TOWSON UNIVERSITY
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EFFICACY OF VARIOUS POLYMER:DRUG RATIOS OF MOLECULARLY HOMOGENEOUS IMPLANTS IN PREVENTING HERPES SIMPLEX VIRUS TYPE-2 INFECTION IN VERO CELLS

by

Sarah Fargis

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Sarah Fargis entitled Efficacy of Various Polymer:Drug Ratios of Molecularly Homogeneous Implants in Preventing Herpes Simplex Virus Type-2 Infection in Vero Cells, has been approved by her committee as satisfactory completion of the requirement for the degree of Master of Science.

Barry Marguli
Chair, Thesis Committee — Date 11/7/13

Elena Ehrlich
Committee Member — Date 11/7/13

Michelle Snyder
Committee Member — Date 11/7/13

Janet V. DeLany
Janet DeLany, DEd
Dean, College of Graduate Education and Research — Date 11/13/13

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ABSTRACT

Efficacy of Various Polymer:Drug Ratios of Molecularly Homogeneous Implants in Preventing Herpes Simplex Type – 2 Infection in Vero Cells

Sarah E. Fargis

Genital herpes, which is typically caused by HSV-2, infects 15-25% of the population worldwide. Infection results in recurrent outbreaks of lesions in the genital area. There is no cure for herpes, but anti-herpetics have been developed to combat infection. These drugs have a low oral bioavailability, a short half-life, and poor patient compliance. To negate these issues, the TUHVL has developed an implant made with drug and the biodegradable polymer polycaprolactone (PCL). However, the ideal ratio of polymer:drug has not been established. This study aims to determine which ratio has the best release kinetics and antiviral activity. Implants were created with various polymer:drug ratios and an *in vitro* study was performed. Samples were collected and analyzed with HPLC to determine the release kinetics. The antiviral effectiveness was determined by monitoring cytopathic effect and plaque assays. Ultimately, we found that the 75:25 PCV implants were the most effective.

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CHAPTER I:

INTRODUCTION

Herpesviridae is a family of double-stranded DNA viruses capable of causing disease in humans and other animals (Davison and Wilkie, 1983; Whitley et al., 1998). Herpesviruses, eight of which infect humans, are categorized into three subfamilies based on differences in their biological properties, the alphaherpesviruses, betaherpesviruses, and gammaherpesviruses (Alba et al., 2001). Alphaherpesviruses have a variable host range, destroy infected cells efficiently, and establish latency in the sensory ganglia (Roizman et al., 2007). The alphaherpesviruses include the human herpes viruses herpes simplex virus type-1 (HSV-1), herpes simplex virus type-2 (HSV-2) and varicella zoster virus (VZV) (Davison and Wilkie, 1983). These viruses are most notable for causing cold sores, genital lesions, and chicken pox, respectively (Whitley et al., 1998). The alphaherpesviruses have many similarities, but HSV-1 and HSV-2 are the most alike and share approximately 50% of their genetic material (Davison and Wilkie, 1983).

HSV Structure, Entry, and Replication

The virions of herpesviruses range in size from 120 to 300 nm, but are similar in structure (Roizman et al., 2007). Virions are composed of four parts: a DNA core, protein capsid, protein-filled tegument, and lipid bilayer envelope. Double-stranded linear DNA, which ranges in size from 120 to 250 kbp, is found within the core (Whitley et al., 1998; Roizman et al., 2007). The core is surrounded by an icosahedral capsid (Whitley et al., 1998). Surrounding the capsid is a protein-rich tegument, which varies in thickness and is primarily responsible for the size difference between virions (Roizman et al., 2007). The

tegument surrounds the capsid and connects it to the outermost phospholipid envelope (Whitley et al., 1998). At least a dozen glycoproteins are present in the envelope and many play a critical role in viral entry (Whitley et al., 1998; Taylor et al., 2007). These glycoproteins, most notably gB, gD, gH and gL, interact with cell surface molecules to allow the envelope of the virus to fuse with the cell plasma membrane (Roizman et al., 2007). Both herpes simplex viruses, HSV-1 and HSV-2, enter cells and replicate similarly. The glycoproteins allow for the entry of HSV into the mucosal epithelia (Muggeridge, 2000). Post-fusion, most of the tegument proteins remain in the cytosol (Ojala et al., 2000). Other proteins, like VP16, get transported to the nucleus with the capsid (Roizman et al., 2007). The capsid is transported along microtubules to the nuclear pore where it releases the viral DNA into the nucleus (Ojala et al., 2000).

Replication

Once inside the nucleus, the HSV genome circularizes (Roizman et al., 2007). Proteins are expressed in a tightly regulated and ordered fashion. RNA polymerase II transcribes the genes in the nucleus and translation occurs in the cytoplasm (Roizman et al., 2007). The first genes to be expressed are the immediate early (IE) genes. These include regulatory proteins that are required to be present for the next step in the cascade of gene expression to occur, the early (E) stage (Roizman et al., 2007). These early stage proteins most notably are involved in DNA replication, like DNA polymerase, and the onset of replication leads to the expression of late (L) gene products (Roizman et al., 2007). The late gene products downregulate early expression and include structural proteins required to generate new capsids (Roizman et al., 2001).

Virion Assembly and Egress

The structural proteins created in the late stage are used to assemble the capsid in the nucleus (Roizman et al., 2001). The capsids are assembled and the progeny DNA is fed into the capsid (Roizman et al., 2001). The capsid ultimately leaves the nucleus and acquires an envelope at multivesicular bodies. The resulting virions bud through the plasma membrane and exit the host cell (Roizman et al., 2007). Ultimately, the infected cell is destroyed and the progeny can infect neighboring cells. Cell death, in conjunction with inflammation from the immune response, results in lesions at the site of infection (Taylor et al., 2007; Roizman et al., 2007).

Latency and Reactivation

All herpesviruses are able to establish a dormant state, known as latency, in the host (Taylor et al., 2007). HSV travels from the primary site of infection, the epithelial cells, by retrograde transport along neurons to the site of latency (Roizman et al., 2007). This site is typically in the trigeminal ganglia for HSV-1 infections and in the sacral ganglia for HSV-2 infections, but the location is ultimately based on the site of primary infection (Taylor et al., 2007). During latency, little to no viral proteins are made (Koelle and Wald, 2000). Stimuli such as stress, fever, ultraviolet light, tissue damage, and immunosuppression can disrupt the dormant state and cause reactivations (Whitley and Roizman, 2001). During reactivation, the virus becomes lytic and travels by anterograde transport down the neuron, replicates, and disease manifests itself at the location of the primary infection again (Koelle and Wald, 2000; Taylor et al., 2007). Infection and replication at the oral mucosa, typical of HSV-1 infection, results in lesions sometimes

called cold sores. Infection and replication at the genital mucosa, typical of HSV-2 infection, is known as genital herpes and results in genital lesions (Whitley et al., 1998).

HSV-2: Transmission and Pathology

Genital herpes is one of the most common venereal diseases. It is caused by HSV-2 in 60-80% of cases and HSV-1 in 20-40% of cases (Malkin, 2004). The seroprevalence of HSV-2 in developed countries is 15-25%, with a seroprevalence of 16.2% in the United States (Xu et al., 2010). There is a direct correlation between the number of sexual partners and the prevalence of HSV-2 (Whitley et al., 1998; Whitley and Roizman, 2001). HSV transmission occurs when a seronegative individual comes in close contact with mucosal secretions, mucosal membranes, or lesions from a seropositive individual who has a lesion or is currently shedding the virus (Koelle and Wald, 2000). Asymptomatic shedding, or subclinical shedding, occurs when HSV is detectable, but no lesions are present (Koelle and Wald, 2000). Studies show that HSV is typically transmitted during this asymptomatic period of viral shedding (Koelle and Wald, 2000). Because latent virus remains through the life of the host, there is no cure for herpes, but treatments have been developed to combat the infection.

Treatment

The antiviral medications typically prescribed to treat herpes are the nucleoside analogues acyclovir and penciclovir, more commonly known as Zovirax® and Denavir®, respectively (Bacon et al., 2003). Daily oral administration of acyclovir is known to decrease the frequency and extent of both symptomatic and asymptomatic shedding in HSV-2 seropositive individuals (Wood et al., 1992). Both acyclovir and penciclovir are

effective in decreasing the length of time a lesion is present and in resolving the associated pain more rapidly (Raborn et al., 2002). The anti-herpetics work by selectively inhibiting viral replication in HSV-infected cells (Bacon et al., 2003). All cells take up the antiviral, but only cells infected with HSV have a virally encoded thymidine kinase that is able to convert the drugs to their monophosphate derivatives (Wood et al., 1992). This event does not occur substantially in uninfected cells (Wood et al., 1992). Cellular enzymes disphosphorylate and triphosphorylate the drug, ultimately resulting in drug concentrations 40 to 100 times higher in cells infected with HSV (Wood et al., 1992). The triphosphorylated drug competes with deoxyguanosine triphosphate as a DNA polymerase substrate (Wood et al., 1992). The viral polymerase has a 100-fold greater affinity for the ACV and PCV than the cellular polymerase, so very little drug gets incorporated into the cellular DNA (Wood et al., 1992). Acyclovir and penciclovir triphosphate lack the 3' hydroxyl group, so the DNA chain cannot get elongated, ultimately leading to the termination of DNA synthesis (Wood et al., 1992).

Despite the strikingly similar mode of action of the two antivirals, there are some critical differences. Acyclovir is known to have a low oral bioavailability of 15-30% (Wood et al., 1992), and the oral bioavailability of PCV is 1-5% (Moomaw et al., 2003). The poor oral bioavailability explains why penciclovir is only available as a topical treatment. To combat the oral bioavailability issue, ACV and PCV pro-drugs have been developed (Bacon et al., 2003). These pro-drugs are altered, inactive, forms of the drug that get converted to the active form by the body. Valacyclovir, a valine-esterified ACV, has an oral bioavailability of 55% (Tyring et al., 1998). Famciclovir, the PCV pro-drug,

has an increased oral bioavailability of 75-77%. (Tyring et al., 1998) All four drugs are widely prescribed for HSV treatment (Bacon et al., 2003).

In addition to low bioavailability, ACV has a short half-life of approximately three hours (Wood et al., 1992). The renal clearance rate is slower and the triphosphate is more stable with penciclovir, so the half-life of the latter is 10 to 20-fold longer (Bacon et al., 2003). In the cells, the quantity of penciclovir triphosphate is greater than that of acyclovir triphosphate because of the decreased cellular excretion rate (Bacon et al., 2003). These differences ultimately make penciclovir an ideal drug for treating herpes. Another notable difference is the veterinary application of PCV compared to ACV. Feline herpes virus type-1 (FHV-1) is a common cause of eye and upper respiratory infections in cats (Riviere and Papich, 2009). ACV is not effective against FHV and is toxic to cats, but PCV can safely be used for FHV treatment in cats (Riviere and Papich, 2009). However, the relatively short half-life and low bioavailability of both ACV and PCV are a hindrance. Because of these issues, ACV and PCV need to be administered several times a day in order to be effective (De Miranda et al., 1983). This constant administration is critical so that the level of drug will be above the therapeutic threshold. If the level of drug in the tissue falls below this threshold, the virus can replicate and potentially acquire a mutation that provides resistance to the drug. However, most patients have difficulty abiding to the strict dosing regimen (Fife et al., 1997). As a result, patients are not seeing the level of effectiveness when the drugs are delivered even under ideal circumstances (Fife et al., 1997). In order to see a profound difference in the number of reactivations of the virus, the delivery method needs to be altered.

Controlled Release Devices

The Towson University Herpes Virus Lab (TUHVL) developed an alternative delivery device, a silicone implant containing acyclovir, for the treatment of herpes. This long-term solution requires virtually no patient compliance, which would overcome one of the main limitations in the treatment of herpes. Studies have shown that this rod-shaped silicone implant released ACV at a constant rate of approximately 1 μg/day (Johnson et al., 2007). In an animal model of recrudescence, HSV-1 infected SKH-1 mice were exposed to UV light; in these experiments the implant significantly suppressed reactivations (Johnson et al., 2007).

Long-term drug delivery devices that work for years have been successfully used before, most notably with Norplant®, which was used to release levonorgestrel for birth control (Sivin et al., 1980). The Norplant® system was composed of six silicone implants that were surgically placed in the arm and approved to last for five years (Campbell and Brautbar, 1995). In the first month, hormone release was approximately 85 μg/day (Sivin, 1994). This decreased to about 30 μg/day for the duration that the implants were in the body (Sivin, 1994; Campbell and Brautbar, 1995). However, one of the main problems was removing the device from the patient after five years (Haugen et al., 1996). The removal caused many complications and was described to be generally unpleasant (Sun et al., 2005). A better solution is using an implant made of biodegradable material, like polycaprolactone (PCL) (Sun et al., 2005).

Polycaprolactone is FDA-approved (Woodruff and Hutmacher, 2010) and degrades more slowly than other biodegradable polymers, making PCL ideal for long-

term drug delivery (Sun et al., 2005). The breakdown of the PCL occurs in two stages called surface erosion and bulk erosion, respectively. During the first stage, molecular weight is decreased, but there is no loss of mass or deformation (Sun et al., 2005). During the second stage, hydrolysis of the ester linkages results in PCL degradation (Sun et al., 2005). Ultimately, the PCL pieces get adsorbed and excreted by the body (Sun et al., 2005).

SID Implants

The TUHVL has successfully made ACV/PCL implants using a method we named Suspension of Insoluble Drug (SID). To create these implants, heat and constant stirring was used to dissolve PCL in acetone (Nelson, 2012). Once the PCL dissolved, ACV was added, the mixture was continuously stirred, and then was left to dry overnight (Nelson, 2012). The resulting mixture was then heated in a 76 °C water bath until it was pliable, then extruded into 10-gauge needles (Nelson, 2012). The mixture was left in the needles to solidify overnight (Nelson, 2012). The rod-shaped implants were then pushed out of the needles and cut to the appropriate size of 15 mm (Nelson, 2012). These implants were tested at various temperatures and pH in a 60-day *in vitro* study to determine the release kinetics of drug from them (Nelson, 2012). In all of the samples, the first 14 days had very inconsistent release, known as a burst period (Nelson, 2012). During days 15 to 60, a decrease in the release rate occurred for all of the samples (Nelson, 2012). Overall, the pH did not have a significant effect on the amount of drug released (Nelson, 2012). All the implants had a decline in the amount of drug released daily, but the implants maintained at 37 °C had both inconsistent release and significant

variation; the amount of drug released decreased daily until no more drug was being released at 60 days (Nelson, 2012), although the average drug released was greater from these implants than it was from the silicone implants.

Therefore, the release from these SID implants was not optimal (Nelson, 2012). The drug release did not exhibit zero-order kinetics like the silicone implants. The release kinetics can be explained by the method, SID, used to generate the implants. Acyclovir, and other drug relatives, are difficult to dissolve in neutral solutions and are poorly soluble in most organic solvents. When the ACV was added to the PCL/acetone mixture, the drug did not dissolve. Instead, a colloidal suspension was created. This colloidal suspension dried overnight and the ACV never distributed in a truly homogenous fashion. The resulting implant had chunks of drug interspersed with chunks of polymer. This meant that pockets of drug were released in sporadic bursts, explaining the inconsistent release and large variation seen in the *in vitro* study. Instead of surface erosion, which is ideal for a controlled delivery device, uneven bulk erosion was occurring most likely throughout the study. Ultimately, the undissolved ACV led to a non- molecularly homogeneous implant that affected the distribution of drug, release kinetics, and structural stability. To improve the release kinetics, we expected that the ACV must be completely dissolved in solution during the mixing phase.

VASE Implants

In 2012, the TUHVL created a novel method to dissolve the ACV in solution called Volatile Acid-Solvent Evaporation (VASE). This method consists of dissolving PCL in acetonitrile with slight heat and constant agitation with a stir bar (Badin, 2013).

Once the PCL is dissolved, drug is added to the mixture (Badin, 2013). This creates a colloidal suspension because the ACV (or PCV) is not dissolved (Badin, 2013), similar to SID technology. To solubilize the drug, the pH is brought down below the pKa of ACV using a volatile acid, formic acid, through dropwise addition to the stirring solution (Badin, 2013). Once the solution turns clear, indicating that the drug is dissolved, the mixture is left to dry in the fume hood for several days while stirring (Badin, 2013). The resulting mixture is placed in a Speed-Vac for at least two hours to remove any residual formic acid (Badin, 2013). The mixture is heated, extruded into needles, dried overnight, and the implants are pushed out and cut to size, similar to the way implants were created from the SID method (Nelson, 2012). Scanning electron microscopy showed that these VASE implants are molecularly homogeneous, while the SID implants are not molecularly homogeneous (Badin, 2013). Placing ACV/PCL VASE implants in cell culture showed both that they suppressed primary HSV-1 infection and that the formic acid treatment did not adversely affect the cells (Badin, 2013). However, the optimal drug to polymer ratio for the implants is still unclear.

This thesis addresses this question by performing an *in vitro* study to determine both the release kinetics and antiviral efficacy of the various polymer:drug ratios. Additionally, an experiment was performed to determine whether there was a difference in release kinetics between ACV versus PCV in VASE implants. Ultimately, the results from this study will be used to select the ideal implants for use in HSV-2-infected guinea pigs. Should these implants be proven to be effective, this can ultimately reduce the

number of reactivations for individuals infected with HSV-2 and for those potentially infected with other herpesviruses.

CHAPTER II:

MATERIALS AND METHODS

Implant Development Using the VASE Method

Biodegradable implants were constructed with polycaprolactone (PCL) (Sigma-Aldrich, St. Louis, MO) and either acyclovir (ACV) (Advanced Scientific & Chemical Inc., Ft. Lauderdale, FL) or penciclovir (PCV) (Advanced Scientific & Chemical Inc.). The PCL-PCV implants were made in a 70:30 polymer:drug ratio. The PCL-PCV implants were made in the various polymer:drug ratios 70:30, 75:25, 80:20, 85:15, 90:10, 95:5, and 100:0. The quantity of PCL and drug used to make the implants is shown in Table 1.

PCL was added to 25 mL of acetonitrile (Sigma-Aldrich) and exposed to 37 °C heat and constant agitation from a magnetic stir bar until the polymer completely dissolved. The drug, ACV or PCV, was then added to the solution. Formic acid (Sigma-Aldrich, St. Louis, MO) was added to the solution dropwise until the drug solubilized and the solution turned clear. The mixture was constantly agitated with a magnetic stir bar and left under the chemical fume hood until the mixture was completely dry. This took approximately three days and allowed the formic acid to evaporate. To remove any residual formic acid, the mixture was transferred into 20 mL glass scintillation vials (Thermo Fisher Scientific, Inc., Madison, WI) and covered with a Kimwipe (Kimberly-Clark, Mississauga, Ontario). The bottles were placed in a Thermo Savant Speed-Vac Model RH 40-11 (Thermo Fisher Scientific, Inc.) for two hours to remove any residual formic acid.

To create the implants, the PCL-drug mixture was transferred into 15 mL Eppendorf Combitips (Thermo Fisher Scientific, Inc.) and placed in a 75 °C bead bath. Once the mixture changed from a solid to a gel consistency, which took approximately thirty minutes, the Combitip was removed from the bead bath. The gel was extruded into a hollow, stainless steel, 2 mm, 10-gauge needles (Painful Pleasures, Hanover, MD) and left at room temperature to solidify overnight. The rod-shaped implants were extruded out of the needle and cut to 15 mm using a razor blade.

Implant Sterilization

Implants were submerged in 10 mL of 70% ethanol for five minutes, and transferred to a new basin with fresh 70% ethanol for an additional five minutes. Then implants were transferred to a basin containing 10 mL of fresh PBS (Thermo Fisher Scientific, Inc.) four more times. The implants were submerged for 10 minutes for the first three basins, and five minutes for the last basin. Lastly, the implants were transferred to an empty basin and left to dry in a biosafety cabinet overnight. The sterile implants were transferred into sterile 1.5 mL microcentrifuge tubes for storage.

Drug Release Study in PBS

A release study was done to compare the release from PCL-ACV implants to that of the PCL-PCV implants. Three 70:30 PCL-ACV implants and three 70:30 PCL-PCV implants were created and sterilized. Each well in a six well plate (BD Biosciences, Durham, NC) was filled with 7 mL of PBS. One implant, either PCL-ACV or PCL-PCV, was added to each well. The six well plate was stored in a 37 °C incubator with 5% CO_2. Twenty-four hours later, the PBS was further mixed. One mL of the PBS was transferred

to a microcentrifuge tube and stored at -20 °C for subsequent HPLC analysis. The remaining 6 mL of PBS was discarded and 7 mL of fresh PBS was put into the well. Collection continued daily until day 15, where it began to occur weekly, until day 63.

Cells and Virus

African green monkey kidney (Vero) cells (ATCC #CCL-81, gift of Prashant Desai, Johns Hopkins University School of Medicine) were the cell line used for cell culture studies. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1% Glutamax (Gibco, Carlsbad, CA) and 1% antibiotic/antimycotic (Hyclone). The cells were grown in T-75 polystyrene cell culture flasks (BD Falcon, Franklin Lakes, NJ) in approximately 25 mL of complete DMEM, as described above. Cells were maintained in a 37 °C incubator with 5% CO_2.

HSV-2 MS (ATCC#VR-540, ATCC, Manassas, VA) strain was used in the cell culture study. The virus was stored at -80 °C.

Cell Culture Study

A cell culture study was done to determine the most effective drug to polymer ratio. A 24-well culture plate (BD Biosciences) was seeded with 1.6×10^5 Vero cells per well, in 1 mL of DMEM per well. The cells were grown overnight in a 37 °C incubator with 5% CO_2. Twenty-four hours later, the 1 mL of media from each well was collected and stored at -80 °C for later HPLC and plaque assay analysis. One mL of fresh DMEM and a single implant were added to each well. This was done with each PCL to PCV ratio (70:30, 75:25, 80:20, 85:15, 90:10, 95:5, 100:0). Control wells did not receive an implant.

The next day, the media were collected, stored, and fresh media was added, as above. Wells were either infected with 100 pfu of HSV-2 (MS) or left uninfected. The uninfected control group served to show that the implant did not have adverse effects on the Vero cells, particularly due to the formic acid treatment. Up until 3 days post-infection (d.p.i.), the media were collected, stored, and changed. At three d.p.i., the cells were observed with an Accu-Scope 3032 microscope and images were captured using a Sony Cybershot DSC-H2 12x optical zoom digital camera. Multiple shots of each well at different focal planes were taken and combined using Zerene Stacker software (Zerene Systems, Richland, WA).

HPLC analysis

HPLC was used to quantify the level of drug released from each implant. The samples from the PBS release study and the cell culture study were prepared differently.

For the release study in PBS, 150 µL of the sample was transferred into a 2 mL Verex vial (Phenomenex, Torrance, CA) containing 1350 µL of acetonitrile. PCV standards with the following amounts were created: 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL, 1000 µg/mL, 1500 µg/mL, and 2000 µg/mL. The samples and standards were run on a Waters system (Waters Corporation, Milford, MA) through a Luna HILIC 3 micron 15 x 100 mm column (Phenomenex, Torrance, CA). The conditions were 90% acetonitrile/10% formic acid (0.1%), run isocratically, for the mobile phase.

For the cell culture study, 150 µL of the sample was transferred to a microcentrifuge tube. Ten micrograms of ACV were added to the sample to serve as an

internal control. These samples underwent methanol precipitation with 1 mL of methanol, were centrifuged, and the supernatant was transferred to a fresh tube. Samples were dried for ten hours in a Speed-Vac. The resulting pellets were resuspended in 1500 μL of a 90% acetonitrile 10% water solution. This solution was passed through a 0.45 μ filter (Phenomenex) into Verex vials and run in an identical fashion to the samples described above.

Plaque Assay

The samples from the cell culture study were analyzed with plaque assays to determine the viral titer. A 12-well cell culture plate (BD Biosciences) was seeded with 4 x 10^6 Vero cells per tray, with 1 mL of DMEM in each well. Twenty-four hours later, the medium was removed from each well and 50 μL of sample was added to each well. As needed, samples were serially diluted. The sample was placed in a 37 °C incubator for one hour during the absorption period. Every ten minutes during the absorption period, the tray was moved forward and backward to evenly distribute the virus. Methylcellulose overlay (gift of Prashant Desai, Johns Hopkins University School of Medicine) was prepared by boiling 100 mL of PBS and adding 2.5g of methylcellulose powder (Sigma Aldrich). The resulting mixture was autoclaved and chilled on ice. Once the suspension turned clear, 375 mL of DMEM containing 5% FBS was added to the PBS/methylcellulose mixture and the methylcellulose overlay was stored at 4 °C. After sixty minutes, 1 mL of methylcellulose overlay was added to each well and the tray was incubated at 37 °C for three days. After three days, the methylcellulose was discarded and 1 mL of 1% crystal violet (Sigma-Aldrich) in 50% ethanol was added to each well. After

thirty minutes, the stain was washed off using water. Trays were set out to dry and the number of plaques was counted. Countable wells contained between 10 and 100 plaques.

Statistical Analysis

A repeated measures ANOVA was performed on all of the data sets including drug release in PBS, drug release in Vero cells, and viral titers. For all of the data sets, the data were entered into JMP (Version 11.0) (SAS Institute, Cary, NC). For analyzing the drug release in PBS and in Vero cells, the drug concentration at each time point was entered as the Y variable, and the group was added to construct the model effects. Repeated measures ANOVA was selected and the alpha level was set at 0.05. For analyzing the viral titers, the titer at each time point was entered as the Y variable, and the group was added to construct the model effects. Repeated measures ANOVA was selected and the alpha level was set at 0.05.

CHAPTER III:

RESULTS

Release Kinetics of ACV Versus PCV in VASE Implants

To determine whether there was a difference in the release kinetics of ACV versus PCV in VASE implants, both types of implants were created in a 70:30 polymer:drug ratio. Each implant was placed in a well containing PBS. The PBS was collected and replaced daily with fresh PBS for the first 15 days. Sample collection continued weekly, through week nine, and the drug levels were quantified with HPLC. For the first 15 days, the average drug released from the ACV implants was 82.22 ± 38.22 µg/mL per day and 112.07 ± 39.87 µg/mL per day from the PCV implants (Figure 1). For both types of implants, the drug release decreased over time (Figure 1). A repeated measures ANOVA determined that there was a significant interaction seen between drug type and time ($P=0.0089$). Although time ($P<0.0001$) but not drug type ($P=0.4717$) had a significant effect on the release rate, these variables appear to work synergistically rather than independently of each other.

After 15 days, the PBS collection resumed weekly up through week nine. For weeks three through nine, the average drug released from the ACV implants was 206.4 ± 48.42 µg/mL per week (29.49 µg/mL per day) and 188.6 ± 104.8 µg/mL per week (26.94 µg/mL per day) from the PCV implants (Figure 2). The implants releasing PCV started off releasing more drug than the ACV implants, but by week nine, the release from the ACV implants appeared more steady than the PCV release (Figure 2). The maximum drug release from the ACV implants occurred on day 1, with 195.2 µg/mL per day. The

drug release slowly declined through week nine, where release dropped to approximately 21.44 µg/mL per day calculated based on the weekly data. The maximum drug release from the PCV implants occurred on day 2, with 205.2 µg/mL per day. The drug release slowly declined through week nine, where the released dropped to approximately 5.114 µg/mL per day calculated with the weekly data. A repeated measures ANOVA was performed on the weekly data and determined that time ($P<0.0001$) but not drug type ($P=0.7648$) was significant. There was no significant interaction between time and drug type ($P=0.3224$).

Release Kinetics of Various Polymer:Drug Ratios of PCV Implants in Vero Cells

To determine the most effective polymer:drug ratio, implants were made with various PCL to PCV ratios (w:w 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30). Vero cells were plated and allowed to attach overnight. Media were collected and changed daily for later analysis by HPLC and plaque assay. After 24 hours, recorded as day 1, one implant was added to each well, except for control wells, which did not receive an implant. The next day, day 2, cells were either infected with HSV-2 or left uninfected as a negative control. The subsequent days after infection are referred to as days post-infection (d.p.i). This study collects samples through 3 d.p.i., the equivalent of study day 5, when sample collection ends. Every day through day 5, the media were collected, stored, and replaced. For all of the implants, the amount of drug release was greatest in the beginning, day 2 or 3, and then the release began to decrease (Figure 3). The amount of release varied depending on the amount of drug present in the implant (Figure 3). The HPLC results revealed that the 75:25 implants released the most drug with an average of

426.73 ± 55.83 μg/mL per day (Figure 3). The 80:20 and 85:15 implants followed with the second and third highest release rates of 321.7 ± 35.21 μg/mL per day and 256.9 ± 45.3 μg/mL per day, respectively (Figure 3). Surprisingly, although the 70:30 implants had the most drug, they had the 4th highest release rate at 215.68 ± 62.02 μg/mL per day (Figure 3). The 90:10 and 95:5 both had a very slow release rate at 26.20 ± 26.03 μg/mL per day and 9.45 ± 11.54 μg/mL per day, respectively. In both of the 90:10 and 95:5 implants, the release rate approached zero μg/mL per day by day 5. As expected, the PCL implant released no drug. A repeated measures ANOVA determined that there was a significant interaction between time and polymer: drug ratio ($P<0.0001$). Although time ($P<0.0001$) and polymer:drug ratio ($P<0.0001$) had a significant effect on release rate, the interaction term determined that these variables appear to work synergistically rather than independently of each other.

Cytopathic Effect in Vero Cells

Three days post-infection, multiple images of each well at different focal planes were taken and collapsed into a single image using Zerene Stacker software. HSV-2-infected Vero cells change morphology, creating CPE that is visible under the microscope. Regardless of treatment, all of the cells that were not infected remained healthy (Figure 4A-H). As expected, the Vero cells infected with HSV-2 and treated with nothing, PCL, 95:5, and 90:10 exhibited extensive CPE (Figure 4I-L). The Vero cells infected with HSV-2 and treated with the 80:20, 85:15, and 70:30 all exhibited minimal CPE (Figure 4M, N, and P). Lastly, the Vero cells infected with HSV-2 and treated with the 75:25 implants exhibited no CPE (Figure 4O).

Plaque Assay

To corroborate the CPE data, plaque assays were performed to determine the viral titer in the samples. Vero cells were seeded and allowed to attach overnight. The next day, virus was added to each well and given an hour absorption period. Methylcellulose overlay was added and the tray was kept in a 37 °C incubator for three days. After three days, the methylcellulose was discarded, the wells were stained with crystal violet, and the number of plaques were counted. The plaque assay data corroborated the CPE data (Figure 5, Table 2). The highest titers were present in the wells receiving no treatment and the PCL alone implant (Figure 5, Table 2). On day five, which was 3 d.p.i., the wells with no treatment had a titer of $1.69 \times 10^7 \pm 1.43 \times 10^6$ pfu/mL, while the PCL alone group had a titer of $1.83 \times 10^7 \pm 2.01 \times 10^7$ pfu/mL (Figure 5, Table 2). Not surprisingly, the HSV-2 titer was always lower in the wells receiving an implant with drug (Figure 5 Table 2). The 95:5 and 90:10 implants allowed a titer of $7.93 \times 10^5 \pm 1.33 \times 10^5$ pfu/mL and $1.01 \times 10^5 \pm 5.85 \times 10^4$ pfu/mL, respectively (Figure 5, Table 2). Much like the HPLC data, the next most effective implants were the 70:30 implants, allowing a titer of $6.16 \times 10^4 \pm 2.16 \times 10^4$ pfu/mL (Figure 5, Table 2). Treatment with 85:15 and 80:20 implants resulted in cells with an even lower titer of $1.18 \times 10^4 \pm 3.93 \times 10^3$ and $5.63 \times 10^2 \pm 5.60 \times 10^2$ pfu/mL, respectively (Figure 5, Table 2). Lastly, and in support of the CPE data and HPLC data, the most effective implants were the 75:25 implants, allowing a titer of only $1.23 \times 10^2 \pm 1.20 \times 10^2$ pfu/mL (Figure 5, Table 2). Note that a higher drug concentration in the implant does not necessarily lead to lower virus titers in cells (Figure 5, Table 2)

A repeated measures ANOVA determined that there was a significant interaction between polymer:drug ratio and time with respect to virus titers ($P<0.0001$). Although both time ($P<0.0001$) and treatment group ($P<0.0001$) both had a significant effect on the virus titer, these variables appear to work synergistically rather than independently of each other.

Figure 1: Average release of drug (ACV or PCV) every 24 hours from implants kept in PBS at 37 °C. Both ACV (indicated by blue diamonds on the graph) and PCV (indicated by red squares on the graph) implants were made in 70:30 polymer:drug ratios. A 6-well plate was filled with 7 mL of PBS. One implant, either PCL-ACV or PCL-PCV, was added to each well. There were three replicates of each type of implant. The PBS was collected every 24 hours and replaced with fresh PBS. HPLC analysis was performed to determine the drug concentration in each sample.

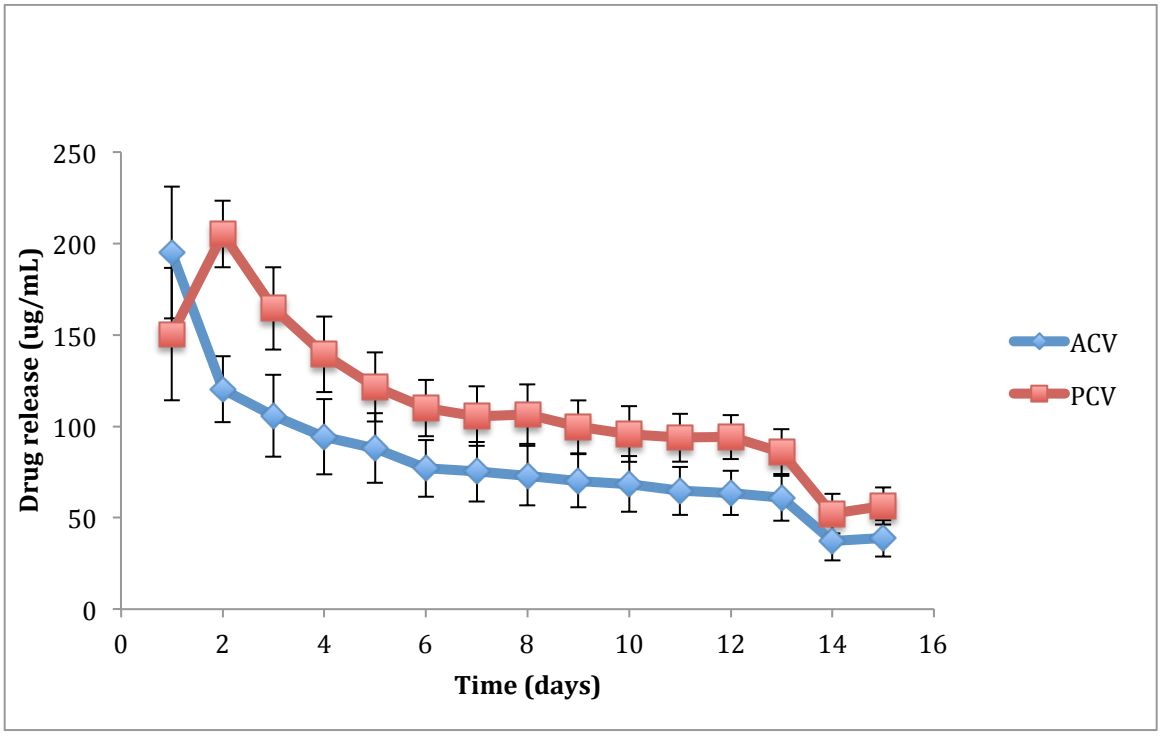
250
200
150
100
50
0
Drug release (ug/mL)
0
2
4
6
8
10
12
14
16
Time (days)
ACV
PCV

Figure 2: Average release of drug (ACV or PCV) every week beginning at week 3 from implants kept in PBS at 37 °C. Both ACV (indicated by blue diamonds on the graph) and PCV (indicated by red squares on the graph) implants were made in 70:30 polymer:drug ratios. A six well plate was filled with 7 mL of PBS. One implant, either PCL-ACV or PCL-PCV, was added to each well. There were three replicates of each type of implant. The PBS was collected once a week and replaced with fresh PBS. HPLC analysis was performed to determine the drug concentration in each sample.

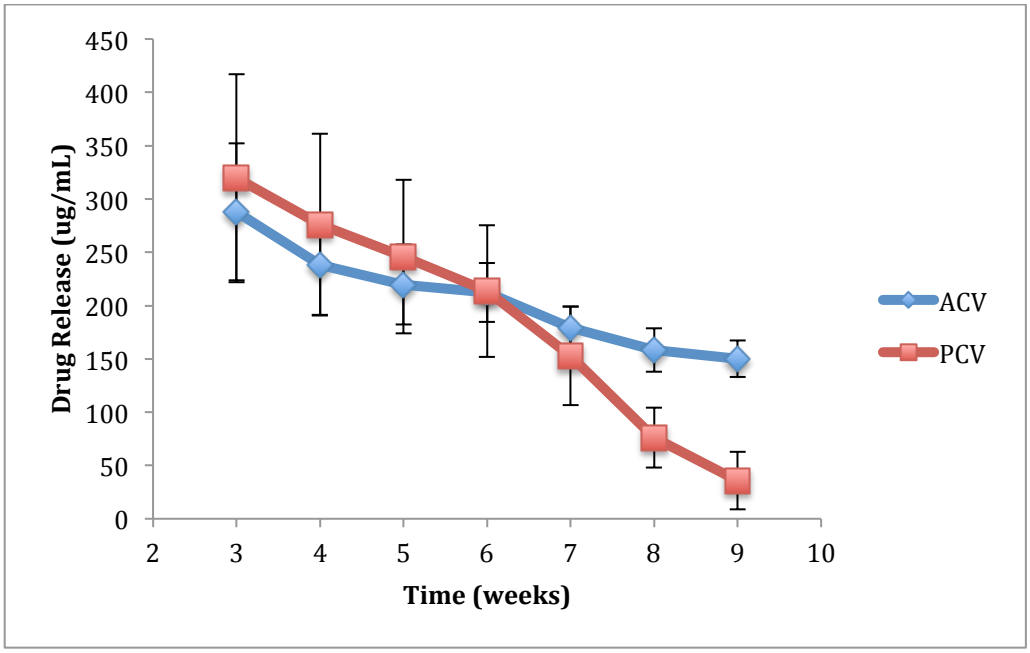
450
400
350
300
250
200
150
100
50
0
Drug Release (ug/mL)
2
3
4
5
6
7
8
9
10
Time (weeks)
ACV
PCV

Figure 3: The average release of drug from PCV implants made of various polymer:drug ratios. PCV implants were made in the following polymer:drug ratios: 100:0 (red), 95:5 (green), 90:10 (purple), 85:15 (teal), 80:20 (orange), 75:25 (light blue), and 70:30 (light red). Infected control wells received no implant (blue). Vero cells were plated overnight and one implant was added to each well. There were three replicates of each type of implant. The next day, the wells were infected with 100 pfu of HSV-2 (MS). The media were collected and replaced with fresh media daily up through day 5. The media were analyzed by HPLC to determine the drug levels.

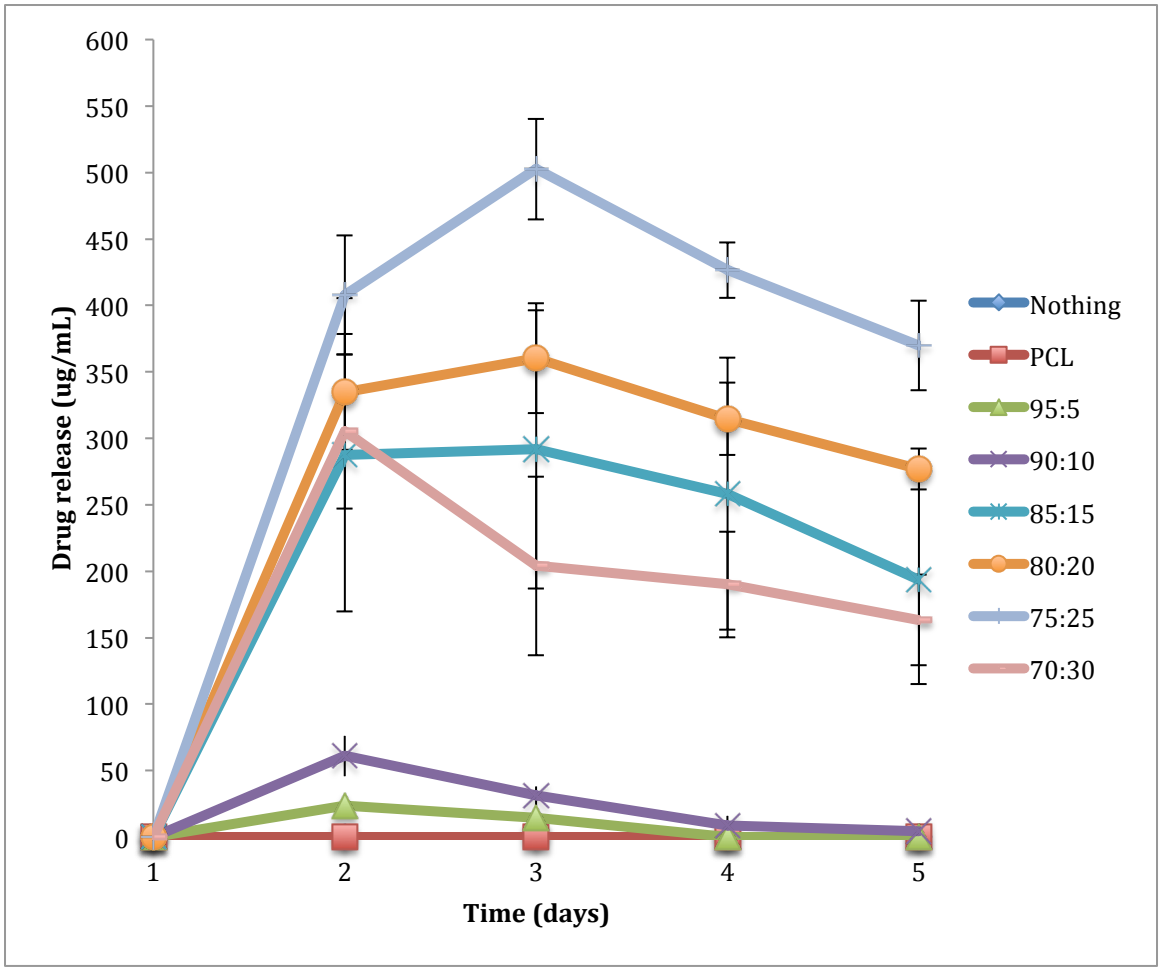
600
550
500
450
400
350
300
250
200
150
100
50
0
Drug release (ug/mL)
1
2
3
4
5
Time (days)
Nothing
PCL
95:5
90:10
85:15
80:20
75:25
70:30

Figure 4: Vero cells treated with implants with various polymer:drug ratios and imaged 3 days post HSV-2 infection. Vero cells were plated overnight and the wells were treated with nothing (A and I) or implants with the polymer: drug ratio 100:0 (B and J), 95:5 (C and K), 90:10 (D and L), 85:15 (E and M), 80:20 (F and N), 75:25 (G and O), or 70:30 (H and P), The next day, the cells were either left uninfected (A-H) or infected with HSV-2 (MS) (I-P). The media were collected and replaced with fresh media daily up through day 5 (3 d.p.i.). At 3 d.p.i., multiple shots of each well at different focal planes were taken and collapsed into a single image using Zerene Stacker software.

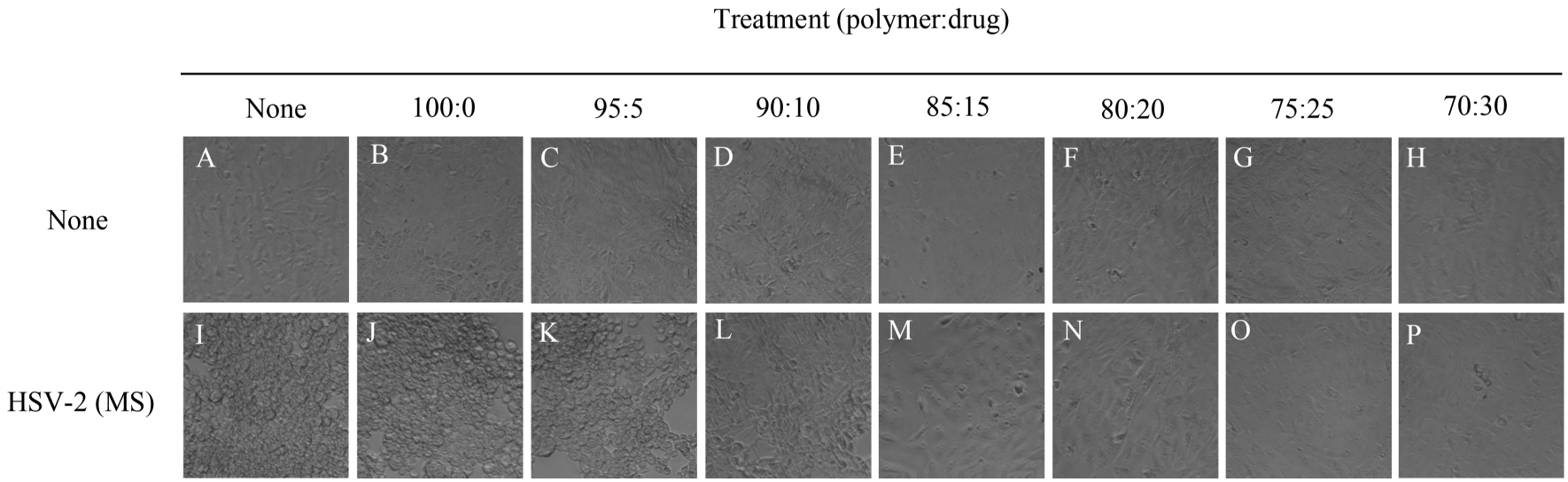
Treatment (polymer:drug)
None
100:0
95:5
90:10
85:15
80:20
75:25
70:30
None
HSV-2 (MS)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 5: The average HSV-2 titer (in logs) from Vero cells infected with HSV-2 and treated with PCV implants containing various polymer:drug ratios. PCV implants were made in the following polymer:drug ratios: 100:0 (red), 95:5 (green), 90:10 (purple), 85:15 (teal), 80:20 (orange), 75:25 (light blue), and 70:30 (light red). Infected control wells received no implant (blue). Vero cells were plated overnight and one implant was added to each well. There were three replicates of each type of implant. The next day, the wells were infected with HSV-2 (MS) or left uninfected. The media were collected and replaced with fresh media daily and a plaque assay was used to determine the viral titer in each of the wells.

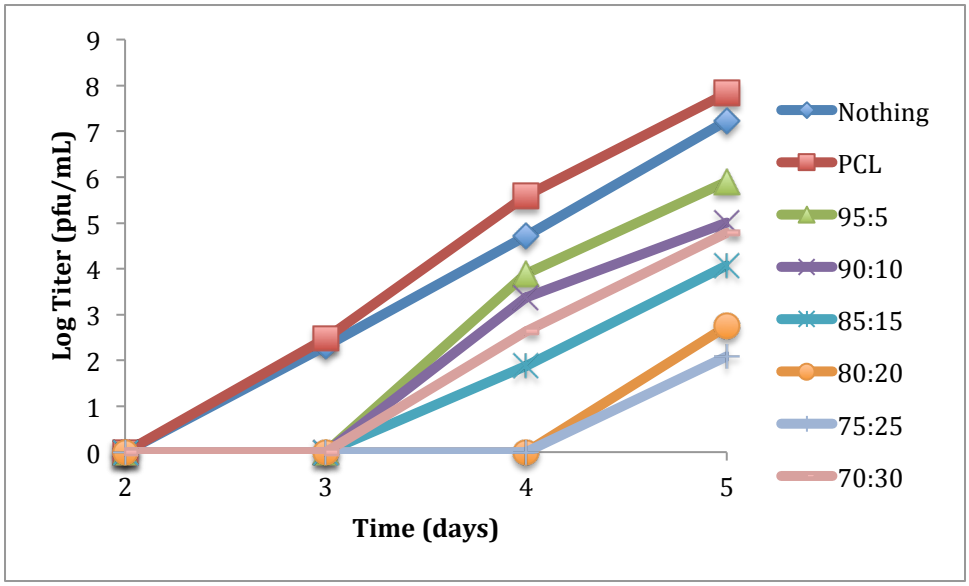
Log Titer (pfu/mL)
9
8
7
6
5
4
3
2
1
0
2
3
4
5
Time (days)
Nothing
PCL
95:5
90:10
85:15
80:20
75:25
70:30

Table 1: The amount of polymer (PCL) and drug (ACV or PCV) used to generate implants with various polymer:drug ratios. The implants are made in w:w 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, and 70:30 polymer:drug ratios.

Polymer:drug Ratio	PCL (grams)	Drug (grams)
100:0	5.00	0.00
95:5	4.75	0.25
90:10	4.50	0.50
85:15	4.25	0.75
80:20	4.00	1.00
75:25	3.75	1.25
70:30	3.50	1.50

Table 2: The average HSV-2 titer from Vero cells infected with HSV-2 and treated with PCV implants containing various polymer:drug ratios. Data is representative of three replicates. Vero cells were plated overnight and one implant was added to each well. The next day, the wells were infected with HSV-2 (MS) or left uninfected. The media were collected and replaced with fresh media daily and a plaque assay was used to determine the viral titer in each of the wells.

Treatment	Day 3 Titer (pfu/mL)	Day 4 Titer (pfu/mL)	Day 5 Titer (pfu/mL)
Nothing	$2.06 \times 10^2 \pm 1.85 \times 10^2$	$5.20 \times 10^4 \pm 7.00 \times 10^3$	$1.69 \times 10^7 \pm 1.43 \times 10^6$
PCL	$3.07 \times 10^2 \pm 1.21 \times 10^2$	$3.90 \times 10^5 \pm 2.29 \times 10^5$	$6.83 \times 10^7 \pm 2.01 \times 10^7$
95:5	0 ± 0	$7.73 \times 10^3 \pm 6.50 \times 10^2$	$7.93 \times 10^5 \pm 1.33 \times 10^5$
90:10	0 ± 0	$2.40 \times 10^3 \pm 1.67 \times 10^3$	$1.01 \times 10^5 \pm 5.85 \times 10^4$
85:15	0 ± 0	$7.66 \times 10^1 \pm 1.32 \times 10^2$	$1.18 \times 10^4 \pm 3.93 \times 10^3$
80:20	0 ± 0	0 ± 0	$5.63 \times 10^2 \pm 5.60 \times 10^2$
75:25	0 ± 0	0 ± 0	$1.23 \times 10^2 \pm 1.20 \times 10^2$
70:30	0 ± 0	$4.36 \times 10^2 \pm 2.10 \times 10^2$	$6.16 \times 10^4 \pm 2.16 \times 10^4$

CHAPTER IV:

DISCUSSION

This thesis compared the release kinetics between implants made of ACV versus PCV, in a 70:30 polymer:drug ratio, in a 9 week-long kinetic study. We found that the release of drug from both ACV and PCV implants significantly decreased over time (Figures 1 and 2). This is not surprising for a controlled delivery device made of biodegradable material, as polymer breakdown is expected to occur over time and this phenomenon has been seen in other drug delivery devices, including those using PCL. In an *in vitro* study, an implant made of PCL and loaded with levonorgestrel released 21.1 ± 1.1 mg/day on day 2, and the release decreased to 15.7 ± 0.9 mg/day on day 12 (Ma et al., 2006). Initially, we selected to test the polymer:drug ratio of 70:30 because the 70:30 ACV implants had been shown to suppress primary HSV-1 infection, based on lack of CPE, in Vero cells (Nelson, 2012; Badin, 2013). Although not significant, the ACV implants in the current study had less drug release in the initial 15 days compared to release from the PCV implants. Since the PCV drug release was greater than that of ACV, we expected 70:30 PCV implants to have been effective at suppressing HSV-2 infection in Vero cells, likely to the same levels of efficacy as the 70:30 ACV implants against HSV-1 infection *in vitro*. This was not the case, as the 70:30 PCV implants had evidence of CPE in the cell culture study. Although both ACV and PCV are structurally similar and have identical mechanisms of action, the discrepancy in the antiviral efficacy may be due to differences in the drug, the virus, and/or the cell type. On average, both HSV-1 and HSV-2 strains have greater susceptibility to ACV compared to PCV in Vero

cells (Leary et al., 2002). The reverse is true for several other cell lines including human amnion cells (WISH), human diploid cells (WI-38), and embryonic cells (VA-13) (Leary et al., 2002). HSV-2 strains typically have higher half maximal inhibitory concentration (IC_{50}) values than HSV-1 strains for both ACV and PCV (Leary et al., 2002). So it is not surprising that both ACV and PCV implants, although made with the same polymer:drug ratio, have different antiviral efficacies based on the drug, virus type, and cell line used.

Regardless of drug type, it is critical that the implant releases an amount of drug greater than the therapeutic threshold. Should the drug levels decrease below the therapeutic threshold, the virus would have a chance to replicate and potentially gain resistance to the drug. The IC_{50} for PCV is usually two-fold greater than that of ACV (Piret and Boivin, 2011). For HSV-2, the ACV IC_{50} ranges from 0.03 to 2.2 μg/mL and therefore the PCV IC_{50} ranges from 0.06 to 4.4 μg/mL (Piret and Biovin, 2011). As explained above, those values are typically lower for HSV-1. Using the slope derived from the weekly drug release data (Figure 2), the 70:30 PCV implants would last approximately 8 weeks until the drug release rate would drop below the IC_{50}; the 70:30 ACV implants would last approximately 15 weeks given the same criterion. However, collecting samples weekly may generate mass action effects that are not seen with daily collection, in which real maximum drug release rates are not seen based on an infinite sink that would be found in vivo. Regardless, the release we observed is not ideal, as we want our treatment to be long-term, over the course of years and not weeks.

This kinetics study looked exclusively at the 70:30 implants. However, the next part of this thesis explored which polymer:drug ratio was the most effective; curiously, it

was not 70:30 like we hypothesized. This may explain why the release was not as expected in the first kinetic study. All wells that were exposed to some amount of PCV had drug release that varied based on the polymer:drug ratio of the implant. In all of these implants, the drug release decreased over time, most likely due to degradation of the PCL. PCL degradation occurs in two stages. In the first stage, there is no deformation, but a decrease in molecular weight (Sun et al., 2005). In the second stage, material begins to break into pieces (Sun et al., 2005). This decrease in drug release is a direct result of all of the accessible drug being released. Inaccessible drug may still be in the implant that may get released during the second stage of breakdown. As the PCL degraded, the 95:5 and 90:10 implants had the lowest drug release rate (Figure 3), which was not surprising because the amount of PCV in those implants was minimal. The most drug and the highest release rate were from the 75:25 implants (Figure 3). This was unexpected because the 75:25 implants had less drug than the 70:30 implants (Table 1). In fact, the 75:25, 80:20, and 85:15 implants all had greater drug levels and release rates than the 70:30 implants had (Figure 3).

This finding, a higher percentage of drug in a controlled delivery device releasing less drug than one with a lower percentage of drug, is undocumented in the literature. Perhaps this phenomenon has to do with the molecular properties of the drug, polymer, media, and the interactions of each of these variables. Tarafder and colleagues address the importance of the interaction between drug and polymer vs. the interaction between drug and the media (Tarafder et al., 2013). Maybe with the 75:25 implants (and higher amounts of drug), the drug has more favorable interactions with the media than with the

implant. This would explain why the implant releases the drug at higher rates into the media. However, at the 70:30 polymer:drug ratio, maybe there are more favorable interactions between the drug and the implant and the drug is less likely to be released into the media. While important phenomena to explore in order to better understand the ultimate pharmacologic implications of this technology, this particular oddity is beyond the scope of this thesis.

Alternatively, yet, it is also possible that not enough formic acid was used to dissolve the drug in the 70:30 ratio, making a nonhomogeneous mixture. The homogeneity could be confirmed with SEM analysis. Prior SEM imaging of 70:30 ACV implants confirmed that VASE creates homogeneous implants, but that homogeneity may vary based on the drug type used. Implants of higher polymer:drug ratios should be generated to determine if increasing the polymer:drug ratio results in an even lower actual drug release, as seen in the 70:30 vs. 70:25 implants. By creating 65:35 and 60:40 implants and performing a kinetic study, it would be possible to see if the trend continues at these higher polymer:drug ratios. In addition, these implants should be analyzed with SEM to see whether they are molecularly homogeneous.

Since these 75:25 implants were shown to be the most effective, a long-term kinetic study should be performed to get an accurate measurement of drug release. This study should collect samples daily to avoid any potential mass action effects. The long-term kinetic study in this thesis was done on the 70:30 implants, under the now mistaken assumption that they would release the most drug. We expect that the release profile from the 75:25 implants will be significantly different from that of the 70:30 implants over 60

days, though, because the amount of drug released from the 75:25 implants was different from the amount of drug released from the 70:30 implants during the 5-day cell culture study. Based on these results, it will be possible to determine a more accurate time frame that the implants could exist in the body, releasing therapeutic amounts of drug above the minimal therapeutic threshold.

The next part of this thesis addressed how the amount of drug released correlated with antiviral efficacy. We found that the Vero cells not infected with HSV-2 and left untreated appeared normal and healthy (Figure 4A-H). The cells not infected and treated with an implant had the same appearance. This confirmed that the formic acid treatment was not harmful to the cells because the Speed-Vac treatment removed most, if not all, of the formic acid from the implant. The appearance of the Vero cells infected with HSV-2 depended on which treatment was used. The cells treated with nothing, 100:0 implant, 95:5 implant, and 90:10 implant all exhibited extensive CPE (Figure 4I-L). The HPLC data showed that these implants released no drug (no treatment, 100:0 implant) or very little drug (95:5 and 90:10 implants) (Figure 3). So it was not surprising that there was not enough drug to stop virus replication. This allowed HSV-2 to replicate and cause the extensive CPE as seen in the Vero cells (Figure 4). The Vero cells infected with HSV-2 and treated with the 70:30, 85:15, 80:20 implants all exhibited minimal CPE (Figure 4M, N, and P). The HPLC data showed that at 3 d.p.i., these implants released drug ranging between 163 to 276 μg/mL (Figure 3). This amount of drug was enough to inhibit some, but not all, of the HSV-2 replication (Figure 4). There was no visible CPE in the cells receiving the 75:25 implant (Figure 4O). This polymer: drug ratio had the greatest

amount of drug release throughout the study, with 369 μg/mL being released at 3 d.p.i (Figure 3); according to the images this level was enough to inhibit HSV-2 replication (Figure 4).

The next part of the study involved supporting the CPE above data with actual viral titers determined by plaque assays. As expected, the cells receiving no drug (no treatment, polymer alone) had high titers (Figure 5). This is because no drug was present to inhibit virus replication. We expected the cells receiving no treatment and the cells receiving polymer alone to have similar titers. However, the titer for the cells with the PCL implant was four-fold higher than that of the cells receiving no treatment. We therefore hypothesize that the polymer must be stimulating HSV-2 replication. One explanation could be that there was a chemical contaminant present in the PCL, one that encouraged HSV-2 replication. For subsequent studies, a higher-quality medical grade PCL should be used to avoid this potential issue.

All of the cells receiving treatment and infected with HSV-2 had lower titers than the cells receiving no treatment. Mirroring both the HPLC and CPE data, the order of implants that resulted in highest titer to lowest titer remained the same (95:5, 90:10, 70:30, 85:15, 80:20, and 75:25) (Figure 5, Table 2). Not enough drug was released from the 95:5 or 90:10 implants to stop HSV-2 replication, resulting in relatively high titers. There was a substantial amount of drug released from the 70:30, 85:15, and 80:20 implants, resulting in lower titers than those from the cells treated with the 95:5 and 90:10 implants. Lastly, the 75:25 implants had the most antiviral efficacy, evidenced by no visible CPE and the lowest titers (Figures 4O and 5).

It is worth noting that if the amount of drug released from the 75:25 implants was enough to completely stop HSV-2 replication, then the titer at 3 d.p.i. should be 0 and not 123 pfu/mL. This number could be explained because, to minimize error in the plaque assay, only wells containing 10 to 100 plaques were counted. For the 75:25 implants, there were some sample titration wells that had fewer than 10 plaques. Mathematically, these turned into zeros for the calculations because there were too few plaques to be counted for accuracy. At 3 d.p.i., two titration wells had 12 plaques, another had 13 plaques, and three had less than 10 plaques. These three numbers (greater than 10), which may be due to very small errors between measurements, skew the data to result in an actual measurement of 123 pfu/mL. Hence, the increase in pfu/mL may be an artifact due to necessary mathematical accommodations inherent in calculations; the accuracy of these numbers, then, needs to be explored further to see if the result is real.

The other possibility is that the amount of drug released was not enough to completely stop HSV-2 replication, which would not necessarily be surprising because of the prevalence of PCV-resistant viruses in the population. Research has shown that in HSV-2 strains, the mutation frequency is 9- to 16- fold greater than it is in HSV-1 strains (Sarisky et al., 2000). Among six strains of HSV-2, the average PCV resistance was 1% of the virus population (Sarisky et al., 2000). We infected our Vero cells with 100 pfu of HSV-2. Therefore, it is possible that just one virion was PCV-resistant at the start of the study, or even expanded slightly under selective pressure applied by the drug, potentially explaining the 3 d.p.i. titers for the 75:25 implant.

It is critical to investigate the antiviral efficacy of the 75:25 implants past 3 d.p.i. to determine whether the presence of virus at 3 d.p.i. is due to a measurement error, not enough drug, or potentially a drug-resistant virus in the population. After about 3 d.p.i., the Vero cells begin to detach from the bottom of the well, making a long-term study difficult. However, if the same amount of Vero cells are plated, but in larger wells (a 6-well plate instead of a 24-well plate), the study can run longer than 3 d.p.i. This will address the issue of measurement error versus actual replication. If there is actual replication, a plating efficiency assay, as described by Sarisky and colleagues, can be done to determine mutation efficiency (Sarisky et al., 2000). This assay would involve calculating the virus titer in the presence of a significant amount of drug and comparing that to the virus titer calculated in the absence of drug (Sarisky et al., 2000).

There are some broader scope questions that the TUHVL also needs to address. These include where the implant will actually go in the patient and whether drug release from the implant results in local or systemic delivery. We have three thoughts on where the implant should go. The implant may be most effective at the sacral ganglia (site of latency), at the site of primary infection, or perhaps at a distal site. In order to figure this out, animals would need to be split into three groups with one set of animals receiving implants at the site of latency, another set with implants at the primary site of infection, and a third set implanted at a distal site, like the arm. We are also exploring ways to determine whether or not drug delivery from the implant is local or systemic. Currently, guinea pigs have been treated with 75:25 PCV implants. At the end of the study, tissue will be harvested from the location containing the implant and a distal site away from the

implant. These tissues will be homogenized and HPLC will be performed to determine levels of PCV in each area. Results from these experiments could potentially impact decisions for the location for clinical deployment of the implant. Should the drug delivery be local, either the primary site of infection or the site of latency would be most effective. We still need to determine whether the antivirals keep the virus from reactivating in the ganglia or from preventing local disease. If the drug delivery is systemic, than a less invasive, more distal implantation site may be possible. The implantation site may also vary based on whether the treatment is for HSV-1 and HSV-2.

This thesis specifically looked at the efficacy of the implants in HSV-2 infections, but PCV and ACV are effective against other herpesviruses as well. This broadens the applicability of the novel implant to HSV-1, VZV, and FHV-1 infections. PCV implants may be particularly beneficial to FHV-1 infection in cats, because giving cats medication for this common infection is a challenge. Being able to provide them with an implant would abate the difficulty of dosing with daily eye drops.

This thesis provides data supporting the effectiveness of the 75:25 PCL: PCV implants. Using a controlled delivery device to provide drug eliminates the need for patient compliance and provides a continual release of drug. This constant drug release will prevent the drug level from decreasing below the therapeutic threshold, and subsequently promoting the appearance of drug-resistant mutants, while providing relief to HSV-2 symptoms. Should these implants prove to be effective in reducing the number of reactivations in our current study with HSV-2-infected guinea pigs, this treatment has the potential to decrease the HSV-2 transmission rate. By reducing the number of

reactivations, the virus will remain in the latent state longer, where it is unable to be transmitted. Ultimately, the likelihood of transmitting the virus will be greatly reduced.

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CURRICULUM VITAE

Sarah E. Fargis
334 Stevenson Lane Apt C1 • Towson, MD 21204
571.236.8409 • sfargi1@students.towson.edu

EDUCATION

Master of Science, Biology December 2013
Towson University Towson, MD
- GPA: 4.0
- Thesis Advisor: Barry Margulies
- Thesis Title: *Efficacy of Various Polymer: Drug Ratios of Molecularly Homogeneous Implants in Preventing Herpes Simplex Virus Type-2 Infection in Vero Cells*

Bachelor of Science, Biology December 2010
James Madison University Harrisonburg, VA
- Major GPA: 3.7
- Overall GPA: 3.58
- Graduated *cum laude*

PROFESSIONAL EXPERIENCE

Research Assistant May 2012 – Present
Towson University Towson, MD
- qPCR
- Cell culture
- HPLC
- Plaque assay
- Create biodegradable implants
- Measure melting point
- Train new lab members
- Aseptic technique

Adjunct Faculty – Carroll Community College Fall 2013 – Present
Fundamentals of Biology 1 Towson, MD
- Create quizzes and exams
- Hold office hours
- Teach both lecture and laboratory courses
- Grade all assignments

Teaching Assistant – Towson University Fall 2011 – Spring 2012
Medical Microbiology Lab Towson, MD
- Created quizzes and practicals
- Provided step-by-step instructions for completing the labs
- Graded lecture and lab assignments
- Lectured introductory material for the labs

Tissue Bank Coordinator Dec 2010 – Aug 2011
Johns Hopkins University Baltimore, MD

- Processed samples according to lab standard operating protocols
- Fulfilled requests for banked samples
- Trained laboratory technicians
- Processed specimens according to study-specific protocols
- Maintained tumor banking database
- PBMC isolation
- Media preparation
- Shipped samples according to IATA standards

Researcher Aug 2008 – Dec 2010
James Madison University Harrisonburg, VA

- Confocal microscopy
- Plant seed plating
- Trained new lab members
- Data analysis

Laboratory Assistant May 2010 – Aug 2010
James Madison University Harrisonburg, VA

- Media preparation
- PCR reactions
- DNA extraction
- Collected and catalogued seeds

Volunteer May 2008 – Aug 2008
Inova Fair Oaks Hospital Fairfax, VA

- Checked patients out of the hospital by following proper hospital procedure
- Delivered various lab tests to the appropriate departments
- Clerical work that included preparing letters to be mailed and directing phone calls to desired locations

POSTER PRESENTATIONS

James Madison University Harrisonburg, VA

- Spring 2010 Annual Biosymposium (Author and presenter)
  "Inhibiting Protein Phosphorylation Prevents Mitosis in Arabidopsis Roots"
- Spring 2009 Annual Biosymposium (Author and presenter)
  "Live Confocal Imaging of Mitosis in the *Arabidopsis thaliana* Mutant rsw7"

ABRCMS Nashville, TN
"Efficacy of Polycaprolactone/penciclovir Implant on the Prevention of Primary Herpes Simplex Virus – 2 Infection" (Author)