



**MONTCLAIR STATE**  
UNIVERSITY

Montclair State University  
**Montclair State University Digital  
Commons**

---

Theses, Dissertations and Culminating Projects

---

5-2015

## **In Vitro Synergistic Antiviral Activity of Black Tea Theaflavins and Acyclovir on Herpes Simplex Virus Types 1 and 2 in A549 Cells**

Cody James Berkefeld  
*Montclair State University*

Follow this and additional works at: <https://digitalcommons.montclair.edu/etd>



Part of the [Biology Commons](#)

---

### **Recommended Citation**

Berkefeld, Cody James, "In Vitro Synergistic Antiviral Activity of Black Tea Theaflavins and Acyclovir on Herpes Simplex Virus Types 1 and 2 in A549 Cells" (2015). *Theses, Dissertations and Culminating Projects*. 355.

<https://digitalcommons.montclair.edu/etd/355>

This Thesis is brought to you for free and open access by Montclair State University Digital Commons. It has been accepted for inclusion in Theses, Dissertations and Culminating Projects by an authorized administrator of Montclair State University Digital Commons. For more information, please contact [digitalcommons@montclair.edu](mailto:digitalcommons@montclair.edu).

MONTCLAIR STATE UNIVERSITY

*In Vitro* Synergistic Antiviral Activity of Black Tea Theaflavins and Acyclovir on Herpes Simplex Virus Types 1 and 2 in A549 Cells

By

Cody James Berkefeld

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

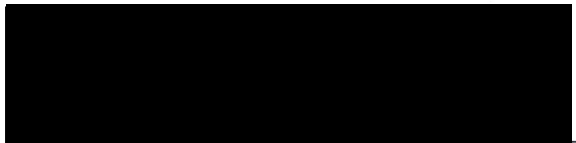
May 2015

College of Science and Mathematics

Thesis Committee:

Biology and Molecular Biology

  
Thesis Sponsor: Dr. Sandra D. Adams

  
Committee Member: Dr. Lee H. Lee

  
Committee Member: Dr. Quinn C. Vega

## Abstract

Herpes simplex virus (HSV) is responsible for one of the most common infections within the population. The primary antiviral used against HSV infections are nucleoside analog drugs such as acyclovir and its deviates. However, in recent years the number of cases of drug resistant HSV has increased, resulting in interest for new novel treatments. Promising antiviral agents are theaflavins found within black tea derived from *Camellia sinensis*. These theaflavins include theaflavin (TF1), theaflavin-3-monogallate (TF2A), theaflavin-3'-monogallate (TF2B), and theaflavin-3-3'-digallate (TF3). Previous studies have supported that theaflavins from black tea, specifically TF3, inhibit the process of viral absorption. Due to this mode of action, black tea theaflavins show potential for synergistic antiviral activity when combined with drugs such as acyclovir, which inhibit viral replication. This study examined the antiviral activity of black tea extract and TF3 with acyclovir on HSV-1 and HSV-2 infections in A549 cells. Cytotoxic analysis was performed with a trypan blue, WST-1 cell proliferation, and ToxGlo assay. Data for each assay supported that concentration of 100  $\mu\text{M}$  of TF3 or 100  $\mu\text{M}$  BTE in combination with 50  $\mu\text{M}$  of acyclovir produce no cytotoxicity in A549 cells. Antiviral activity was measured using a WST-1 based antiviral assay along with a viral ToxGlo assay. In each case theaflavins showed higher antiviral activity when combined with acyclovir, with up to 21.8% increase in viral inhibition. Moreover, the mixture showed higher antiviral activity than acyclovir alone at concentrations of 5  $\mu\text{M}$ . Furthermore, isolated TF3 with acyclovir showed higher levels of viral inhibition than the combination of theaflavins with acyclovir. In conclusion, acyclovir and black tea theaflavins, TF3 in particular, have

shown synergistic activity and may provide an alternative regimen, to decrease emergence of resistant strains of HSV types 1 and 2.



***IN VITRO* SYNERGISTIC ANTIVIRAL ACTIVITY OF BLACK TEA  
THEAFLAVINS AND ACYCLOVIR ON HERPES SIMPOLEX VIRUS TYPES 1  
AND 2 IN A549 CELLS**

**A Thesis**

**Submitted in partial fulfillment of the requirements**

**For the degree of Master of Science**

**By**

**Cody Berkefeld**

**Montclair State University**

**Montclair, NJ**

**2015**

## **Acknowledgments**

This project would not have been possible without the support of several people. Thank you to Dr. Sandra D. Adams for her guidance during this project and her patience dealing with my numerous revisions. It has been an honor to work in your lab for the duration of my thesis. I would also like to thank Dr. Lee H. Lee and Dr. Quinn Vega for offering guidance as members of my thesis committee. Thank you to Montclair State University for providing the facilities to perform my research. Also, thank you to Dr. Ronald Goldberg for allowing the use of his 96 well plate reader, which made the ToxGlo assay possible. Finally, thank you to my friends and family who supported me throughout my time finishing my thesis.

## **Table of Contents**

Abstract .....	iii
Acknowledgments.....	iv
List of Figures .....	vi
List of Tables .....	vii
Introduction .....	1
Review of Literature.....	2
Materials and Methods.....	12
Results .....	17
Discussion .....	29
Conclusion .....	34
References .....	35

## List of Figures

Figure 1: Morphological effects of acyclovir on A549 cells.....	17
Figure 2: Morphological effects of BTE and Acyclovir on A549 cells.....	18
Figure 3: Morphological effects of BTE and Acyclovir on A549 cells.....	19
Figure 4: Trypan blue assay results for BTE, TF3, and ACV cytotoxicity on A549 cells.....	20
Figure 5: Trypan blue assay results for ACV cytotoxicity on A549 cells.....	21
Figure 6: WST-1 assay results for BTE, TF3, and ACV cytotoxicity in A549 cells.....	22
Figure 7: WST-1 assay results for ACV cytotoxicity in A549 cells.....	22
Figure 8: ToxGlo assay results for TF3 and Acyclovir cytotoxicity on A549 cells.....	23
Figure 9: ToxGlo assay results for TF3 and Acyclovir combination cytotoxicity on A549 cells.....	23
Figure 10: Antiviral equation.....	24
Figure 11: WST-1 antiviral assay results for HSV-1 exposed to BTE, TF3, and ACV in A549 cells .....	26
Figure 12: Figure 6: WST-1 antiviral assay results for HSV-2 exposed to BTE, TF3, and ACV in A549 cells.....	26
Figure 13: ToxGlo Assay for TF3 and Acyclovir on HSV-1 infected A549 cells.....	28
Figure 14: ToxGlo Assay for TF3 and Acyclovir on HSV-2 infected A549 cells.....	28



**List of Tables**

**Table 1: WST-1 antiviral assay results for HSV-1 exposed to BTE, TF3, and ACV in A549 cells .....26**

**Table 2: WST-1 antiviral assay results for HSV-2 exposed to BTE, TF3, and ACV in A549 cells .....27**

**Table 3: ToxGlo Assay for TF3 and Acyclovir on HSV-1 and HSV-2 in A549 cells.....28**



## **Introduction**

Herpes simplex virus (HSV) is responsible for one of the most common infections within the human population. Despite its prevalence no cure is available due to HSV's ability to remain in a latent state within the host's neurons, causing sequential outbreaks during reactivation for life. The primary antivirals used to treat outbreaks are nucleoside analog drugs such as acyclovir. However, in recent years the number of cases of drug resistant HSV has increased, resulting in interest for novel treatments. Promising antiviral agents are theaflavins found within black tea derived from *Camellia sinensis*. These theaflavins include theaflavin (TF1), theaflavin-3-monogallate (TF2A), theaflavin-3'-monogallate (TF2B), and theaflavin-3-3'-digallate (TF3). Previous studies have supported that theaflavins from black tea, specifically TF3, interact with viral surface proteins thus interfering with the process of absorption (Leung et al., 2001; Cantatore et al., 2013). Due to this mode of action, black tea theaflavins show potential for synergistic antiviral activity when combined with drugs such as acyclovir, which inhibit DNA replication. The purpose of this study is to examine the antiviral activity of black tea extract and TF3 with acyclovir on HSV-1 and HSV-2 infections in A549 cells. Synergy between the theaflavins and acyclovir could provide new potential regimens for HSV infections, which would decrease the prevalence of resistant strains. Furthermore, a regimen with strong synergy will allow for shorter outbreak times and quicker healing.

## Review of Literature

Herpes simplex virus types 1 and 2 are two human pathogens that cause infection in human epithelial cells. Herpes simplex virus type 1 (HSV-1) is known by most as the cause of lesions on the lips, commonly referred to as cold sores. On the other hand, Herpes simplex virus type 2 (HSV-2) is the typical cause of genital warts and is classified as a sexually transmitted infection. While each virus has a preferred point of infection, both viruses can be found at either location and in fact can cause active outbreaks in any type of epithelial cells of the body (Connell, Cerruti, and Trown, 1985). Once infected, individuals will experience occasional outbreaks for life, leading to psychological and socioeconomic stress. Majority of infections are not life threatening. However, both viruses can cause ocular manifestations, which are known as HSV keratitis and are estimated to infect 500,000 in the United States (Farooq and Shukla, 2012). Disease progression can lead to blindness, causing the disease to be cited as the leading cause of infectious blindness in the developed countries (Liesegang, 2001). Herpes infections can also become life threatening when the virus begins to replicate in the brain, known as herpes encephalitis, causing inflammation (Whitley, 2006). As such, HSV has become a significant public health concern.

The actual cause of genital warts and cold sores are HSV-1 and HSV-2 respectively. Both viruses are members of the *Herpesviridae* family of viruses, specifically within the *Alphaherpesvirinae* subfamily (Roizman and Baines, 1991). Like all members of the family, HSV-1 contains a double stranded DNA genome encased within a complex capsid. The genome consists of 75 protein-coding genes with the highly conserved genes located within the UL sequence and the variable sequences for the strain



located in the US regions (McGeoch et al., 1987; Macdonald et al., 2012). Once the virus enters the body orally through direct contact, the virions migrate to the epithelial cells of the lips and produce lesions through an active lytic infection. However, after the primary infection has run its course the virus migrates to the neurons where it enters a dormant state until the virus receives an activation signal. Once the virus has been reactivated the virus enters the lytic pathway once more and virions migrate down the axon through retrograde axon transport to the epithelial cells causing the lesions to appear once more (Webre et al., 2012). The virus is able to switch between active and dormant states through this process of reactivation, while in the latent state the virus is hidden within the host cell's DNA, and therefore becomes impervious to current treatment options. This causes herpes infections to become lifelong with no currently available cure. Current treatments reduce virus titers during outbreaks to shorten periods of infection.

Estimates show that 45% to 98% of individuals within the human population are seropositive for HSV-1. Of the United States population 40% to 63% are seropositive with higher percentages found within adults over 60 years old (Spruance, 1992; Fatahzadeh and Schwartz, 2007). On the other hand, HSV-2 shows 16% to 21.8% seropositive adults in the United States (Malkin, 2004). In both cases the viruses are seen more commonly in women and non-Hispanic blacks who showed 20.9% and 39.2% seropositive, respectively, for HSV-2. Although, studies have indicated that prevalence of HSV-2 is almost negligible in individuals that are not sexually active, mainly due to the viruses preferred area of expression and mode of transmission. Rates of HSV-2 infection are also highly age dependent. Children under 15 have been shown to have the lowest reported cases of seropositive, with the number of cases increasing around puberty,

plateauing at around 40 years old. On the other hand, the prevalence of HSV-1 is found equally in the population and increases with age, with less than 40% of the population seropositive at 15 years and increasing linear fashion, leading to 60-90% seropositive positive in older adults. It is estimated with increased availability of type specific HSV ELISA's in recent years that the number of seropositive cases may be higher than previously anticipated (Smith and Robinson, 2002).

The high prevalence seen in HSV is due to the lack of a cure and to the virus's ability to remain dormant in the neurons, known as latency. The latent stage of HSV-1 is the result of an alteration in the genetic expression pathway of the virus. During lytic infections, VP16 activates the expression of the immediate early genes ICP0 and ICP4, which then cause the production of early genes, DNA replication, and structural genes in a transcriptional cascade (Wildy, Field and Nash, 1982). On the other hand, during latency HSV-1 expresses only a limited number of gene products, which are collectively known as LATs (Allen et al., 2011). These LATs interact with the genome and ensure the expression of the immediate early genes ICP0 and ICP4 does not occur. Thus the virus remains in a latent state where no new virions are produced. Other functions of the LATs have been proposed, such as an ability to promote neuronal survival in latently infected cells (Webre et al., 2012). Unfortunately, the virus does not remain in the latent state indefinitely. Several appropriate stimuli, such as stressor, weakening of the immune system, or heat can cause the virus to exit the latent stage and begin to replicate. During this process, the newly established virions utilize anterograde transport to travel down the axon and re-infect the local epithelial cells (Hafezi et al., 2012). For the patient, this results in the reappearance of a cold sore lesion.



The frequency of reactivation has been found to depend on several factors, all of which have a direct correlation with the immune system. One such factor is the age and health of the host. As a person ages the immune system tends to weaken in a process known as immunosenescence (Bennett et al., 2012). As the natural process of immunosenescence occurs, the body is unable to properly fight off infections, leading to increased illness. While in the latent state the HSV-1 will not reactivate as long as the immune system is healthy and active (Bennett et al., 2012). Instead, when the immune system is weakened by another infection HSV-1 tends to reestablish a lytic infection within the host through some unknown mechanism. However, it has been shown that HSV-1 can be reactivated through heat, thus it has been theorized that the increased body temperature acts as a signal to the latent HSV-1 and causes it to reactivate (Stowe, Peek, Cutchin, and Goodwin, 2012). Thus, the body temperature acts as a signal, telling the virus that the host immune system is compromised and it is a prime time to establish an active infection. As the person increases in age, the immune system declines, leading to more infections, and thus more signals for the HSV-1 to enter the lytic pathway through reactivation. Any potential cure of HSV infections would be required to not only eliminate existing viral titers but aid in the removal of latent virus as well.

Despite both viruses causing similar symptoms, HSV-1 and HVS-2 have several key differences stemming from the evolutionary divergence of the viruses roughly 8 million years ago. The most notable differences between the two viruses are the cell tropism. HSV-1 tends to produce outbreaks on the lips, while HSV-2 is usually seen in the pubic region, which is primarily due to virus producing virions more efficiently from specific ganglia. HSV-1 tends to enter latency within the trigeminal ganglia, while HSV-



2 tends to enter latency in the lumbar-sacral ganglia. The gene product found in latency, LATs, have been shown to effect the location of the efficiency of the site-specific reactivation. The region coding LATs in HSV-1 and HSV-2 are significantly different, which contributes to this difference in reactivation location (Yoshikawa et al., 1996). Alignment of the DNA sequences shows an 83% identical nucleotide alignment. Most of this alignment occurs within the coding regions, and as a result the two viruses contain nearly all of the same protein products. However, one gene in particular is heavily altered between the two viruses. The US4 gene, which is responsible for encoding glycoprotein G, contains large deletions in HSV-1, as compared to HSV-2. It has been proposed that this difference in glycoprotein is responsible for the difference in cell tropism observed with these viruses (Dolan et al., 1997). Differences in protein products, even minor, can lead to altered response to treatments and requires individual testing of antivirals for both HSV-1 and HSV-2 for effective treatments.

Currently, the frontline drug for herpes simplex infections is acyclovir [9-(2-hydroxyethoxymethyl) guanine] and its derivatives. Acyclovir is stable for oral and topical application with 15-30% bioavailability and is also administered intravenously for severe infections. Since its development in 1981 derivatives of the drug have been developed with increased bioavailability, specifically valacyclovir with 54% bioavailability (Frobert et al., 2014). In its native state acyclovir is inactive, however when exposed to viral thymidine kinase acyclovir is converted into acyclovir monophosphate, which is phosphorylated twice by host kinase and nucleoside diphosphate kinase to produce acyclovir triphosphate. Acyclovir triphosphate competes with 2-deoxyguanosine triphosphate (dGTP) for incorporation by viral DNA polymerase

as a nucleoside on the free 3' hydroxyl of the growing viral DNA chain. However, binding of the next deoxynucleoside 5'-triphosphate to the primer template produces a dead end complex, preventing further nucleotide addition. Furthermore, the 3', 5'-exonuclease activity of HSV DNA polymerase I cannot remove acyclovir triphosphate leading to incomplete viral replication (Frobert et al., 2014, Reardon and Spector, 1989). Since Herpes DNA polymerase is 100 times more likely to incorporate an acyclovir triphosphate than cellular DNA polymerase it is considered highly selective. This selectivity is further heightened due to the inability of acyclovir to become active without the presence of viral kinases thus acyclovir shows minimal cytotoxicity to uninfected cells.

Despite the success of acyclovir, in recent years there have been increasing reports of acyclovir resistant strains of HSV, specifically in immunosuppressed patients. These include patients with HIV infections, post-transplant surgery, and congenital immunodeficiency. A study conducted in France has shown that over the past 10 years the number of cases of resistance has increased from 3.8% in 2002-2006 to 15.7% in 2007-2010 in immunocompromised patients. Prevalence in other regions of the world can range from 2.5-10% in immunocompromised patients. For immunocompetent patients the rates of resistance are significantly lower, with only .3% reported in the United States. In immunocompetent patients the occurrence of resistance has not been shown to alter the clinical outcome. Rates of resistance in immunocompetent patients are usually associated in patient with genital herpes, herpes keratitis, or herpes encephalitis with fewer reported cases of facial herpes (Piret and Boivin, 2011). Therefore, the number of incidence of resistance may be higher in the immunocompetent population than previously recorded.



Under typical conditions acyclovir prevents replication of HSV and any survivors are eliminated by the immune system. In immunosuppressed patients the immune system cannot aid acyclovir, leading to increased prevalence of resistant strains (Piret and Boivin, 2011, Frobert et al., 2014). While resistance primarily develops in immunosuppressed individuals, it can easily spread into new hosts, leading to prevalence of resistant strains in the general population.

Resistance to acyclovir is the result of mutations in viral thymidine kinase or viral DNA polymerase I. Alterations in either gene would result in inadequate conversion of acyclovir to its active form or incorporation of acyclovir triphosphate. Mutations to thymidine kinase arise in the UL23 gene for both HSV-1 and HSV-2. Majority of the cases of thymidine kinase resistance show a deletion or addition within homopolymer repeats of guanines or cytosines. These alterations cause frameshifts of the entire gene sequence and therefore produce a nonfunctional truncated enzyme. DNA polymerase I mutations, while less common, are the results of alterations to the UL30 and UL42 genes. Mutations are typically single amino acid substitutions located within regions II and III of the polymerase. These areas are associated with recognition and binding of nucleotides. Regardless, either mutation would lead to decreased effectiveness of acyclovir on HSV infections. Ninety-five percent of clinical resistant strains are the result of thymidine kinase mutations rather than DNA polymerase I (Piret and Boivin, 2011, Pottage and Kessler, 1995; Larder and Darby, 1982). As such, derivatives of acyclovir become ineffective for treatment and different treatment options must be explored. Novel drug therapies are being researched to use alongside acyclovir to decrease the prevalence of such resistant strains.

Throughout the world natural product remedies have been historically used to treat a wide variety of illnesses. One such example is tea, which has been shown to be a powerful antioxidant, to reduce hydrogen peroxide induced oxidative damage, and inhibit growth of bacteria and viruses among other benefits (Yang et al., 2012; Tanaka et al., 2014; Jeon et al., 2014; Cantatore et al., 2013). Commercially consumed tea is typically derived from the plant *Camellia sinensis*, which is the second most consumed beverage in the world, with a per capita worldwide consumption of .12 liters per day (Graham, 1992). Traditionally preparation involves complete, incomplete, or no fermentation of a leaf resulting in green, oolong, and black tea respectively. This process involves letting the leaves air dry, which leads to oxidation. While the fermentation process does lead to differences in taste and aroma, it also leads to different polyphenol composition (Graham, 2001). In green tea catechins predominate while in black tea theaflavins are more common. The theaflavins found within black tea can further be identified as theaflavin (TF1), theaflavin-3-monogallate (TF2A), theaflavin-3'-monogallate (TF2B), and theaflavin-3-3'-digallate (TF3) (Leung et al., 2001). While the chemical structure is slightly varied, each of the theaflavins are stable at neutral pH and highly reactive (Issacs and Xu, 2013). TF3, unlike the other theaflavins, contains two gallate rings, which provide several additional hydroxyl groups. These additional hydroxyl groups allow TF3 to be a more power antioxidant than the other theaflavins. Furthermore, this additional reactivity may support that theaflavins derived from black tea are more potent at viral inhibition than catechols of green tea.

The antiviral activity of theaflavins and catechols has been examined in recent studies, showing inhibition in viruses such as herpes, human immunodeficiency virus



(HIV), influenza, and hepatitis C (Yang et al., 2014; Yang et al., 2012; Issac et al., 2011; Cantatore et al., 2013). Specifically the mode of action of these tea phenols is via interaction with essential viral proteins. For HSV-1 and HSV-2 glycoprotein B, a necessary protein for viral attachment and entry, appears to be the target. Upon interaction glycoprotein mediated membrane fusion is disrupted and viral titers decrease substantially (Elion, 2011). Furthermore, this mechanism of inhibition is not only seen in members of the herpes simplex family. Similar inhibition of enveloped proteins required for fusion has been observed in HIV and enteroviruses, thus supporting that tea phenols are a potential general antiviral agent (Elion, 2011).

Ideally a drug regimen to maximize herpes inhibition would require increased efficiency, bioavailability, and reduced potential for resistance. Such potential exists in using a regimen of drugs that work in synergy rather than a single inhibitor. By applying two drugs that utilize different mechanisms, viral inhibition can increase to levels not seen with either alone. Furthermore, the prevalence of resistance decreases due to the requirement for multiple mutations in the viral genome to decrease effectiveness in two different pathways. Based on this definition the phenols from tea leaves could provide such synergy when combined with existing drugs such as acyclovir. The mode of inhibition of acyclovir is dependent on the viral DNA synthesis, while tea phenols are shown to act on viral absorption. Theoretically, by acting on different modes of action the two should show a degree of synergy. The proposed study will examine such synergy between theaflavins of black tea and acyclovir. By evaluating the antiviral activity of theaflavins and acyclovir alone it will be possible to evaluate if a combination of the two reagents can lead to higher antiviral activity than either alone. If synergy is observed then



a new drug regimen can be utilized for HSV infections and decrease the prevalence of resistance and offer a more effective treatment for current patients.

## **Materials and Methods**

### **Cells**

Viruses were harvested and propagated in A549 cells [American Type Culture Collection (ATCC) CCL-185, Manassas, VA], a human lung epithelial line. Cells were maintained in a T25 flask at 5% CO<sub>2</sub> at 37°C in Ham's F12-K (Kaighn's) media (Life Technologies, Grand Island, NY), supplemented with 10% Fetal Bovine Serum (FBS) as well as 10 µg/ml gentamicin.

### **Virus maintenance**

Virus stocks of HSV-1 UL-46 that contains a green fluorescent protein (GFP) insert fused to the tegument protein (Willard, 2002)(ATCC, Manassas, VA) and HSV-2 (VR-1779) (ATCC, Manassas, VA) were maintained for the experiment. Passage of virus was performed in a T25 flask of A549 cells. A monolayer of cells were infected and checked daily for complete cytopathic effect. Media was then collected and centrifuged briefly to remove cellular debris. Resulting supernatant was transferred to microcentrifuge tubes and stored at -80°C for the course of the experiment.

### **Black tea extract preparation**

BTE ≥ 80% theaflavin (10 mg) (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in 1 ml of dimethyl sulfoxide (DMSO), giving a final concentration of 14 mM BTE stock solution. Solution was maintained in microcentrifuge tubes stored at 4°C. Concentrations of 25, 50, 75, and 100 µM were prepared through dilution with Ham's F12-K media as needed.

### **TF3 preparation**

TF3  $\geq$ 98% (1 mg) (Nacalai USA Inc, San Diego, CA, USA) was dissolved in 1 mL of DMSO giving a concentration of 1.1511 mM stock solution. TF3 solution was maintained in microcentrifuge tubes at  $-20^{\circ}\text{C}$ . Concentrations of 25, 50, 75, and 100  $\mu\text{M}$  were prepared through dilution with Ham's F12K media as needed.

### **Acyclovir preparation**

Acyclovir (.22521 mg) (Spectrum Chemicals, Gardena, CA, USA) was dissolved in 1 mL DMSO giving a concentration of 1M. Acyclovir stock solution was maintained in microcentrifuge tubes at  $4^{\circ}\text{C}$ . Concentrations of 0.1 to 50  $\mu\text{M}$  were prepared through dilution with Ham's F12K media as needed.

### **Assays**

#### **Cytotoxicity**

A549 cells were seeded into 6 well plates and allowed to incubate over 24 hours to reach 80% confluence. Once at the desired confluence 100  $\mu\text{L}$  of varying concentrations of BTE (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ), TF3 (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ), or acyclovir (1  $\mu\text{M}$  to 50  $\mu\text{M}$ ) were added to each well. Reagents were allowed to absorb over one hour, aspirated, and replaced with 100  $\mu\text{L}$  Hams F12-K media. Cells were incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 24 hours, at which point they were observed at 400x magnification through a phase contrast microscope. Morphological differences were assessed through comparison between a negative control of cells grown in media only. The same protocol was applied for combinations of BTE or TF3, at concentrations of 25 to 100  $\mu\text{M}$ , with 5  $\mu\text{M}$  of acyclovir.

#### **Trypan Blue**



A549 cells were seeded into 6 well plates and allowed to propagate over 24 hours to reach 80% confluence. At this time 100  $\mu\text{L}$  of various concentrations of BTE (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ), TF3 (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ), or acyclovir (.1  $\mu\text{M}$  to 50  $\mu\text{M}$ ) were added to each well. Furthermore, combination of TF3 (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ) or BTE (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ) with 5  $\mu\text{M}$  acyclovir was tested. After one hour, wells were aspirated and replaced with 100  $\mu\text{L}$  Ham's F12-K media. Cells were allowed to propagate over 24 hours at 37°C and 5%  $\text{CO}_2$ . Cells were then exposed to 250  $\mu\text{L}$  trypsin for five minutes to facilitate cell detachment. Trypsin was inactivated with 1 mL of media and cell solution placed into centrifuge tubes. Cells were then centrifuged for 5 minutes and 2 ml of the supernatant was removed. Remaining solution was briefly vortex and .5 ml of trypan blue was added. Ten  $\mu\text{L}$  of cell suspension was added to a hemocytometer and ratio of dead cells to live cells was recorded.

### **WST-1 Cell Proliferation**

Ten  $\mu\text{L}$  of A549 cell suspension were seeded into wells of a 96 well plate along with 90  $\mu\text{L}$  of Ham's F12-K media and allowed to propagate to 80% confluence over 24 hours. At this time BTE (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ), TF3 (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ), or acyclovir (1  $\mu\text{M}$  to 50  $\mu\text{M}$ ) was added to respective wells. Also, combination of TF3 (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ) or BTE (25  $\mu\text{M}$  to 100  $\mu\text{M}$ ) with 5  $\mu\text{M}$  acyclovir were tested. After one hour unabsorbed reagents were aspirated and 100  $\mu\text{L}$  of media was added to each well. Seventy-two hours later, 10  $\mu\text{L}$  of WST-1 reagent (Roche Diagnostics, Indianapolis, IN, USA) was added to each well and allowed to sit for one hour, with intermediate rocking every 15 minutes, in an incubator set to 37°C and 5%  $\text{CO}_2$ . The plate was then read

through a 96 well plate reader set at 450 nm and absorbance was recorded. Assay was performed in triplicate.

### **ToxGlo Cytotoxicity**

A 96 well plate was seeded with 10 $\mu$ L of A549 cell solution and allowed to propagate for 24 hours. Media was removed and 100  $\mu$ L of various concentrations of TF3, ranging from 25  $\mu$ M to 100  $\mu$ M, were added to the respective well. Combination of TF3 ranging from 25  $\mu$ M to 100  $\mu$ M with 5  $\mu$ M acyclovir were also tested. After one-hour absorbance the compounds were removed and 100  $\mu$ L of Ham's F12-K media was added to each well. After 72 hours, 100  $\mu$ L of ToxGlo reagent (Promega Corp., Madison, WI) was added to each well and the plate was read by a luminometer. ATP to relative light unit (RLU) conversion was obtained through plating ATP at concentrations of 1 mM to .14 nM on a 96 well plate. ToxGlo reagent (100  $\mu$ L) was added to each well and RLU was recorded. The assay was performed in triplicate.

### **WST-1 Antiviral Assay**

A549 cells were seeded into a 96 well plate and allowed to propagate to 80% confluence over 24 hours. At this point 100  $\mu$ L of virus stock, either HSV-1 or HSV-2, was combined with 100  $\mu$ L of various concentrations of BTE (25  $\mu$ M to 100  $\mu$ M), TF3 (25  $\mu$ M to 100  $\mu$ M), or acyclovir (5  $\mu$ M). For synergy, virus was mixed with TF3 or BTE, ranging from 25  $\mu$ M to 100  $\mu$ M, combined with concentrations of acyclovir maintained at 5  $\mu$ M. After one hour of treatment with the reagents, 100  $\mu$ L of the virus solution was added to respective wells on the 96 well plate for one hour. Unabsorbed virus was then aspirated and replaced with 100  $\mu$ L of Ham's F12K media. After 72 hours of infection, 10  $\mu$ L of WST-1 reagent was added to each well with rocking every 15



minutes. After one hour the plate was read through a 96 well plate reader set at 450 nm. Antiviral activity was determined through comparison of a negative control (media treated cells) and a positive control (untreated virus and cells). The assay was performed in triplicate.

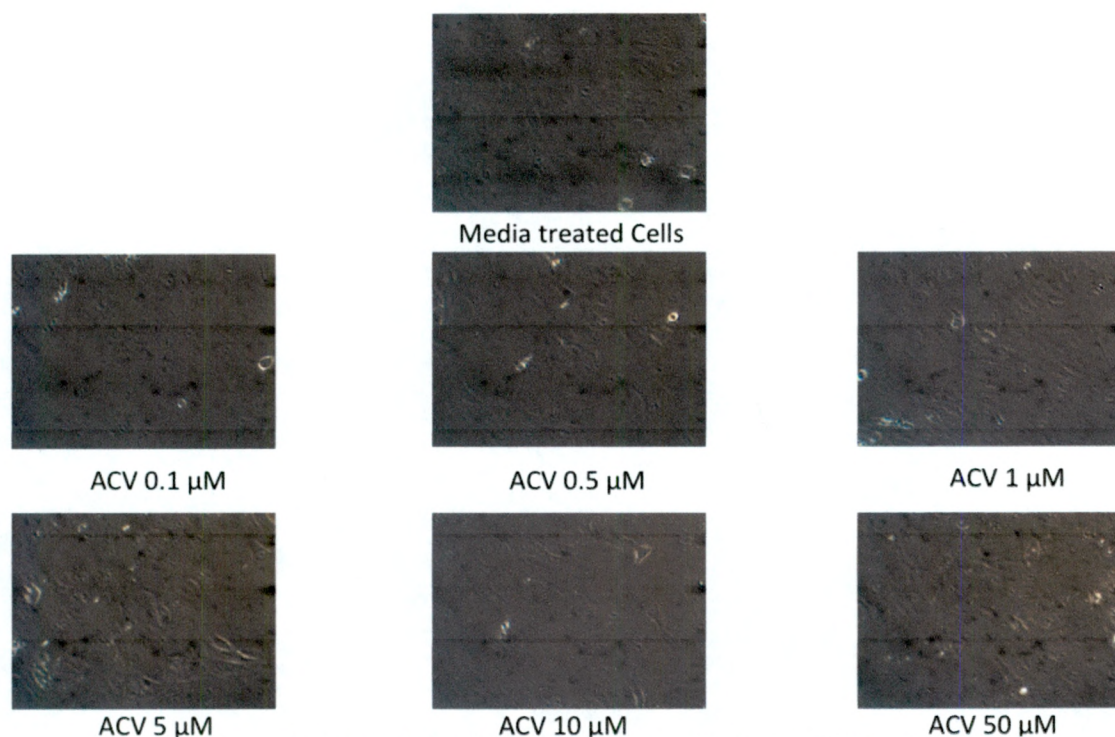
### **Viral ToxGlo**

Cells were seeded into a 96 well plate and allowed to reach 80% confluence over 24 hours. One hundred  $\mu\text{L}$  of virus, either HSV-1 or HSV-2, was then treated with 100  $\mu\text{L}$  of TF3 with concentrations ranging from 100  $\mu\text{M}$  to 6.25  $\mu\text{M}$  or BTE, 100  $\mu\text{M}$  to 6.25  $\mu\text{M}$ , combined with 5  $\mu\text{M}$  acyclovir. After one hour of treatment with the compounds, 100  $\mu\text{L}$  of the viral solution was added to the 96 well plate. After one hour absorption the solution was aspirated and 100  $\mu\text{L}$  of Ham's F12-K media was added with intermittent rocking every 15 minutes. After 72 hours, 100  $\mu\text{L}$  of ToxGlo reagent was added to each well and allowed to sit for one hour. The plate was then read through a luminometer and RLU values of each well were recorded. The assay was performed in triplicate

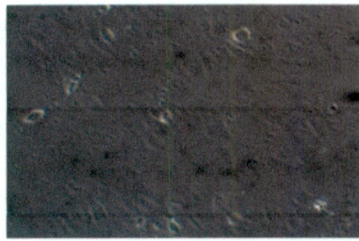
## Results

### **BTE and TF3 concentrations up to 100 $\mu\text{M}$ and acyclovir concentrations up to 50 $\mu\text{M}$ do not alter cell morphology**

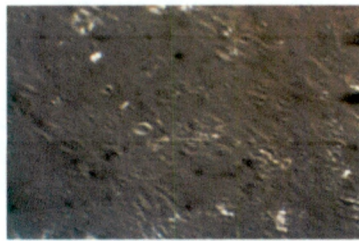
A549 cells were treated with a range of BTE or TF3 at concentrations from 25  $\mu\text{M}$  to 100  $\mu\text{M}$ . Cells were observed for morphological changes over the course of 48 hours. For each concentration of BTE and TF3, up to the maximum concentration of 100  $\mu\text{M}$ , no morphological changes were observed. Changes were evaluated by comparison to a positive control of cells treated with only Ham's F12-k media. For acyclovir, no altered morphology was observed for concentrations of 0.1 to 50  $\mu\text{M}$ . For the highest concentration of the combination of TF3 or BTE (100  $\mu\text{M}$ ) with the highest concentration of acyclovir (50  $\mu\text{M}$ ) there showed no significant change in cell morphology (Figures 1,2,3).



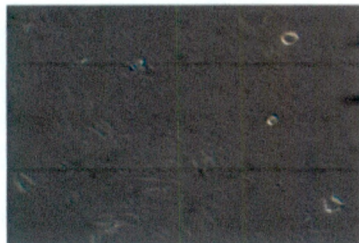
**Figure 1: Morphological effect of acyclovir on A549 cells**



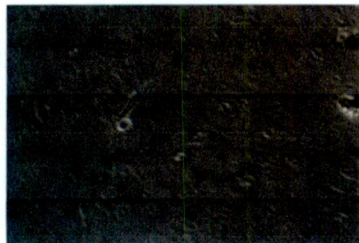
BTE 25  $\mu\text{M}$



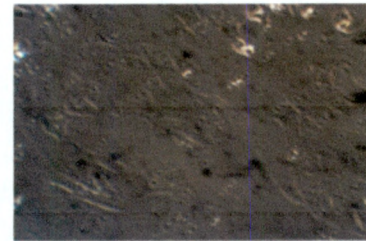
BTE 50  $\mu\text{M}$



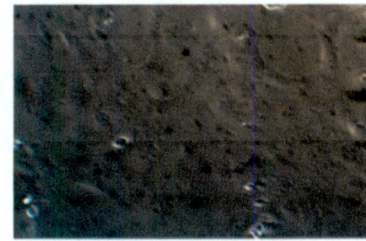
BTE 75  $\mu\text{M}$



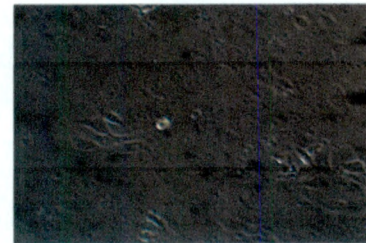
BTE 100  $\mu\text{M}$



BTE 25  $\mu\text{M}$  + 5  $\mu\text{M}$  ACV



BTE 50  $\mu\text{M}$  + 5  $\mu\text{M}$  ACV



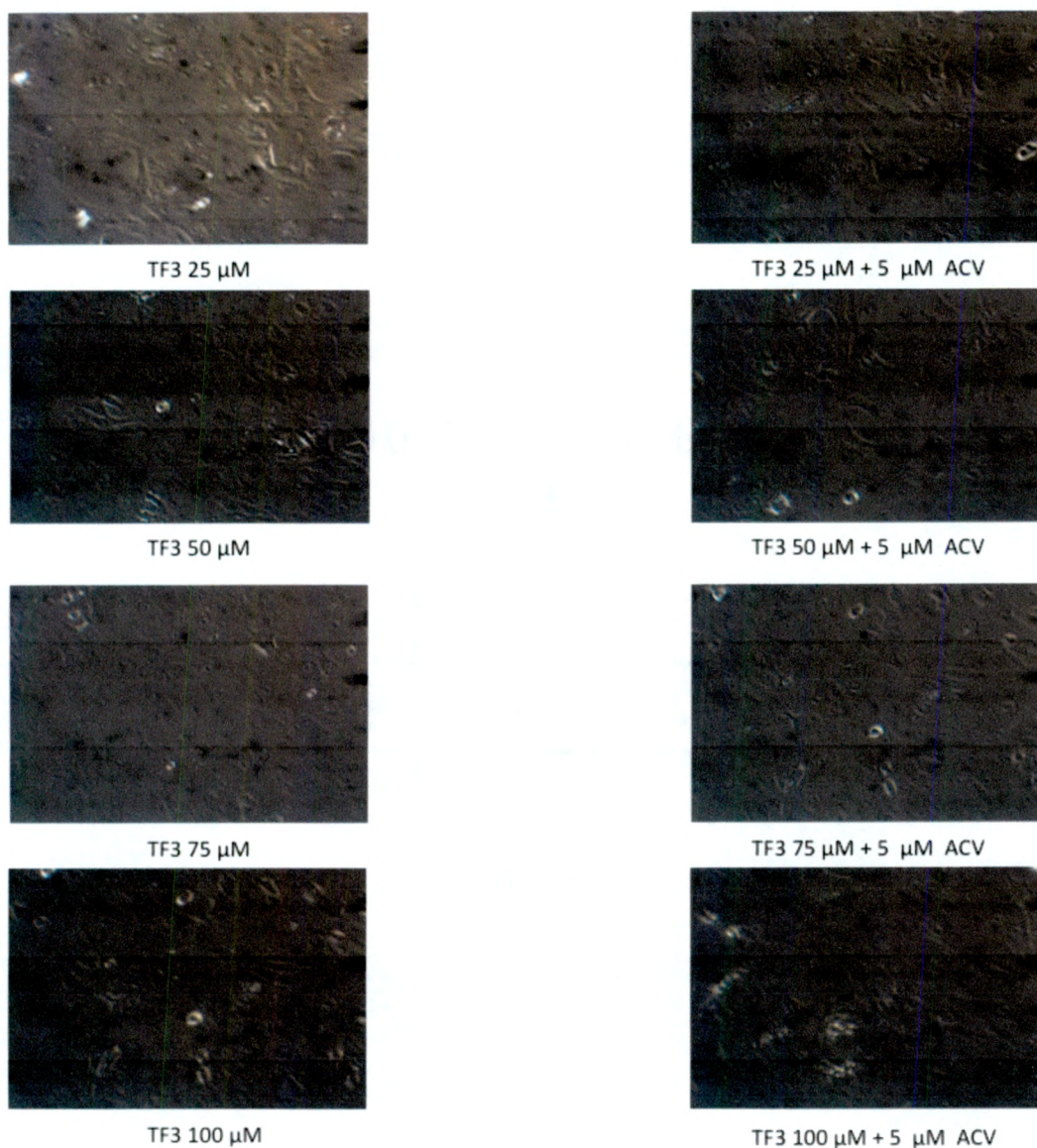
BTE 75  $\mu\text{M}$  + 5  $\mu\text{M}$  ACV



BTE 100  $\mu\text{M}$  + 5  $\mu\text{M}$  ACV

**Figure 2: Morphological effect of BTE and acyclovir on A549 cells**



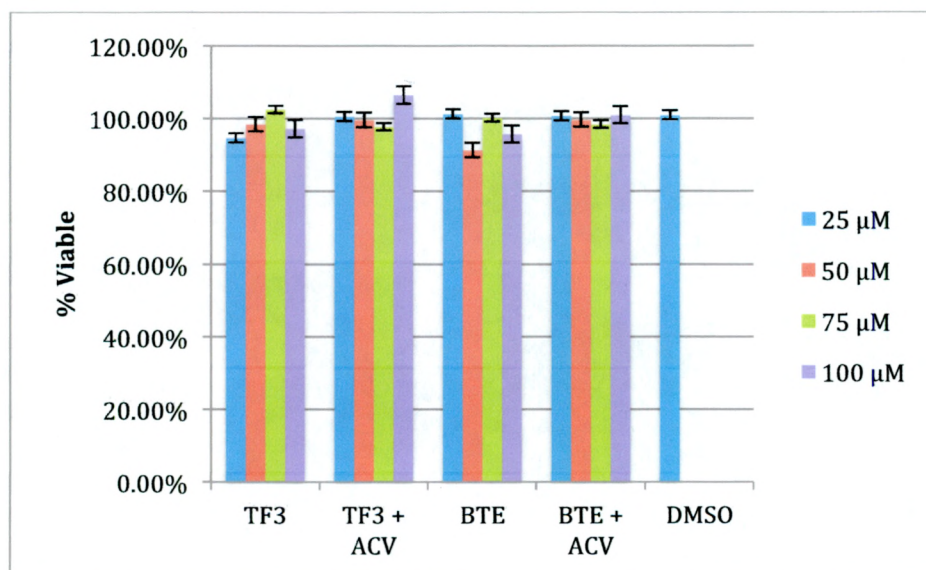


**Figure 3: Morphological effects of TF3 and acyclovir on A549 cells**

**BTE and TF3 at 100  $\mu\text{M}$  and acyclovir at 50  $\mu\text{M}$  do not alter cell viability**

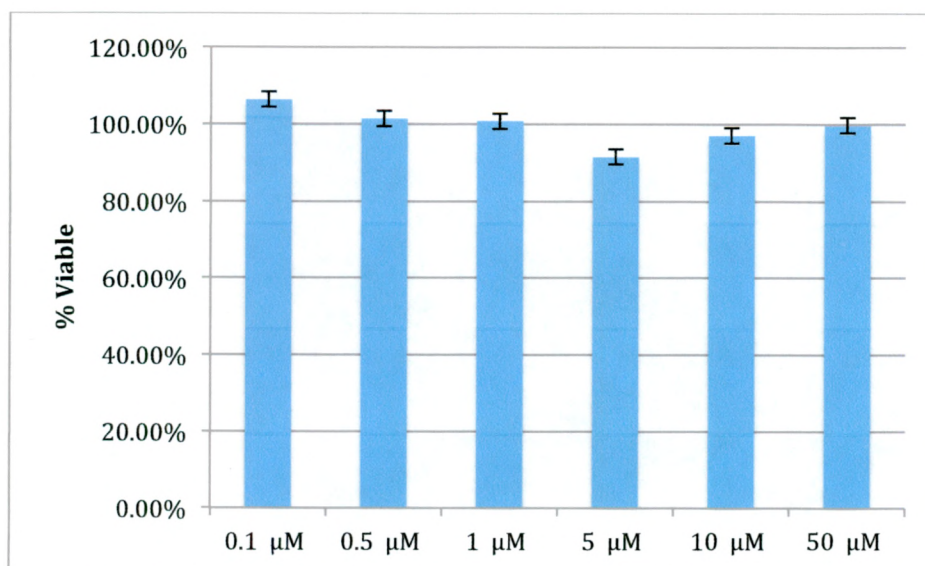
Viability was quantitatively determined with a trypan blue assay. With a hemocytometer the number of live and dead cells was evaluated and a ratio was determined for the percent of cells that were viable after a 24-hour period of treatment with BTE, TF3, or acyclovir. For both TF3 and BTE the percent of viable cells remained consistent with the positive control of cells treated with 10% FBS media. Furthermore,

cells that were treated with a combination of acyclovir and BTE or TF3 showed no significant difference in viability as compared to the positive control. For each, as the concentration of reagent increased, no difference in cell viability was observed. DMSO concentrations used as a solvent,  $\leq 0.5\%$ , showed similar viability as compared to the positive control (Figure 2). Acyclovir by itself was tested at a range of concentrations from 0.1  $\mu\text{M}$  to 50  $\mu\text{M}$ . For each concentration there was similar percent of viable cells as compared to the cells treated with media control (figure 4).



**Figure 4: Trypan blue assay results for BTE, TF3, and ACV cytotoxicity on A549 cells.**



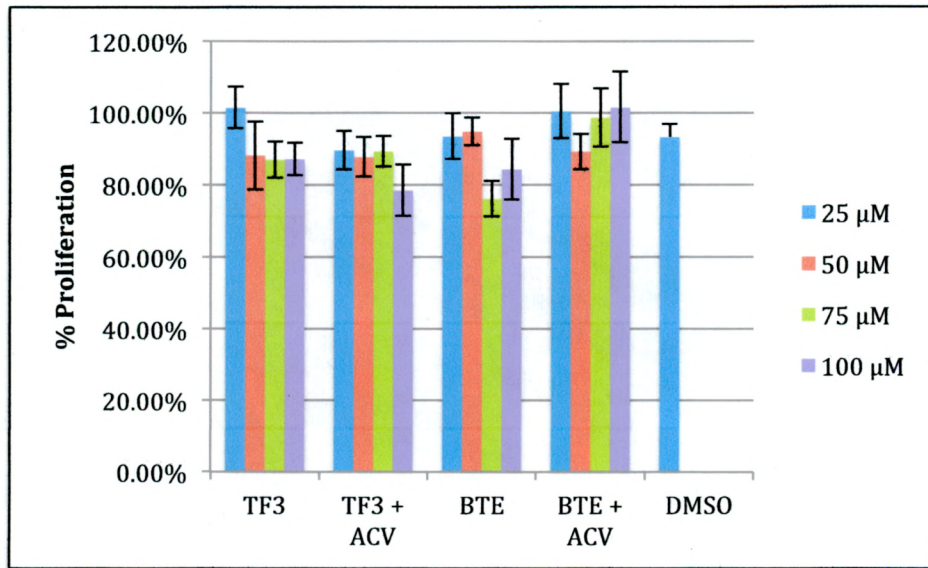


**Figure 5: Trypan blue assay results for ACV cytotoxicity on A549 cells.**

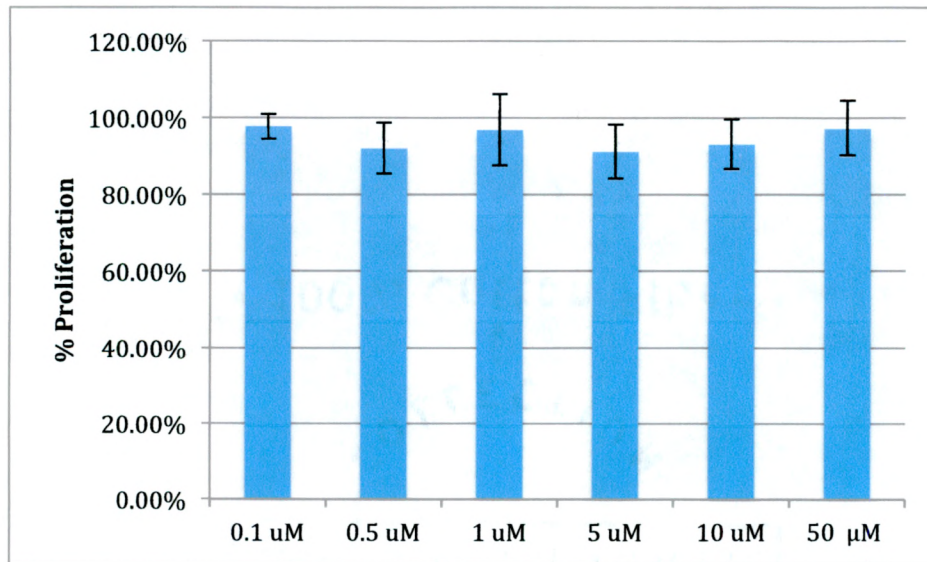
**WST-1 proliferation assay supports BTE, TF3, and acyclovir are not toxic to A549 cells.**

To evaluate if cell proliferation was altered by BTE, TF3, or acyclovir a WST-1 proliferation assay was performed. For each tea reagent a range of concentrations from 25 μM to 100 μM was used and a percent proliferation was calculated through comparison to the negative control of cells treated with 10% FBS media. If the reagents are cytotoxic then the concentration of ATP will decrease within a cell, giving lower absorbance. Data observed support the findings from the trypan blue assay. TF3 and BTE each showed over 85% viability for each concentration, up to 100 μM. For the combinations of TF3 and BTE with 5 μM of acyclovir there were similar data observed, with no viability below 85%. DMSO at concentrations less than 0.5% showed 93% viability when compared to the control (Figure 6). Acyclovir was also tested at concentrations from 0.1 μM to 50 μM. All concentrations showed similar percentage of viable cells, with none dropping below 90% viability (Figure 7).





**Figure 6: WST-1 assay results for BTE, TF3, and ACV cytotoxicity in A549 cells.**

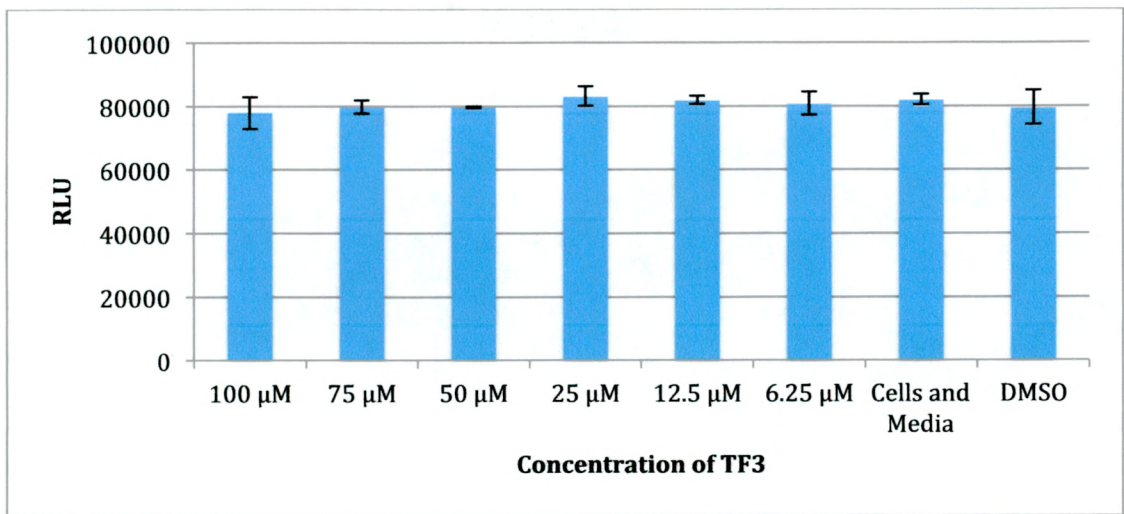


**Figure 7: WST-1 assay results for ACV cytotoxicity in A549 cells.**

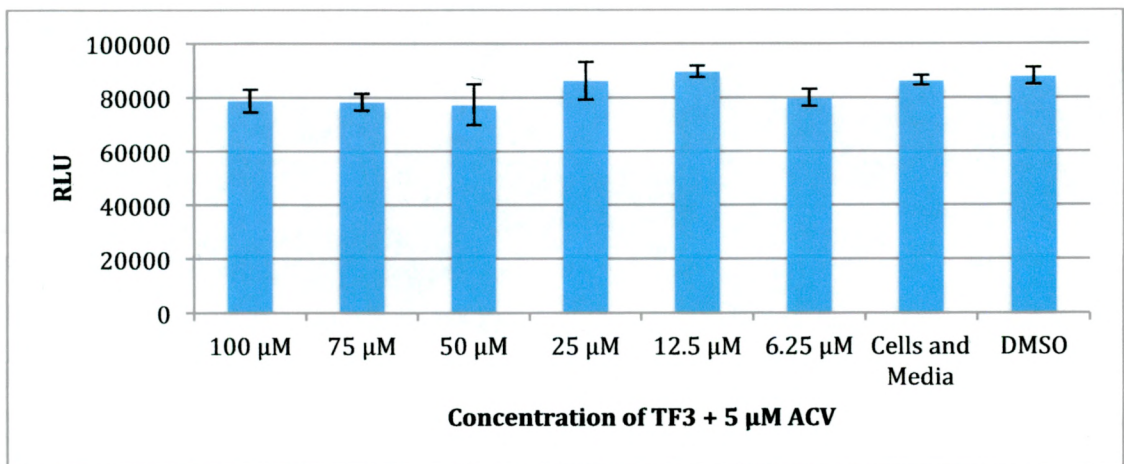
### **ToxGlo Proliferation**

To confirm the cytotoxicity data, a ToxGlo assay was performed. Data collected showed similar RLU values between each concentration and the positive control of cells with media only. TF3 concentrations from up to 100 μM showed no significant decrease

in RLU over the negative control, nor did acyclovir at concentrations up to 50  $\mu\text{M}$ . Furthermore, DMSO, at concentrations used to dissolve reagents  $>0.5\%$ , showed no difference in RLU in comparison to the positive control (Figure 8). For the combination of 5  $\mu\text{M}$  of acyclovir with various concentrations of TF3 ranging from 6.25  $\mu\text{M}$  to 100  $\mu\text{M}$ , there showed no difference in RLU compared to the control. This data is in parallel with the data collected from both the viability and proliferation assays.



**Figure 8: ToxGlo assay results for TF3 and Acyclovir cytotoxicity on A549 cells**



**Figure 9: ToxGlo assay results for TF3 and Acyclovir combination cytotoxicity on A549 cells**



### **TF3 reduces HSV-1 and HSV-2 titers more effectively than 100 μM BTE**

Antiviral properties of the tea reagents were quantitatively evaluated with a WST-1 assay since infected cells would lead to ATP depletion, thus giving lower absorbance readings. The first reagent tested was BTE at concentrations ranging from 25 μM to 100 μM. At the lowest concentration of BTE, 25 μM, the percent inhibition was only 28.6%. However, with increased concentration to the maximum concentration of 100 μM BTE, the viral inhibition increased to 65.8% for HSV-1. Viral inhibition was calculated through comparison of a negative control (uninfected cells) and a positive control (infected cells with no tea reagents), to the absorbance seen in the experimental wells as determined by the equations seen below (Figure 10). For HSV-2, 25 μM had a viral inhibition of 36.8% while 100 μM of BTE showed a 68.7% viral inhibition. On the other hand, the isolated TF3 showed viral inhibition of 80.4% at 100 μM for HSV-1 and 78.1% at 100 μM for HSV-2. For each concentration there is increased viral inhibition for TF3 over BTE. For HSV-1 there was a 22.8% increase in antiviral activity following treatment with TF3 as compared to BTE and a 13.74% increase for HSV-2 (Figures 11 and 12).

$$\frac{(\textit{Experimental well} - \textit{positive control})}{(\textit{Negative control} - \textit{positive control})}$$

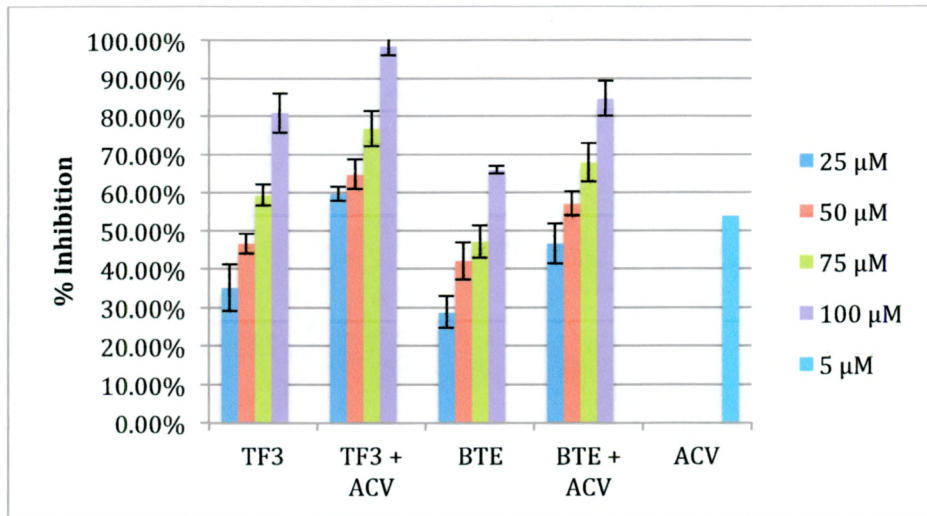
**Figure 10: Antiviral equation**

### **Acyclovir has greater synergy with TF3 than BTE against HSV-1 and HSV-2**

After determining the baseline of the antiviral activity of the tea reagents and acyclovir, the synergy between the tea reagents and acyclovir was evaluated. Due to acyclovir being a potent antiviral in its own rights, it was determined that the maximum concentration of acyclovir used in the cytotoxicity screening should not be used. At such



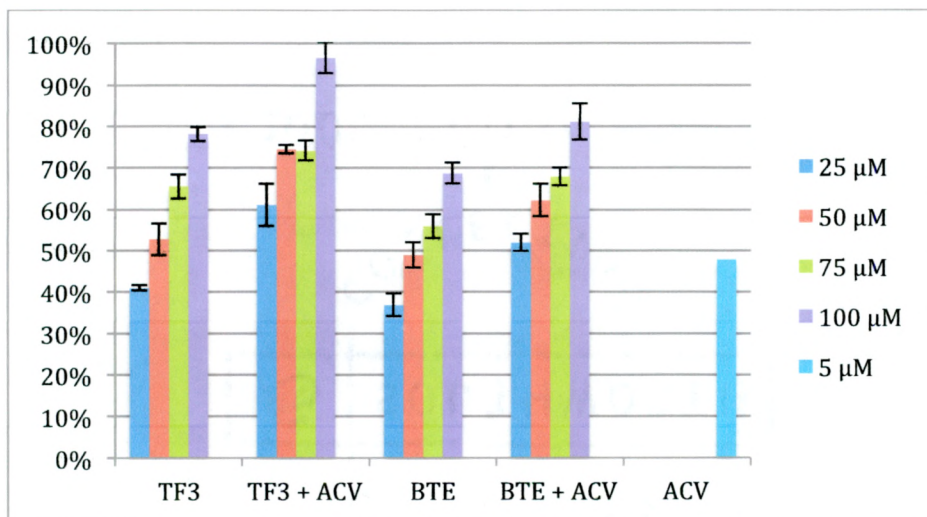
a high concentration the synergy data would be eclipsed by the antiviral activity of acyclovir alone and would provide little insight on the synergistic value. Therefore screening was performed to find the EC50. Previous studies indicated that 5  $\mu\text{M}$  is the EC50 of acyclovir, which was confirmed through a WST-1 assay (Andersen, Jenssen, and Gutteberg, 2002). At the EC50 it would be easier to properly evaluate the antiviral activity of the combined reagents. Data for HSV-1 and HSV-2 indicated that the viral inhibition was 53.8% and 47.8%, respectively. For each synergy evaluation the concentration of acyclovir was 5  $\mu\text{M}$  with a varying level of BTE or TF3 ranging from concentrations of 25  $\mu\text{M}$  to 100  $\mu\text{M}$ . TF3 showed higher synergy with acyclovir, giving higher viral inhibition at each concentration as compared to the BTE. The lowest concentration of BTE, 25  $\mu\text{M}$ , showed 46.5% and 51.9% viral inhibition for HSV-1 and HSV-2, respectively. While at the highest concentration, 100  $\mu\text{M}$ , the viral inhibition was 84.6% and 81.1% for HSV-1 and HSV-2, respectively. However, for TF3 at 25  $\mu\text{M}$  viral inhibition was 59.6% for HSV-1 and 61.0% for HSV-2. On the other hand, 100  $\mu\text{M}$  of TF3 with acyclovir showed 98.4% and 96.4% viral inhibition for HSV-1 and HSV-2, respectively (Figure 11 and 12, Tables 1 and 2). At the highest concentration of TF3, 100  $\mu\text{M}$ , there was a 21.8% increase in viral inhibition with the addition of acyclovir for HSV-1 and 23.3% increases for HSV-2. For BTE viral inhibition increased by 28.7% for HSV-1 and 18% for HSV-2.



**Figure 11: WST-1 antiviral assay results for HSV-1 exposed to BTE, TF3, and ACV in A549 cells**

	TF3	TF3 + ACV	BTE	BTE + ACV
25 μM	35.12±6.1%	59.68±1.85%	28.68±4.14%	46.54±5.26%
50 μM	46.56±2.54%	64.73±3.9%	42.03±4.86%	57.09±6.15%
75 μM	59.37±2.75%	76.73±4.67%	47.09±4.20%	67.86±5.07%
100 μM	80.84±5.25%	98.39±10.72%	65.86±0.98%	84.68±4.64%

**Table 1: WST-1 antiviral assay results for HSV-1 exposed to BTE, TF3, and ACV in A549 cells**



**Figure 12: WST-1 antiviral assay results for HSV-2 exposed to BTE, TF3, and ACV in A549 cells**

	TF3	TF3 + ACV	BTE	BTE + ACV
25 $\mu$ M	40.95 $\pm$ 0.68%	61.05 $\pm$ 3.80%	36.82 $\pm$ 2.94%	51.93 $\pm$ 6.54%
50 $\mu$ M	52.72 $\pm$ 5.14%	74.49 $\pm$ 0.98%	48.90 $\pm$ 2.37%	62.23 $\pm$ 3.74%
75 $\mu$ M	65.53 $\pm$ 2.75%	74.14 $\pm$ 3.06%	55.88 $\pm$ 2.93%	67.91 $\pm$ 2.52%
100 $\mu$ M	78.14 $\pm$ 2.14%	96.46 $\pm$ 3.96%	68.72 $\pm$ 2.14%	81.09 $\pm$ 4.36%

**Table 2: WST-1 antiviral assay results for HSV-2 exposed to BTE, TF3, and ACV in A549 cells**

**Viral ToxGlo assay confirms acyclovir has greater synergy with TF3 than BTE against HSV-1 and HSV-2**

To confirm these results a viral ToxGlo assay was performed, which provided a higher degree of accuracy for viral inhibition measurements. For this assay only TF3 with acyclovir was tested. Five  $\mu$ M of acyclovir was combined with various concentrations of TF3 ranging from 6.25 to 100  $\mu$ M. As the concentration of TF3 increased the viral inhibition increased. Maximum viral inhibition occurred at 100  $\mu$ M TF3 with 91.5% viral inhibition for HSV-1 (Figure 13, Table 3) and 95.7% viral inhibition for HSV-2 (Figure 14, Table 3). At concentrations of 12.5 and 6.25  $\mu$ M the percent inhibition plateaued at approximately 50%, similar to the viral inhibition of acyclovir alone as seen in the WST-1 antiviral assay.



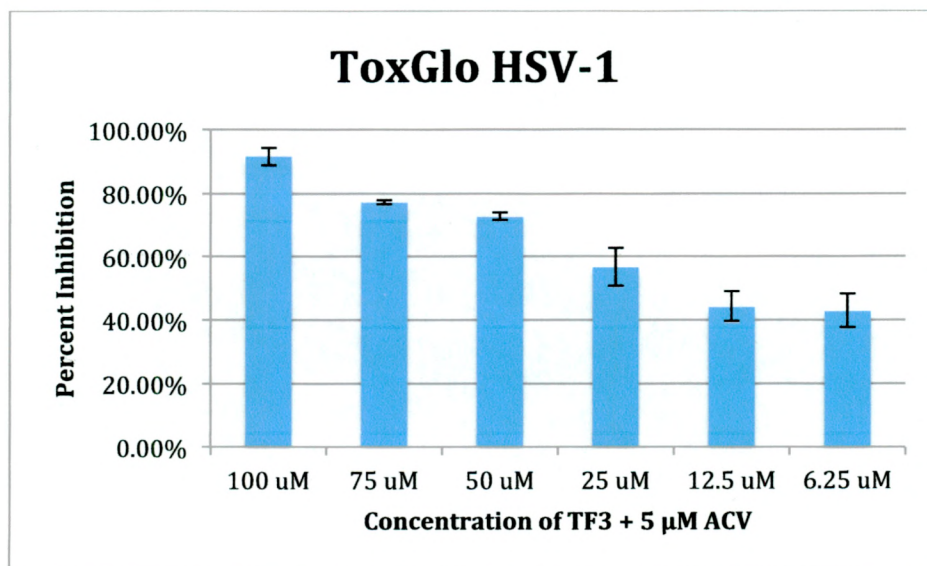


Figure 13: ToxGlo Assay for TF3 and Acyclovir on HSV-1 infected A549 cells

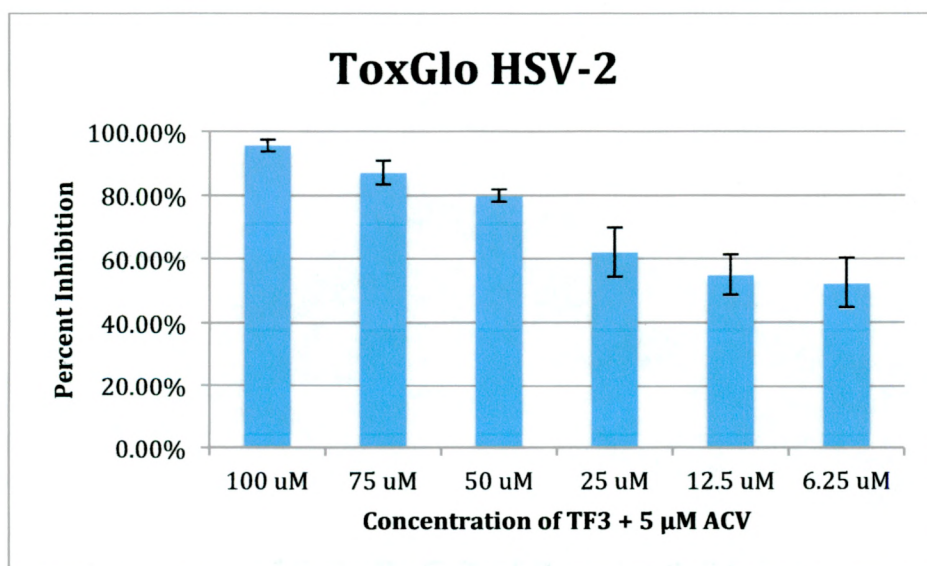


Figure 14: ToxGlo Assay for TF3 and Acyclovir on HSV-2 infected A549 cells

HSV-1					
100 μM	75 μM	50 μM	25 μM	12.5 μM	6.25 μM
91.56 ± 2.79%	77.13 ± 0.65%	72.62 ± 1.19%	56.59 ± 6.00%	44.11 ± 4.73%	42.76 ± 5.31%
HSV-2					
100 μM	75 μM	50 μM	25 μM	12.5 μM	6.25 μM
95.59 ± 1.92%	86.99 ± 3.79%	79.75 ± 1.95%	62.00 ± 7.73%	54.82 ± 6.42%	52.25 ± 7.79%

Table 3: ToxGlo Assay for TF3 and Acyclovir on HSV-1 and HSV-2 in A549 cells

## Discussion

HSV-1 and HSV-2 are responsible for one of the most common infections in the human population, infecting between 45 to 98% of the population (Xu et al., 2006; Spruance, 1992). Infections lead to painful lesions on the lips or genitals, referred to as cold sores and genital warts, respectively. While not life threatening, the disease does lead to psychological impact on the infected due to recurrent outbreaks throughout the individuals life. In some cases, ocular manifestations of the disease, known as herpes keratitis can lead to blindness. Due to these symptoms herpes infections are a notable health concern in the population and novel treatment options are actively sought.

Tealeaves derived from *Camellia sinensis* have been well known for their health benefits when consumed. Within black tea, theaflavins known collectively as black tea extract have shown to be powerful antioxidants (Luczaj and Skrzydlewska, 2007; Yang et al., 2007). Furthermore, these tea theaflavins have shown protective properties against cardiovascular disease and cancer (Leung et al., 2001). It is through these antioxidant properties that theaflavins may be causing viral inhibition. Among the mixture of theaflavins, one in particular TF3 is the most powerful antioxidant due to the chemical structure. TF3, unlike the other theaflavins, contains two gallate groups, while the others simply have one gallate group. Therefore, it is proposed during this study that TF3 may have a higher antiviral activity than the mixture of theaflavins found in BTE.

The primary drug used for the treatment of HSV-1 and HSV-2 infections is acyclovir and its derivatives. This class of drugs works through converting into an active state upon exposure to HSV infected cells. Viral thymidine kinase causes a conformational change into acyclovir monophosphate, which is converted by cellular



kinases into acyclovir triphosphate. Acyclovir triphosphate can then be incorporated into the growing viral DNA chain, which due to the lack of a 3' hydroxyl group, leads to stalling of viral replication (Reardon and Spector, 1989). Due to the common mechanism of inhibition for acyclovir and its derivate, a mutation in viral thymidine kinase or polymerase can lead to resistance. Therefore, novel treatments to aid in the effectiveness of acyclovir need to be developed.

The purpose of this study was to assess BTE and TF3 for antiviral properties and examine if these theaflavins exhibit synergy when used in combination with acyclovir. This hypothesis stems from the findings that theaflavins from *Camellia sinensis* inhibit viral titers through disruption of the absorption process (Cantatore et al., 2013; Lui et al., 2005). Since acyclovir is known to inhibit HSV during DNA replication, it is reasonable to assume that the two reagents would show a degree of synergy since the mode of action for inhibition is entirely different.

In order for theaflavins and acyclovir to be an effective treatment option for HSV infections it was necessary to assess if the reagents contained cytotoxic properties. Data collected during the study indicates that both BTE and TF3 are not cytotoxic to A549 cells at concentrations up to 100  $\mu\text{M}$  (Figures 4, 6, and 8). Furthermore, the lack of cytotoxicity is similarly seen in TF3 or BTE combined with up to 50  $\mu\text{M}$  of acyclovir (Figures 5 and 7). This suggests that theaflavins and acyclovir do not interact to produce a product that would be toxic to human cells. At maximum concentrations ATP concentrations remain stable for each reagent tested, indicating proper cell health during treatments.



In addition to the lack of cytotoxicity at 100  $\mu\text{M}$ , the theaflavins found in black tea are stable at neutral pH, more so than catechins found in green tea that also have antiviral properties (Henning, Choo, and Heber, 2008; Su et al., 2003). As such, theaflavins may be a preferred treatment option than other components derived from tea. Furthermore, the increased stability would be more suitable for development of a topical treatment designed for active lesions during outbreaks.

Inhibition of the virus was measured quantitatively through the use of a WST-1 proliferation assay. The antiviral assay supported that BTE and TF3 significantly inhibit the replication cycle of HSV-1 and HSV-2 at concentrations that are not cytotoxic when treated for one hour (Figures 11 and 12). Data also indicate that TF3 is a more potent inhibitor of both viruses than the BTE (Figures 11 and 12). This increased antiviral activity is most probably the result of the additional gallate group on TF3, which provides additional hydroxyl groups. Additional hydroxyl groups would aid in reactivity of the compound, providing increased antiviral properties not seen in the mixture of theaflavins. These findings were confirmed through the use of a viral ToxGlo assay, which quantified ATP reduction due to cytopathetic effect (Figures 13 and 14).

When acyclovir was combined with treatments of theaflavins the percent of viral inhibition increased in each case, at a maximum of 21.8 and 23.3% increase in antiviral activity for TF3 and acyclovir on HSV-1 and HSV-2, respectively (Figures 11 and 12). This suggests that a synergistic relationship is present for black tea theaflavins and acyclovir. The presence of synergy suggests that acyclovir and theaflavins lack a common mechanism. This higher level of viral inhibition may potentially provide the

treatment option required to aid in outbreaks and decrease prevalence of resistance strains that have been emerging in the population.

While the research presented supports the synergistic antiviral properties of TF3 and acyclovir, the exact mechanism were not observed. Understanding the mechanisms of inhibition by the combination of reagents and TF3 alone could result in a more effective treatment and expand on the reasoning for the synergy observed. PCR data for treated cells would show the effects of the black tea theaflavins on inhibition of the viral replication process. Prior studies have indicated that this inhibition is most likely during the process of viral absorption (Cantatore et al., 2013). Application of qPCR on treated viral DNA will provide quantitative insight on amplification of viral DNA. By applying qPCR over a time course study it will be possible to observe at what timeframes in the viral life cycle theaflavins and acyclovir are most effective, Furthermore, qPCR and PCR data will support at which stage in the replication cycle the viral inhibition is occurring, which will allow for a proper understanding of the mechanisms behind this inhibition. To further confirm previous evidence that theaflavins work at the absorption stage of replication, an in-depth analysis of treated virus under observation of scanning electron microscope (SEM) should be conducted. SEM imaging can show the physical alterations to virions during the treatment, such as disrupted envelopes. Observations of such alterations would confirm the nature of inhibition seen during theaflavin exposure.

Results shown support that treatment of virus with TF3 and acyclovir show the highest inhibition of HSV-1 and HSV-2. The increased stability of TF3 along with the higher antiviral properties observed make it an attractive option for a safe topical treatment. Along with the synergy observed the mixture of acyclovir and TF3 might

become a better treatment option in immunocompromised patients to decrease the emergence of resistant strains.



## **Conclusion**

Here it has been shown that black tea theaflavins and acyclovir are not cytotoxic to human cells through both a cell viability and proliferation assay. In addition, it has been shown that TF3 and BTE decrease viral replication at concentrations of 25  $\mu\text{M}$  and higher for both HSV-1 and HSV-2. Although both are powerful antioxidants, TF3 has shown to have higher antiviral properties than the combination of theaflavins found within BTE. The antiviral properties can further be heightened through the addition of 5  $\mu\text{M}$  acyclovir to either BTE or TF3. This increase supports that synergy is present between both theaflavins and acyclovir. As the prevalence of acyclovir resistant herpes increases in immunocompromised patients, the need for additional novel treatments becomes necessary. The synergistic relationship observed between theaflavins and acyclovir may provide such a prophylactic treatment option to decreasing the rate of new resistant cases.

## References

- Allen SJ, Hamrah P, Gate D, Mott RK, Mantopoulos D, Zheng L, Town T, Jones T, Von Andrian UH, Freeman GJ, Sharpe AH, Mohamed L, Ahmed R, Wechsler SL, and Ghiasi H: The Role of LAT in Increased CD8+ T Cell Exhaustion in Trigeminal Ganglia of Mice Latently Infected with Herpes Simplex Virus 1. *Journal of Virology* 2011, 85(9): 4184-197.
- Andersen JH, Jenssen H, and Gutteberg TJ: Lactoferrin and lactoferricin inhibit Herpes simplex 1 and 2 infection and exhibit synergy when combined with acyclovir. *Antiviral Research* 2003, 58(3): 209-215.
- Bradley H, Markowitz LE, Gibson T, and McQuillan GM: Seroprevalence of Herpes Simplex Virus Types 1 and 2 United States, 1999–2010. *Journal of Infectious Diseases* 2013, 89(33):
- Cantatore A, Randall SD, Traum D, and Adams SD: Effect of Black Tea Extract on Herpes Simplex Virus-1 Infection of Cultured Cells. *BMC Complementary and Alternative Medicine*. 2013, 13(1): 139.
- Ciesek S, Von Hahn T, Colpitts C, Schang LM, Friesland M, Steinmann J, Manns MP, Ott M, Wedemeyer H, Meuleman P, Pietschmann T, and Steinmann E: The Green Tea Polyphenol, Epigallocatechin-3-gallate, Inhibits Hepatitis C Virus Entry. *Hepatology* 2011, 54(6): 1947-955.
- Connell EV, Cerruti RL, and Trown PW. Synergistic Activity of Combinations of Recombinant Human Alpha Interferon and Acyclovir, Administered Concomitantly and in Sequence, against a Lethal Herpes Simplex Virus Type 1 Infection in Mice. *Antimicrobial Agents and Chemotherapy* 1985, 28(1): 1-4.
- Darby G, Field HJ, and Salisbury SA. Altered Substrate Specificity of Herpes Simplex Virus Thymidine Kinase Confers Acyclovir-resistance. *Nature* 1981, 289(5793): 81-83.
- Dolan A, Jamieson FE, Cunningham C, Barnett BC, and McGeoch DJ. The Genome Sequence of Herpes Simplex Virus Type 2. *Journal of Virology* 1997 72(3): 2010-021.
- Elion, GB. The Biochemistry and Mechanism of Action of Acyclovir. *Journal of Antimicrobial Chemotherapy* 1983, 12.Suppl B: 9-17.
- Farooq, AV, and Shukla D. Herpes Simplex Epithelial and Stromal Keratitis: An Epidemiologic Update. *Survey of Ophthalmology* 2012, 57(5): 448-62.
- Fatahzadeh M, and Schwartz RA. Human Herpes Simplex Virus Infections: Epidemiology, Pathogenesis, Symptomatology, Diagnosis, and Management. *Journal of the American Academy of Dermatology* 2007, 57(5): 737-63.



Frobert E, Burrell S, Ducastelle-Lepretre S, Billaud G, Ader F, Casalegno J, Nave V, Boutolleau D, Michallet M, Lina B, and Morfin F. Resistance of Herpes Simplex Viruses to Acyclovir: An Update from a Ten-year Survey in France. *Antiviral Research* 2014, 111: 36-41.

Graham, HN. Green Tea Composition, Consumption, and Polyphenol Chemistry. *Preventive Medicine* 1992, 21(3): 334-50.

Henning SM, Choo JJ, and Heber D. Nongallated compared with gallated flavan-3-ols in green and black tea are more bioavailable. *Journal of Nutrition* 2008, 138: 1529S-1534S

Isaacs CE, and Xu W. Theaflavin-3,3'-Digallate and Lactic Acid Combinations Reduce Herpes Simplex Virus Infectivity. *Antimicrobial Agents and Chemotherapy* 2013, 57(8): 3806-814.

Isaacs CE, Xu W, Merz G, Hillier S, Rohan L, and Wen GY. Digallate Dimers of (-)-Epigallocatechin Gallate Inactivate Herpes Simplex Virus. *Antimicrobial Agents and Chemotherapy* 2011 55(12): 5646-653.

Jeon J, Kim JH, Lee CK, Oh CH, and Song HJ. The Antimicrobial Activity of (-)-Epigallocatechin-3-Gallate and Green Tea Extracts against *Pseudomonas Aeruginosa* and *Escherichia Coli* Isolated from Skin Wounds. *Annals of Dermatology* 2014, 26(5): 564-69.

Larder BA, and Darby G. Properties of a Novel Thymidine Kinase Induced by an Acyclovir-resistant Herpes Simplex Virus Type 1 Mutant. *Journal of Virology* 1982, 42(2): 649-58.

Leung LK, Su Y, Chen R, Zhang Z, Huang Y, and Chen ZY. Theaflavins in Black Tea and Catechins in Green Tea Are Equally Effective Antioxidants. *Journal of Nutrition* 2001, 131(9): 2248-51.

Ya LS, Leung LK, Huang Y, and Chen Z. Stability of Tea Theaflavins and Catechins. *Food Chemistry* 2003, 83(2): 189-95.

Liesegang TJ. Herpes simplex virus epidemiology and ocular importance. *Cornea* 2001, 20(1): 1-13.

Liu S, Lu H, Zhao Q, He Y, Niu J, Debnath AK, Wu S, Jiang S. Theaflavin derivatives in black tea and catechin derivatives in green tea inhibit HIV-1 entry by targeting gp41. *Biochim Biophys Acta* 2005, 1723.: 270-281

Macdonald SJ, Mostafa HM, Morrison LA, and Davido DJ. Genome Sequence of Herpes Simplex Virus 1 Strain KOS. *Journal of Virology* 2012, 86(11): 6371-372.



- Malkin JE. Epidemiology of genital herpes simplex virus infection in developed countries. *Herpes* 2004, 11(Suppl 1): 2A–23A.
- McGeoch DJ, Moss HWM, Mcnab D, and Frame MC. DNA Sequence and Genetic Content of the HindIII L Region in the Short Unique Component of the Herpes Simplex Virus Type 2 Genome: Identification of the Gene Encoding Glycoprotein G, and Evolutionary Comparisons. *Journal of General Virology* 1987, 68(1): 19-38.
- Molina M, Romaguera RA, Valentine J, and Tao G. Seroprevalence of herpes simplex virus 2 among Hispanics in the USA: National Health and Nutrition Examination Survey, 2007-2008. *International Journal of STD & AIDS* 2011, 22(7): 387-90.
- Nakayama M, Suzuki K, Toda M, Okubo S, Hara Y, and Shimamura T. Inhibition of the Infectivity of Influenza Virus by Tea Polyphenols. *Antiviral Research* 1993, 21(4): 289-99.
- Oliveira A, Adams SD, Lee L, Murray SR, Hsu SD, Hammond JR, Dickinson D, Chen P, Chu TC. Inhibition of herpes simplex virus type 1 with the modified green tea polyphenol palmitoyl-epigallocatechin gallate. *Food Chemistry Toxicology* 2012 52.: 207-215.
- Piret J, and Boivin G. Resistance of Herpes Simplex Viruses to Nucleoside Analogues: Mechanisms, Prevalence, and Management. *Antimicrobial Agents and Chemotherapy* 2011, 55(2): 459-72
- Pottage, JC, and Kessler HA. Herpes Simplex Virus Resistance to Acyclovir: Clinical Relevance. *Infectious Agents and Disease* 1995, 4(3): 115-24.
- Reardon JE, and Spector T. Herpes Simplex Virus Type 1 DNA Polymerase Mechanism of Inhibition by Acyclovir Triphosphate. *Journal of Biological Chemistry* 1989, 264(13): 7405-411.
- Roizman B. and Baines J. The diversity and unity of Herpesviridae. *Comparative Immunology, Microbiology and Infectious Diseases* 1991, 14: 63-79
- Safrin S. Treatment of Acyclovir-Resistant Herpes Simplex Virus Infections in Patients with AIDS. *Journal of Acquired Immune Deficiency Syndromes* 1992, 5(1): 29-32.
- Smith JS, and Robinson NJ. Age-Specific Prevalence of Infection with Herpes Simplex Virus Types 2 and 1: A Global Review. *The Journal of Infectious Diseases* 2002, 186(S1): S3-S28.
- Spruance SL. The natural history of recurrent oro-facial herpes simplex virus infection. *Seminars in Dermatology* 1992, 11:200-6



Su YL, Leung LK, Huang Y, and Chen ZY. Stability of tea theaflavins and catechins. *Food Chemistry* 2003, 83: 189-195

Tanaka Y, Kritia M, Abe Y, Miyata S, Tagashira M, and Maeda-Yamamoto M. Metabolic Stability and Inhibitory Effect of O-methylated Theaflavins on H<sub>2</sub>O<sub>2</sub>-induced Oxidative Damage in Human HepG2 Cells. *Bioscience, Biotechnology, and Biochemistry* 2014, 78(7): 1140-146.

Wang C, Li Y. Research progress on property and application of theaflavins. *African Journal of Biotechnology* 2006, 5: 213-218

Webre JM, Hill JM, Nolan NM, Clement C, McFerrin HE, Bhattacharjee PS, Hsia V, Neumann DM, Foster TP, and Lukiw WJ. Rabbit and Mouse Models of HSV-1 Latency, Reactivation, and Recurrent Eye Diseases. *Journal of Biomedicine and Biotechnology* 2012: 21-39.

Willard M. Rapid directional translocations in virus replication. *Journal of Virology*. 2002, 76(10): 5220–5232

Whitley RJ. Herpes Simplex Encephalitis: Adolescents and Adults. *Antiviral Research* 2006, 71(2-3): 141-48.

Yang J, Li L, Tan S, Jin H, Qiu J, Mao O, Li R, Xia C, Jiang Z, Jiang S, and Liu S. A Natural Theaflavins Preparation Inhibits HIV-1 Infection by Targeting the Entry Step: Potential Applications for Preventing HIV-1 Infection. *Fitoterapia* 2012, 83(2): 348-55.

Yang Z, Bai L, Huang W, Li X, Zhao S, Zhong N, and Jiang Z. Comparison of in Vitro Antiviral Activity of Tea Polyphenols against Influenza A and B Viruses and Structure–activity Relationship Analysis. *Fitoterapia* 2014, 93: 47-53.

Yoshikawa T, Hill JM, Stanberry LR, Bourne N, Kurawadwala JF, and Krause PR. The Characteristic Site-specific Reactivation Phenotypes of HSV-1 and HSV-2 Depend upon the Latency-associated Transcript Region." *Journal of Experimental Medicine* 1996, 184(2): 659-64.