effective against either virus. IDU was the only drug that showed a statistical difference between EC_{50} for the two the viruses. The IDU EC_{50} for HVP2 OU1-76 was significantly different than the BV EC_{50} value, but the HVP2 X313 EC_{50} value was not significantly different from that or HVP2 OU1-76 or BV. Since at least one of the HVP2 stains tested was similar to BV, HVP2 sensitivity was still predictive of BV drug sensitivity. Overall, there was good predictive drug sensitivity.

The *in vitro* data suggest that HVP2 X313 may be better than HVP2 OU1-76 as a predictive model for BV E90-136 drug sensitivity. EC_{50} values for HVP2 X313 were closer to those of BV than were HVP2 OU1-76 EC_{50} values for five drugs: ACV, AraA, CDV, GCV, and TFT. The most pronounced difference was for ACV: the difference between the X313 and BV EC_{50} values was only 6.61 μg/ml and this difference was almost negated when the standard error is considered. The OU1-76 ACV EC_{50} value was 16.05 μg/ml higher than that of BV, a difference outside of the range of compensation by standard error. For PCV all three viruses had EC_{50} values close to one other. EDU was the only drug for which HVP2 OU1-76 (not HVP2 X313) had an EC_{50} value that was more predictive of the BV EC_{50} value.

Interestingly, AraA had a lower EC_{50} than ACV. ACV replaced AraA for treatment of herpesvirus infections because it had lower toxicity than AraA. However,

based on the *in vitro* AraA may be more effective against BV than ACV. The *in vitro* data confirm previous evidence of AraA having a lower EC_{50} than ACV (Focher). It would be interesting to test AraA *in vivo* in our scarification model. Given the complete lack of ACV efficacy in the mouse BV model, AraA may well prove more effective than ACV. Future consideration should be given to more in depth study of AraA efficacy against BV; the efficacy of AraA may outweigh the higher toxicity as compared to ACV.

Drug testing experiments were not without complications. A major one was that different lots of EDU had great variance in efficacy from very high to almost none at all. When EDU was obtained from GLSynthesis it was very effective, with 20 μg/ml often giving 100% in plaque reduction. However, when EDU was obtained from Sigma-Aldrich there was little to no reduction in plaque numbers and the drug was ineffective. Samples of Sigma-Aldrich EDU were sent to Dr. Wright at GLSynthesis to be examined by mass spectrometry, and the ineffective Sigma-Aldrich EDU was found to be identical to the effective GLSynthesis EDU. At this time we have no explanation as to why the EDU efficacy was so different between the two sources. We chose to use the GLSynthesis EDU data to determine the *in vitro* EC_{50} values and for use in the *in vivo* experiments.

While this study tested the *in vitro* predictive nature of drug sensitivities using EC_{50} values, the lack of efficacy was also important. Four drugs showed very low efficacy: PFA, BVDU, BUdR, and HBPG. All four of these drugs were ineffective against all three viruses tested. This is important because although these drugs were not effective, the HVP2 model was still predictive of sensitivity of BV to these drugs.

Five drugs that were effective and were possible candidates for use in systemic therapy were tested in the *in vivo* model: ACV, PCV, GCV, EDU and CDV. IDU and TFT have low EC_{50} values, but their toxicity levels were too close to their EC_{50} to be considered as possible systemic treatment options for BV patients. However, IDU and TFT are not without therapeutic possibility. The drugs are both currently licensed for topical use in treatment of HSV infections. If a possible BV exposure is from a bite or scratch, the topical use of IDU or TFT at the site of infection may be a viable prophylactic treatment option. IDU and TFT both had very low EC_{50} values; the high potency of these drugs may be able to stop or greatly reduce initial replication of BV at the site of infection, thereby lessening the chance of BV entering the nervous system and thus neurological involvement.

The *in vivo* model was first tested using the three most commonly used HSV antivirals: ACV, PCV, and GCV. For these three drugs, *in vivo* efficacy against HVP2 paralleled efficacy against BV. ACV and PCV did not protect from death, even at the highest non-toxic doses tested, and both drugs only delayed death by a few days as compared to no-drug controls. In contrast, the two highest doses of GCV tested provided full protection from death. This was true for both HVP2 and BV.

The current first choice treatment drug ACV did not protect mice from death against HVP2 or BV. HVP2 OU1-76 and BV infected mice receiving ACV at 150 mg/kg/day progressed to death by 7-9 DPI. The HVP2 X313 infected mice survived longer with ACV at 150 mg/kg/day, but only until 11 DPI. Thus, ACV was ineffective against all three viruses.

The outcome of the ACV *in vivo* experiments were not a surprise since the ACV EC_{50} value showed ACV did not have a high potency *in vitro*, and it could be expected to have low efficacy *in vivo* as well. More surprising was the lack of efficacy of PCV. In the *in vitro* experiments PCV and GCV had EC_{50} values that were very close, but *in vivo* PCV was not effective whereas GCV was. Even the highest PCV dose did not protect from death, although it did delay death by an extra week.

PCV at 200 mg/kg/day protected BV infected mice from death through 14 DPI, but all mice died by 21 DPI. The PCV dose of 200 mg/kg/day was the only PCV dose to show any mouse survival at 14 DPI; lower doses of PCV (100 and 50 mg/kg/day) resulted in complete death by 8 DPI. Similar to BV, HVP2 X313 infected mice receiving PCV at 200 mg/kg/day were able to survive past 14 DPI, but eventually all died by 21 DPI. PCV at 100 mg/kg/day was able to delay death until 13 DPI, while with 50 mg/kg/day all mice were dead by 10 DPI. For HVP2 OU1-76 infected mice given PCV at 200 mg/kg/day, all mice died by 10 DPI. At lower concentrations of PCV (100 and 50 mg/kg/day) all mice died by 8-9 DPI.

Although *in vitro* testing of PCV and GCV resulted in similar efficacy for the two drugs, unlike PCV, full protection from death was afforded by GCV. At 100 mg/kg/day both BV and HVP2 X313 infected mice were fully protected from death. As GCV doses decreased, there was a concomitant increase in neurological signs. In contrast, HVP2 OU1-76 infected mice displayed a sharp increase in mortality with doses starting at $\leq$50 mg/kg/day. At 14 DPI with a GCV dose of 50 mg/kg/day, BV had 2/8 mice die, X313 had 3/8 die, and OU1-76 had 6/8 die; at 25 mg/kg/day both BV and X313 had 50% death and OU1-76 had 7/8 die. Looking at all GCV doses tested with the three viruses, as the

GCV dose decreased the score of neurological disease and death both increased. Again, HVP2 X313 was more predictive of BV sensitivity than was HVP2 OU1-76.

The lower efficacy GCV showed against OU1-76 resulted in a higher EC_{50} value of 52.64 mg/kg/day, while BV and X313 had very similar EC_{50} values (26.85 and 28.14 mg/kg/day). The higher EC_{50} value for OU1-76 was significantly different from the BV EC_{50}, but interestingly, it was not significantly different from the X313 EC_{50}. While OU1-76 had a higher EC_{50} value, it was still relatively predictive of BV (only 2-fold higher). HVP2 X313 was not significantly different from BV for any drugs tested *in vivo*, and so was more predictive of BV drug sensitivity than OU1-76 was. Small differences aside, both strains of HVP2 did show similar drug sensitivity to that of BV.

Based on *in vitro* test results, we tested two additional drugs in the *in vivo* mouse model: EDU and CDV. The expectation for EDU *in vivo* efficacy was short-lived as all the mice died as fast as the control mice. Even though the EC_{50} values of EDU were similar to those of PCV and GCV, the highest tested dose of EDU was 400 mg/kg/day (as compared to doses in the 100-200 mg/kg/day range for PCV and GCV) and was completely ineffective.

In contrast to EDU, the CDV experiments were so effective that the results of the first experiment were questioned. In all *in vivo* experiments, mice were experimentally infected with ~10 LD_{50} of virus. Even with such a high virus dose, mice receiving the three highest concentrations of CDV (200, 100 and 50 mg/kg/day) did not show any signs of infection, even lesions at the site of inoculation were not observed. Mice receiving a dose of 25 mg/kg/day had an average neurological score of only 1 (abnormal adduction of the leg). The lowest concentration tested in the first experiment (6 mg/kg/day) resulted

in only 50% mortality at 14 DPI, and only one additional mouse died by 21 DPI. A second experiment testing additional lower doses of CDV had to be performed to determine the EC_{50} value.

Although in most *in vivo* experiments, HVP2 X313 was more predictive of BV than HVP2 OU1-76, CDV was the exception. In the *in vitro* experiments OU1-76 had a lower EC_{50} value (3.14 μg/ml) as compared to the X313 EC_{50} (8.36 μg/ml). The higher efficacy of CDV against OU1-76 was also seen in the *in vivo* experiments. OU1-76 infected mice did not have any signs of infection or death at a CDV dose of 50 mg/kg/day, and neurological symptoms and death only occurred at CDV doses $\leq$12.5 mg/kg/day. In contrast, X313 infected mice developed paresis at 50 mg/kg/day and deaths occurred at 25 mg/kg/day. BV was similar to OU1-76 in having no signs of infection with CDV at 50 mg/kg/day, but BV appeared to be more sensitive to CDV as there were deaths only at doses of $\leq$6 mg/kg/day. The CDV EC_{50} values determined for the three viruses were all at or below 10 mg/kg/day and within 3.6 mg/kg/day of each other, and there was no statistically difference among the three EC_{50} values. In the CDV experiment HVP2 was very predictive of BV drug sensitivity in that all the viruses responded with similar sensitivity.

CDV is so effective that it has raised a problem not previously encountered. CDV appears to be able to stop virus replication soon enough that there was no stimulation of the host immune system and thus no viral antibodies to be detected by ELISA. Consequently, there was no way to confirm infection in the groups receiving high CDV doses. In lower CDV doses where neurological involvement was evident, mice did test positive for anti-viral IgG. In all previous years of mouse experiments, only rarely has a

surviving mouse not developed anti-viral antibodies (due to failed skin scarification and/or virus application). It was unlikely that all 8 mice in each group, and even more so three complete CDV dose groups, would all fail to become infected. It was even more unlikely that both HVP2 and BV infected mice, inoculated at different times by different investigations, would both fail to be infected. We therefore interpreted the negative ELISA results as being reflective of CDV being highly effective. If CDV is investigated further in the future, another form of testing needs to be used to demonstrate proof of infection. One possible method to confirm proper virus application is to preform immunohistochemistry for presence of viral antigen, virus isolation, or real-time PCR on skin samples taken from the site of infection in CDV dosed mice at early times after infection.

Even though data were collected in all experiments through 21 DPI, results have only been shown through 14 DPI. This is because after 14 DPI there appears to be a difference between HVP2 and BV. BV mice stabilize by 14 DPI and the group average neurological scores remain steady or can even decrease slightly over time. BV infected mice develop neurological involvement quickly, followed by either a stabilization of neurological involvement or a rapid advance to death. If mice survive past the 6-7 DPI time of the height of infection, they usually lived through the whole 21-day experiment. This suggests that if BV replication is hindered in the early stages of the infection, little to no progression of the infection occurs. This is different from HVP2. Based on neurological signs, HVP2 infection takes an extra 1-2 days to develop as compared to BV. After 14 DPI, HVP2 infected mice continue to experience an increase in neurological scores and mortality continues to climb. Therefore, it appears that even if

initial viral replication is controlled, once drug therapy ends HVP2 may still be able to recover and the infection progresses. Thus, BV infection is effectively controlled, wherea it appears to only temporarily inhibit replication of HVP2.

The difference in the viral progression past 14 DPI led us to conclude that HVP2 is not a viable model for BV drug sensitivity past 2 weeks PI. However, for this conclusion to be confirmed, more testing needs to be done. In previous *in vivo* studies, drug therapy was needed for 14 days to protect rabbits from BV (Boulter), as compared to the 7 day regime we used in our mouse model. It could be possible that if drugs were given for an additional 7 days that HVP2 infection would parallel BV infections even through 21 DPI.

The delayed drug mouse experiment takes the initial findings and goes one step further in making the mouse model more reflective of human BV infections. Humans that become infected with BV do not start antiviral therapy before the incidence of exposure occurs. If anything, patients may wait several days before they seek treatment. Some patients do not even seek medical attention until lesions are present at the site of infection. The delayed drug study was designed to mimic the delay of treatment onset that can be present in human BV infections. GCV and CDV were tested because in the initial drug sensitivity experiments both were effective, allowing at least two doses rates to have full survival from death. Delayed start of drug therapy confirmed the efficacy of GCV against BV, and the efficacy of CDV against BV was recognized.

The results of the GCV delayed drug therapy onset experiment will be the first discussed. Therapy started at -1 DPI was used as a control, with drug therapy started on the day of infection and at one-day intervals after being examined. BV drug therapy was

tested out to a delay of 4 DPI, with all mice in the 3 DPI and 4 DPI groups dying. Due to the one-day delay in presentation of neurological symptoms, HVP2 was tested until 5 DPI. Similar to BV, the two latest days of therapy onset all died (4 and 5 DPI groups). Within the groups that lived, the responses were similar to the sensitivity experiments. BV infected mice experienced some neurological involvement, but the group average scores stabilized after the height of infection at 7 DPI. However, HVP2 infected mice had a steady increase in the group average scores of neurological involvement between 7-10 DPI. As seen previously, if the initial BV infection in mice is effectively suppressed then the mice survived, while HVP2 infection is only temporarily inhibited and eventually symptoms slowly increase in severity.

Looking at the results from the HVP2 delayed GCV therapy, a problem is evident: the control group starting therapy at -1 DPI does not have the expected results based on initial drug dose controls that also started therapy at -1 DPI. The delayed drug therapy group should parallel groups receiving the same dose in the drug sensitivity experiment, meaning there should be 100% survival. Instead, the -1 DPI delayed therapy group had 50 % mortality. There is no explanation of why this happened, and so repeating this experiment is necessary. If the delayed control group of -1 DPI have full survival, then this current control group could be explained as variation of a biological system.

CDV was also tested in the same delayed therapy mouse model. When the onset of CDV therapy was delayed in BV infected mice there were three groups (-1, 0, and 1 DPI) in which there was no evidence of BV infection based on both symptoms of neurological involvement and lesions at the site of infection. There was again a clear break in efficacy, with therapy delayed $\geq$3 DPI resulting in 100% mortality. The results

of the HVP2 delayed onset experiment was similar to the BV experiment in that groups receiving therapy ≤2 DPI had full survival. However, similar to the GCV delayed experiment, even groups that lived had an increase in neurological scores over time.

What is interesting about the CDV delayed experiment was the day on which the die-off occurred. Usually BV infected mice die a day before HVP2 infected mice, but in this experiment HVP2 and BV infected mice exhibited a similar time course of infection. For both viruses all mice in ≤1 DPI groups lived, mortality was 37.5 % in the 2 DPI group, and mortality was 100% in ≥3 DPI groups. This is the first time HVP2 infected mice had mortality that followed the same time line as BV infected mice. These results are the strongest evidence in the predictive nature of the HVP2 model.

The BV mortality in the delay therapy experiments alludes to a problem not yet addressed: the limits of CDV drug therapy. The results of the drug sensitivity experiments make CDV seem like a promising therapy for BV treatment, but the delayed therapy experiment may have exposed a shortcoming of CDV. Whereas in the GCV delayed experiment BV infected mice had full survival if therapy was started at ≤3 DPI, with CDV delayed therapy the mice only had full survival when therapy was started at ≤1 DPI. This may be indicative of CDV not being as effective as GCV once the virus has established an infection. This suggests that the strength of CDV is to stop the infection before it starts, and not in slowing an infection already present in nervous tissue.

The delayed drug experiment served to show the amount of time that can elapse before drug therapy can start while still protecting from death. For HVP2 this window of time was between 3 and 4 DPI. One possibility is that this time represents the point at which the virus enters or establishes itself in the CNS. When we collected nervous tissue

from HVP2 infected mice we found that the presence of virus in nervous tissue correlated with mouse mortality. No virus was present in the DRG or the lumbar spinal cord at ≤2 DPI, but on 3 DPI virus was present in the DRG, and on 4 DPI virus was present in both the DRG and spinal cord. This indicates that virus reaching the CNS marks the point of no return for the onset of drug therapy. What is not known is exactly when in the pathogenic process or viral invasion what tissue represents the deciding factor. It is unknown if virus can be stopped once it reaches the DRG, or if it is considered terminal only once it reaches the spinal cord. This question is very important when dealing with human BV infection. A more in-depth study is needed to fully understand the progression of virus and expected mortality.

CONCLUSIONS

The current recommended first choice drug for treatment of BV infections, ACV, did not provide protection from death in mice and only delayed death by approximately one day. While showing efficacy *in vitro*, PCV was not effective *in vivo*, providing only a slight delay of death. This was also true of EDU, where *in vitro* efficacy did not translate to protection from disease or death in the mouse model. GCV could provide full protection against death, with all mice receiving higher doses surviving to the end of study. CDV was the high point of the study, stopping viral replication before it was even able to establish an active infection. The delayed therapy experiments illustrated the importance of immediate drug therapy after exposure to BV, and the corresponding virus isolation experiment demonstrated that once the virus has invaded the CNS the infection is not amenable to effective treatment with any of the drugs tested.

This study was undertaken to test the hypothesis that HVP2 is a predictive model of drug sensitivity for BV. With all the similarities of sensitivity between both the HVP2 strains and BV, both *in vitro* and *in vivo*, we consider HVP2 to be a reliably predictive model system for preliminary drug sensitivity testing of BV, and that this model has been fully validated.

Moving forward with this conclusion of validity, implications in the treatment of BV infection can been made. The first is that ACV should no longer be recommended as initial therapy for treating a BV infection. Currently ACV is recommended for use until

signs of disease are present, and then therapy is switched to the more effective GCV. But as the delayed therapy experiment demonstrated, this may be too late to effectively treat BV infection. Instead, an oral prodrug form of GCV should be used from the beginning, as this therapy appears to be very effective at controlling the virus infection before it spreads into the nervous system. Also, the topical use of IDU or TFT for prophylactic treatment of BV infections should be examined more closely. The use of IDU or TFT would be very effective in this application, and if so should be added to the recommended treatment guidelines. Lastly, what could be the most important finding, is that CDV showed high efficacy for treatment of BV infections if given early after infection. Now that a model for BV exists, CDV should be study more in-depth to determine the full scale of therapy this drug may possess.

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