5-2011

Modified Green Tea Polyphenol, EGCG-ester, as a Novel Approach to Inhibit Herpes Simplex Virus Infections

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Modified Green Tea Polyphenol, EGCG-ester, as a Novel Approach to Inhibit Herpes Simplex Virus Infections

by

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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 2011

College/School College of Science and Mathematics

Department Molecular Biology

[redacted]

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Abstract

HSV-1 DNA with the green fluorescent protein (GFP) introduced into the UL46 gene was used to investigate the effects of green tea polyphenols on the virus. Two different green tea extracts have been isolated and modified, EGCG and EGCG-ester. These extracts with concentration of 12.5, 25, 50, 75 and 100μM were used to determine its effects on cell morphology, cell proliferation and cell viability. The results indicated that the maximum non-toxic concentration is 75μM for both polyphenols. GFP expression, DAPI DNA stain and Lysosome activation stain were also used to observe the morphological, and cellular changes in the infected cells. The results suggested that EGCG-ester treated infected cultures did not show any changes compared with non-infected cells. Each extract was further used to study its effect on HSV1, by plaque forming unit (PFU) assay and GFP expression. EGCG treated virus had a titer that is ten fold lower than the control, and EGCG-ester completely inhibited the PFU. Quantitative study using RT-PCR indicated that EGCG-ester at 75μM inhibited 99.46% of infection when compared to the control.

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Montclair, NJ

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Acknowledgments

I would like to express my most sincere appreciation and gratefulness to both my advisors Dr. Lee Lee and Dr. Sandra Adams for their continuous guidance and support during my graduate studies. They have been a constant inspiration to me, and true examples of powerful, knowledgeable women in the science field.

I am also thankful for my committee members Dr. Quinn Vega, Dr. Lee Lee and Dr. Sandra Adams, whose valuable insights contributed immensely to the final product of my research. I would also like to thank Dr. Tin-Chun Chu for her time, assistance, and expertise in the Real Time PCR experiment conducted. I must also thank Dr. Lee Lee, Dr. Sean Murray and Jeff Hammond for assisting me with the fluorescence microscope and for helping interpret the data acquired. I would also like to thank Dr. Steven Hsu for sharing his purified samples of green tea polyphenols with me, and foremost for believing in my potential.

Special thanks to Dr. Robert Prezant, Dean of CSAM, for providing me the opportunity to receive the Novartis Scholarship. Lastly, I would like to thank Novartis for the fund received for my graduate studies.

Finally, I dedicate this thesis to the ones I will be forever grateful, my parents, Ricardo and Alessandra de Oliveira.

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Introduction

Background on Herpes Simplex Virus

The word Herpes was first used by Hippocrates 2,500 years ago to describe skin diseases. But it was not until 1694, that Richard Morton first described what we now recognize as Herpes Simplex Virus infection. Finally, in 1921, researchers first understood that the skin lesions observed from HSV diseases contained infectious particles and could be passed from human to humans[36]. Out of more than 80 herpesviruses currently known, 8 of them have been identified as potential human pathogens[10].

Herpes simplex virus (HSV) types 1 and 2 belong to the family *Herpesviridae,* subfamily *Alphaherpesviridae*, and the genus *Simplexvirus*. HSV1 and 2 have been constant threats to the human populations around the world and are among the most common infectious diseases in humans[35, 38]. During the course of one's life, there is a high percentage of chance that one will either become infected with HSV1 or HSV2 and or will meet someone who has either virus. It is believed that when life started on earth, there was only one Herpes simplex virus and this virus infected monkeys and the great apes both orally and or sexually. Since monkeys often mix oral and genitalia secretions, it was common to transfer viral particles from one area to another. When the Herpes virus developed a way to infect humans, the virus was forced to adapt to changes in personal and sexual behavior. Given that humans have a more erect posture compared to monkeys, it became much more difficult to mix oral and genital secretions, thus allowing evolution to take place and giving rise to two different but very related viruses[36]. Since then, HSV-

1 and HSV-2 have coevolved with the human population, and have successfully created a variety of ways to overpass its host immune system[35].

HSV Demographics

The prevalence of HSV infections varies between countries, within regions within a country, an even within subgroups of populations within a region[8]. In the United States, 17% of individuals, ages 14 - 49 were seropositive for HSV-2 between 1999 and 2004. These data represent a slight decrease from 1988 – 1994 (21%). The percentage of individuals in the same age groups and during the same time periods who were seropositive for HSV-1 also declined (57.7% from 62%). However, this decline was accompanied by an increase in genital infections caused by HSV-1[38].

The risk of acquiring HSV infections increases with several factors such as older age, lower socioeconomic status, poor education, and high number of sexual partners[10]. In the United States, half a million people show new symptoms of HSV infections each year and the number of first time reported cases keeps increasing every year (fig.1) [34]. Due to the seriousness of the diseases caused by both HSV1 and 2, controlling HSV spread and the number of people infected every year is considered a significant public health concern [36].

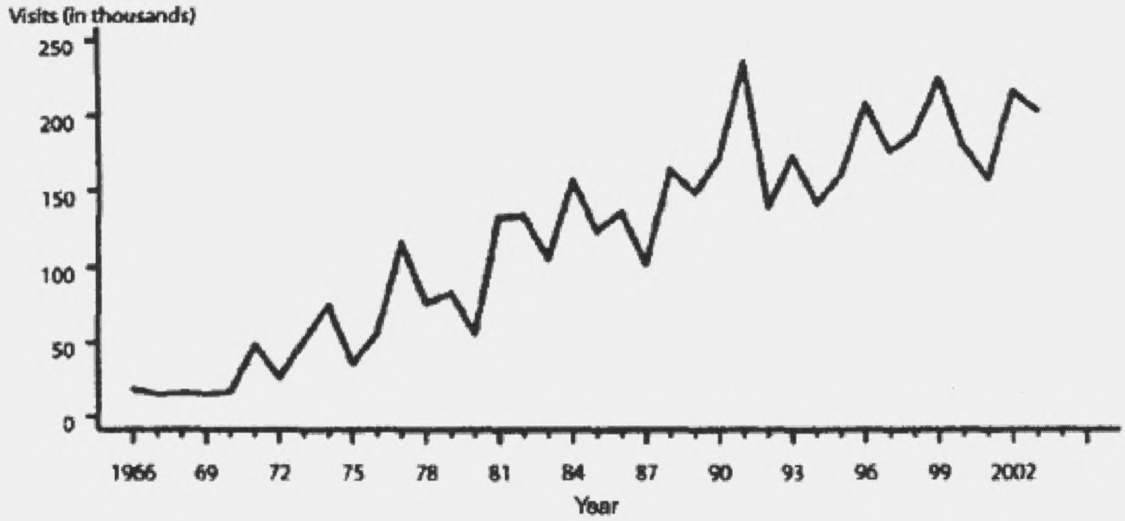


Figure 1. Number of People Reporting First Case of Genital Herpes at Doctor's Offices Within the United States[34].

The Impact of HSV to Human Life

Frequent HSV outbreaks cause discomfort and have a major psychological and social impact in infected individuals. Those infected by HSV are often concerned about passing on the virus to eventual partners as well as how they are seen by the outside world. This in turn can have a negative impact in their social lives and can cause an array of emotional reactions. Although most HSV infections are asymptomatic, when symptoms do appear, lesions are painful, recurrent, and ulcerative[21, 29]. HSV-1 can cause cold sores and lesions of the mouth and lip, as well as herpes keratitis, a leading cause of corneal blindness in the United States. HSV-1 has also emerged as a leading inducer of genital herpes in developing countries. HSV-2 is the source of most cases of genital herpes, and can, in rare cases, cause encephalitis[36, 38]. Thus, the diseases caused by HSV infections can have a major impact in one's life and are often the cause of great turmoil.

HSV Structure

HSV viruses measure approximately 200nm in diameter. HSV1 and 2 are enveloped, double stranded DNA viruses of about 152Kb in length, consisting of two segments of DNA, known as unique long (Ul) and unique short (Us) regions[13]. The virion consists of 3 major structures (fig.2); an outer portion called the envelope, which includes 11 glycoproteins (gB gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM), a tegument layer composed of 15 proteins, and an icosahedral capsid enclosing the viral DNA as well as 4 structural proteins [11, 13, 42].

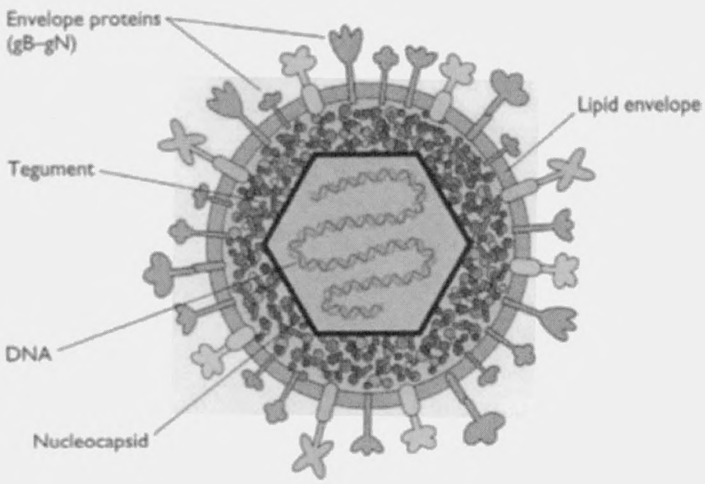


Figure 2. HSV Structure[47]

HSV Life Cycle

As soon as a person is exposed to HSV, a critical series of events within cellular, molecular and immune system biology takes place. Several glycoproteins located on the virus envelope are responsible for cell recognition, cell fusion, and eventually cell entry[35, 37]. The first contact of HSV with its host cell is by binding to heparin sulfate chains contained on the cell surface proteoglycans. The viral glycoproteins B and C assist this binding reaction as glycoprotein D is recruited to bind to one the host cell receptors. Once glycoprotein D binds to the cell receptor, glycoproteins B, H and L form a fusion complex along with glycoprotein D and the cell receptor. This fusion complex is what allows the virion's plasma membrane to fuse to the host cell plasma membrane and subsequently viral nucleocapsid and tegument entry. Consequently, although glycoprotein D is essential for cell recognition and receptor binding, all five glycoproteins are needed for successful virus adsorption and fusion[5, 35].

When a short lysine-rich region (KPKKNKKPK) within glycoprotein B of HSV-1 was removed in an experiment with Vero cells, heparin sulfate could not be bound by the receptors[18]. Although glycoproteins B and C are not required during the first binding at heparan sulfate chains, they do make the process more efficient. Thus, not only gD, but also this lysine-rich region within gB seems to be indispensable for competent viral entry into the host cell[5, 37].

Glycoprotein D recognizes and may bind to one of several host receptors. These include, HVEM (Herpesvirus entry mediator), a member of the TNF-receptor family; nectin- 1 or nectin-2, members of the immunoglobulin superfamily; and locations on the cell surface made by the reaction of heparin sulfate and 3-O-sulfotransferases[1,32,35]. The structure of HSV-1 glycoprotein D has been obtained by x-ray crystallography, and several amino acid residues within gD were seen to be critical for the binding of the receptors HVEM and nectin-1[5, 20, 41]. HSV-1 and HSV-2 glycoprotein D have been shown to contain 82% amino acid similarity. Thus, it is believed that they posses the same or similar functionality[35].

Once inside the cell, HSV will take over the cellular transport machinery in order to have access to the internal cellular compartments. Viral particles move throughout different regions of the cytoplasm in an extremely fast manner allowing viral components to reach their destinations in a very efficient way[42]. The viral particles are sent to the nucleus through the nucleopores where the viral genome will enter and viral transcription and replication will begin. HSV uses microtubules to travel by retrograde transport to the nucleus with the help of the dynein motor system[3, 13, 36]. Being able to enter the nucleus is essential for viral transcription, translation, replication, and packaging of the DNA into progeny nucleocapsids (fig.3). Interestingly, in flat cells such as Vero cells, the process of transporting viral particles through the usage of microtubules may not be necessary in order to achieve a successful infection. Vero cells may make use of diffusion to transport the virion to the nucleus[24].

HSV-1 and HSV-2 infect epithelial cells during lytic infection and move to sensory neurons in latent infections. During the latent infection, viruses stay in a dormant state within nerve cells until they are triggered into the lytic cycle[17, 36]. This allows HSV to permanently survive and replicate for the lifetime of the HSV infected patient[17, 44]. For the virus to become latent, viral particles have to travel from nerve axons at the initial site of infection to the sensory ganglia[3]. The latently infected nerve cells do not replicate HSV's DNA, but they make mRNA of a short sequence of the genome known as the latency associated transcript (LAT). A study done in which this sequence was removed showed that viruses were not able to cause recurrent infections[17].

These latently infected cells may be unreactive for a long period of time, but can reactivate at anytime during one's life course. Currently, it is not known what is the cause of virus reactivation, but there are several factors that may be associated with reactivation, such as stress, heat, cold, ultraviolet light, emotional responses, and pituitary or adrenal hormones. When the virus is reactivated, the viral genome travels by anterograde transport in axons, to the epithelium where, viral replication will occur[10, 35].

Although the only known natural host for HSV infection is humans, cultured cells from a variety of different animals, such as Vero cells from green monkey kidney cells, can also be infected by HSV in the laboratory[11]. In vitro experiments with HSV virions do not involve the immune system of an animal, allowing the virus to infect cells efficiently. Thus, several cells contain at least one of the receptors necessary for viral entry by the envelope glycoprotein gD of HSV[35].

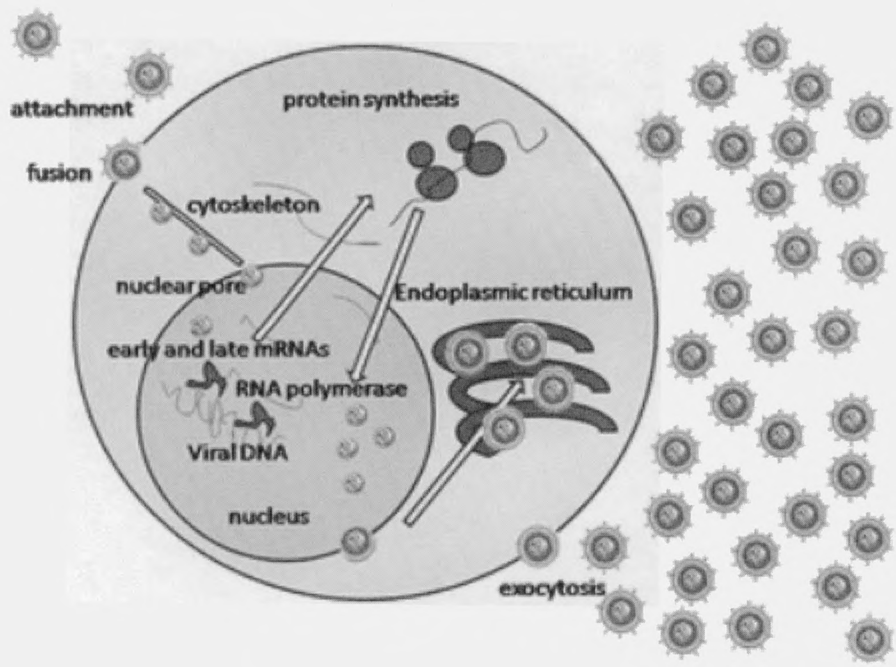


Figure 3. HSV Life Cycle[46]

HSV Transmission and Diagnoses

Transmission of HSV infection is done by intimate contact and the exchange of body fluids containing the virus. The virus enters the host through a lesion in an infected person and infects another who has had close contact with the infected person. Even when there are no apparent symptoms, the infected individual may be shedding the virus[21]. People who have been infected with HSV are estimated to be unknowingly spreading the virus 1 day in every 20 days[36].

In immunocompromised hosts and newborns, the risk of transmitting HSV is even greater, leading to eventual fatalities[4]. The great majority of HSV-2 cases are misdiagnosed, contributing to the spread of the disease and the further discomfort of the patient. Because symptoms are not always present, a clinical examination of the patient as the only form of analysis is not sufficient. Laboratory tests need to be conducted in order to have a more accurate evaluation of an infected individual[8]. There are several procedures currently done to diagnose HSV. Isolation of HSV in tissue culture is the preferred diagnostic study, but the use of PCR to amplify HSV DNA in the cerebrospinal fluid is a faster way to obtain results. Samples can also be analyzed using immunohistochemical methods for the detection of viral proteins[10, 28]. There is no cure for the disease caused by HSV infections, thus by correctly diagnosing an infection, one is able to educate and encourage a patient to contribute to the control of HSV[8].

Treatment of HSV

The current antiviral drugs of preference for the treatment of oral or genital HSV infections are acyclovir (Zovirax, Glaxo SmithKline, Research Triangle Park, NC), valacyclovir (Valtrex, Glaxo SmithKline, Research Triangle Park, NC), penciclovir

(Denavir, Novaris Pharma GmbH, Wehr, Germany), and famiciclovir (Famvir, Novartis Pharmaceuticals Corporation, East Hanover, NJ). These drugs are often given orally for 7 to 10 days. Both valacyclovir and famciclovir break down in the body into active forms of the medicine (acyclovir and penciclovir). By being analogs of nucleosides, these drugs are able to shut off viral replication[4, 10, 23].

The HSV thymine kinase phosphorylates acyclovir, and the host cell further phosphorylates it resulting in an active acyclovir triphosphate. Active acyclovir can then inhibit viral DNA polymerase, preventing viral DNA elongation. The problem with using these drugs is that they are efficient only until HSV begins to alter its thymine kinase and becomes resistant to acyclovir and the other drugs that work as analogs of nucleosides[12, 19].

The mode of action of famiciclovir is also by inhibiting viral DNA polymerase in a process similar to acyclovir, although with less efficacy. Famiciclovir is able to obtain higher concentrations within cells and has a longer-half life compared to acyclovir. It can also be given less frequently than acyclovir. Lastly, Cidofovir, an acyclic nucleoside 5'-monophosphate, is phosphorylated by the host cell kinases and is able to inhibit the viral DNA polymerase in this manner. HSV resistance to cidofovir may also arise when the DNA polymerase gene is mutated[4, 36]. Because of the great resistance that arises from taking these different drugs, new and more effective medications need to be developed in order to prevent the shedding of HSV[23].

The Link Between HSV and HIV

Although HSV-2 is the most common way of acquiring genital herpes, HSV-1 has also been linked to genital herpes in developing countries[38]. Genital herpes increases the risk of sexually acquired HIV infections, and is therefore considered a serious public health concern[27,39]. Lesions and or blisters in the skin caused when the HSV lytic cycle is active allows for an easy route of HIV transmission during sexual activity. When a person is suffering from HSV outbreaks, white blood cells travel to the site of infection, but these white blood cells are in turn an easy means for HIV to gain access into another person's body[34, 41]. In a study conducted with 224 couples in which only one of the partners was HIV seropositive, it was established that HIV RNA is often seen in HSV lesions. The scientists also noticed that when HSV is reactivated, the possibility of HIV viral loads increasing is immense. Lastly, when human CD4 cells are coinfected with both HSV and HIV, replication of HIV is greatly enhanced[26].

Unfortunately, anti-HSV drugs currently on the market have not proven to be effective against both HSV and HIV. Thus, people with Herpes have yet no choice but to protect themselves at all times, until a better and more efficient antiviral topical application is made[10, 38].

Green Tea and the Search for Microbicides

Topical microbicides are currently being researched and tested for intravaginal usage against HSV and other sexually transmitted diseases. Scientists have been trying to come up with an effective vaccine against HSV as well, but it has proven to be extremely challenging since HSV establishes latency and an infection may arise even if the immune system becomes activated[2, 36]. As a result of the emergence of drug resistance and the absence of an effective HSV vaccine, new antiviral drugs need to be developed. More importantly, HSV infection could be significantly reduced if effective agents for prevention are developed.

Green tea has been an important food in the life of Chinese people for decades now. Green tea consumption flourished in China after it was said to cure the people in the troops of General Zhu Ge Liang (181-234 A.D.). A few decades later, green tea became an essential part of the Chinese culture. In recent years, green tea has gained great support through scientific discoveries, and scientists have now started to test the benefits of green tea in trying to combat different types of illnesses. Diseases such as cancer, diabetes, obesity, influenza, and Herpes Simplex Viral Infections, once thought impossible to treat and or cure, have been given hope with the advance of research and the use of innovative compounds such as green tea polyphenols[7]. Therefore, there is a huge interest in its antimicrobial and antiviral potential. Green tea polyphenols have been shown to possess antimicrobial properties, including HSV, and can thus be a good candidate in HSV prevention[16].

Epigallocatechin gallate (EGCG)

Green tea is made from the *Camellia sinensis* plant. It is rich in catechin polyphenols, in particular, epigallocatechin gallate (EGCG). EGCG has not been found in any other plant, but is the main catechin found in tea[30]. EGCG is on the FDA's list as a safe consumption product[25]. EGCG has received much attention due to its antiviral, antimicrobial, and anticancer activities. Scientists have shown that EGCG is able to inhibit HIV by inhibiting the binding of an envelope glycoprotein (gp120) to its receptor (CD4) [43]. Influenza is also inhibited by EGCG through interaction with the hemagglutinin envelope glycoprotein, which may lead to an alteration in the envelope structure[34]. In addition, EGCG also inhibits hepatitis B by interfering with viral DNA synthesis and thus stopping viral replication[15].

While green tea has also been the subject of several other studies, virologists have tried to focus on whether it has the ability to inhibit different viral infections. In a study conducted with Vero cells and both HSV-1 and HSV-2, researchers concluded that EGCG successfully inhibited HSV infection in a concentration dependent manner. Other green tea catechins were also tested, but only EGCG produced the inhibitory effect. Results also showed that treating Vero cells treated with EGCG following adsorption and entry of HSV-1 does not inhibit the viral production. EGCG has to be applied before the virus is adsorbed in order for an effect to be seen. Also, after treatment of Vero cells with EGCG, the envelope of HSV virions was damaged. As a result, EGCG seemed to have a direct effect on the inhibition of HSV[16].

When ECGC treated HSV-1 and non-treated HSV-1 were immunogold labeled with antibodies against gB, gD, and a capsid protein, there was a 30% and a 40% drop in the treated compared to untreated virions. Therefore, once the virus is treated with EGCG its envelope glycoproteins have a decreased ability to bind to the monoclonal antibodies[16].

There is no doubt now that the EGCG compound in green tea inhibits HSV, but a problem one would face when preparing a topical application with EGCG is that it is highly unstable and oxidizes very quickly, losing its antiviral abilities long before one would be able to apply it. Most of the studies done with EGCG, have to be done with freshly prepared EGCG, otherwise it looses it potent antiviral activity[6, 7]. Also, since EGCG is water soluble, one would not be able to benefit from it as a topical application.

Epigallocatechin gallate-ester (EGCG-ester)

However, it has been proposed that fatty acid esters of the polyphenols (EGCG-ester) can be used as an effective antiviral agent as an ingredient in lipophilic preparations for HSV prevention[22]. Lipid esters of EGCG can be formed either enzymatically or chemically[6]. Data generated from experiments using influenza virus showed that the esters of EGCG are 24 times more effective to inhibit the infection and inactivate influenza virus. Since a high concentration of EGCG is needed in order for an antiviral effect to be seen in influenza, researchers thought of a way to increase its lipid membrane permeability, its chemical stability, and slow down its metabolism· Thus, they introduced an ester chain to EGCG and were able to greatly enhance its anti-influenza virus activity[22]. The same procedure can potentially be applied to HSV.

Both HSV1 and HSV2 pose a serious threat to human populations around the world, and the number of people infected with the disease each year has been shown to increase. The therapeutic usage of EGCG has been suggested previously, but EGCG in its original form would not be adequate to be implemented in a topical application without rapid oxidation and lost of antiviral activity. EGCG-ester, on the other hand, would be an ideal candidate for this purpose.

EGCG-Esterification

The structure of EGCG-ester was purified previously by a team of researchers in China. This was accomplished from a catalytic esterification between green tea polyphenols and C_{16}-fatty acid. The esterification was obtained by mixing 4 grams of green tea polyphenols and 6.5 grams of hexadecanoyl chloride. Next, 50mLs of ethyl acetate and a catalyst at 40°C were added to the mixture. After 3 hours of stirring, the solution was washed three times with 30mLs of deionized water. The organic layer was then allowed to evaporate and further dried by using a vacuum at 40°C. This resulted in 8.7g of powder product. The reaction can be seen in figure 4[6].

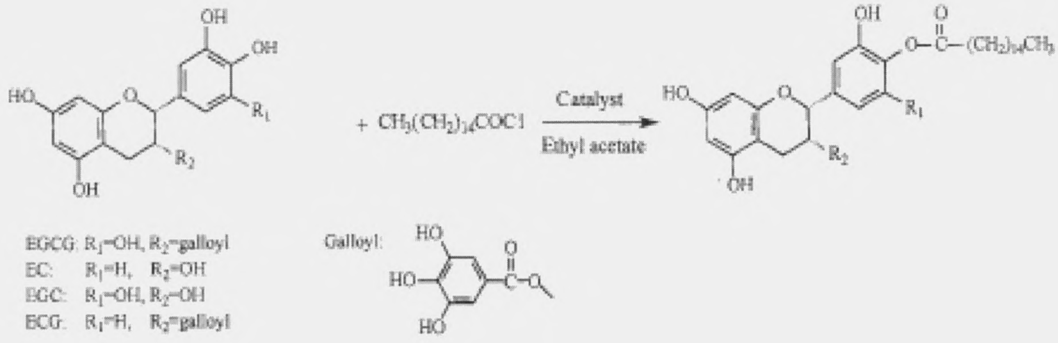


Figure 4. A likely Esterification Between GTP and Hexadecanoyl Chloride [6].

Next, high current chromatography separation was used to purify the EGCG-ester product. A two-phase solvent composed of (1:1) n-hexane-ethyl acetate-methanol-water was used in the separation column. Five grams of EGCG-ester was dissolved in 50mL of the upper phase solution. After purification and HPLC analysis, it was seen that EGCG-ester was successfully purified. The structure of EGCG acyl-derivative can be seen in figure 5[6].

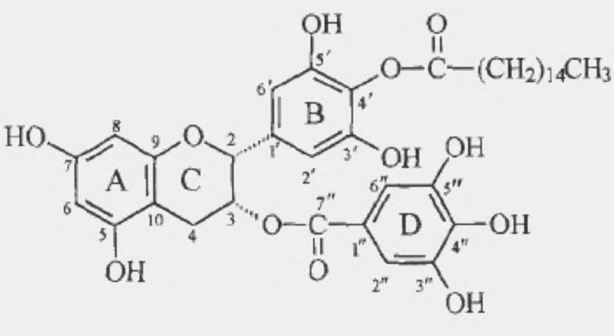


Figure 5. EGCG-acyl Derivative [6].

GHSV-UL46

With a green fluorescence protein tagged HSV, it is possible to study the HSV viral life cycle, and study potential effects of lipophilic tea polyphenol on *in vitro* HSV infections[42]. EGCG-ester may well be a novel and more effective form of EGCG for topical applications. If ester-modified EGCG is proven to have similar or better results against HSV infections, future animal and human studies can then be conducted toward a stable, and effective topical application to prevent human herpes simplex viral infection. Sexually transmitted HSV infects the host cell very quickly; therefore, an efficient antiviral drug needs to be made in order to inhibit HSV prior to the initial viral infection[16].

A HSV1 virus model has been modified by a team of researchers at Washington University School of Medicine. A green fluorescent protein was attached to the viral gene UL46 that encodes tegument protein VP11/12. A schematic representation can be seen in figure 6. The GFP sequence was added to the UL46 gene by using homologous recombination vectors and primers designed to amplify the desired sequences[42].

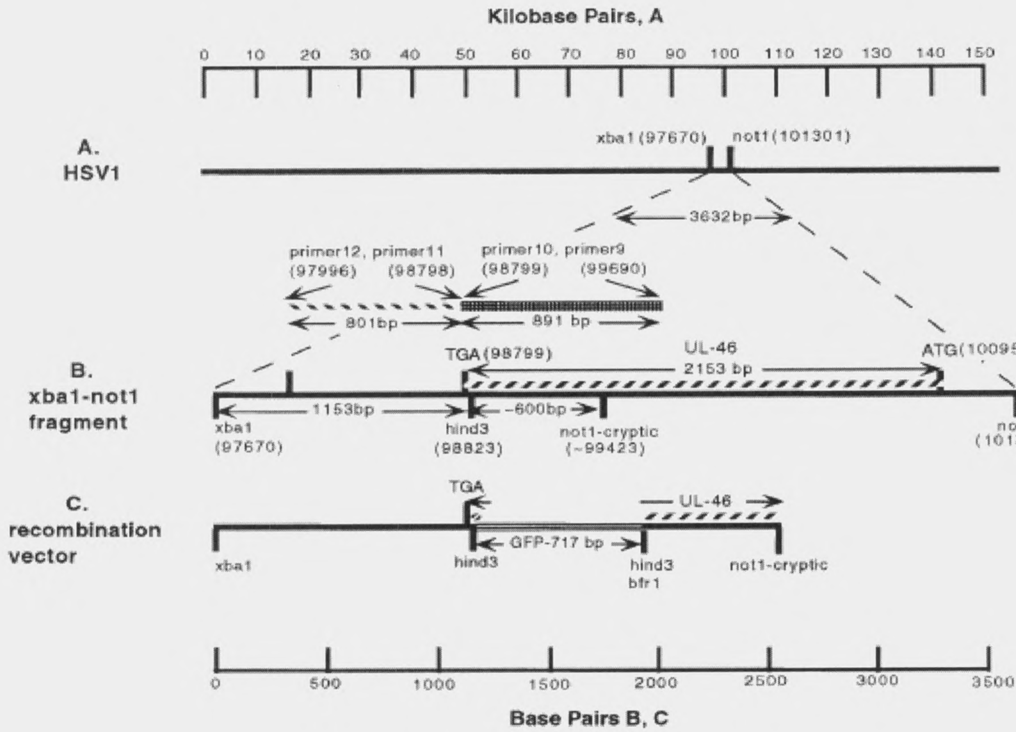


Figure 6. Schematic of How GFP was Introduced to the Gene UL46 of HSV1[42].

Research Proposal

The objectives of this research are as follows:

I. Identify and analyze any effect EGCG or ECGC-ester may have on in *vitro* cultures of VERO cells (green monkey kidney cells) and later on A549 cells.

II. Identify, analyze and compare any effects EGCG or EGCG-ester may have on HSV1 infections.

III. To provide insight into molecular mechanisms of EGCG and EGCG-ester in HSV1.

Successful completion of this project should elucidate the potential inhibitory effect of EGCG and EGCG-ester may have against HSV1. The results of this in vitro study will be of great significance as they may be used to develop a topical application against HSV infections.

Materials and Methods

Cells Culture Maintenance

Vero cells were purchased from ATCC (Manassas, VA) and were cultured in T25 flasks containing Dulbecco Minimal Essential Media (DMEM) with 5% Fetal Bovine Serum (FBS) and 1ug/mL gentamycin at 37ºC and 5% CO_2 until confluent. Cell growth was carefully monitored using an ACCU-SCOPE phase contrast microscope with attached Micrometrics digital camera and Micrometric SE Premium software. To maintain the cultures, confluent monolayers of Vero cells were trypsinized with 500μL of Trypsin/ EDTA for 5 minutes. 4.5mLs of media was then added to the T-25 flask and cells were subcultured into 6 well plates or other T-25 flasks and incubated until confluent.

HSV1-UL46 Virus Maintenance

HSV1-UL46 virus was purchased from ATCC (Manassas, VA). Passage of virus was done in T-25 flasks and cells were allowed to reach complete cytopathic effect (CPE). The media was then collected into 15mLs tornado tubes and it was centrifuged at 1000rpm for 10 minutes to remove cellular debris. The supernatant containing virus was then kept under -80°C until needed.

Preparation of Green Tea Polyphenols Solutions

Samples of EGCG and EGCG-ester were generously given to our laboratoty by Dr. Stephen Hsu from the Medical College of Georgia. The two different polyphenols tested were EGCG and EGCG-ester. The concentration span we used was 12.5, 25, 50, 75, and 100μM.

Cytotoxicity Assay

In the first cytotoxicity experiment, Vero cells were plated in 6 well plates with different concentrations (12.5, 25, 50, 75, 100μM), of EGCG or EGCG-ester. Cells were studied for morphological and proliferation changes 48hrs later using an ACCU-Scope 3002 microscope with a camera attached.

The next experiment, Vero cells were plated in 6 well plates and after a period of 24 hours, different concentrations (12.5, 25, 50, 75, 100μM) of EGCG or EGCG-ester were added to each well respectively. After one hour, the polyphenols were aspirated and the cells were washed with PBS. Cells were studied for morphological and proliferation changes 24hrs later using an ACCU-Scope 3002 microscope with a camera attached.

Cell Viability Assay

Vero cells were plated in 6 well plates and after a period of 24 hours different concentrations of EGCG or EGCG-ester (12.5, 25, 50, 75, 100μM) were added to each well, respectively. After one hour, the polyphenols were aspirated and the cells were washed with PBS. DMEM media was put back into each well and cells were incubated for 24hrs. Cells were then counted using a hemocytometer and trypan blue which stains the dead cells blue and leaves the live cells white.

Cell Proliferation Assay

Cell proliferation kit (G5421, Promega Corp.) was used. This is a colorimetric method for determining cell proliferation. This kit contains a tetrazolium compound and an electron-coupling reagent (phenazine methosulfate). Only live cells will be able to bioreduce the tetrazolium compound into soluble formazan. Thus, the amount of formazan formed and measured at 490nm absorbance is directly proportional to the number of live cells. Cells were plated into 96 well plates and after 24 hrs they were treated with different concentrations (12.5, 25, 50, 75, 100μM) of polyphenols and allowed to adsorb for 1 hour. Polyphenols were then aspirateded and 100μL of fresh DMEM was added back to each well. 24 hours later, 20μL of the MTS reagent was added to every 100μL of cells in media. The plates were incubated at 37°C and 5% CO_2 for 4 hours, and then the absorbance was read using a plate reader.

Viral Titer Study Using Plaque Assay

Vero cells were plated on 6 well plates and allowed to reach confluence. HSV virions were treated for 1 hour with the respective concentration of polyphenols (12.5, 25, 50, 75, 100μM) prior to cell infection. Cells were then infected with HSV and allowed to adsorb for 1 hour. Viruses that had not been absorbed were then aspirated. Plates were then overlaid with a nutrient medium-containing agar. Plaques were visualized by staining cells with crystal violet, observed, and counted within 50 hours.

***Esherichia coli* Ampicillin-Resistant Plasmids w. Green Fluorescent Protein (GFP)**

An ampicillin-resistant strain of *Esherichia coli* was isolated from a pure colony that was originally grown on Luria Broth (LB) in the presence of ampicillin and L-arabinose. The colonies were transferred onto new LB that had been supplemented with 100μl of ampicillin and 100μl of L-arabinose prior to transferring the colonies. The concentration of ampicillin used was 100ng/mL. The concentration of L-arabinose was created using serial dilutions and set in a 1:10 ratio to create a solution of 5% percent.

GFP Expression Study Using a Fluorometer

GFP expression was studied in *Escherichia coli* that contained GFP plasmid, and *Escherichia coli* without GFP plasmid. The fluorometer used was a Tuner Digital

Fluorometer- Model 450. Samples were put into 3mLs of H_2O and serial dilutions were performed from the first 3 mLs of each sample. A standard dilution curve was obtained from the serial dilutions.

Next, Vero cells were grown on T-75 flasks and allowed to reach confluence. HSV1 virions were treated for 3 hours with 75μM of polyphenols prior to cell infection. Cells were then infected with treated and non-treated HSV1-UL46 and allowed to adsorb for 1 hour. Viruses that had not been absorbed were then removed by washing the cells twice with PBS. The cells were then incubated at 37°C for 12hrs. Cells were then trypsinized and pelleted. Finally, cells were resuspended with 3mLs of H_2O and analyzed for GFP expression using a fluorometer. 3ml of water was used as the blank sample. The Gain knob was set to 1000 for higher fluorescence sensitivity.

Fluorescence Microscopy Study

Cells were grown on glass cover slips and allowed to reach confluence. They were then infected with either control HSV or previously treated HSV for 1 hour. Time course studies were performed in which the virus was allowed to adsorb for 1 hour and then aspirated. Cells were washed with PBS and media was added to each well. After a period of 8, 10 and 12 hours, cells were then fixed with a 1:1 acetone and methanol solution and visualized under a Zeiss Axiovision fluorescence microscope using differential interference contrast settings.

DNA Extraction from HSV1 Infected Cells

Cells were grown on 60mm plates and allowed to reach confluency. Cells were then infected with HSV1 treated and HSV1 non-treated for 1 hour at 37°C and 5% CO_2. After absorption time, cells were washed with PBS and media was added to the plates.

After 12 hours, cells were trypsinized and DNA was extracted using the DNeasy Blood and Tissue Handbook (Qiagen 2006). DNA concentration was then measured by using a Nanodrop Specrophotometer.

Polymerase Chain Reaction

Nine sets of primers were designed to prime different regions of the HSV1 genome based on published sequences. Two sets of primers were designed to target HSV1 glycoprotein D. These include 5'-AGACGTCCGGAAACAACCCTACAA-3' for the forward and 5'-ACACAATTCCGCAAATGACCAGGG-3' for the reverse. The second set includes 5'-TTGTTTGTCGTCATAGTGGGCCTC-3' for the forward and 5'TGGATCGACGGTATGTGCCAGTTT-3' for the reverse. Next, two sets of primers were designed to target HSV1 glycoprotein B. They are the following: 5'AGATTCTGCGGTACTGCGATCACT-3' for the forward and 5'-ACGGAACACAAACAAGCACGGATG-3' for the reverse. The second set includes 5'-AGCTGATTATCGCCACCACACTCT3'-for the forward and 5'TGGCGTTGATCTTGTCGATCACCT-3' for the reverse. A third set of primers had been previously designed and published on the book *Herpes Simplex Virus Protocols* by S. Moira Brown and Alasdair R. MacLean. The set includes 5'ATTCTCCTCCGACGCCATATCCACCTT-3' for the forward and 5'-AGAAAGCCCCCATTGGCCAGGTAGT-3' for the reverse.

A set of primers was designed to target the Green Fluorescent protein attached to the UL46 gene of HSV1. It includes 5'-TGACCCTGAAGTTCATCTGCACCA-3' for the forward and 5'-AACTCCAGCAGGACCATGTGAT-3' for the reverse. A second set of primers designed for the GFP had been previously designed[42]. It includes 5'-

GTCAAAGCTTAAGATGGTGAGCAAGG-3' for the forward and 5'-CTTGAAGCTTCTTGTACAGCTCGTCC-3' for the reverse. Finally, two sets of primers were designed to target HSV1 tegument protein VP11/12 that corresponds to the UL46 gene. They are the following: 5'-ACCAAGCCTTGATGCTCAACTCCA-3' for the forward and 5'-ACAACACGGTTCCCGAGAGTTTGA-3' for the reverse. The second set includes5'-ACCAAGCCTTGATGCTCAACTCCA-3' for the forward and 5'ACACAACACGGTTCCCGAGAGTTT-3' for the reverse.

The reaction mix included 1μL of the extracted DNA, 1μL of forward and 1μL of reverse primers, 12.5μL of Master Mix, and 9.5μL of diH2O. The mix was put into PCR tubes and placed into a Labnet MultiGene II thermal cycler (Labnet International, Edison NJ). The reaction profile was initial denaturation at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 30 seconds. The last step included a final extension period at 72°C for 10 minutes. Once the cycle was over, samples were cooled down to 4° and then stored at -20°C freezer for future analysis via agarose gel electrophoresis.

Analysis of PCR Products

Polymerase chain reaction products were analyzed and visualized on 1% agarose gels. Each gel was made by weighing 0.5g of agarose (USB Corporation, Cat No 32802) and combining it with 50mLs of 1X TAE (Tris-Acetate- EDTA) buffer. The mixture was heated for 1 minute in a microwave until the agarose completely dissolved. The mixture was then poured into a gel rig and allowed to solidify. A gel comb was used on one end of the gel in order to produce the wells. Once the gel had solidified samples were loaded into each well (2μL of 10X loading dye with 10μL of PCR product). A Hi-Lo DNA marker was loaded into the first well. The gel was run at 115V for 1 hour. The gel was then stained with Ethidium Bromide for 15 minutes and washed with water for another 15 minutes. The gel was then analyzed under UV light using Kodak Image Station 440 CF (Perkin Elmer Life Sciences, Waltham,MA).

Real Time Polymerase Chain Reaction

A set of primer that primes HSV1 glycoprotein D was designed for use in Real time polymerase chain reaction. This set includes 5"-CAACCCTACAACCTGACCATC-3' for the forward and 5'TTGTAGGAGCATTCGGTGTAC-3'for the reverse. Each tube (except the negative controls- no DNA) contained 10μL of Fast SYBR green master mix (ABI Fast SYBR Green Master Mix), 1μL of forward primer, 1μL of reverse primer, 1μL of genomic DNA and 6μL of Di H_20. The samples were run on an ABI StepOnePlus Real-Time PCR System. The Run methods were: a holding stage at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 30 seconds. Next, the melting curve stage included 95°C for 15 seconds, followed by 60°C for 1 minute, and 95°C for 15 seconds.

Results and Discussion

1. Cytotoxicity Study of EGCG/Vero Cells and EGCG-ester/Vero Cells Without Removing Polyphenols

In order to determine the proper concentrations of EGCG and EGCG-ester to be used for treating HSV1, the effect of polyphenols on the cells were studied. Different concentrations of EGCG and EGCG-ester (12.5, 25, 50, 75, 100μM) were evaluated and both the growth and morphology of the cells were observed at 48hrs. In this experiment, the polyphenols were added at the same time as the cells were plated and were not removed subsequently. The results are shown in figures 7 and 8.

EGCG- 48hrs

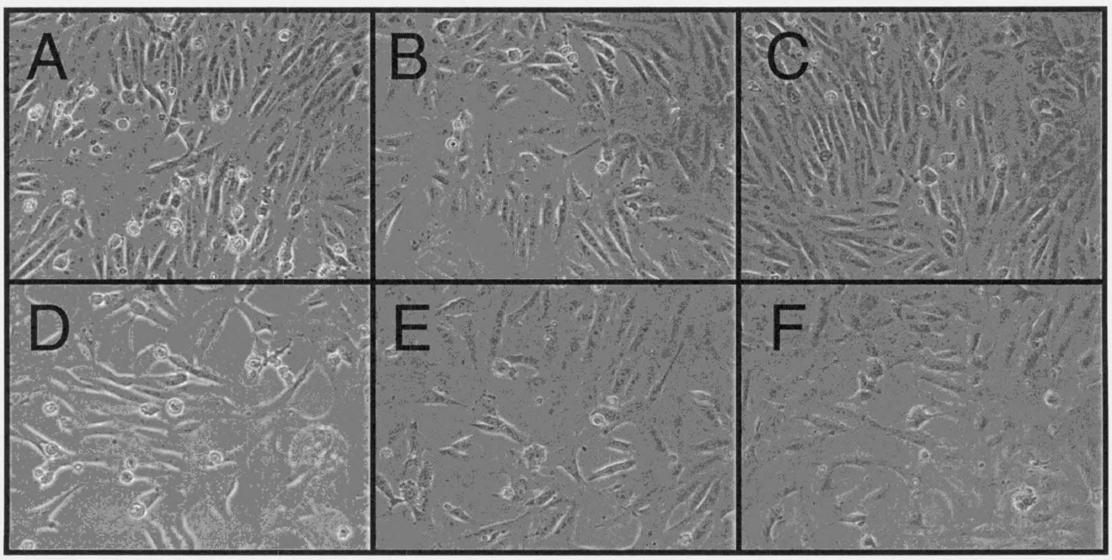


Figure 7. Microscopic Observation (200X) of Cytotoxicity Study of Vero Cells Treated with Different Concentrations of EGCG (A) 0μM; (B) 12.5μM ;(C) 25μM; (D) 50μM; (E) 75μM; (F) 100μM.

EGCG-ester 48hrs

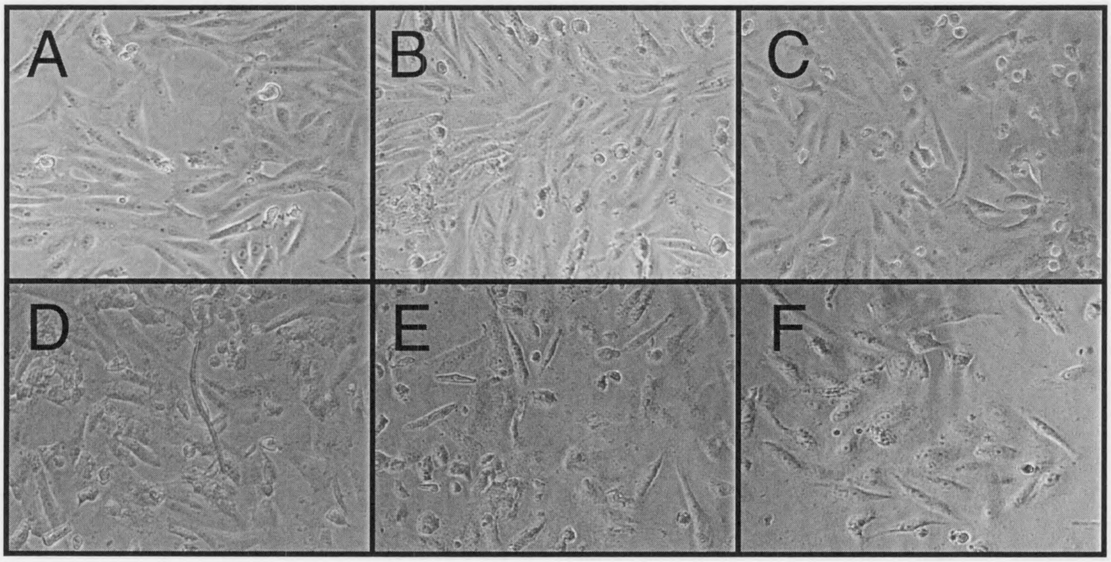


Figure 8. Microscopic Observation (200X) of Cytotoxicity Study of Vero Cells Treated with Different Concentrations of EGCG-ester (A) 0μM; (B) 12.5μM ;(C) 25μM; (D) 50μM; (E) 75μM; (F) 100μM

Experiments to assess the cytotoxicity of green tea polyphenols for cultured eukaryotic cells indicate a moderate toxic behavior of these polyphenols in cell cultures. The results suggest that when EGCG is added to Vero cells, there are no morphological changes seen in the concentrations used. In the presence of EGCG-ester the maximum nontoxic concentration was evaluated to be 75μM. At the concentration of 100μM, cell morphology is affected to a certain extent.

2. Cytotoxicity Study of EGCG/Vero Cells with Removing the Polyphenols (24hrs):

The effect of different concentrations of EGCG-ester on Vero cells was also observed at 24 hrs. Since in the procedures for HSV1 infected cultures, after 1 hr adsorption, the media and unabsorbed virus are aspirated, it is important to determine the effect of polyphenols under similar conditions. In this study, Vero cells were plated first for 24 hours and then different concentrations of EGCG-ester (12.5, 25, 50, 75, 100μM) were added to the cells and allowed to adsorb for 1 hour. EGCG-ester was then aspired, and cells were observed 24 hours later. The results are shown in figure 9.

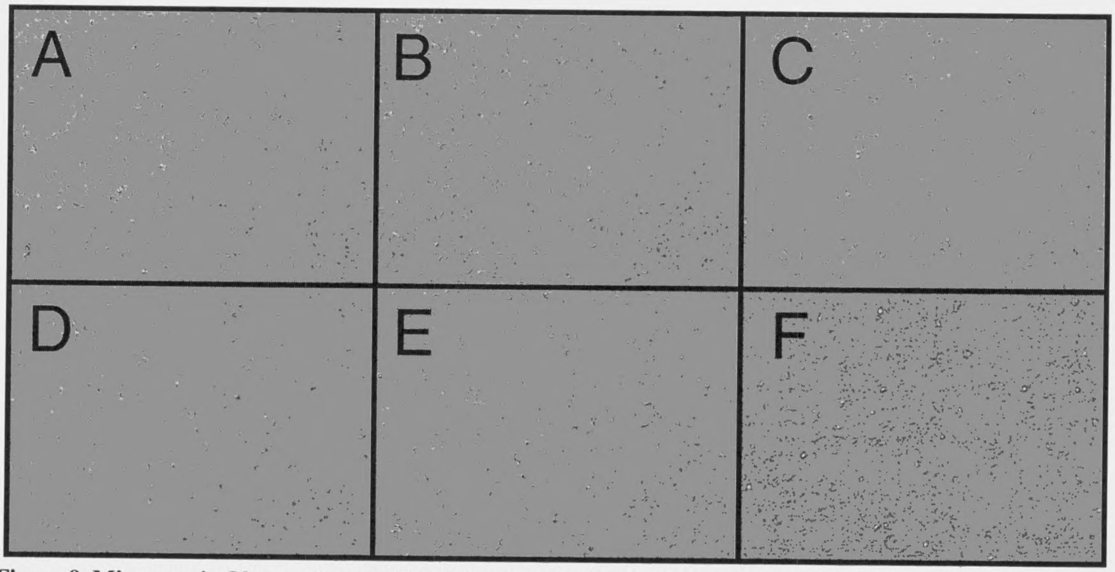


Figure 9. Microscopic Observation (100X) of Cytotoxicity Study of Vero Cells Treated with Different Concentrations of EGCG-ester (A) 0μM; (B) 12.5μM; (C) 25μM; (D) 50μM; (E) 75μM; (F) 100μM.

As indicated in figure 9, as the concentration of EGCG-ester is increased, cell morphology was not greatly affected. This is therefore the method used for subsequent experiments.

3. Cell Viability Study of EGCG-ester on Vero Cells

The cytotoxicity study indicated that EGCG and EGCG-ester at concentration of 12.5 to 75μM do not change cell morphology. The cell viability was then determined by using trypan blue and hemacytometer direct cell count to detect the effect of EGCG-ester on Vero cells. The results are shown in figure 10 and table 1.

As the concentration of EGCG-ester is increased, the percentage of cell death is not changed. Therefore, the maximum nontoxic concentration, 75μM EGCG-ester, can be used to treat HSV1 and study its inhibitory effects. Figure 11 illustrates a graph of hemacytometer count of live cells that appear white and dead cells that stain blue.

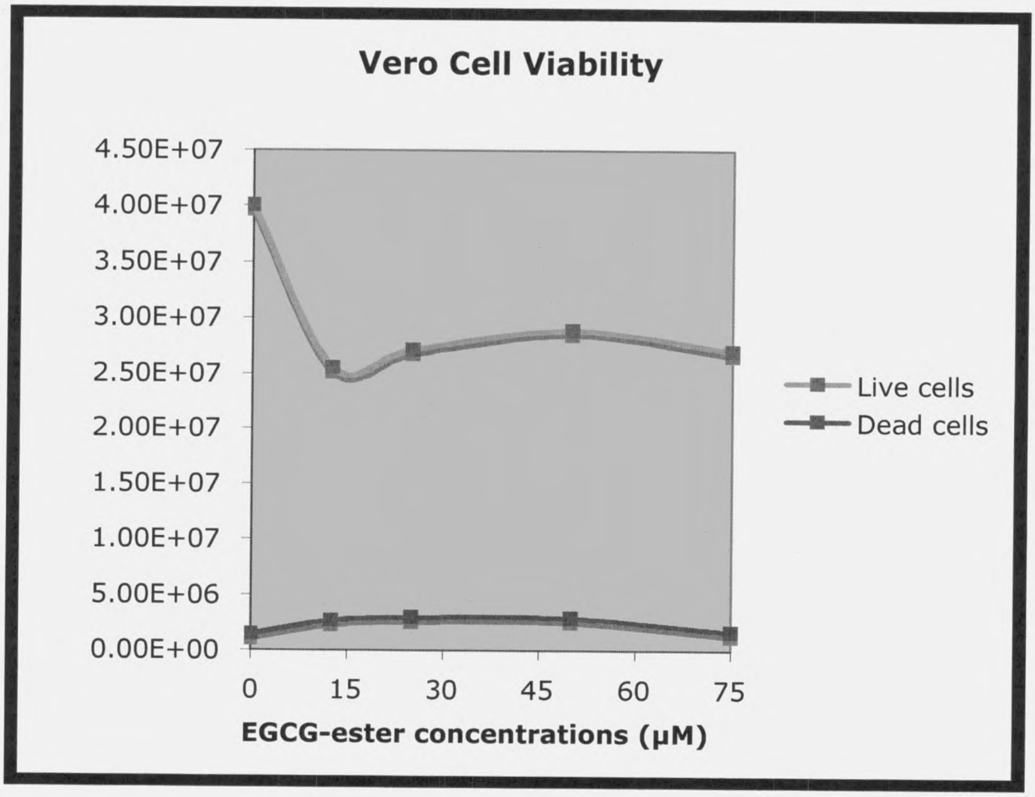


Figure 10. Cell Viability Studies of Vero Cells Treated with Different Concentrations of EGCG-ester

Live cells
Dead cells

	0µM	12.5µM	25µM	50µM	75µM
Live cells	4.01E+07	2.56E+07	2.72E+07	2.89E+07	2.71E+07
Dead cells	1.45E+06	2.70E+06	2.95E+06	2.90E+06	1.70E+06
% of death	3.60%	10%	10%	10%	10%

Table 1. Cell Viability Data of Vero Cells Treated with Different Concentrations of EGCG-ester

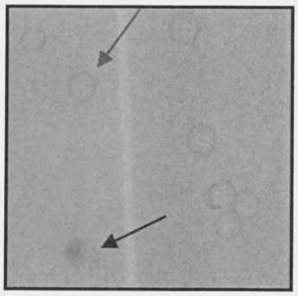


Figure 11. Microscopic Observation under 400X of Vero Cells on a Hemacytometer.

→ **Live cells**
→ **Dead cells**

4. Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS)

The previous results suggested that EGCG-ester does not show significant effect on Vero cell cytotoxicity and viability. In this study, cell proliferation was examined under the same conditions as described before. Each experiment was assayed in triplicates, and absorbance of this colorimetric assay was recorded at 490nm using a 96 well ELISA plate reader. The results are shown in table and figure 12.

A	EGCG	EGCG	EGCG	EGCG	EGCG	Control
	12.5uM	**25uM**	**50uM**	**75uM**	**100uM**	
	0.601	0.616	0.589	0.652	0.730	0.780
	0.615	0.628	0.632	0.645	0.688	0.793
	0.606	0.620	0.587	0.645	0.677	0.752
Mean	**0.607**	**0.621**	**0.603**	**0.647**	**0.698**	**0.775**

B	EGCG-ester	EGCG-ester	EGCG-ester	EGCG-ester	EGCG-ester	Control
	12.5uM	**25uM**	**50uM**	**75uM**	**100uM**	
	0.642	0.668	0.663	0.582	0.691	0.780
	0.647	0.651	0.698	0.689	0.741	0.793
	0.574	0.621	0.667	0.687	0.719	0.752
Mean	**0.621**	**0.647**	**0.676**	**0.653**	**0.717**	**0.775**

Table 2. Cell Proliferation Data with Mean and Standard deviation of Vero Cells Treated with Different Concentrations of EGCG and EGCG-Ester (A) EGCG/Vero (B) EGCG-ester/Vero.

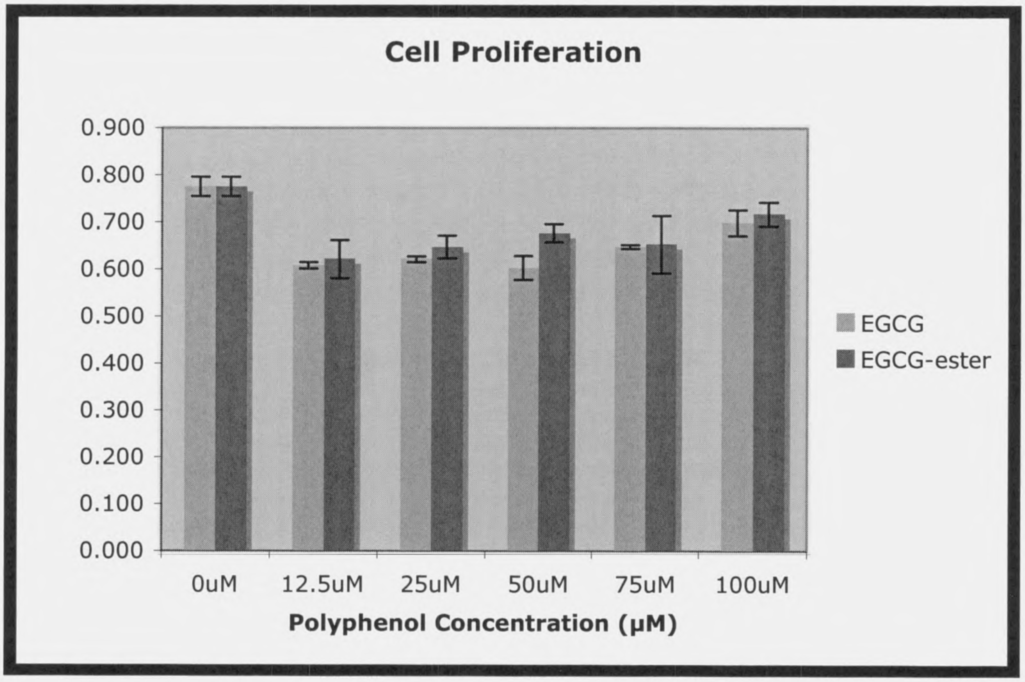


Figure 12. Cell proliferation Studies of Vero Cells Treated with Different Concentrations of EGCG and EGCG-ester

EGCG/Vero cells- 48hrs

EGCG-ester/Vero cells- 48hrs

The proliferation assay results in table 2 and figure 12 indicate that both EGCG and EGCG-ester are not inhibiting cell proliferation to a great extent. In comparison to controls, cells exhibited only a small decrease in levels of 490nm absorbance. This indicates that most of the cells are inducing high reduction of MTS tetrazolium to formazan and therefore retain high cell viability. As seen, increases in the concentrations of EGCG and EGCG-ester up to 75μM do not lead to a significant reduction in cell proliferation.

5. The Effect of EGCG and EGCG-ester on the Production of HSV1 Particles

Viral titer was determined to study the effect of 50μM of EGCG and EGCG-ester on the viral particle production by using plaque assay. Serial dilutions of viral lysates were performed from 10^{-1} to 10^{-7} and cells were then infected respectively with the dilutions. Results can be seen in figure 13. In the HSV1/Vero cells experiment, plaques were observed from 10^{-3} to 10^{-5} viral lysate dilution and the viral titer was 1.25x 10^{6} PFU/ml. In the 50μM EGCG-HSV1/ Vero cells experiment, plaques were only observed from 10^{-3} to 10^{-4}, and the viral titer was $1x10^{5}$ PFU/ml. Lastly, in the 50μM EGCG-ester-HSV1/Vero cells experiment, there was no plaque formation seen in any of the viral lysate dilutions. The results are summarized in figure 14, which demonstrates, a 10-fold decrease when comparing HSV1/ Vero titer to EGCG-HSV1/Vero titer. It also shows an even larger decrease in EGCG-ester HSV1/Vero titer compared to HSV1/Vero titer. This indicates that EGCG-ester is more potent in inhibiting HSV1 when compared to EGCG.

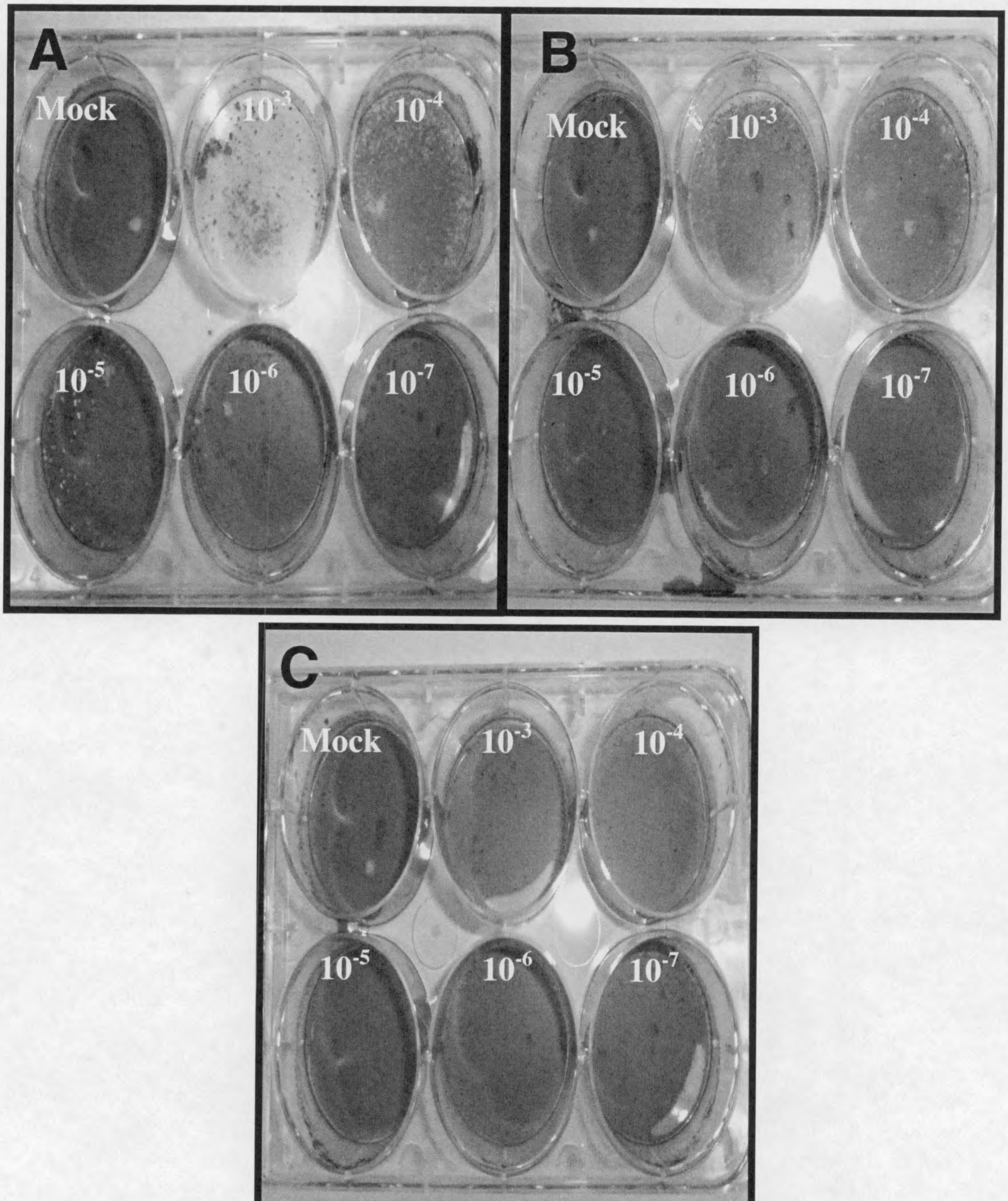


Figure 13. Plaque Assays of HSV1, HSV1 Treated 50μM EGCG , HSV1 Treated 50μM EGCG-ester. (A) Titer of HSV1/Vero (10^{-3} to 10^{-7}); (B) Titer of 50μM EGCG- HSV1/Vero (10^{-3} to 10^{-7}; (C) Titer of 50μM EGCG-ester HSV1/Vero (10^{-3} to 10^{-7}).

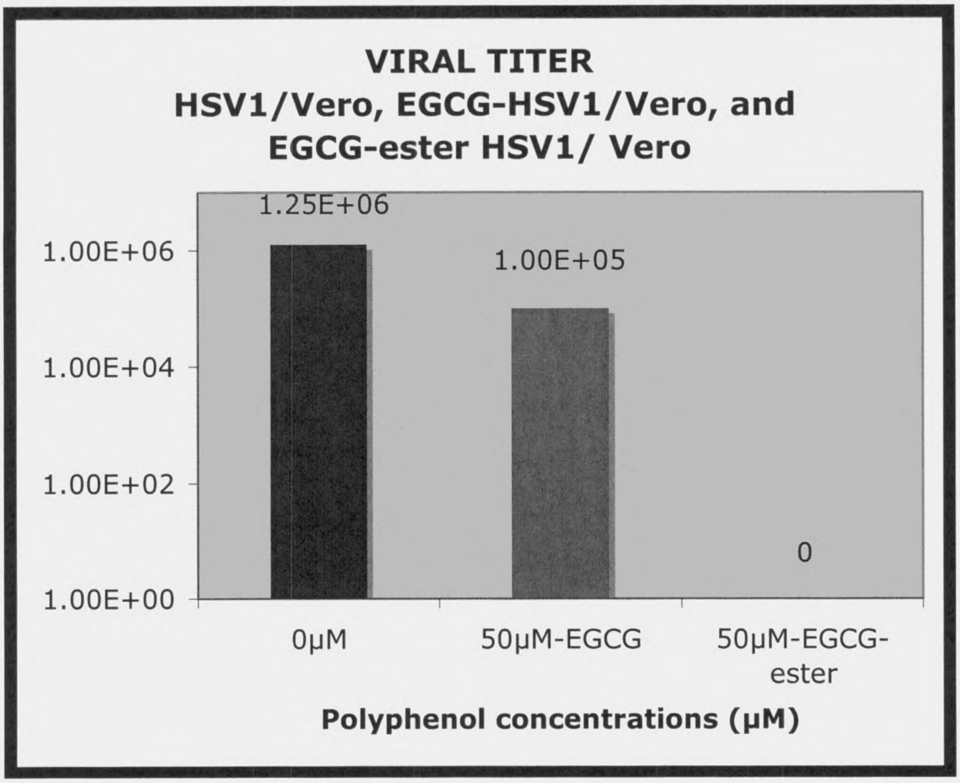


Figure 14. Viral Titer Comparison of HSV1, HSV1 Treated 50µM EGCG, HSV1 Treated 50µM EGCG-ester.

In order to obtain the minimum inhibitory concentration of EGCG-ester on HSV1, different concentrations (12.5,25,50,75µM) of EGCG-ester were used at lower dilution of viral lysate (10^{-1} and10^{-2}). The results are shown in figure 15. Figure 15(A) shows the 10^{1} dilution and 15(B) shows the 10^{-2} dilution. The results indicated that as the concentration of EGCG-ester is increased, HSV1 titer is decreased. As seen in figure 16, at a concentration of 50µM EGCG-ester and above, the HSV1 ability to form plaques is reduced by >99%.

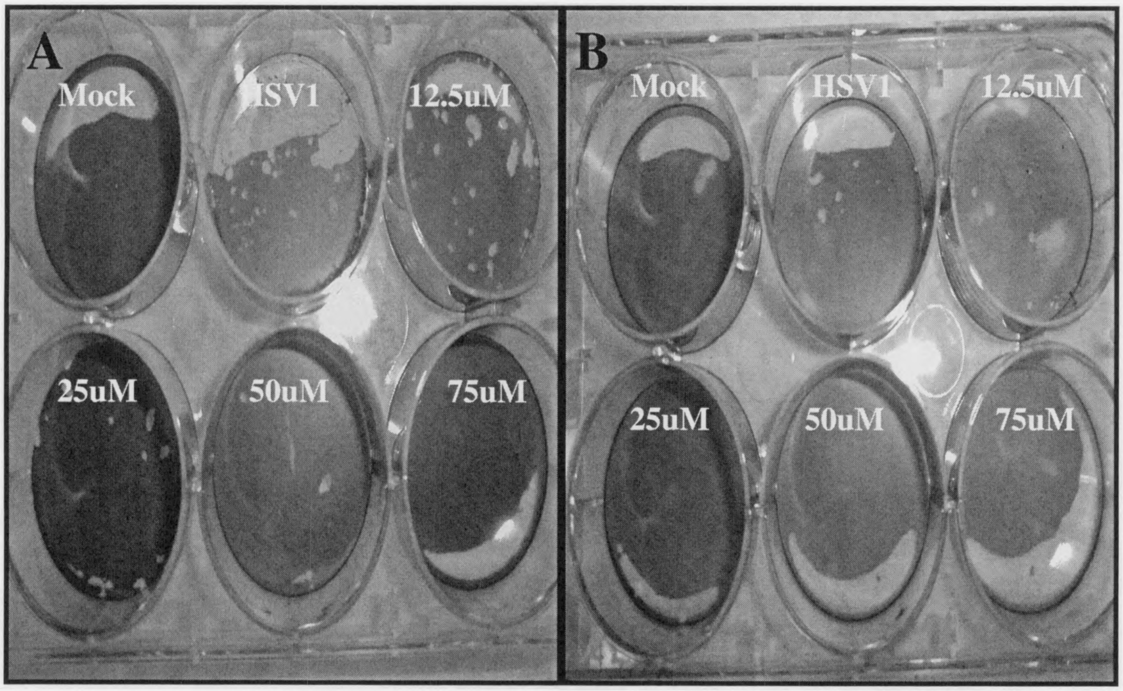


Figure 15. Viral Titer of HSV1/Vero and Different Concentrations of EGCG-ester HSV1/Vero. (A) HSV1/Vero and Different Concentrations of EGCG-ester HSV1/Vero at 10^{-1} Dilution. (B) HSV1/Vero and Different Concentrations of EGCG-ester HSV1/Vero at 10^{-2} Dilution.

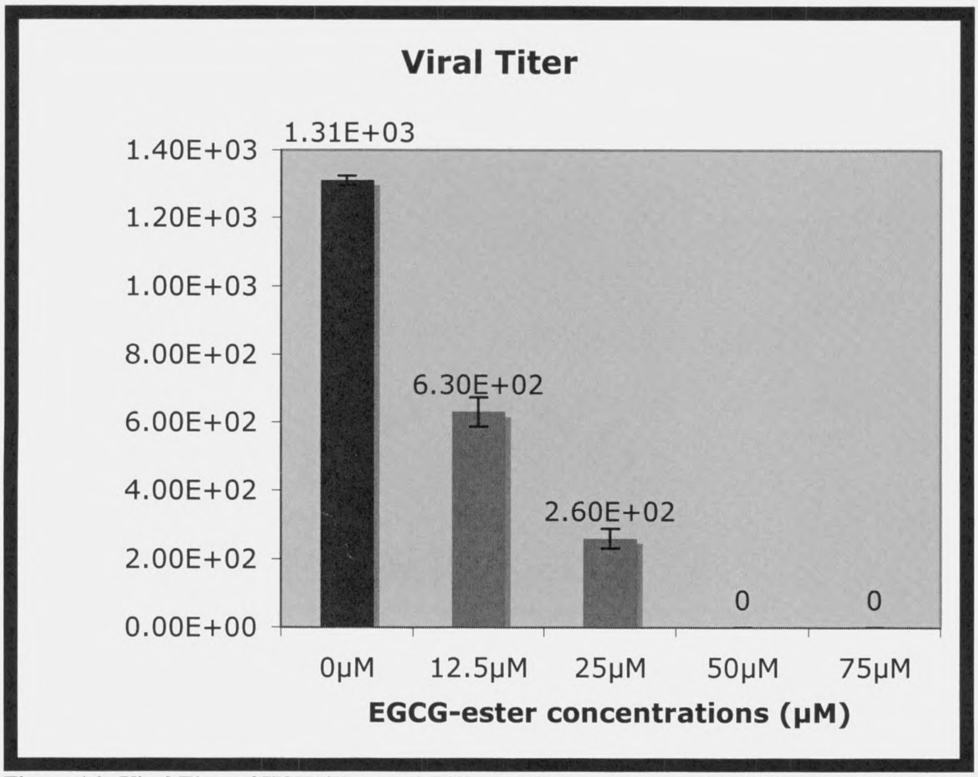


Figure 16. Viral Titer of HSV1/Vero and Different Concentrations of EGCG-ester-HSV1/Vero

6. Study of Green Fluorescence Protein Expression in HSV1/Vero Cells and EGCG-ester-HSV1/Vero Cells

The viral model system used in this study, HSV-GFPUL46, carries a green fluorescence protein (GFP) tag on the HSV1 UL46 gene. In this study, a fluorometer was used to measure the expression of green fluorescence protein in infected Vero cells. In order to quantitatively determine the GFP expression in cells, a standard dilution curve was generated with *Escherichia coli* (*E.coli*) containing a GFP insert. *Escherichia coli* with no GFP insert was also used as a negative control. Serial dilutions of *E.coli* cultures were prepared as described in the Materials and Methods section. The results are shown in table 4 and figure 17.

As seen in the results, a linear dilution curve was successfully generated with serial diluted cultures of *E.coli* with GFP. The negative control shows very low readings. This experiment demonstrated that the use of the fluorometer is appropriate to provide a quantitative measure for GFP expression.

Ecoli+GFP	Ecoli-GFP	Dilutions
1000	22	1/1
541	13	1/2
284	7	1/4
148	4	1/8
75	1	1/16
35	0	1/32
17	0	1/64
8	0	1/128
4	0	1/356
2	0	1/712

Table 4. Fluorometer Data of GFP Expression in *E.coli* with a GFP Plasmid Insert and E.coli with no GFP Plasmid Insert.

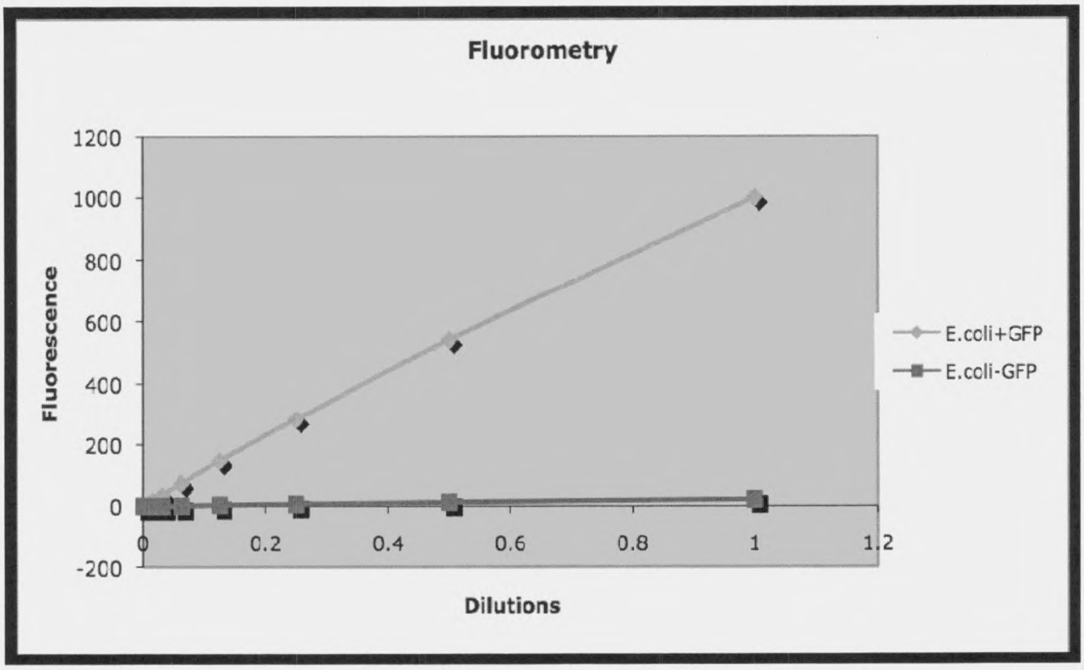


Figure 17. Fluorometer Study of GFP Expression.

***E.coli* with GFP plasmid.**

***E.coli* with no GFP plasmid.**

This experiment demonstrates that the fluorometer can be used to study and quantitatively measure GFP expression in both HSV1/Vero cells and EGCG-ester-HSV1/Vero cells. In this experiment, Vero cells only, HSV1/Vero cells, and 75μM EGCG-ester-HSV1/Vero cells were used to quantitatively measure GFP expression. The results are shown in figure 18.

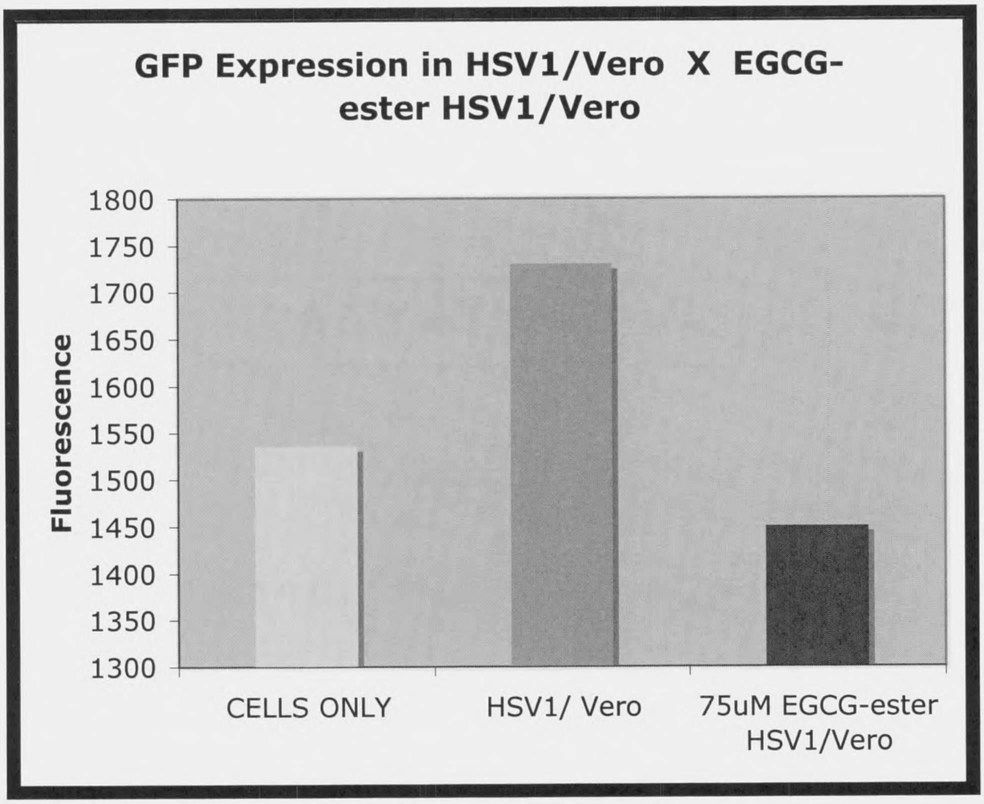


Figure 18. Fluorometer Study of HSV1 GFP Expression

Cells only
HSV1/Vero
75uM EGCG-ester HSV1/Vero

As seen, GFP expression is much lower in 75μM-EGCG-ester HSV1/ Vero cells compared to HSV1/Vero cells. Thus, expression of GFP as part of viral biosynthesis is decreased when HSV1 is treated with EGCG-ester.

7. Fluorescence Microscopy Observation of HSV1/Vero Cells and EGCG-ester-HSV1/Vero Cells

The lytic cycle of HSV1/ Vero cells was observed at 8, 10, 12 hours post-infection to study the molecular changes within the infected cells. HSV1/Vero cells were used as positive controls and Vero cells were used as negative control. 75μM EGCG-ester-HSV1/Vero cells were also monitored and compared to controls. GFP-HSV1, DAPI stained nucleus and lysosome stain were used to identify cytophatic effects on Vero cells. The course study was performed in triplicates and the representative fluorescence images are shown in figures 19-26.

A single cell without HSV1 infection and EGCG-ester treatment are showed in figure 19. The images are used as a reference to compare to all the treated samples. In the image with DAPI stained nucleus, there is very smooth margin and no granules are observed within the nucleus. In the green fluorescence image, there is an obvious green background, but no green fluorescence particles. In the lysosome stain, although there is a red background, there are no obvious fluorescence red particles inside the cells.

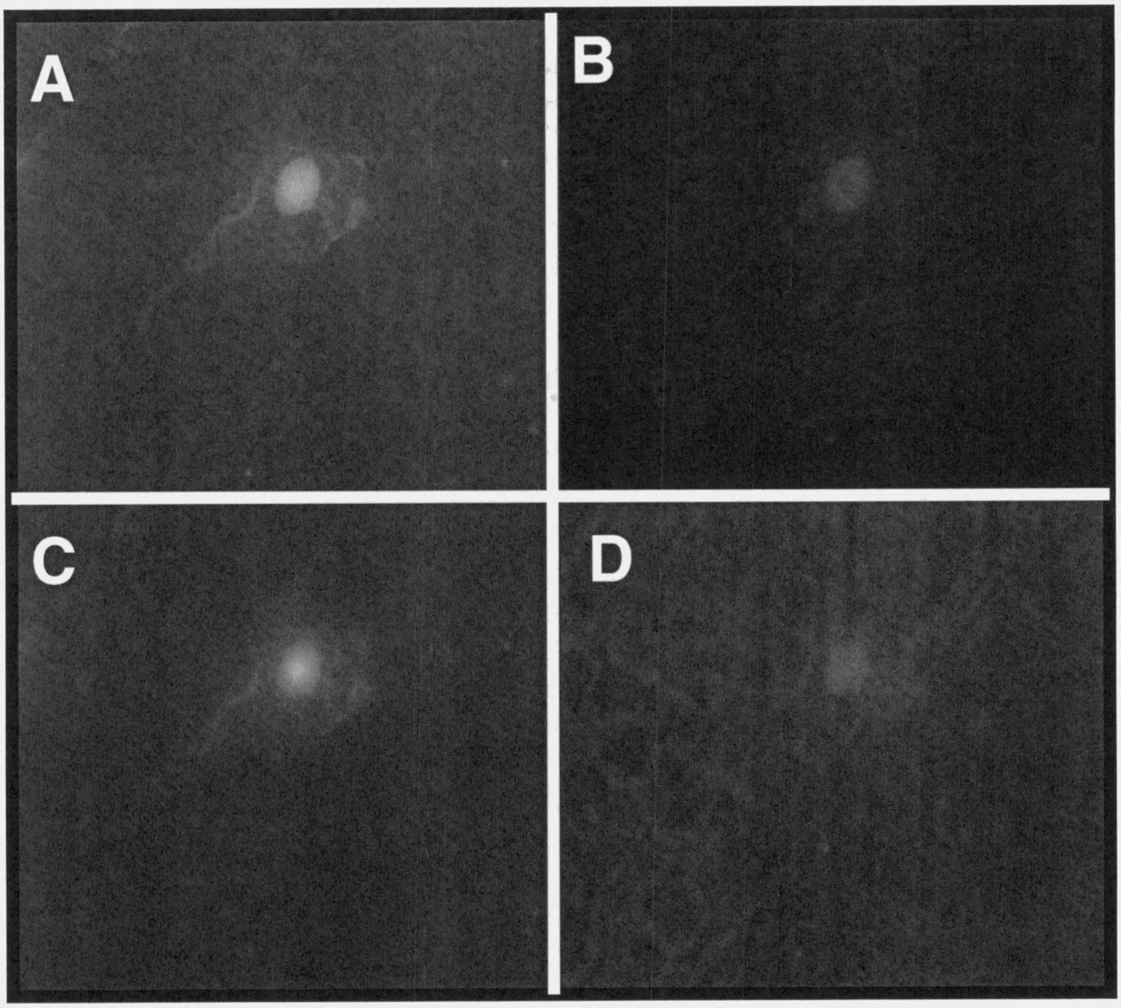


Figure 19: Fluorescence Microscopic Observation of Single Vero Cell at 400X. (A) Overlay image of GFP and DAPI stain; (B) DAPI stain; (C) GFP; (D) Lysosome stain.

Next, uninfected Vero cells in a monolayer without any treatment were observed under 400X magnification. The images of the phase contrast microscopy, DAPI, GFP, DAPI+GFP, DAPI+GFP+Lysosome stains and an all stain-overlaid image are shown in figure 20. Overall, figure 20 provides information of the cell morphology and the background for all the fluorescence staining.

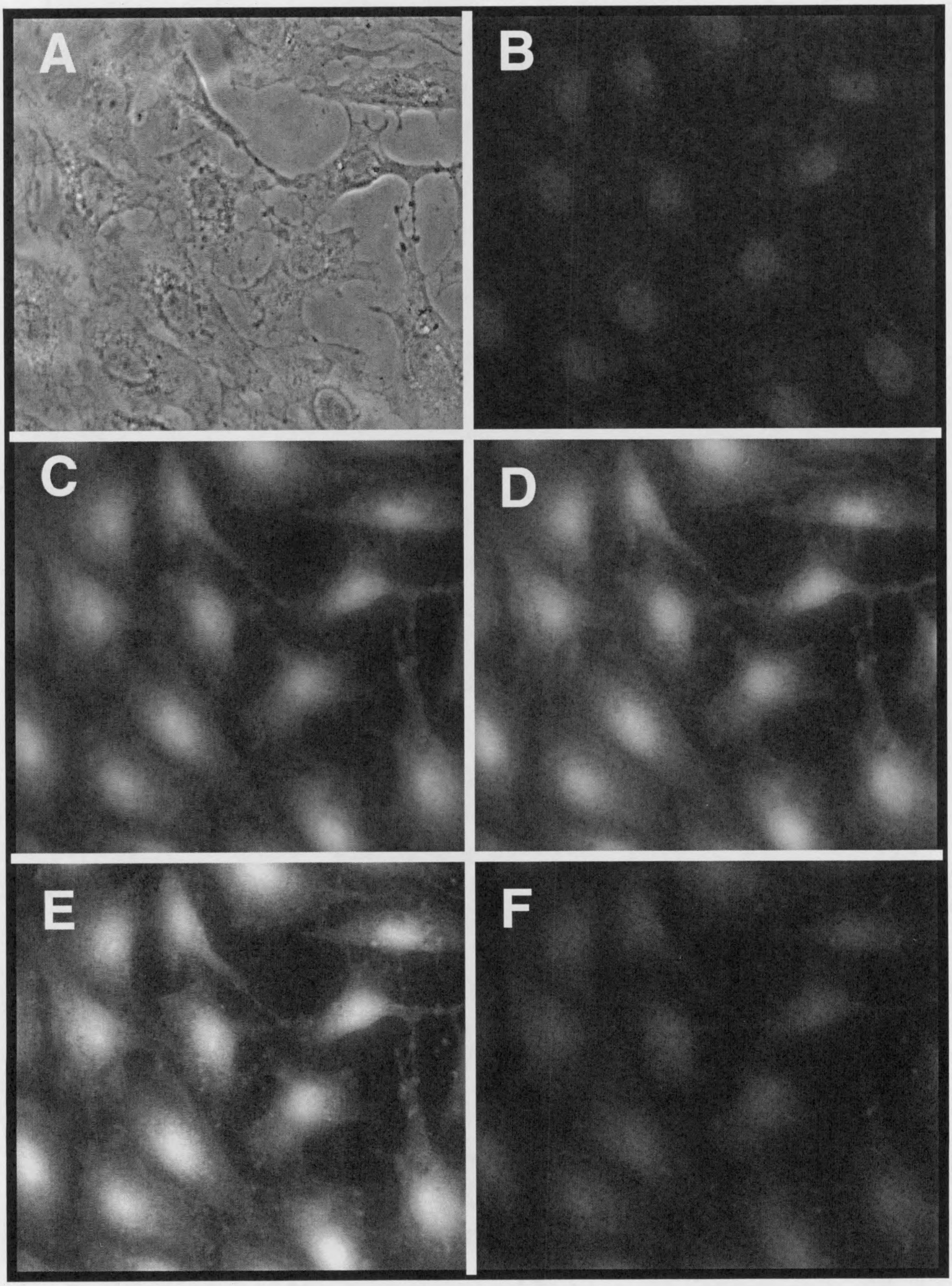
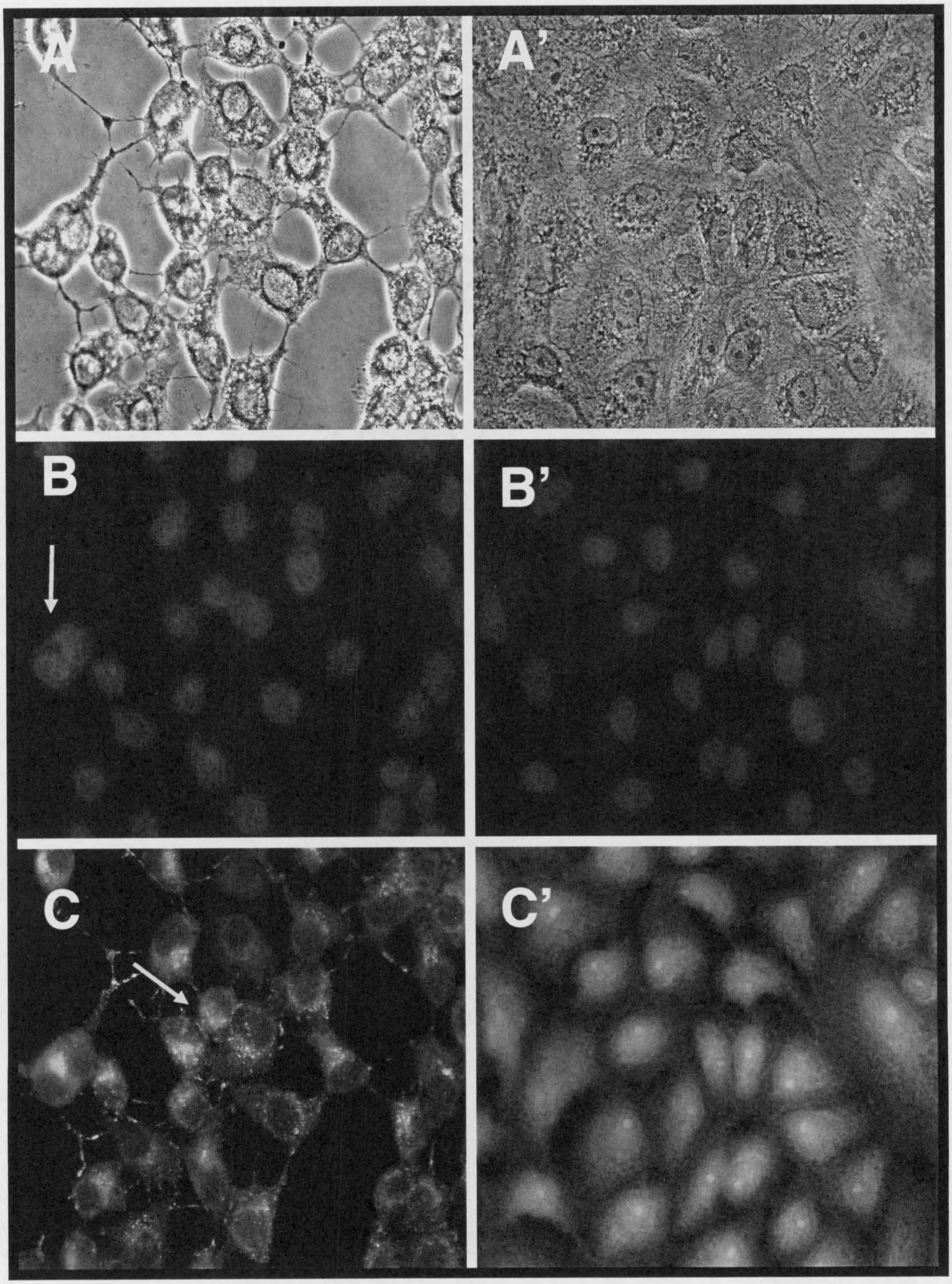


Figure 20. Fluorescence Microscopic Observations of Vero Cells Monolayer (400x). (A) Phase contrast; (B) DAPI stain; (C) GFP; (D) GFP+DAPI; (E) GFP+DAPI+Lysosome (F) Lysosome stain.

HSV1/Vero cells and EGCG-ester-HSV1/Vero cells were observed 8 hrs post infection as shown in figure 21. Phase contrast image for HSV1/Vero cells are very different from EGCG-ester-HSV1/Vero cells. The cell morphology was greatly changed, indicating lytic viral infection in the HSV1/Vero cells. Cell morphology of EGCG-ester-HSV1/Vero is very similar to the cells alone image, illustrated in figure 20. The results imply that HSV1 was not able to continue with its lytic cycle when treated with EGCG-ester.

When staining the HSV1/Vero cells with DAPI stain, there is an obvious difference in the cell nucleus compared to the EGCG-ester-HSV1/Vero cells. Significant granulation as well as demargination is only seen in the HSV1/Vero cells, demonstrating a normal occurrence of viral infection as indicated by the yellow arrow in figure 15(B). EGCG-ester- HSV1/vero cells still maintained their nuclear integrity and show similar appearance to the cells only image.

Green fluorescence images clearly indicate a significant amount of GFP expression in the HSV1/Vero cells but almost none in the EGCG-ester-HSV1/Vero cells as indicated by the yellow arrow in figure 21(C). In addition, the lysosome stain demonstrates lysosome activation in the HSV1/vero cells but not in the EGC-ester-HSV1/vero cells, as indicated by the yellow arrow in figure 21(D). Since there is a strong background in the fluorescence stains, black and white images (figures 24-26) were also obtained to further illustrate the phenomenon observed in figure 21.


A
A'
B
B'
C
C'

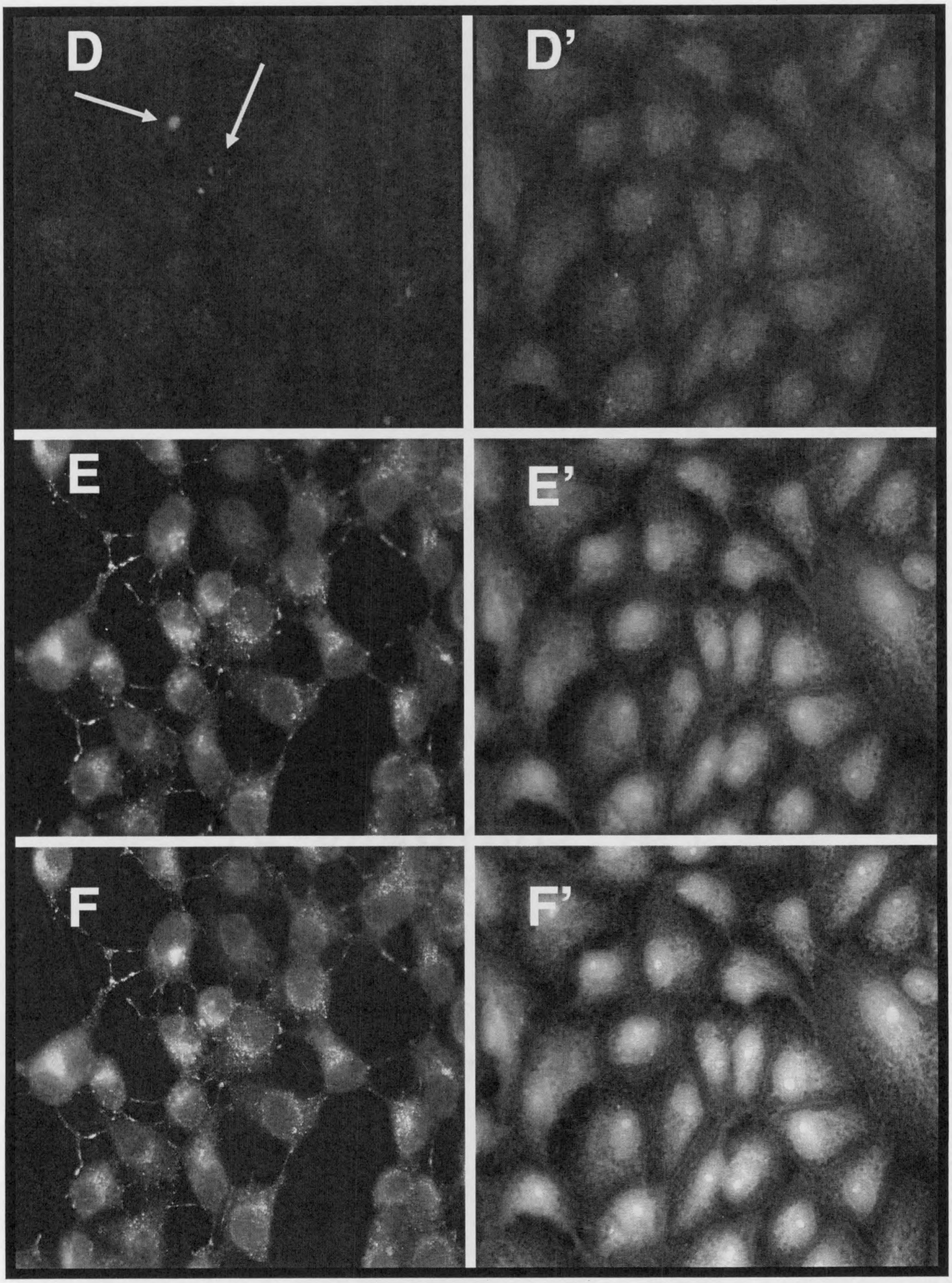
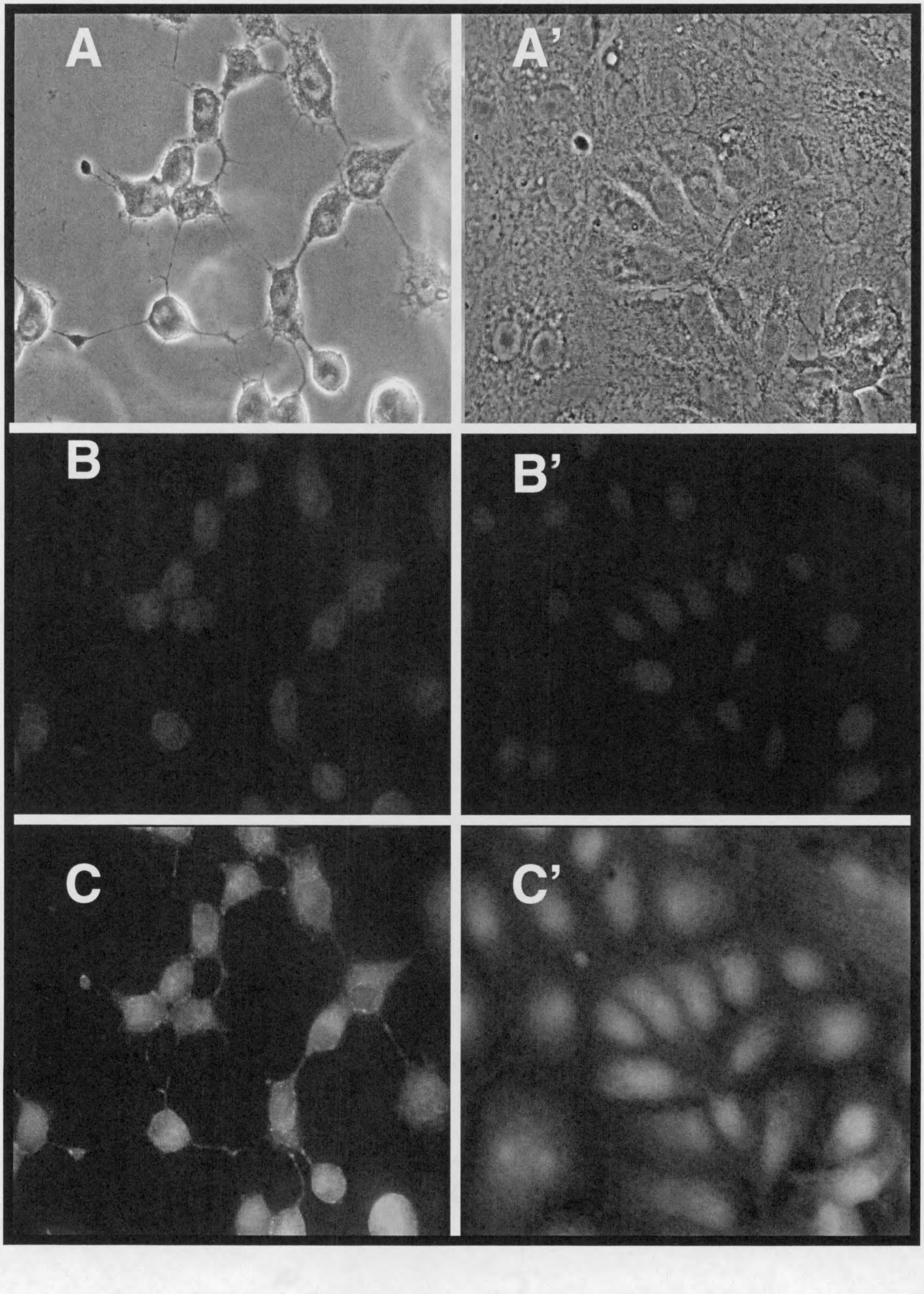


Figure 21. Fluorescence Microscopy Data of 8hrs Post-Infection for HSV1 Infected Vero Cells and EGCG-ester Treated Infected Vero Cells (A) Phase Contrast of HSV1/Vero; (A') Phase Contrast of EGCG-ester-HSV1/Vero; (B) DAPI stain of HSV1/Vero; (B') DAPI stain of EGCG-ester-HSV1/Vero; (C) GFP of HSV1/Vero; (C') GFP of EGCG-ester-HSV1/Vero; (D) Lysosome stain of HSV1/Vero; (D') Lysosome stain of EGCG-ester-HSV1/Vero; (E) GFP+DAPI of HSV1/Vero; (E') GFP+DAPI of EGCG-ester-HSV1/Vero; (F) GFP+DAPI+lysosome of HSV1/Vero; F) GFP+DAPI+lysosome of EGCG-ester-HSV1/Vero.

In the 10 hours post infection images, the cell morphology is significantly changed in HSV1/Vero cells, even more so than 8 hours post infection. EGCG-ester-HSV1/Vero cells look very similar to the cells only image. In the HSV1/Vero cells DAPI stain, there is even further granulation and demargination in the cell nucleus. Green fluorescence particles are also observed in the HSV1/Vero cells only. Lysosome stain showed no significant activation for HSV1/Vero cells in the 10 hours post infection. On the contrary, EGCG-ester-HSV1/Vero cells demonstrate similar results to the 8 hours post infection in all of the fluorescence images.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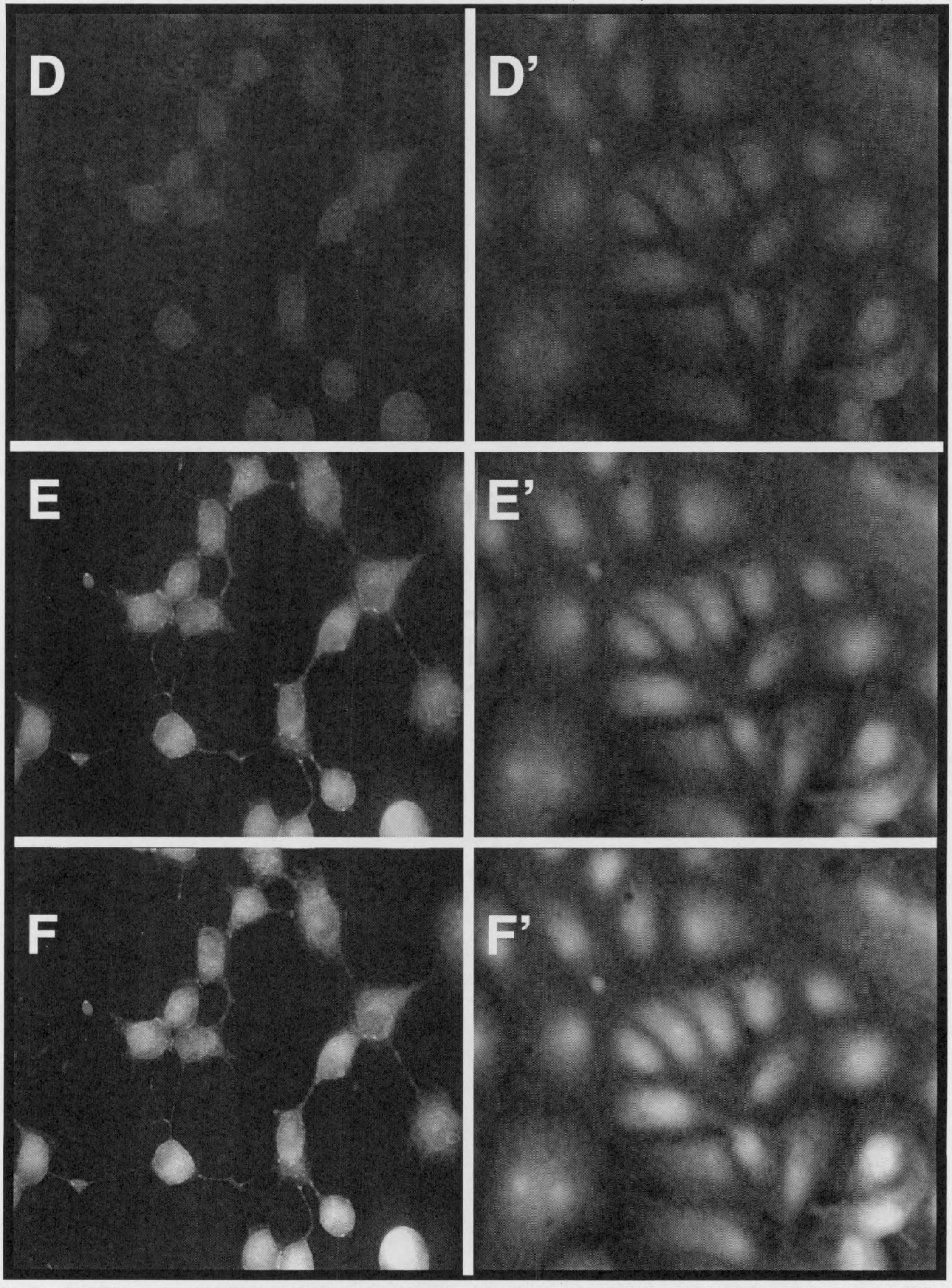
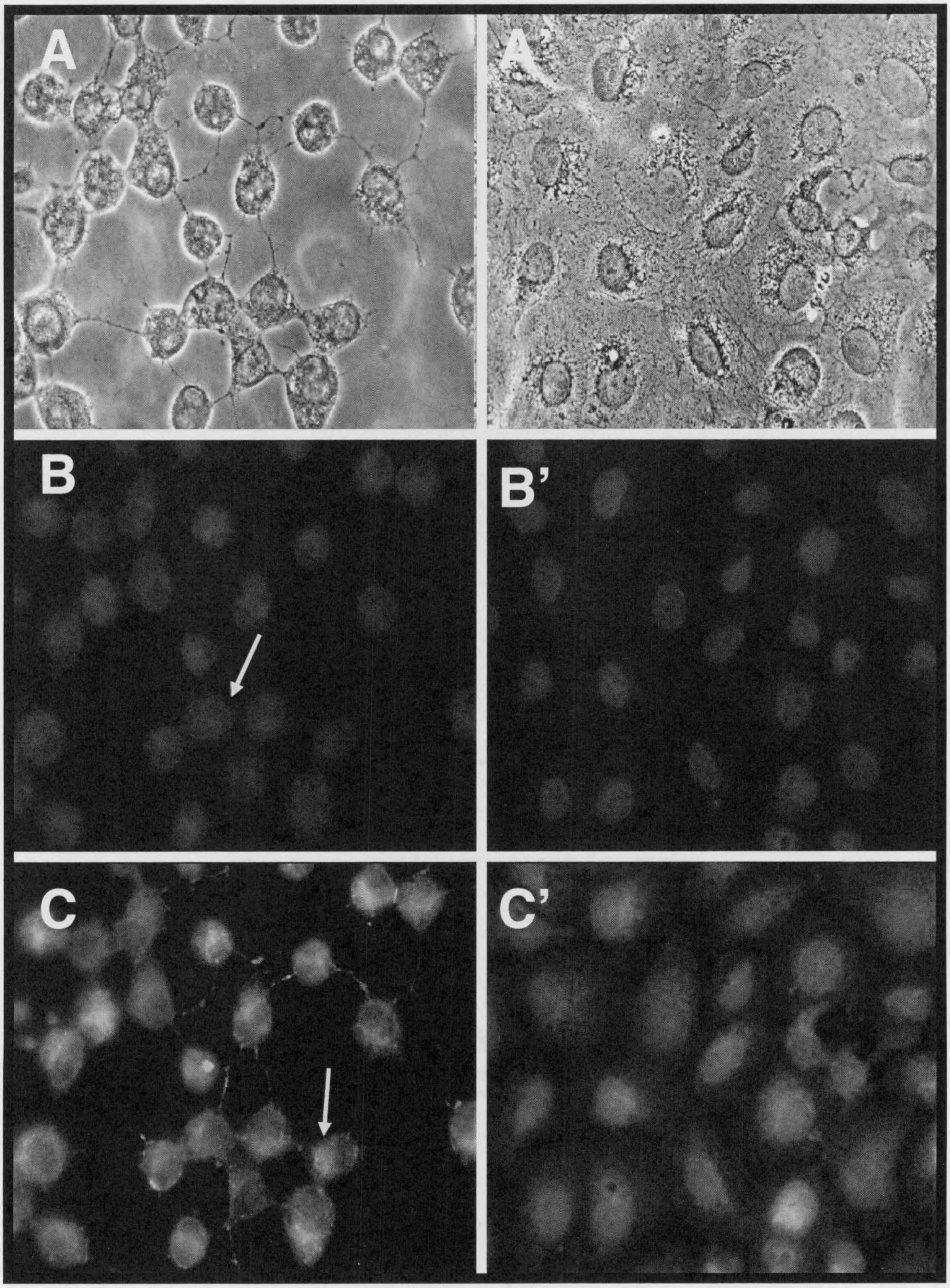


Figure 22. Fluorescence Microscopy Data of 10hrs Post- Infection for HSV1 Infected Vero Cells and EGCG-ester Treated Infected Vero Cells (A) Phase Contrast of HSV1/Vero; (A') Phase Contrast of EGCG-ester-HSV1/Vero; (B) DAPI stain of HSV1/Vero; (B') DAPI stain of EGCG-ester-HSV1/Vero; (C) GFP of HSV1/Vero; (C') GFP of EGCG-ester-HSV1/Vero; (D) Lysosome stain of HSV1/Vero; (D') Lysosome stain of EGCG-ester-HSV1/Vero; (E) GFP+DAPI of HSV1/Vero; (E') GFP+DAPI of EGCG-ester-HSV1/Vero; (F) GFP+DAPI+lysosome of HSV1/Vero; F) GFP+DAPI+lysosome of EGCG-ester-HSV1/Vero.

In the 12 hours post infection experiment, the cell morphology remained similar to cells at 8 and 10 hrs post infection. More granulation and demargination are seen at this stage of infection in the HSV1/Vero cells when stained with DAPI. Green fluorescence particles are also observed. No lysosome activation was observed. EGCG-ester HSV1/Vero cells shows similar results to the 8 and 10 hours post infection cells in all the fluorescence images as well as similar to the cells only images.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C'

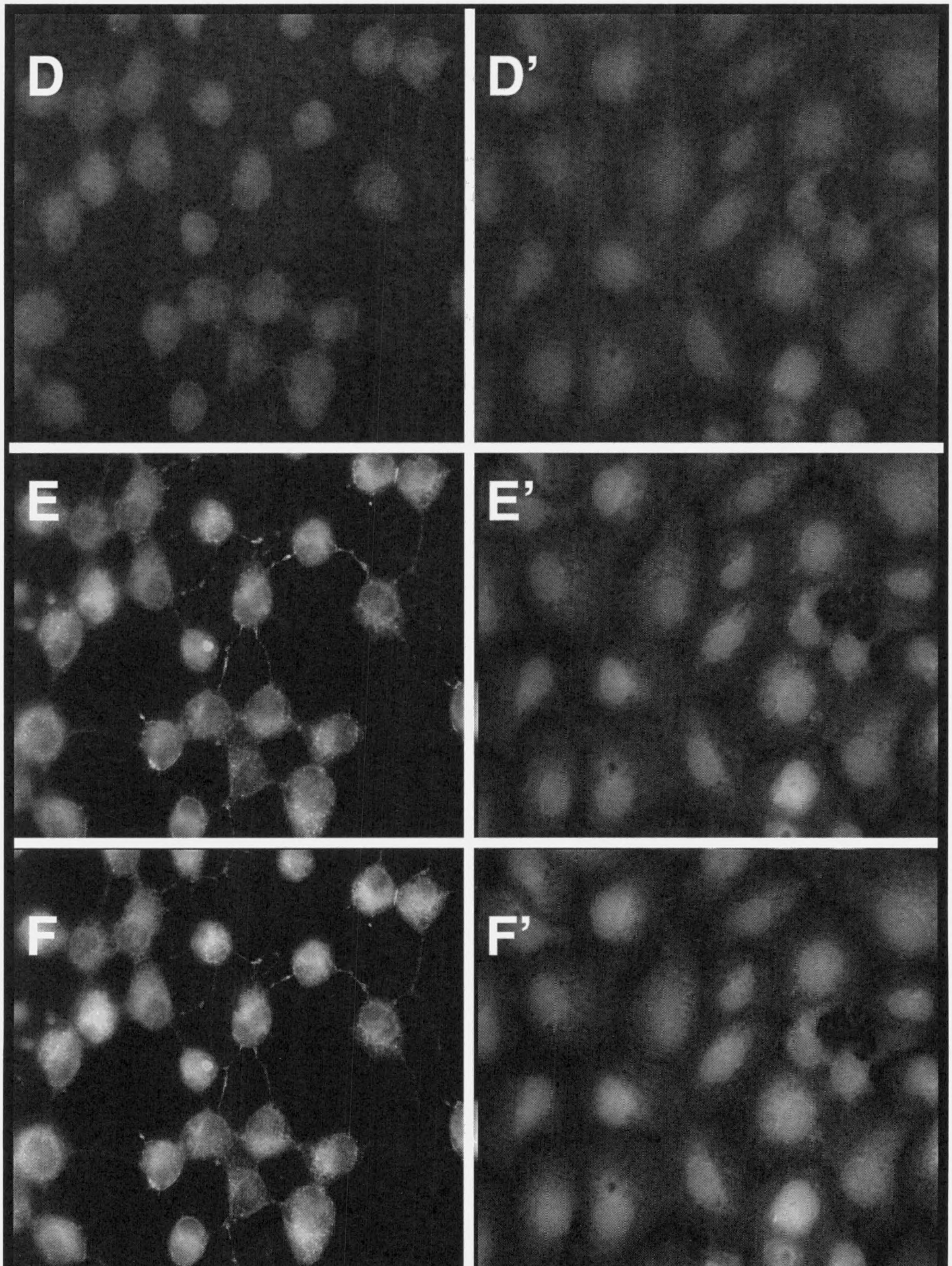


Figure 23. Fluorescence Microscopy Data of 12hrs Post- Infection for HSV1 Infected Vero Cells and EGCG-ester Treated Infected Vero Cells (A) Phase Contrast of HSV1/Vero; (A’) Phase Contrast of EGCG-ester-HSV1/Vero; (B) DAPI stain of HSV1/Vero; (B’) DAPI stain of EGCG-ester-HSV1/Vero; (C) GFP of HSV1/Vero; (C’) GFP of EGCG-ester-HSV1/Vero; (D) Lysosome stain of HSV1/Vero; (D’) Lysosome stain of EGCG-ester-HSV1/Vero; (E) GFP+DAPI of HSV1/Vero; (E’) GFP+DAPI of EGCG-ester-HSV1/Vero; (F) GFP+DAPI+lysosome of HSV1/Vero; F) GFP+DAPI+lysosome of EGCG-ester-HSV1/Vero.

In summary, when comparing and contrasting the results of the fluorescence microscopy, it is clear to the observer the differences between HSV1/Vero cells and 75μM EGCG-ester HSV1/Vero cells. There are visible viral particles in the cells infected with HSV1 and almost no virions in the cells infected with EGCG-ester -HSV1.

The nucleus of the cells is also very different. In the cells infected with HSV1, the margin of cells is lost, and there is granulation of the chromosomes. In the cells infected EGCG-ester-HSV1, the nucleus of the cells were not affected. The comparison of GFP at 8, 10 and 12 hours post infection as well as the comparison of DAPI and lysosome stain at 8,10 and 12 hours post infection in black and white images are shown in figure 18.

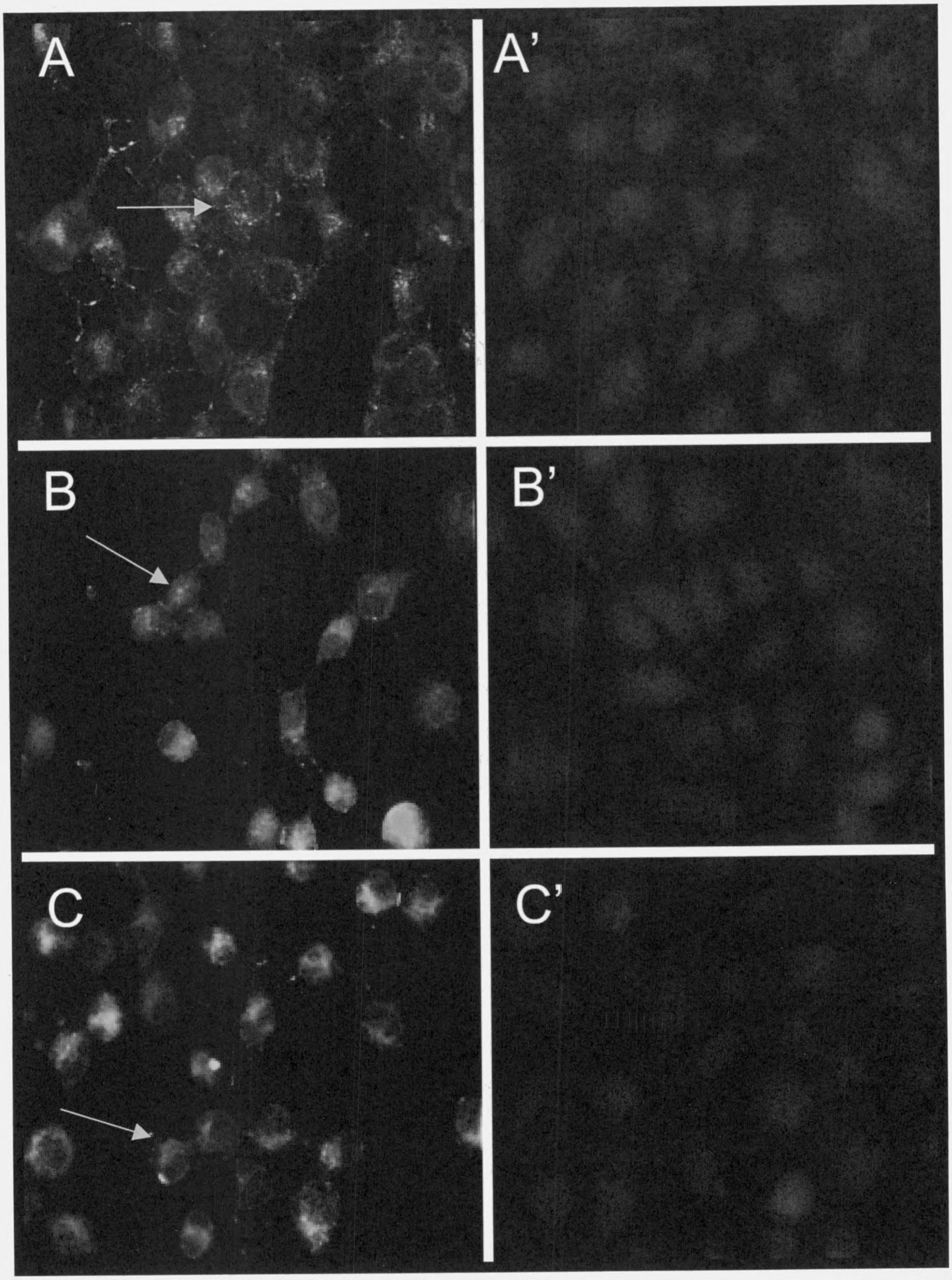


Figure 24. Fluorescence Microscopy Data of GFP expression at 8-12 hrs Post-Infection for HSV1 Infected Vero Cells and EGCG-ester Treated Infected Vero Cells (A) 8hrs HSV1/Vero; (A') 8hrs EGCG-ester-HSV1/Vero; (B) 10hrs HSV1/Vero; (B') 10hrs EGCG-ester-HSV1/Vero; (C) 12hrs HSV1/Vero; (C') 10hrs EGCG-ester-HSV1/Vero.

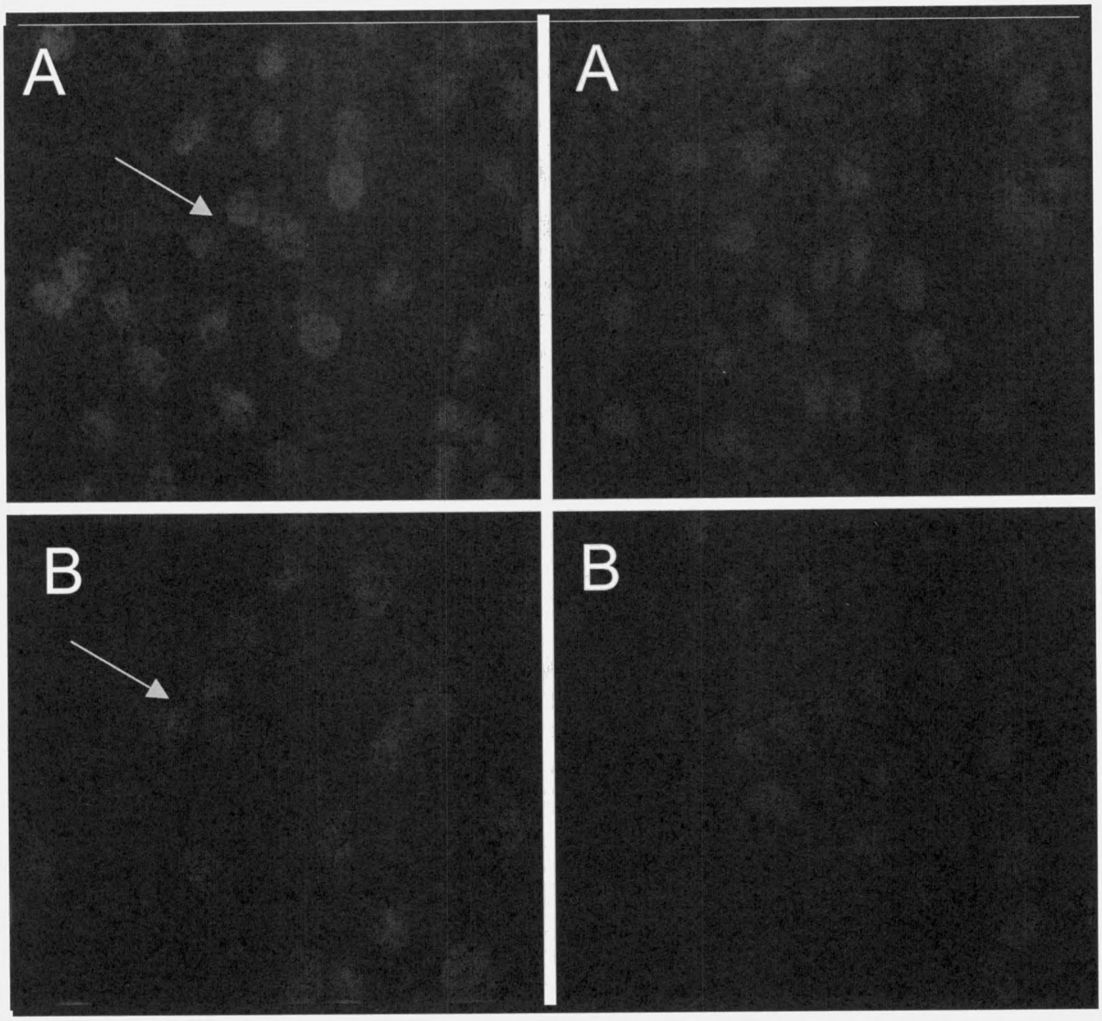


Figure 25. Fluorescence Microscopy Data with DAPI Stain at 8-10 hrs Post-Infection for HSV1 Infected Vero Cells and EGCG-ester Treated Infected Vero Cells (A) 8hrs HSV1/Vero; (A') 8hrs EGCG-ester-HSV1/Vero; (B) 10hrs HSV1/Vero; (B') 10hrs EGCG-ester-HSV1/Vero.

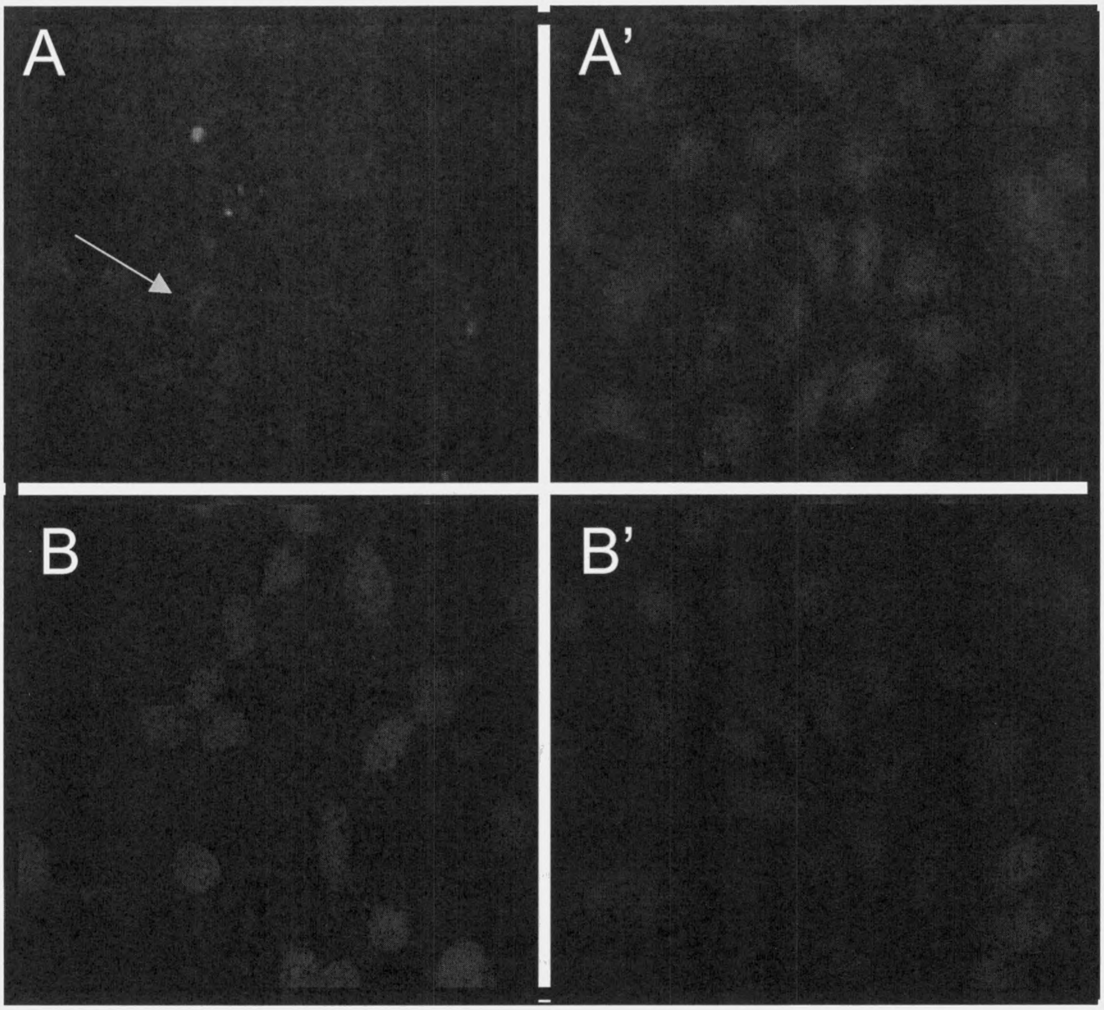


Figure 26. Fluorescence Microscopy Data with Lysosome Stain at 8-10 hrs Post-Infection for HSV1 Infected Vero Cells and EGCG-ester Treated Infected Vero Cells (A) 8hrs HSV1/Vero; (A') 8hrs EGCG-ester-HSV1/Vero; (B) 10hrs HSV1/Vero; (B') 10hrs EGCG-ester-HSV1/Vero.

Green fluorescence particles are seen from 8 to 12 hours post infection in the HSV1/Vero cells (figure 24). The amount of nuclear granulation and demargination are increased from 8 to 10 hours post infection in the HSV1/Vero cells as seen in figure 25. In figure 26, it is clearly indicated that the lysosome activation can be seen at 8 hours but decreases at 10 hours post infection in HSV1/Vero cells. These results correlate well with reported events in HSV1/Vero cells lytic infection[34]. None of this was observed in the

EGCG-ester HSV1/Vero cells indicating that EGCG-ester at 75μM is able to inhibit HSV1 infections.

8. Molecular Mechanisms of EGCG-ester on HSV1/Vero Cells

The results of each experiment performed indicate that EGCG-ester is able to inhibit HSV1 viral production and no significant viral biosynthesis is observed in EGCG-ester HSV1/Vero cells. In order to understand the molecular mechanism of EGCG-ester inhibiton on HSV1/Vero cells, genome-specific primers from HSV1 genome were designed as seen in table 5.

Primer Design For PCR

Primers	Nucleotide sequence (5' to 3')	Tm	Amplicon
gD1	F.AGACGTCCGGAAACAACCCTACAA	64.6	752
	R.ACACAATTCCGCAAATGACCAGGG	64.6	
gD2	F. TTGTTTGTCGTCATAGTGGGCCTC	64.6	938
	R. TGGATCGACGGTATGTGCCAGTTT	64.6	
gB1	F. AGATTCTGCGGTACTGCGATCACT	64.6	986
	R.ACGGAACACAAACAAGCACGGATG	64.6	
gB2	F. AGCTGATTATCGCCACCACACTCT	64.6	910
	R.TGGCGTTGATCTTGTCGATCACCT	64.6	
gB3	F. ATTCTCCTCCGACGCCATATCCACCTT	67.6	191
	R. AGAAAGCCCCCATTGGCCAGGTAGT	67.9	
GFP1	F.GTCAAAGCTTAAGATGGTGAGCAAGG	64.6	544
	R. CTTGAAGCTTCTTGTACAGCTCGTCC	66.2	
GFP2	F. TGACCCTGAAGTTCATCTGCACCA	64.6	717
	R. AACTCCAGCAGGACCATGTGAT	62.7	
VP11/12	F. ACCAAGCCTTGATGCTCAACTCCA	64.6	957
	R. ACAACACGGTTCCCGAGAGTTTGA	64.6	
VP11/12	F. ACCAAGCCTTGATGCTCAACTCCA	64.6	959
	R. ACACAACACGGTTCCCGAGAGTTT	64.6	

Table 5. Primers Used in Polymerase Chain Reaction for Analysis of HSV1/ Vero Cells DNA.

The primer sets were used to prime DNA isolated from HSV1/Vero and EGCG-ester HSV1/Vero cells. The PCR product was analyzed using 1% gel electrophoresis and the results are shown in figure 27. All the primers were able to prime the DNA samples and the amplicons have the expected sizes.

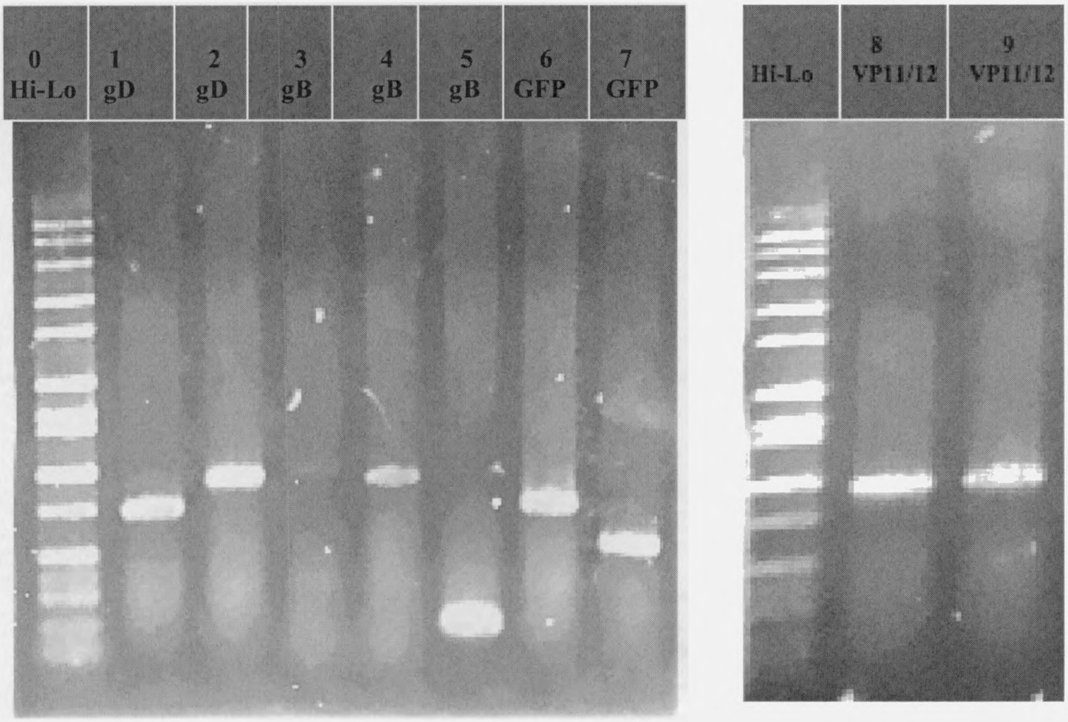


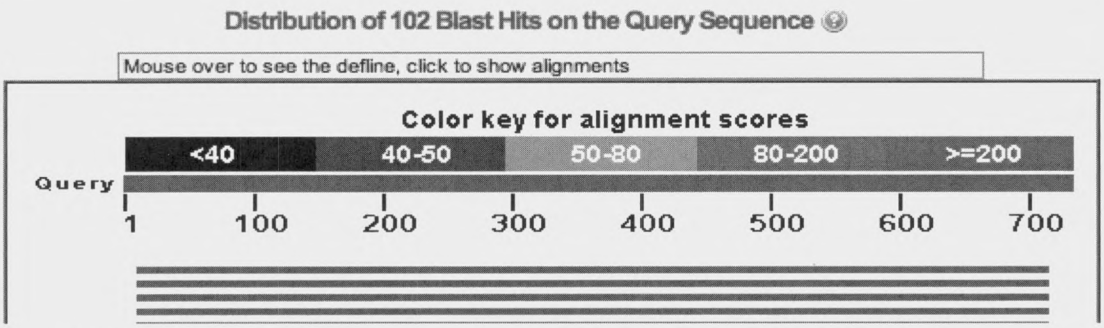
Figure 27. Priming of HSV1/Vero Cells DNA with Different Designed Primers.

9. Sequencing of PCR products

The PCR products for each primer set 1-9 have been sequenced and analyzed using NCBI homology search. The results indicated high homology to the reported HSV1 sequence as seen on figures 28-36. Therefore, the designed primers can be used to successfully study the molecular mechanisms of inhibition of EGCG-ester on HSV1/Vero cells.

Glycoprotein D- Primer1F- 733bps

GTCATGCCATGCTCGGATGGGAGGCACTGTGCTATCCCCATCACGGTCATGGAGTACACCGAATGC
TCCTACAACAAGTCTCTGGGGGCCTGTCCCATCCGAACGCAGCCCCGCTGGAACTACTATGACAGC
TTCAGCGCCGTCAGCGAGGATAACCTGGGGTTCCTGATGCACGCCCCCGCGTTTGAGACCGCCGGC
ACGTACCTGCGGCTCGTGAAGATAAACGACTGGACGGAGATTACACAGTTTATCCTGGAGCACCGA
GCCAAGGGCTCCTGTAAGTACGCCCTCCCGCTGCGCATCCCCCCGTCAGCCTGCCTCTCCCCCCAGG
CCTACCAGCAGGGGGTGACGGTGGACAGCATCGGGATGCTGCCCCGCTTCATCCCCGAGAACCAGC
GCACCGTCGCCGTATACAGCTTGAAGATCGCCGGGTGGCACGGGCCCAAGGCCCCATACACGAGCA
CCCTGCTGCCCCCGGAGCTGTCCGAGACCCCCAACGCCACGCAGCCAGAACTCGCCCCGGAAGACC
CCGAGGATTCGGCCCTCTTGGAGGACCCCGTGGGGACGGTGGCGCCGCAAATCCCACCAAACTGGC
ACATCCCGTCGATCCAGGACGCCGCGACGCCTTACCATCCCCCGGCCACCCCGAACAACATGGGCC
TGATCGCCGGCGCGGTGGGCGGCAGTCTCCTGGCAGCCCTGGTCATTGGGAAATTTTTGTATATA
AAAAAA



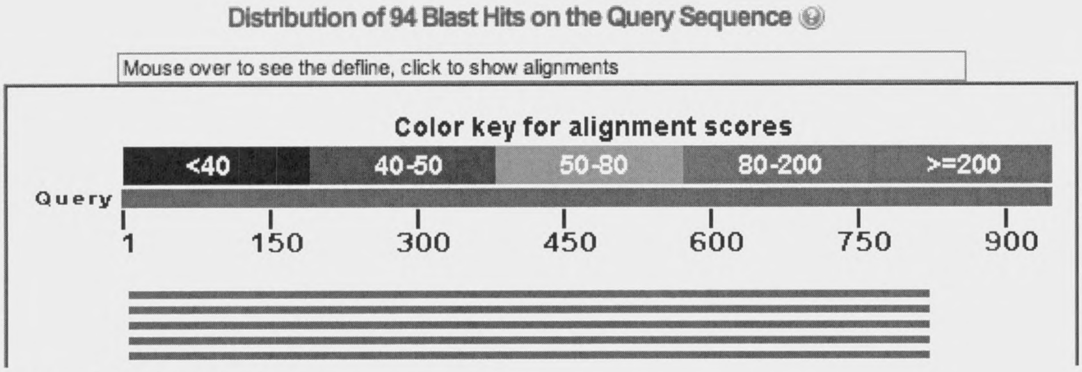
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
gi|122831528|EF177451.1	Human herpesvirus 1 strain gC-39-R6 glycoprotein D (US6	1252	1252	96%	0.0	99%
gi|121277018|EF157321.1	Human herpesvirus 1 strain KOSc(AC4) glycoprotein D (US	1252	1252	96%	0.0	99%
gi|121276[illegible]	Human herpesvirus 1 strain KOSc(AC3,AC6) glycoprotein D	1252	1252	96%	0.0	99%
gi|121276981|EF157319.1	Human herpesvirus 1 strain KOSc glycoprotein D (US6) ge	1252	1252	96%	0.0	99%
gi|330193|L09244.1	Herpes simplex virus type 1 glycoprotein D gene, complete	1252	1252	96%	0.0	99%

Figure 28. Blast Search Results for Retrieved Sequence of Glycoprotein D Primer 1F.

Glycoprotein D- Primer 2F- 947bps

AGGCTGCGCGATATGCTTGGCGGATGCTCTCTCAGATGGCCGACCCCAATCGCTTTCGCGGCAAAG
ACCTTCCGGTCCTGGACCAGCTGACCGACCCTCCGGGGGTCCGGCGCGTGTACCACATCCAGGCGG
GCCTACCGGACCCGTTCCAGCCCCCCAGCCTCCCGATCACGGTTTACTACGCCGTGTTGGAGCGCGC
CTGCCGCAGCGTGCTCCTAAACGCACCGTCGGGAGGCCCCCCCAGATTGTCCGGCGGGGGCCCTCC
GAAGGACGTTCCGGAAACAACCCTACAACCTGACCATCGCTTGGTTTCGGATGGGAGGCAACTGTG
CTATCCCCATCACGGTCATGGAGTACACCGAAATGCTCCTACAACAAGTCTCTGGGGGCCTGTCCCA
TCCGAAAACGGGCCCCGCGGGAAAAATAAAAAAAAAAATTTTCCGGGGCCGGAGGAAAAAAAACC
GGGGGTTTATAAAGGGGGGGGGGGTTGAAAAAATACGCCGGCACGTACCTGCGGTTCGTGGAAG
ATAAAAAGTGACGGAGATTAATTTTATTGGGCCGGCCGGGCCCGTAGTACGCCCTCCGCTGCGCAT
CCCCCCCGTCAGCCTTGCCTCTCCCCCCCAGGCCTAACAGCAAGGGGGGTGAACGGTGGGAACAGC
AATCGGAATGGCTGGCCCCGCTTTCAATCCCCCGAGAAAACCAAGCCGCAACCGGTCGCCCGGTAA
TTACAGGCTTGGAAAGGATCGCCGGGTAGACAACGGGGCCCCAAAGGCCCCATACAACGTAGTCA
CCCTTGGCTTGCCGCCCGGAGCTGTTCCGAGAACTTCTCAATGGCTCACGCGCAGCCGGGAAAGTTC
GTCTCCGGCAAGAACACGAGAGAATTCGTCCCATCATTGGAAGAGGCCTAGTGCGCTACGGTGTTG
CGCGCTGTCATCATGATTATGTC



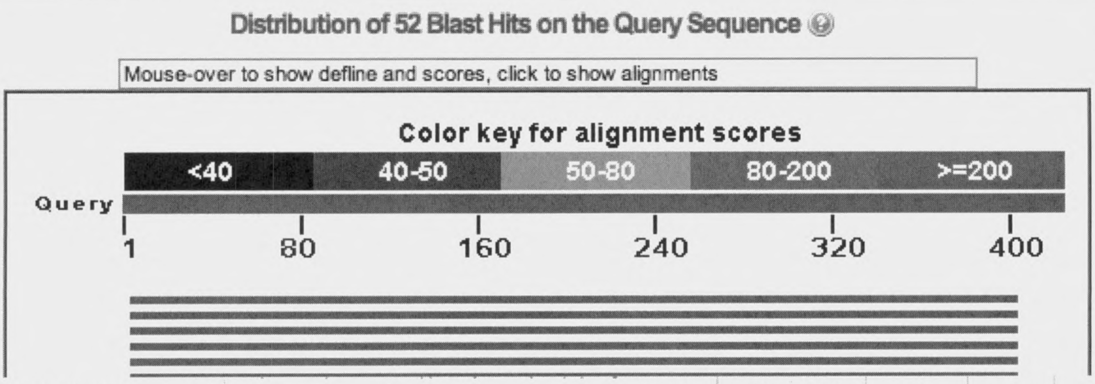
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
gi|121277035|EF157322.1	Human herpesvirus 1 strain KOSc(C2) glycoprotein D (US6	744	744	85%	0.0	83%
gi|121276981|EF157319.1	Human herpesvirus 1 strain KOSc glycoprotein D (US6) ge	744	744	85%	0.0	83%
gi|330100|J02217.1	HSV1 glycoprotein D gene	744	744	85%	0.0	83%
gi|330066|L09243.1	Herpes simplex virus type 1 glycoprotein D gene, complete	744	744	85%	0.0	83%

Figure 29. Blast Search Results for Retrieved Sequence of Glycoprotein D Primer 2F.

Glycoprotein B- Primer 1F- 425 bps

CAGGCATCACACCATCACAGACCATCATCGTAGAGTACCGCCGTCCCCCAGCGTGCAGGCGGCGGC
CGCGTGGTAGAGCAGGCGGGGGGGCGTGGAGCGTGGCAGACCGCCATGGACGCGTGGACGCGCAT
CCGTGCGCGTTTTCCTCCATGTAGGGGAACCTGAAACTGGTAGCGGCCCGTAAGGCCGCCCGCCCA
GGAACATGTGGGGGTTAAGCATAGCAAGTACTACGGCAGTGCCGGGTAACACCGTGTGTTTCAGCG
CGGCAACTGGCGCAGCCTTGTGGGTAGCAAACACGCCGTGCCCGGCCTTTATTTTTGACGGACCCG
GCAGATCGTTCAAGCCGGACGCGGGCCGGGTTTAGTAGCGCGGCAGGGACCTCGGCGAGGAGACA
GGAAGCTCGCTATCAGACTATCCGGGTAATT

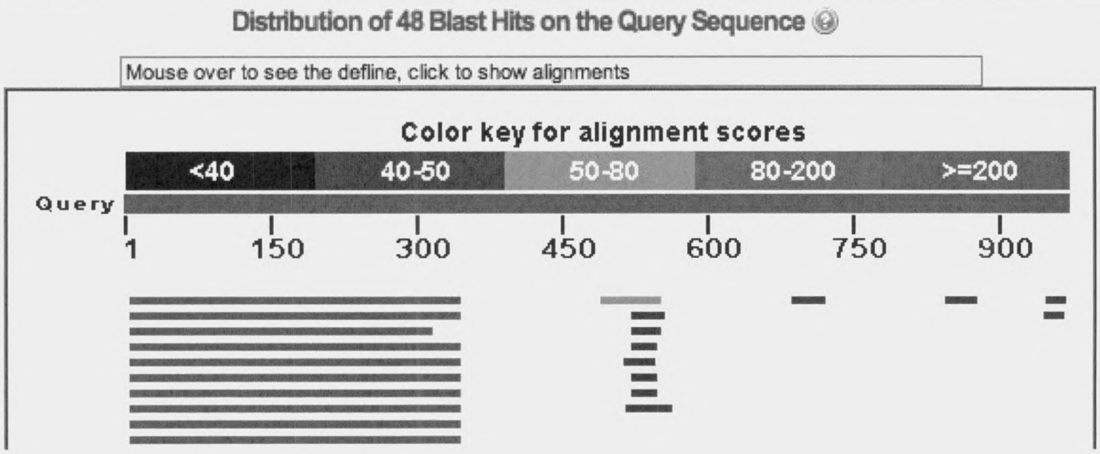


gi|330109|M21629.1	Herpes simplex virus type 1 glycoprotein B gene (gB-1), c	223	223	94%	1e-54	75%
gi|6572414|Z86099.2	Herpes simplex virus type 2 (strain HG52), complete geno	149	149	85%	2e-32	72%

Figure 30. Blast Search Results for Retrieved Sequence of Glycoprotein B Primer 1F.

Glycoprotein B- Primer 2F- 967 bps

TTCTCCTAACGGGTGCGCGGACTCGAGAGCGCCGCCCGGACTGCAGCCGCCGACCTCCGAAGTCGT
TACAGCAAGACGCGCGGCGAATATCTCACGTACGACTCCGACTGTCCGCTGTTGGCCATCGTCGAG
AGCGCCCCCGACGGCTGTATCGGCCCCCGGTCGGTCGTGGTCTACGACCGCGAAGTTTTTCTCGATC
CTCTACTCGGTCCTCCAGCACCTCGCCCCCAGGCTACCTGACGGGGGTTACGACGGGCCCCGTAGCC
CCGGCATTGCACAAGGCCCCCCCCTCGGGGGGGCCGCCGGGGGTTCGCCGTTAGGGTCCCTTTGGG
GGTGGAGGGGGGGGTTTGGGTTTTTCGGGTTTTTTGCCCATTTTCCCGTTACGGCTTGGGGTTGGGG
GCGGCCCCAGGGGGGGGGAGGGGGGGGGATTGGAAATTCCGCCGGCCCCCCCCCTTGTGGCCCCCC
CCCAAGGGGGGGGGGCGGGAAGGGGAAAAAAAAAAAAAAACCAAAAAAACCCGGGGCCCCCCCC
CCCCCCACCCGAAAAAAGCCAAATTGGTGGAGGGGGGGGGGCCCCCCTGGGGGGGGACCCCGTGG
GGGAAATTGGGGGGTAAAAAAACCCCTTTTATTTTCTTGGGGGGGGCCCCCCCCCCAGGGGGCCTG
GGGGGTCCAATTTAAGAAACCCACAAAATTTTCCGTACCGGCCCAAGGATACAAAAATTTACCGAG
GACCCCCGGGGGGGTTTTTTTAGAAAAAAAACCCCCCCCCCAAAAATTTCGAGGGCCCAAAGTGTA
AAAAAAAAACGCCCTGCCGTTTTTTACAGAATATTTTTCTTTGCCGAAACCTTAACTCCTCCAATTTT
TAAGGGGAGATTTTTTTAAAGACACGCCCCCTCTCTTCCTTTTTTCGAGGAGGGGGTAAATAGAACA
TAAGTATCGCGCCCATAAAAAAAAAAGATAGAATAAAAAGG



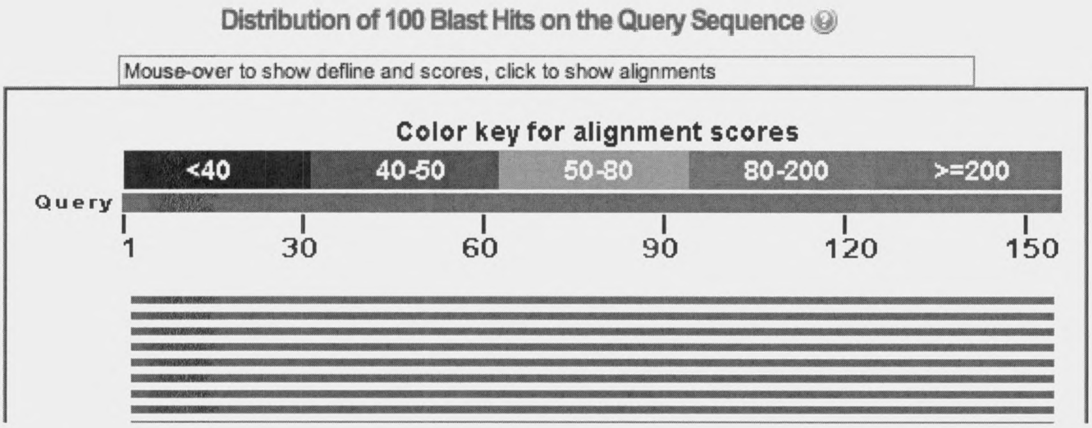
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
gi|330082|K01760.1	HSV1 (KOS) glycoprotein B gene, complete cds	407	407	35%	1e-109	88%
gi|114318788|DQ889502.1	Human herpesvirus 1 strain HF clone 10, partial sequence	398	398	35%	5e-107	87%
gi|60416|Y00453.1	Herpes simplex virus type 1 late gene ICP 18.5	398	398	31%	5e-107	90%
gi|290766081|GU734772.1	Human herpesvirus 1 strain H129, complete genome	394	394	35%	6e-106	87%
gi|222478328|FJ593289.1	Human herpesvirus 1 transgenic strain 17, complete genor	394	394	35%	6e-106	87%
gi|1944536|X14112.1	Human herpesvirus 1 complete genome	394	394	35%	6e-106	87%
gi|290766003|GU734771.1	Human herpesvirus 1 strain F, complete genome	389	389	35%	3e-104	86%
gi|330089|K03541.1	HSV-1 (Patton) glycoprotein B gene, complete cds	389	389	35%	3e-104	86%

Figure 31. Blast Search Results for Retrieved Sequence of Glycoprotein B Primer 2F.

Glycoprotein B- Primer 3F- 156bps

CCACCAGACCGAGTACCCGCTCTCGCGCGTGACCTGGGGACTGCATCGGCAAGGACGCCCGCGAC
GCCATGGACCGCATCTTCGCCCGCAGGTACAACGCGACGCACATCAAGGTGGGCCAGCCGCAGTAC
TACCTGGCCAATGGGGGCTTTCTT

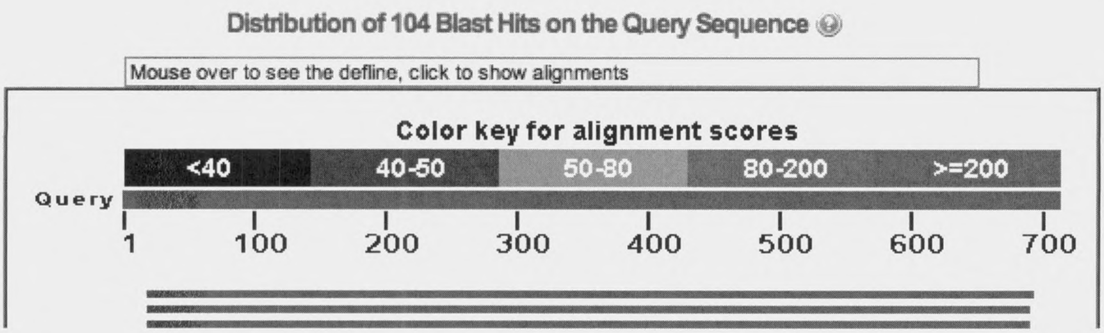


gi|7839508|AF259899.1	Human herpesvirus 1 glycoprotein B (UL27) gene, complet	266	266	98%	3e-68	98%
gi|330109|M21629.1	Herpes simplex virus type 1 glycoprotein B gene (gB-1), c	266	266	98%	3e-68	98%
gi|330089|K03541.1	HSV-1 (Patton) glycoprotein B gene, complete cds	266	266	98%	3e-68	98%
gi|330087|K02720.1	HSV1 (mutant strain tsB5), glycoprotein B (gB) gene	266	266	98%	3e-68	98%

Figure 32. Blast Search Results for Retrieved Sequence of Glycoprotein B Primer 3F.

GFP- Primer 1F- 714bps

GAGGCCGGATCACGGGTGTGCCATCCTGGTCGAGCTGGACGGCGACGTAACGGCCACAAGTTCAGC
GTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC
GGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCC
GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGG
AGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCG
ACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGG
CACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGG
CATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTA
CCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCA
GTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGC
CGCCGGGATCACTCTCGGCATGGACGAGCTGTACTGAAAACTCTCTTTGAGGAAA



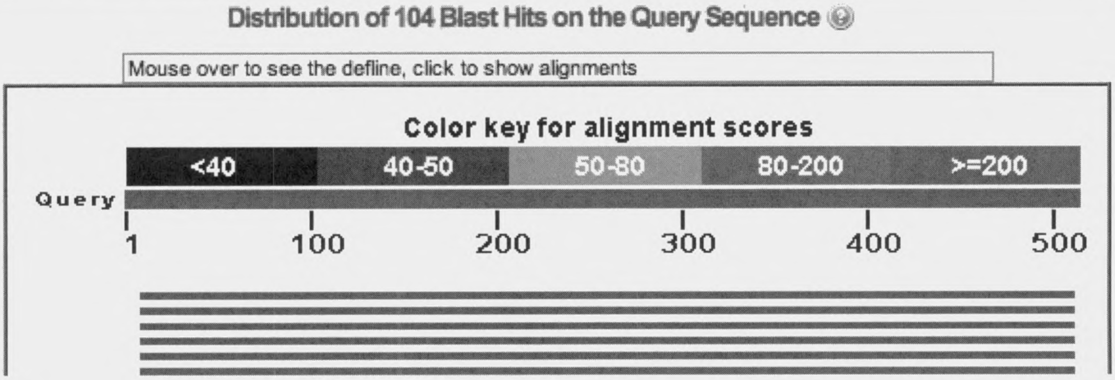
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
gi|193884059|AB375115.1	Cloning vector pInSRT-GFPhFDC.SP DNA, complete sequen	1209	1209	94%	0.0	99%
gi|328672377|HM367072.1	Synthetic construct ArchT-GFP gene, complete cds	1207	1207	94%	0.0	99%

Figure 33. Blast Search Results for Retrieved Sequence of GFP Primer 1F.

GFP- Primer 2F- 513bps

CGCCGCCGTCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCC
CGACCACAAGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCAC
CATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCT
GGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC
TGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAG
GTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAG
AACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCC
CTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCTGCCGGGAGTTAA



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
gi|328672377|HM367072.1	Synthetic construct ArchT-GFP gene, complete cds	883	883	97%	0.0	99%
gi|327360350|FR846927.1	Synthetic construct for ACTA2-BKbeta1-E gene	883	883	97%	0.0	99%
gi|326910732|JF275063.1	Synthetic construct plasmid pBIT GST/EGFP fusion protein	883	883	97%	0.0	99%
gi|325699378|HQ895843.1	Cloning vector pGEM/hM33_UL33-GFP, complete sequence	883	883	97%	0.0	99%

Figure 34. Blast Search Results for Retrieved Sequence of GFP 2F.

VP11/12- Primer 1- 964bps

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TCCGCGTGGGGGCGTCATCGTGGGGACAGGGGGGCGGTGGTCCGACAGAACGCTCCTGGCTGTCCA
CCGCGGCCCGCAGATACTCGTTGTTCAGGCTGTCGGTGGCCCAGACGCCGTACCCGGTGAGGGTCG
CGTTGATGATATACTGGGCGTGGTGATGGACGATCGACAGAACCTCCACCGTGGATACCACGGTAT
CCACGGTCCCGTACGTACCGCCGCTCCGCTTGCCGGTCTGCCACAGGTTGGCTAGGCACGTCAGGTG
GCCCAGGACGTCGCTGACCGCCGCCCTGAGCGCCATGCACTGCATGGAGCCGGTCGTGCCGCTGGA
ACCCCGGTCCAAATGGCGCGCGAACGTTTCCGCGGCCCCTTCCGGGCTGCCGCCGAAGCGGAAGG
AACCGGGCAATTGGAGGGACTCAGCCGGTGACATACGTGCTTGTCCGTCGTCAACGGCATCCAGGA
GGCCCACCGGTACAGCACGGAAACGTAGGCCAGGAGCTCCGTTAAGCCGCATTGCGGTGTCGGTCC
TGGGACGTTTTGGGCCCCCCGGGGCGCATAAGGAACATGTACTGCTGAATCCAATGGAGGGGCGTC
GCGCAGCCGGCCCACGGTGGCGGCTTATTTGGGCCCCCGCGCCCCCGCCTTTTAAACGGGGTGCCG
CGCCAAGCCACTTTTGGGGCCGGGTTGGCCCGCAACAACCACGTGAAAGGCTGGGGCTCCGCAGTC
GCCCACGGGGTCCTTCGGGAAACGTCAAGGCCGGCTGGGCCACAACCGTCTGCAGGTACTTCAAGT
ACTGCGTGGAGGATGGCGCGCTCAAACTGGGCCGCTCTGGTCAGCTCCACCTTCGCCCAGCGCTGG
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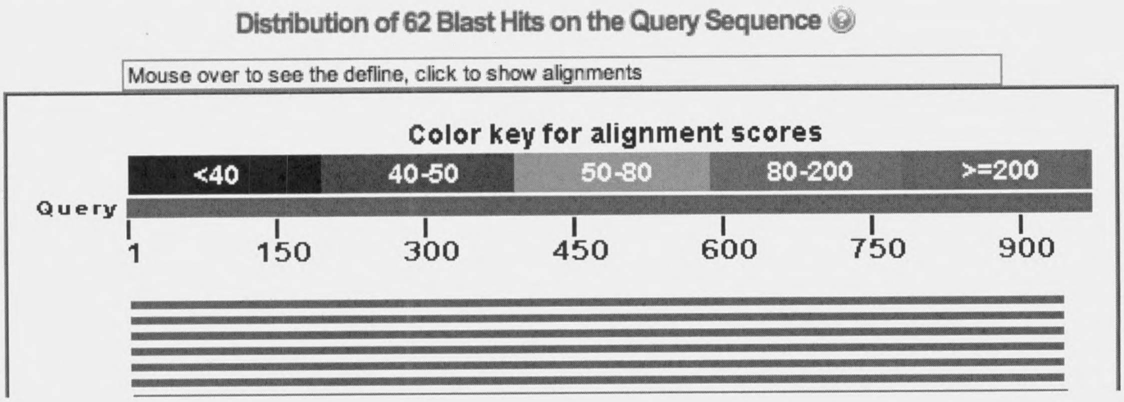

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
gi|222478328|FJ593289.1	Human herpesvirus 1 transgenic strain 17, complete genor	1180	1180	96%	0.0	90%
gi|1944536|X14112.1	Human herpesvirus 1 complete genome	1180	1180	96%	0.0	90%
gi|290766081|GU734772.1	Human herpesvirus 1 strain H129, complete genome	1177	1177	96%	0.0	90%
gi|290766003|GU734771.1	Human herpesvirus 1 strain F, complete genome	1177	1177	96%	0.0	90%
gi|330056|M15621.1	HSV1 (strain F) alpha-trans-inducing factor genes, complet	1177	1177	96%	0.0	90%
gi|114318788|DQ889502.1	Human herpesvirus 1 strain HF clone 10, partial sequence	1171	1171	96%	0.0	90%
gi|154744672|EU029143.1	Human herpesvirus 2 isolate subject ID VRC9154 specimer	776	776	96%	0.0	81%
gi|154744670|EU029142.1	Human herpesvirus 2 isolate subject ID GW13903 specime	776	776	96%	0.0	81%
gi|154744668|EU029141.1	Human herpesvirus 2 isolate subject ID GW20219 specime	776	776	96%	0.0	81%
gi|154744666|EU029140.1	Human herpesvirus 2 isolate subject ID VRC8339 specimer	776	776	96%	0.0	81%
gi|154744664|EU029139.1	Human herpesvirus 2 isolate subject ID GW9821 specimen	776	776	96%	0.0	81%
gi|154744662|EU029138.1	Human herpesvirus 2 isolate subject ID GW4317 specimen	776	776	96%	0.0	81%
gi|154744660|EU029137.1	Human herpesvirus 2 isolate subject ID VRC11098 specime	776	776	96%	0.0	81%
gi|161789583|EU281624.1	Human herpesvirus 2 strain 186 UL46 (UL46) gene, comple	773	773	96%	0.0	81%

Figure 35. Blast Search Results for Retrieved Sequence of VP11/12 Primer 1F

VP11/12- Primer 2- 964bps

```
TTCAGCTGGGGGCGTCATCGTGCGGACAGGGGGGCGGTGGTCCGACAGAAACGCTCCTGGCTGTCC
ACCGCGGCCCGCAGATACTCGTTGTTCAGGCTGTCGGTGGCCCAGACGCCGTACCCGGTGAGGGTC
GCGTTGATGATATACTGGGCGTGGTGATGGACGATCGACAGAACCTCCACCGTGGATACCACGGTA
TCCACGGTCCCGTACGTACCGCCGCTCCGCTTGCCGGTCTGCCACAGGTTGGCTAGGCACGTCAGGT
GGCCCAGGACGTCGCTGACCGCCGCCCTGAGCGCCATGCACTGCATGGAGCCGGTCGTGCCGCTGG
GACCCCGGTCCAGATGGGGGCGCGAACGTTTCCGGGGGGCCCCTCCGGCCTGCCGCCGAGCGGAAG
GAACCGGGCAATTGGAGGGACTCAGCCGGGGACATACGTGCTTTGTCCGTCGTCCACAGCATCAGG
GACGCCCACGGTTACAGCACGGAAACGTAGCCCAGGACCTCTTTGACCCGCAGTGCGGTTTCGGTC
CTGGGGCGACTTGGTCCCCCCGGGCCCCATAAGGAACATGTACTGCTGAATCCAATGGAAGGGCGT
CGGCCAGCCCGGCCAGGGTGGCGGCTAATTTGGGCCGCCGGCGCCCCCGCTTTTGAACGGGGGTGC
GCGCCAGCGTTTTTGGGGCCGGGGGTGGGCCCGCGCCACCACGTGAAGGCCGGGGTCCGCAGTCCC
CCCACGGGGTCTTGGGGAATGTCAGGGCGGTGGGAACCACCGTTCGGCGGTACTTTCCGGAACGGG
CGTGAAGGATGGCGCGGCTCAAACTGGACCGCGGGGCAGTCTCCACTTTCGCCCAAGCGCCTGGGT
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AATCTCTCGGAGACGTGGTGTGATAGTATATAA
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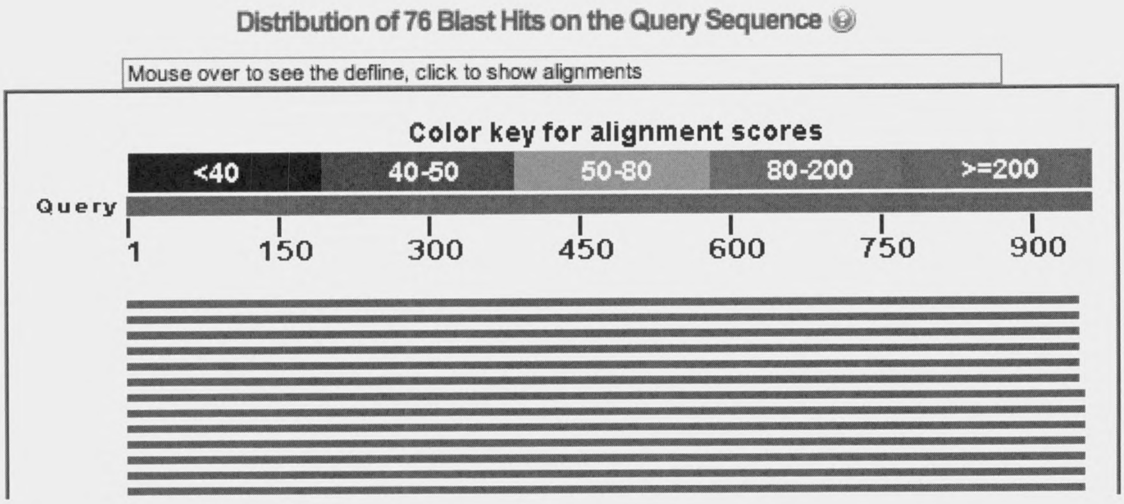


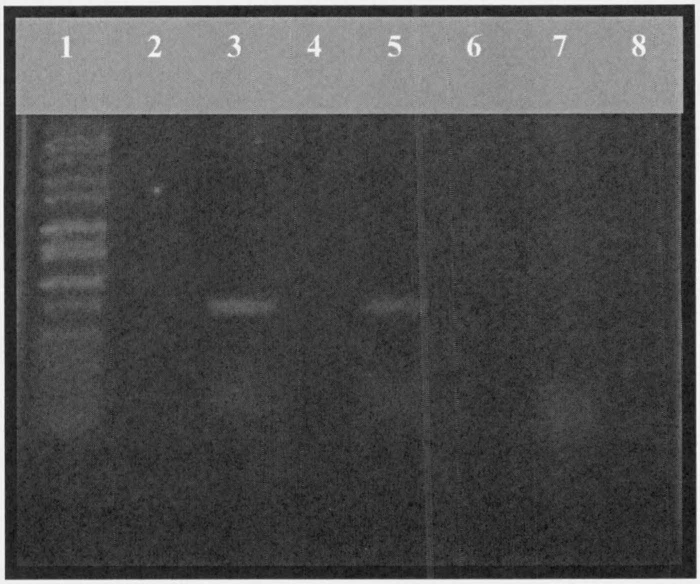
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
gi|222478328|FJ593289.1	Human herpesvirus 1 transgenic strain 17, complete genor	1144	1144	98%	0.0	89%
gi|1944536|X14112.1	Human herpesvirus 1 complete genome	1144	1144	98%	0.0	89%
gi|290766081|GU734772.1	Human herpesvirus 1 strain H129, complete genome	1139	1139	98%	0.0	89%
gi|114318788|DQ889502.1	Human herpesvirus 1 strain HF clone 10, partial sequence	1135	1135	98%	0.0	89%
gi|290766003|GU734771.1	Human herpesvirus 1 strain F, complete genome	1130	1130	98%	0.0	88%
gi|330056|M15621.1	HSV1 (strain F) alpha-trans-inducing factor genes, complet	1130	1130	98%	0.0	88%
gi|154744674|EU029144.1	Human herpesvirus 2 isolate subject ID VRC7494 specimer	728	728	99%	0.0	79%
gi|154744672|EU029143.1	Human herpesvirus 2 isolate subject ID VRC9154 specimer	728	728	99%	0.0	79%
gi|154744670|EU029142.1	Human herpesvirus 2 isolate subject ID GW13903 specimei	728	728	99%	0.0	79%
gi|154744668|EU029141.1	Human herpesvirus 2 isolate subject ID GW20219 specimei	728	728	99%	0.0	79%
gi|154744666|EU029140.1	Human herpesvirus 2 isolate subject ID VRC8339 specimer	728	728	99%	0.0	79%
gi|154744664|EU029139.1	Human herpesvirus 2 isolate subject ID GW9821 specimen	728	728	99%	0.0	79%
gi|154744662|EU029138.1	Human herpesvirus 2 isolate subject ID GW4317 specimen	728	728	99%	0.0	79%
gi|154744660|EU029137.1	Human herpesvirus 2 isolate subject ID VRC11098 specime	728	728	99%	0.0	79%
gi|161789583|EU281624.1	Human herpesvirus 2 strain 186 UL46 (UL46) gene, comple	724	724	99%	0.0	79%

Figure 36. Blast Search Results for Retrieved Sequence of VP11/12 Primer 2F

10. Comparison of Amplicons of Glycoprotein D, GFP, and VP11/12 in HSV1/Vero Cells and EGCG-ester-HSV1/Vero Cells Using PCR-Based Assay.

DNA extracted from HSV1/Vero cells and EGCG-ester HSV1/Vero cells was purified and isolated and the amount and purity was determined by Nanodrop spectrophotometer ND1000 as shown in figure 39. Since the concentration of DNA in each samples is similar in quantity, 1μL was used from each sample with constant amount of primer for glycoprotein D to do a comparatively PCR-based assay. The results are shown in figure 37. By using a Kodak Image analyzer 440CF, the band intensity of each experiment was measured (figure 38). It is indicated that 75μM EGCG-ester HSV1/Vero intensity of PCR product was less than the intensity of HSV1/vero cells. Further experiment was carried out using primers for glycoprotein D, GFP and VP11/12 to perform PCR-based assay. The results shown in figure 40 demonstrate that when HSV1 is treated with 75 μM EGCG, the DNA band intensity is decreased. When HSV1 is treated with75 μM EGCG-ester the DNA band intensity is further decreased. This implies that fewer HSV1 particles were able to infect Vero cells when treated with EGCG and even more when treated with EGCG-ester as compared to Vero cells infected with HSV1 only. However, in order to have a quantitative measure, further analyses needed to be carried out by using Real Time PCR.



Band intensity:

Control: 445.92, 588.24, 541.73
avg: 525.29

75uM: 353.87, 438.47, 407.82
avg: 400.05

Figure 38. Band Intensities from Kodak Image Station

Figure 37. Polymerase Chain Reaction Priming Glycoprotein D of HSV. Lane (3)HSV1/Vero; Lane(5) 75µM EGCG-ester-HSV1/Vero; Lane (7) Vero Cells Only; Lane (8) No DNA Negative Control.

Nanodrop:
Control: 566.4 ng/µL
75µM EGCG-ester: 560.1 ng/µL
Cells only: 562.8 ng/µL

Figure 39. DNA Concentrations of DNA Extraction

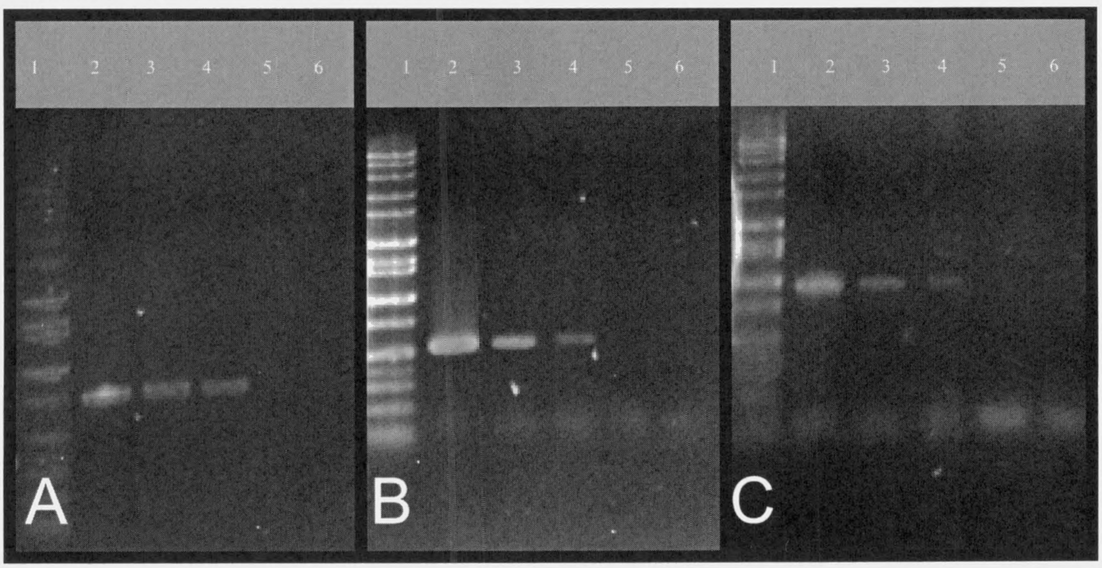


Figure 40. (A)Polymerase Chain Reaction Priming Glycoprotein D of HSV1. Lane (2)HSV1/Vero; Lane(3) 75µM EGCG-HSV1/Vero; Lane (4) 75µM EGCG-ester-HSV1/Vero; Lane (5)Vero Cells only; Lane (6) No DNA negative control. (B)Polymerase Chain Reaction Priming GFP tag in HSV1UL46. Lane (2)HSV1/Vero; Lane(3) 75µM EGCG-HSV1/Vero; Lane (4) 75µM EGCG-ester-HSV1/Vero; Lane (5)Vero Cells only; Lane (6) No DNA Negative Control. (C)Polymerase Chain Reaction Priming of VP11/12 Tegument Protein of HSV1. Lane (2)HSV1/Vero; Lane(3) 75µM EGCG-HSV1/Vero; Lane (4) 75µM EGCG-ester-HSV1/Vero; Lane (5)Vero Cells only; Lane (6) No DNA Negative Control.

11. Quantitative Study of Glycoprotein D in HSV1/ Vero Cells and EGCG-ester-HSV1/Vero Cells by Using Real Time PCR.

The previous experiment suggested that the amplification of Glycoprotein D is less in EGCG-ester-HSV1/Vero cells compared to HSV1/Vero cells, therefore it is important to carry out quantitative study of Glycoprotein by using Real time PCR. Special primers were designed for this study as shown in table 6. The process of real time PCR works by collecting fluorescence data at the end of each cycle of the reaction. The SYBR green dye used in the experiment binds to the double stranded PCR product causing the PCR product to fluoresce. As the reaction continues the instrument recalls the threshold for each sample. The threshold cycle (Ct) is the critical cycle at which the first significance increase in fluorescence is detected. Once the PCR cycles ended, the data were collected and the Ct values for each of the samples were analyzed and the relative quantity (RQ) of fluorescence was reported. The standard for comparing Ct values is that a difference in one Ct is equivalent to a two-fold difference in the amount of DNA. 100ng/μL of DNA was used for all samples; cells only, HSV1/Vero, and EGCG-ester HSV1/Vero. The results are shown in figures 41 and 42.

Primer Design For RT-PCR

Primers	Nucleotide sequence (5' to 3')	Tm	Amplicon
gD1	F. CAACCCTACAACCTGACCATC	62.6	100bps
	R. TTGTAGGAGCATTCGGTGTAC	60.6	100bps

Table 6. Real time PCR Primers for Priming Glycoprotein D on HSV1

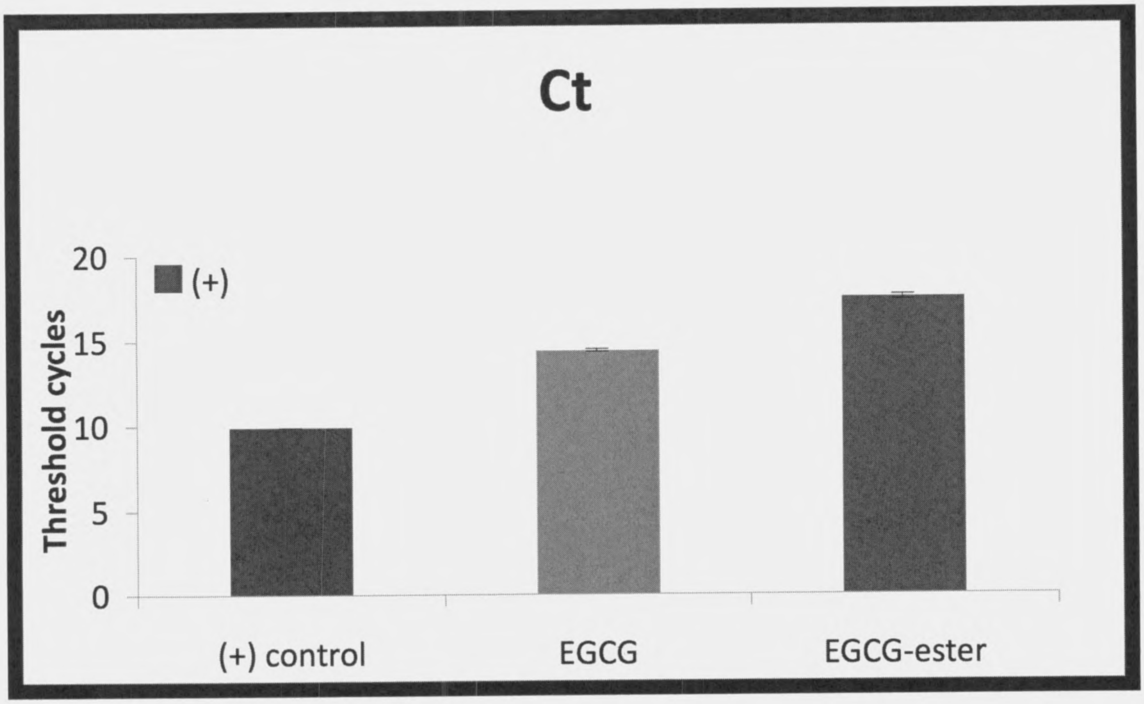


Figure 41. Real Time PCR Data of HSV1 Glycoprotein D.

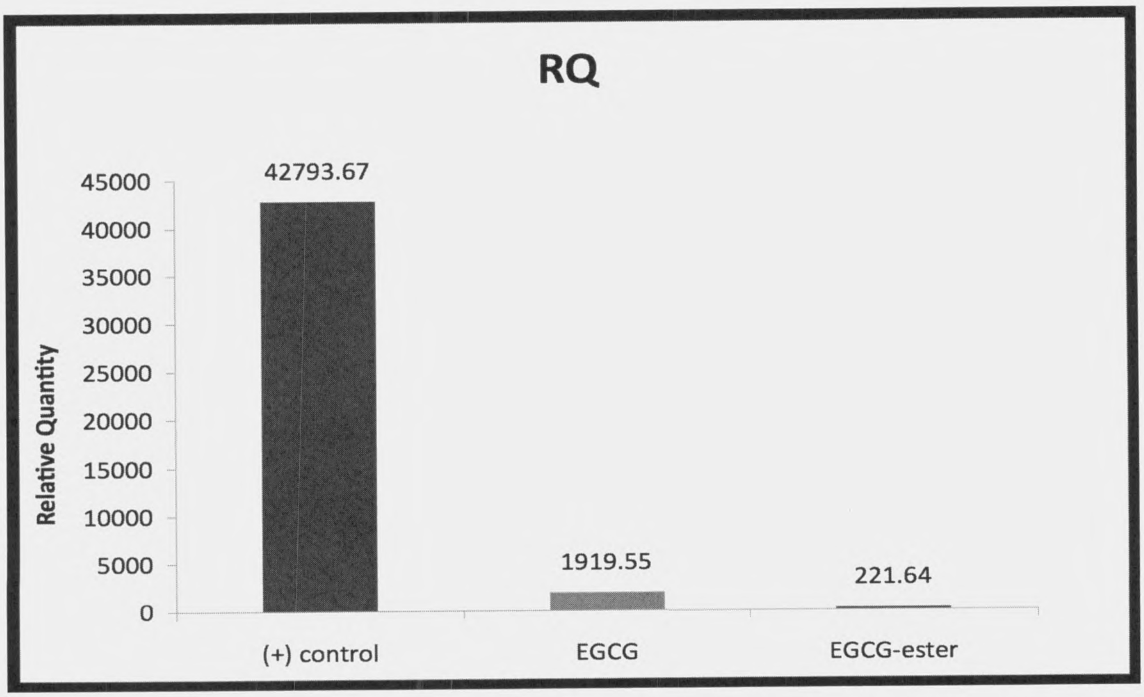


Figure 42. Relative Quantity of HSV1 Glycoprotein D Amplification in HSV1/Vero, EGCG-HSV1/Vero and EGCG-ester-HSV1/Vero Cells.

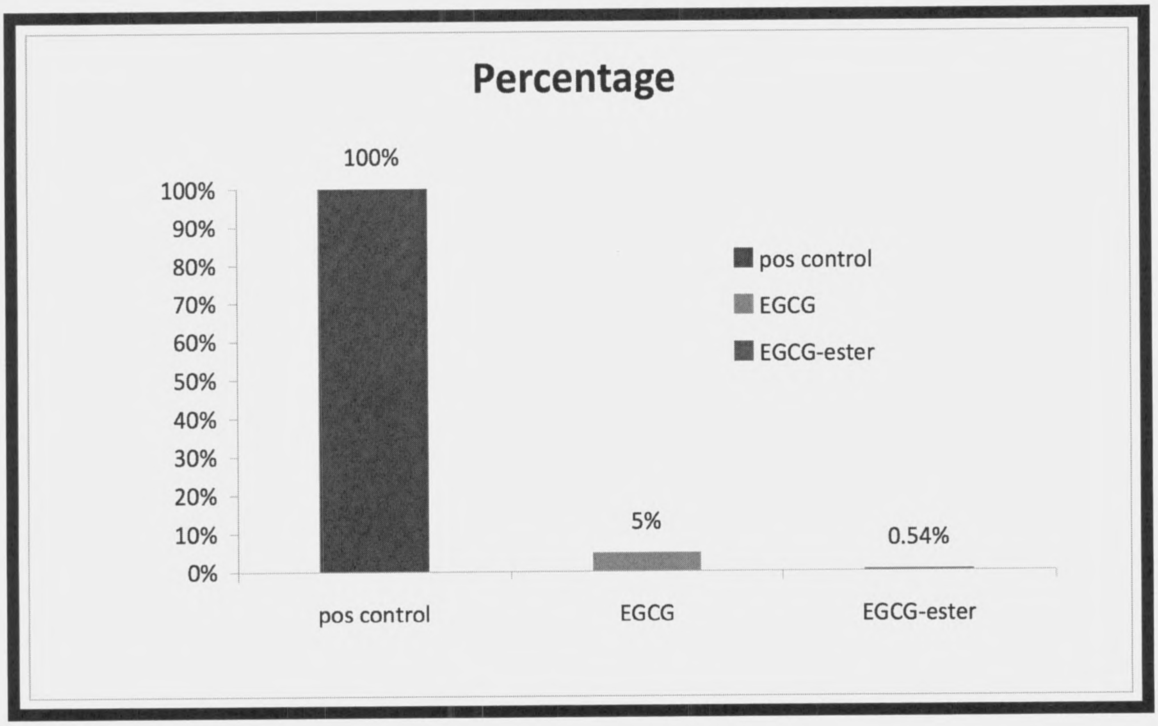


Figure 43. Percentage of HSV1/ Vero Infection, EGCG-HSV1/Vero and EGCG-ester-HSV1/Vero Cells.

The results show a 32-fold difference in the amount of glycoprotein D of EGCG-HSV1/Vero to HSV1/Vero and a 256-fold difference from HSV1/vero to EGCG-ester HSV1/ Vero. Our results suggest that EGCG inhibits HSV1 95% and EGCG-ester inhibits HSV1 99.46%(fig 43). Its mode of action seems to be by interfering with the virion envelope glycoproteins or by interfering with viral compounds for viral adsorption and cell entry[34,43]. Apparently, inhibition appears to occur during adsorption but not after penetration of the virus (figure 44). By using a green fluorescent protein tag, we were able to confirm that GFP expression is greatly decreased in treated virus compared to control virus.

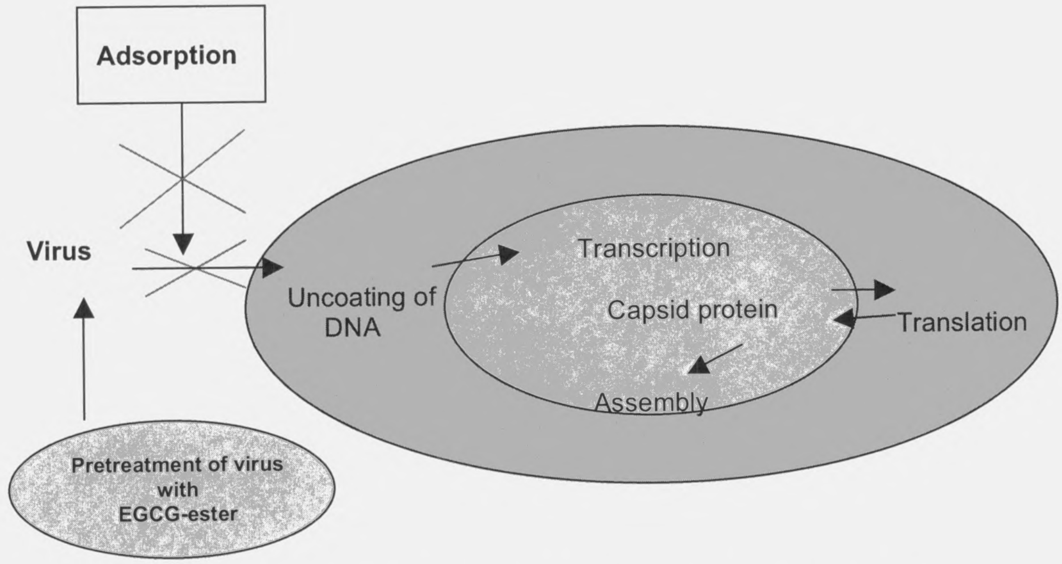


Figure 44. Schematic Representation of Possible Mode of Action of EGCG-ester on HSV1.

Conclusions

The effect of EGCG and EGCG-ester on HSV1 and Vero cells have been studied using different approaches to establish the concentrations that do not affect the host cells but efficiently inhibits the virus. First, in the cell cytotoxicity assay, as the concentration of polyphenols increased, cell morphology did change a little. This was seen when polyphenols were added at the same time as cells were plated. On the other hand, when the polyphenols were added for 1 hr after the cells had already been plated for 24hrs, and then consequently removed, cell morphology did not seem to be affected. Cell proliferation was also not significantly impaired as the concentrations of polyphenols increased. In addition, the percentage of cell death did not change much when cells were treated with EGCG-ester compared to control.

The maximum nontoxic concentration was identified to be 75μM for both EGCG and EGCG-ester and these concentrations were then used to study their effect on HSV1 viral production. In the plaque assay, the results showed that when the virus is previously treated with 50μM EGCG, there is a 10-fold decrease in plaque formation. On the other hand, when HSV is treated with EGCG-ester at a concentration of 50μM and above, there are no plaques observed. Different concentrations of EGCG-ester were then used to determine the minimum HSV1 inhibitory concentration. The results suggested that at a concentration of 50μM and above, EGCG-ester completely inhibits PFU formation.

In the fluorometer experiment, GFP expression is much lower at 75μM-EGCG-ester HSV1/ Vero cells compared to HSV1/Vero cells. Thus, expression of GFP as part of viral biosynthesis is decreased when HSV1 is treated with EGCG-ester. In addition, the fluorescent microscopy analyses showed remarkable results with both the GFP and DAPI stain. GFP expression was reduced in the 75μM-EGCG-ester HSV1/ Vero cells as compared to HSV1/Vero cells. Also, when evaluating the morphology of the nuclei of the HSV1/ Vero cells to the EGCG-ester-HSV1/Vero cells several differences are seen. The nuclei of the HSV1/ Vero cells lose their margins during infection, and there is major granulation of the chromosomes. On the contrary, the nuclei of the EGCG-ester-HSV1/Vero cells keep their integrity throughout infection.

Lastly, the PCR based of DNA of assay of glycoprotein D of HSV1/Vero showed higher band intensity as compared to EGCG-ester-HSV1/Vero cells. This infers that there was more viral DNA in the cells infected with HSV1/Vero as compared to 75μM EGCG-ester-HSV1/Vero.

Also, the real time PCR- based assay indicated that there is a 32-fold difference in the amount of DNA of glycoprotein D in EGCG-HSV1/Vero cells compared to the DNA of HSV1/Vero cells and a 256-fold difference from HSV1/Vero cells to EGCG-ester HSV1/ Vero cells. Our results suggest that EGCG-ester inhibits HSV1 to a great extent and is more effective against HSV1 when compared to EGCG alone.

In summary, both EGCG and EGCG-ester can inhibit HSV1, but EGCG-ester has proven to be more potent compared to EGCG in inhibiting the action against HSV1 infections in vitro. Contrary to EGCG, EGCG-ester is a stable compound and is also stable at vaginal pH and would be an ideal candidate for a topical application. An EGCG-ester topical application against HSV1 and possibly HSV2 would benefit millions of people every year. Furthermore, by being able to stop the spread of the disease enables us to possibly stop the link between HSV and HIV. Although further studies need to be conducted in order to completely understand the mode of action of EGCG-ester in humans the results obtained in this study are very promising. The use of natural products may improve the lives of many and thus give patients hope for a better and healthier future.

Future Studies

The results of this study suggested that EGCG-ester is able to effectively inhibit HSV1 in Vero cells (Green monkey kidney cells). Some preliminary results were also obtained by using human A549 cells. Future studies should be carried out to understand the molecular mechanism of EGCG-ester on HSV1. EGCG-ester can be potentially used as a novel approach to inhibit HSV1.

Using the human cell line A549, the same studies should be carried out to determine the optimal concentration of EGCG-ester on cell cytotoxicity. HSV1 inhibition will also be studied by performing plaque assay, fluorometry study, fluorescence microscopy, PCR and real time PCR.

In addition, further studies need to be conducted in order to understand the exact mechanism of EGCG-ester in inhibiting HSV1. EGCG- ester seems to be inhibiting HSV1 cell entry by either binding to the virion glycoproteins or preventing them from participating in viral adsorption and penetration or by interfering with the virus itself, maybe by damaging its envelope and thus disabling the virus from getting into contact with the host cell.

Lastly, all the primers designed to target different regions of HSV1 genome should also be used in real time PCR study. Additional primers should also be designed for genome regions involved in viral replication. Furthermore, reverse transcriptase PCR can be used in different steps of viral infection to look at the expression of genes involved in viral replication, which will help elucidate the molecular mechanisms of EGCG-ester.

The ultimate goal is to use an animal model to study the effect of EGCG-ester on

HSV1 infected animals. This will allow us to further develop a topical application for human usage.

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Appendix

1. Cytotoxicity Study of EGCG/A549 cells and EGCG-ester/A549 cells with Removing the Polyphenols (24hrs):

The effect of different concentrations of EGCG and EGCG-ester on A549 cells was observed at 24 hours. In this study, A549 cells were plated first for 24 hours and then different concentrations of EGCG and EGCG-ester (12.5, 25, 50, 75, 100μM) were added to the cells and allowed to adsorb for 1 hour. EGCG and EGCG-ester was then aspirated, and cells were observed 24 hours later. The results are shown in figures 1 and 2. The results suggest that when EGCG is added to A549 cells, the maximum nontoxic concentration was observed to be 25μM. At the concentration of 50 to 100μM, cell morphology is affected to a certain extent. In the presence of EGCG-ester the maximum nontoxic concentration was evaluated to be 12.5μM.

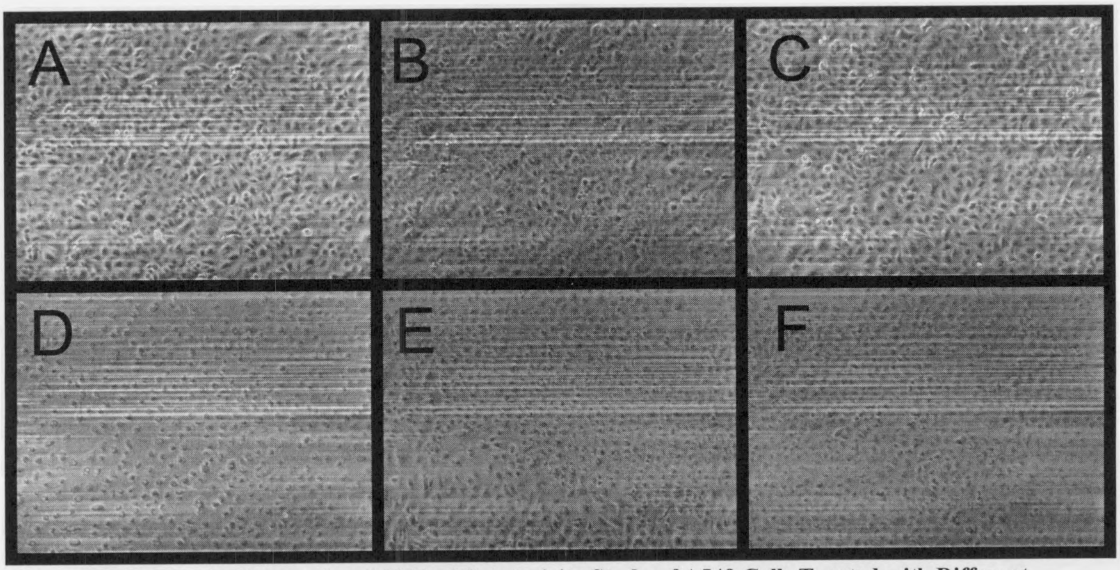

Figure 1. Microscopic Observation (100X) of Cytotoxicity Study of A549 Cells Treated with Different Concentrations of EGCG (A) 0μM; (B) 12.5μM; (C) 25μM; (D) 50μM; (E) 75μM; (F) 100μM.

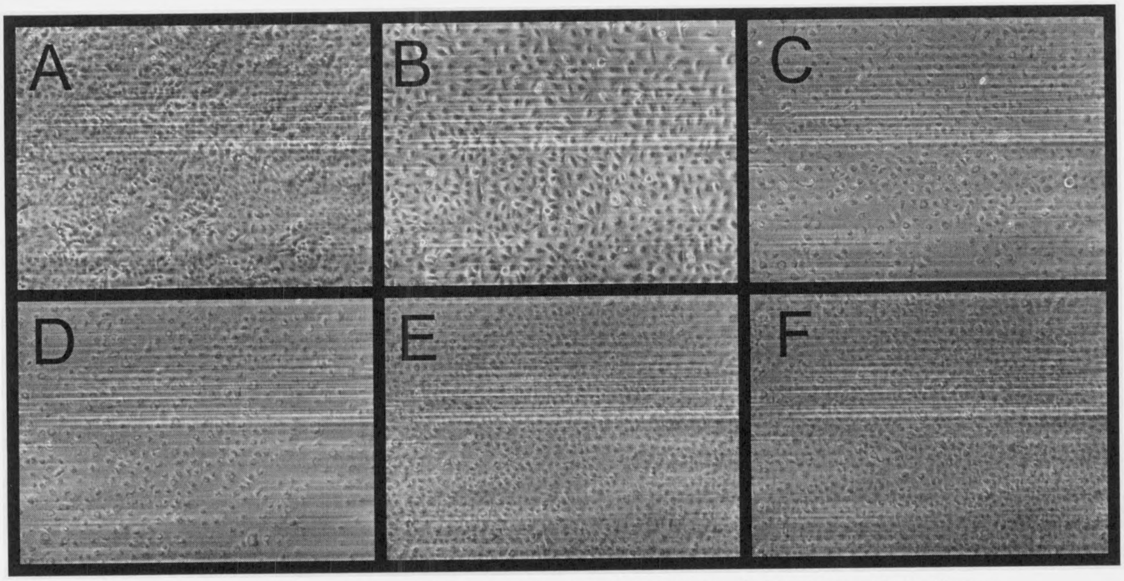

Figure 2. Microscopic Observation (100X) of Cytotoxicity Study of A549cells Treated with Different Concentrations of EGCG-ester (A) 0µM; (B) 12.5µM; (C) 25µM; (D) 50µM; (E) 75µM; (F) 100µM.

2. Fluorescence Microscopy Observation of HSV1/A549 cells and EGCG-ester-HSV1/A549 Cells

The lytic cycle of HSV1/ A549 cells was observed at 8 hours post-infection to study the molecular changes within the infected cells. HSV1/A549 cells were used as positive controls. 75µM EGCG-ester-HSV1/A549 cells were also monitored and compared to control. GFP-HSV1, DAPI stained nucleus and lysosome stain were used to identify cytophatic effects on A549 cells.

Phase contrast image for HSV1/A549 cells did not show a difference in cell morphology, but when staining the HSV1/A549 cells with DAPI stain, there is an obvious difference in the cell nucleus compared to the EGCG-ester-HSV1/A549 cells. Significant granulation as well as demargination is only seen in the HSV1/A549 cells, demonstrating a normal occurrence of viral infection as indicated by the yellow arrow in figure (3). EGCG-ester- HSV1/A549 cells still maintained their nuclear integrity.

Green fluorescence images clearly indicate a significant amount of GFP expression in the HSV1/A549 cells but almost none in the EGCG-ester-HSV1/A549 cells as indicated by the yellow arrow in figure 3(C). In addition, the lysosome stain demonstrates lysosomal activation in the HSV1/A549 cells but not in the EGC-ester-HSV1/A549 cells, as indicated by the yellow arrow in figure 3(D). Further experiments have to be carried out to better understand the effect of EGCG and EGCG ester on HSV1/A549 cells.

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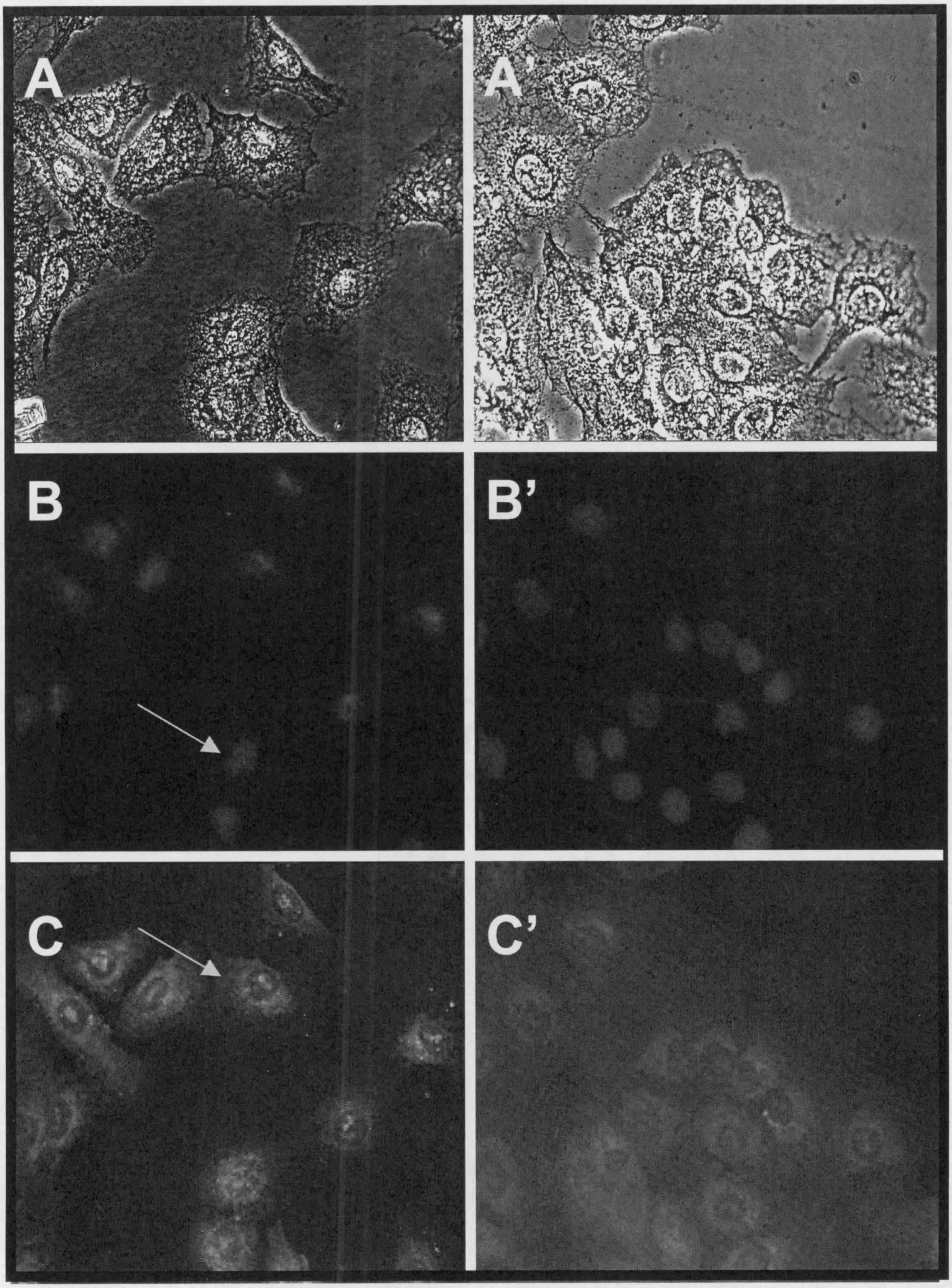

A
A'
B
B'
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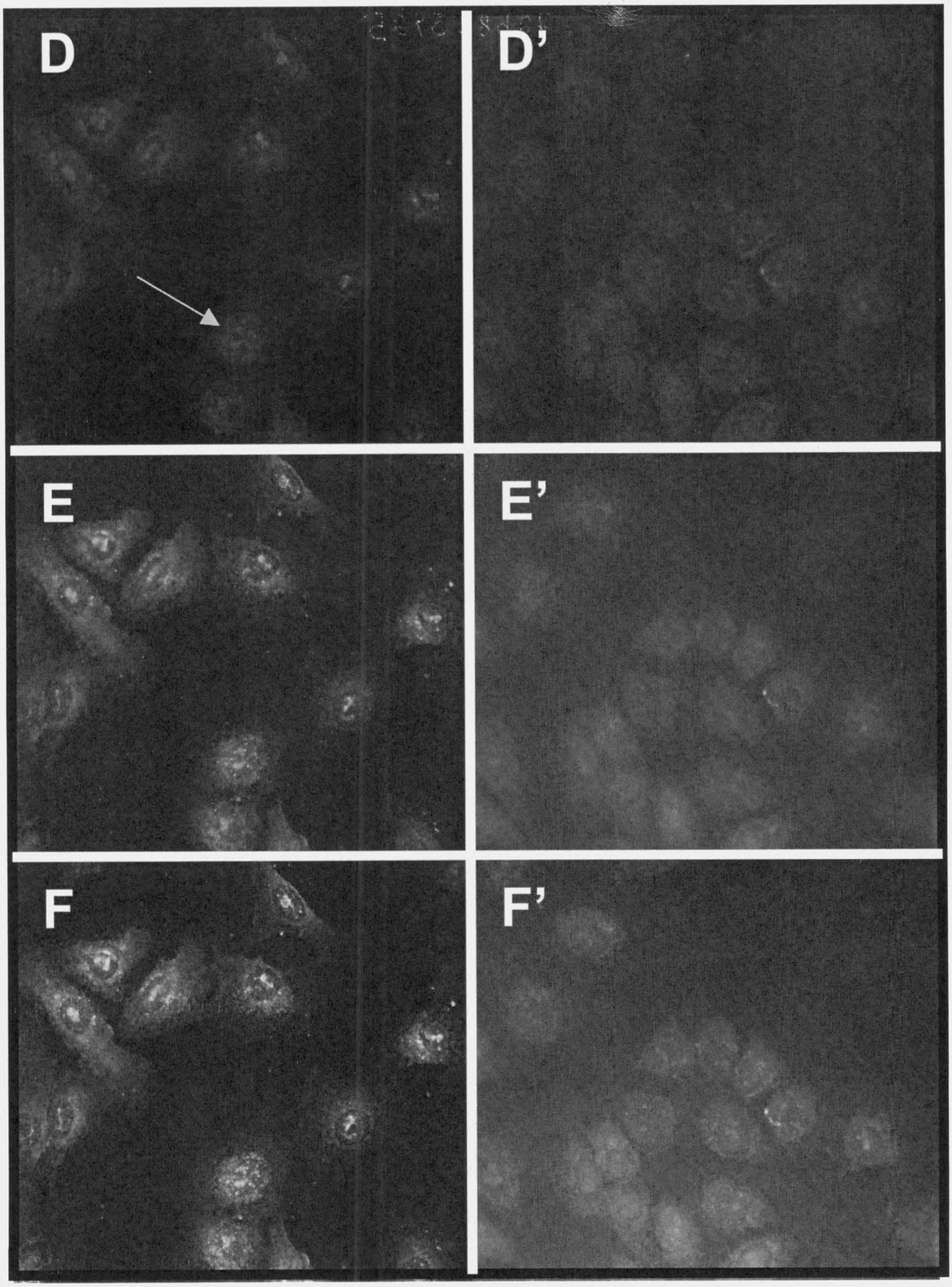

Figure 3. Fluorescence Microscopic Observations of A549 Cells Monolayer (400x). (A) Phase Contrast; (B) DAPI stain; (C) GFP; (D) Lysosome stain (E) GFP+DAPI; (F) GFP+DAPI+Lysosome.