5-2012

The Antiviral Activity of Curcumin on Herpes Simplex Virus-1

Susan A. Pungitore

MONTCLAIR STATE UNIVERSITY

The Antiviral Activity of Curcumin on Herpes Simplex Virus-1

By

Susan A. Pungitore

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 2012

College of Science and Mathematics

Department of Biology and Molecular Biology

[redacted]

Robert Prezant
Dean of College of Science and Mathematics

6/11/12
(date)

Thesis Committee:

[redacted]

Sandra Adams
Thesis Sponsor

[redacted]

Quinn Vega
Committee Member

[redacted]

Carlos Molina
Committee Member

[redacted]

Quinn Vega
Department Chair

Abstract

Herpes simplex virus 1 is a common human pathogen that causes an initial productive infection followed by a lifelong latent infection of sensory neurons. Although the active infection is self-limiting in healthy populations, HSV can pose serious health risks to immunocompromised individuals. Furthermore, drug-resistant strains of HSV-1 have emerged in the human immunodeficiency virus (HIV) afflicted populations, necessitating the development of efficacious treatment options to combat HSV-1 infections. In recent years, plant derived products have gained popularity as promising antiviral agents. Studies of plant-derived compounds, including polysaccharide extracts from algae and hydrolyzable tannins, indicated that antiviral activity may be contributed to the plant extract's inhibition of binding and penetration of the virus in cell cultures. Curcumin, a polyphenol derived from the *Curcuma longa* plant, has gained attention as an antiviral therapeutic agent after research publications suggested the compound exhibits antioxidant, antiproliferative, anti-inflammatory, and microbicidal properties. Curcumin has demonstrated in previous studies to modulate numerous cellular signaling pathways. Additionally, curcumin has been indicated as an effective inhibitor of HSV-2 in HeLa cell cultures and as an inhibitor of HSV-1 infectivity, immediate-early gene expression and replication in Vero cell cultures. The results of this study suggest that curcumin indeed exhibits antiviral properties against HSV-1 in Vero cell cultures. HSV-1 viral particles appeared to successfully bind and penetrate Vero cells. However, the replication of the virus was interrupted after the virus gained entrance to the Vero cells, resulting in a reduction in HSV-1 titer for the Infectious Dose 50 (ID_{50})and diminished signal strength of HSV-1 in curcumin-treated cells as viewed by gel electrophoresis. This

evidence supports the inhibitory effects of curcumin on HSV-1, warranting further investigation into the antiviral capacities of this biologically active plant-derived compound.

THE ANTIVIRAL ACTIVITY OF CURCUMIN ON HERPES SIMPLEX VIRUS-1

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

By

SUSAN A. PUNGITORE

Montclair State University

Montclair, NJ

2012

Table of Contents

Introduction

Herpes simplex virus type 1 (HSV-1) is a member of the *Herpesviridae* family of viruses. Although the *Herpesviridae* family contains over one hundred herpesviruses that can infect various species of fish, birds, horses, and humans, HSV-1 is only one of eight *Herpesviridae* known to infect humans; no animal reservoirs are known (Akhtar & Shukla, 2009). HSV-1 is a relatively large, double-stranded deoxyribonucleic acid (dsDNA) virus that is a member of the subfamily *Alphaherpesviridae*. The *Alphaherpesviridae* are distinguished by a short eighteen hour lifecycle, rapid spread, destruction of host cells, and the establishment of a latent infectious cycle (Mettenleiter, Klupp, & Granow, 2009; Roizman & Baines, 1991). The closest phylogenic relative to the alphaherpesviruses is the varicella-zoster virus, the cause of the common childhood illness chickenpox. Other herpes virus families include the betaherpesviruses, specifically cytomegalovirus, human herpes viruses 6 and 7, and the gammaherpesviruses Epstein-Barr virus and human herpesvirus 8, otherwise known as Kaposi's sarcoma-related virus (Roizman & Baines, 1991). Greater than eighty percent of the adult population in the world possess antibodies to HSV-1 and harbor the latent form of the viral genome in their peripheral nervous systems (Akhtar & Shulka, 2009). The establishment of this lifelong, latent infection that has the ability to reactivate and cause one or more rounds of the disease is a noteworthy hallmark of a herpesvirus infection (Rao et al., 2011). No vaccine has been successfully developed to prohibit the virus from establishing latency in neurons because the intimate contact of peripheral neurons with epithelial cells enables movement of the virus particles in and out of the nervous system

without exposure to circulating antibodies, and this immune avoidance by HSV-1 presents difficulty in developing an effective vaccine against the virus (Rao et al., 2011). HSV-1 is transmitted when mucous membranes or abraded skin is exposed to lesions or mucosal secretions of an individual with an active HSV-1 infection (Fatahzadeh & Schwartz, 2007). Additionally, HSV-1 has been demonstrated to shed onto skin and fomites, including utensils and clothing, permitting unsuspecting individual's exposure to the virus (Fatahzadeh & Schwartz, 2007). HSV-1 can be transmitted from mother to child *in utero* or during childbirth (Fatahzadeh & Schwartz, 2007). HSV-1 mainly infects oral, pharyngeal, and ocular sites. HSV-1 infections include primary herpetic gingivostomatitis (PHGS), herpes simplex labialis (HSL), recurrent intraoral herpes (RIH), genital herpes, Kaposi's varicelliform eruption (KVE), herpes gladitorum, herpetic whitlow, ocular herpes, including stromal keratitis, encephalitis, and neonatal herpes (Fatahzadeh & Schwartz, 2007; Lin et al., 2011). Symptoms associated with HSV-1 infections are dependent upon the host's genetic composition, immune status, site of primary infection, and dose of viral inoculum (Fatahzadeh & Schwartz, 2007). The most common form of HSV-1 infections is orofacial lesions. The lesions develop in and/or around the oral cavity of the host. These lesions are normally preceded or accompanied by a burning/stinging sensation, pain and discomfort. Lesions are formed when vesicles at the infection site rupture to eventually form ulcerations and symptoms can persist for two weeks and viral shedding can continue up to several weeks after the resolution of symptoms (Fatahzadeh & Schwartz, 2007). HSL and RIH are the most common forms of HSV-1 infections. HSL affects the outer vermilion border, the border that marks the transition from lips to skin, and is normally associated with an ulcerative

region's crusting over, forming a scab while RIH affects the hard palate, surrounding gingiva, and keratinized tissues of the oral cavity, producing an ulcerative region more commonly known as a canker sore or aphthous ulcer (Fatahzadeh & Schwartz, 2007). Additionally, HSV-1 can cause genital herpes which is localized to the labia minora and urethra meatus in women and the shaft and glans of the penis in men (Fatahzadeh & Schwartz, 2007). However, most HSV-1 infections are not life-threatening except in the case of neonates and immunocompromised patients.

Classification:

HSV-1 is a dsDNA virus with a diameter of approximately 200 nm. The genome consists of 152kb (Rode et al., 2011; Svobodova, Bell, & Crump, 2012). Additionally, the linear genome contains 30 to 35 different proteins and two main protein coding components—the unique long (U_L) segment, containing 59 genes and a short region—the unique short (U_S) segment, containing 14 genes, and within each of these regions, inverted repeat sequences exist both internally (IRL, IRS) and terminally (TRL, TRS). The HSV-1 genome is able to recombine through the repeat sequences to form four equimolar isomers in which the U_L and U_S sequences are inverted with respect to one another (Wadsworth, Jacob, & Roizman, 1975). Furthermore, the three replication origins (Ori) exist along with 84 open reading frames (Martin, Deb, Klaur, & Deb, 1991). The dsDNA genome resides within the 100-110 nm diameter icosahedral nucleocapsid which is surrounded by a complex proteinaceous tegument layer (Akhtar & Shukla, 2009). This tegument layer consists of greater than 15 known proteins and is in turn surrounded by a lipid envelope which contains approximately 10 viral envelope glycoproteins

(Fatahzadeh & Schwartz, 2007)). These glycoproteins are essential for establishing a productive herpes virus infection.

Infection Cycle:

Productive (Lytic) Infection

Specific transcriptional strategies are characteristic of viruses with DNA genomes during the productive infectious cycle and HSV-1 is no exception. Viral genes are transcribed in a reproducible and precise chronological sequence which ensures efficient gene expression and regulatory functions, ease of transition from one transcriptional stage to the next, and the successful encoding of virion structural proteins (Svobodova et al., 2012). Three categories of genes are represented in HSV-1 genome replication and follow a specific sequential transcriptional cascade upon entry into the host cell—immediate early (α), early (β), and late (γ) (Kalamvoki & Roizman, 2011) α proteins ICP0 (infected cell protein 0) and ICP4 are transported directly to the nucleus and are responsible for regulating the transcription of immediate early genes and for activating the transcription of early genes, β proteins, and interacting with cellular RNA polymerase II (Nagel et al., 2011; Lester & Deluca, 2011). ICP0 is required for efficient viral gene expression, replication, and reactivation from latency (Mostafa et al., 2011). VP 22, a major component of the HSV-1 tegument, has been implicated in the recruitment of ICP0 from the cytosol to ICP0-specific domains in the cytoplasm, a necessary step in the viral assembly pathway (Gillian, Hafezi, & Bernard, 2005; Maringer & Elliott, 2010). Additionally, α proteins counteract host cellular silencing responses and increase the rate of core histone chromatin exchange, promoting HSV-1 gene expression (Conn, Hendzel,

& Schang, 2011). The β proteins ICP8, UL42, and thymidine kinase function primarily in viral DNA replication and production substrates for DNA synthesis (Liptak, Uprichard, & Knipe, 1996). γ proteins gB, gC, gD, and gH are virion structural proteins required for virus assembly and particle egress; γ proteins are produced on and inserted into the membranes of the rough endoplasmic reticulum and γ proteins are also transported to the nucleus for assembly of the nucleocapsid and for DNA packaging (Farnsworth, Goldsmith, & Johnson, 2003; Farnsworth et al., 2007; Rode et al., 2011).

The primary HSV-1 infection begins in epithelial cells at mucosal surfaces. The virus is transmitted through saliva into the eyes or breaks in the skin. The host's intrinsic and innate defenses, including interferon (IFN) and cytokines, limit the spread of the infection at this stage (Mossman & Ashkar, 2005). The virions may infect local immune effector cells, including dendritic and natural killer (NK) cells, and virions spread locally between epithelial cells and may even spread deeper to fibroblasts, capillary endothelial cells, sweat glands, and hair follicles (Mossman & Ashkar, 2005). Antibody neutralization to HSV-1 is non-existent, as the virus is spread through cell-to cell contact, not through the hematogenous or lymphatic route (Akhtar & Shukla, 2009). Virions are released from the basal surface in close proximity to sensory nerve endings. HSV-1 spreads from neuron to neuron at or near sites of synaptic contact (Akhtar & Shukla, 2005; Fatahzadeh & Schwartz, 2007). The virion envelope glycoproteins fuse with cell receptors on plasma membranes of nerve endings in a pH-independent manner, releasing the nucleocapsid with the inner tegument proteins into the axoplasm (Akhtar & Shukla, 2009). HSV-1 virions then bind to the extracellular matrix glycosaminoglycans, including heparin sulfate and/or chrondroitin sulfate via envelope glycoproteins gB and gC (Lin et al.,

2011). Viral protein gD interacts with a second cellular receptors, nectin-1, nectin-2 (members of the immunoglobulin superfamily), and herpesvirus entry mediator (HVEM; a member of the tumor necrosis factor receptor family) which are found primarily at junctions of epithelial cells and the synaptic junctions of neurons and undergoes a conformational change which in turn recruits gH and gL (Akhtar & Shukla, 2009; Lin et al., 2011). Additionally, viral protein gD may interact with a modified heparin sulfate molecule, 3-O-sulfated heparin sulfate, which is found in corneal cells (Akhtar & Shukla, 2009). Viral and plasma membrane fusion is mediated by viral glycoproteins gD, gB, gH, and gL (Akhtar & Shukla, 2009). When membrane fusion occurs, tegument proteins and the HSV-1 nucleocapsid are released into the host cell's cytoplasm (Akhtar & Shukla, 2009). Viral nucleocapsids are then transported on microtubules by dynein motors over long distances to the neuronal cell bodies that innervate the infected peripheral tissue where the viral DNA enters host cell's nucleus through attachment with microtubules (Rode et al., 2011). After viral adsorption, some tegument proteins are transported to the nucleus while others remain in the cytoplasm. The HSV-1 icosahedral nucleocapsid docks at the nuclear pore and releases viral DNA into the host cell's nucleus. Viral tegument protein (VP) 16 interacts with the host cell transcription proteins to stimulate the transcription of the immediate-early (α) genes by the host cell RNA polymerase II (Conn, Hendzel, & Schang, 2011). Some immediate-early mRNAs are spliced and transported to the cytoplasm and translated. VP16 recruits HCF-1. Oct-1 and LSD-1 to response elements of immediate-early promoters while α proteins are transported to the nucleus to activate the transcription of early genes and regulate transcription of immediate-early genes (Kalamvoki & Roizman, 2011). Early gene

transcripts (β) are then transported to the cytoplasm and translated. These β proteins function primarily in viral DNA replication and production of substrates for viral DNA synthesis; some β proteins are transported to the nucleus while some remain in the cytoplasm (Liptak, Uprichard, & Knipe, 1996). Viral DNA synthesis is then initiated at HSV-1 Ori and DNA replication and recombination produces a long, concatemeric DNA template for late-gene expression (Martin, Deb, Klaur, & Deb, 1991). Late proteins (γ) are primarily virion structural proteins which are necessary for virus assembly and particle egress; γ proteins may also insert into the membranes of the rough endoplasmic reticulum (Farnsworth, Goldsmith, & Johnson, 2003; Farnsworth et al., 2007; Rode et al., 2011). Furthermore, membrane proteins may be modified by glycosylation and precursor glycoproteins are transported to the Golgi apparatus for modification and processing while mature glycoproteins are then transported to the plasma membrane of the infected cell. The newly replicated viral DNA is packaged in nucleocapsids and buds from the inner nuclear membrane into the perinuclear lumen along with tegument proteins to form an envelope. These immature enveloped virions fuse with the outer nuclear membrane from within, releasing the nucleocapsid into the cytoplasm while leaving behind viral membrane proteins. The tegument proteins which were added in the nucleus remain with the nucleocapsid. The nucleocapsids bud into late golgi-endosome compartment, acquire an envelope containing a mature viral envelope and proteins and a complete tegument layer. The enveloped HSV-1 particle buds into a vesicle, is transported to the plasma membrane, and is finally released by exocytosis (Akhtar & Shukla, 2009; Lin et al., 2011; Mettenleiter, Klupp, & Granow, 2009). Acute infection commences in the affected ganglions, resulting in ulcerative lesions. Because peripheral nervous system ganglia are

in close proximity to the bloodstream, HSV-1 is exposed to lymphocytes and humoral effectors of the immune system and the infected ganglia become inflamed and populated with lymphocytes and macrophages, leading to cell-mediated immunopathology and the appearance of symptoms (Fatahzadeh & Schwartz, 2007; Mossman & Ashkar, 2005). Cell mediated immunity is required for the resolution of the HSV-1 infection while ganglion infection is resolved within seven to fourteen days after the primary infection ensues and at this point, long term latent infection is established (Fatahzadeh & Schwartz, 2007).

Latent Infection:

The latent HSV-1 infection occurs primarily in the neuronal ganglia of the peripheral nervous system (Held et al., 2011). Neurons do not undergo mitosis, thus no further viral replication is required for HSV-1 to persist in the latent state. Furthermore, HSV-1 rarely spreads to the central nervous system even though the virus is in direct synaptic contact with the peripheral nervous system (Fatahzadeh & Schwartz, 2007). This life cycle is similar to lytic infection, except that the viral DNA circularizes in the nucleus and wraps around nucleosomes (Su et al., 2002). The nucleosome-covered viral genome is tethered in the nucleus to cellular chromatin. The latent HSV-1 genome is transcriptionally silent as only a single pre-mRNA is produced from the latency-associated transcript (LAT) promoter (Wang et al., 2005). LAT RNA is spliced and forms a stable intron in the form of a lariat which is maintained in the cell's nucleus. The spliced LAT mRNA is transported to the cytoplasm where several small open reading frames may be translated. When cellular proteins are produced in response to changes in neuronal physiology

induced by trauma such as tissue and nerve damage, surgery, hormonal changes, fever, fatigue, immunosuppression, steroids, sunlight, and physical and psychological stress, viral replication is initiated as HSV-1 can travel either in the antiretrograde direction and reinfect epithelial tissue or in the retrograde direction to infect synaptically linked neurons, hence the productive infection cycle commences (Fatahzadeh & Schwartz, 2007; Lin et al., 2011). ICP0 phosphorylation and reactivation appears to be a contributing factor of HSV-1 switching from the latent to the lytic cycle (Everett, Parsy, & Orr, 2009; Mostafa et al., 2011).

Diagnosis and Treatment:

The majority of HSV-1 infections are non-life threatening, except in the immunocompromised and neonate population, especially in the case of keratoconjunctivitis, meningitis, and encephalitis (Lin et al., 2011). Detection of HSV-1 normally is performed through the propagation of viral cultures and the subsequent direct cell observations of cytopathetic effect. In addition, immunohistochemistry such as direct fluorescent antibody testing can be incorporated to detect HSV-1 antigens present in specimen The Chemicon SimulFluor® immunofluorescence assay simultaneously diagnoses HSV-1, HSV-2 and varicella-zoster virus (VZV) in vesicular, oral, genital and skin lesions (Chan, Brandt, & Horsman, 2001). Polymerase chain reaction can detect HSV-1 DNA in clinical specimens and is the most precise and sensitive of diagnostic tools especially when determining the subspecies of virus present, and to confirm encephalitis (Fatahzadeh & Schwartz, 2007). Furthermore, after a conclusive diagnosis is

determined, therapeutic antiviral treatments are introduced. These antiviral treatments however only limit the symptoms of HSV-1 by inhibiting viral replication. Normally topical antiviral agents are applied to accessible lesions and inaccessible lesions are treated with systemic antiviral agents, either orally or intravenously. Acyclovir (Zovirax®) is the most commonly prescribed antiviral agent to combat HSV-1, both topically and systemically (Fatahzadeh & Schwartz, 2007). Acyclovir is a synthetic guanosine analogue which displays an affinity for thymidine kinase, a protein encoded by HSV. Acyclovir stops replication if HSV by competitive inhibition of viral DNA polymerase, incorporation into and termination of the growing viral DNA chain, and the inactivation of viral DNA polymerase (Lin et al., 2011). The drug has relatively mild side effects, including nausea, diarrhea, and general malaise although an extremely rare but serious side effect includes renal impairment/failure while acyclovir has low average oral bioavailability, low plasma protein binding, and short plasma elimination half-life, thus requiring frequent dosage for optimum therapeutic value (Arduino & Porter, 2006; Lin et al., 2011). Furthermore, acyclovir resistant strains of HSV have surfaced, especially in HIV-positive patients, warranting the urgent development of new drugs to combat HSV infections (Lin et al., 2011). Foscarnet (Foscavir®), a viral DNA polymerase-targeting inhibitor has been employed in acyclovir-resistant patients but has also been hindered by drug resistance (Danve-Szatanek et al., 2004; Lin et al., 2011). Helicase-primase inhibitors (HPI) have proven promising as new therapeutic agents against HSV. HPI targets the UL5/8/52 helicase-primase complex essential for HSV DNA synthesis. Higher potency against HSV has been established with this class of drugs in both cell culture and animal models, although resistant laboratory strains of HSV

have been reported (Biswas, Miguel, Sukla, & Field, 2009; Biswas, Swift, & Field, 2007). Vaccine development against HSV viral glycoprotein D has proven efficacious, with a recent study suggesting that vaccination was successful against HSV-1 genital infections (Belshe et al., 2012). New topical therapeutic compounds must be identified to inhibit the sexual transmission of HSV disease, especially considering the emergence of drug-resistant strains in the HIV population (Harden, Falshaw, Carnachan, Kern, & Prichard, 2009).

Curcumin:

Curcumin (diferuloylmethane) is a polyphenol extracted from the plant *Curcuma longa,* a member of the ginger family Zingiberaceae. Curcumin is the main chemical component of turmeric powder extracted from the rhizome of this plant. The extract has been employed in Ayurveda, the Indian system of medicine for 6000 plus years (Aggarwal, Kumar, & Bharti, 2003; Beevers & Huang, 2011). Curcumin is a natural yellow orange dye that is commonly used as a spice, flavoring agent, food preservative and coloring agent. Furthermore, studies have demonstrated that curcumin is a potent antioxidant, anti-inflammatory, antiproliferative, and anti-atherosclerotic (Aggarwal, Kumar, & Bharti, 2003; Beevers & Huang, 2011; Si et al., 2007). Curcumin has been shown to inhibit scarring, gallstone, and cataract formation; promote wound healing, muscle regeneration; prevent liver injury and kidney toxicity; exert medicinal benefits against psoriasis, diabetes, multiple sclerosis, Alzheimer's, HIV, septic shock, cardiovascular disease, lung fibrosis, arthritis, and inflammatory bowel disease (Aggarwal, Kumar, & Bharti, 2003; Beevers & Huang, 2011). Additionally, current research suggests that

curcumin may exhibit chemotherapeutic and chemopreventative properties against various cancers, including breast cancer, colon cancer, kidney cancer, liver cancer, leukemia, basal cell carcinoma, prostate cancer, rhabdomyosarcoma, and melanoma (Beevers & Huang, 2011). Curcumin has effectively demonstrated inhibitory properties for the major stages of carcinogenesis including transformation, initiation, promotion, invasion, angiogenesis, and metastasis (Beevers & Huang, 2011). Commercially available curcumin contains three major components: curcumin, demethoxycurcumin (curcumin II), and bis-demethoxycurcumin (curcumin III) which together are known as the cucuminoids (Aggarwal, Kumar, & Bharti, 2003; Beevers & Huang, 2011). Curcumin is insoluble in water but is soluble in the organic solvents methanol and dimethylsulfoxide (DMSO) (Beevers & Huang, 2011). Curcumin has poor gastrointestinal absorption, limiting its bioavailability and effectiveness when administered orally and curcumin toxicity is low, as little side effects were reported in human clinical trials at doses up to 10g/day (Aggarwal, Kumar, & Bharti, 2003). Curcumin has been proven to be phototoxic against bacteria and could possibly be application in the topical phototherapy of psoriasis, cancer, bacterial, and viral diseases (Aggarwal, Kumar, & Bharti, 2003).

Current HSV research has focused on the development of topical microbicides that prove efficacious against the virus, especially in HIV-positive women. Limiting the sexual transmission of the virus has become imperative as the HIV-positive population continues to expand. Plant-derived compounds have displayed antimicrobial activity and remain popular research options in part because of their low toxicity, suggested effectiveness in laboratory studies, and low developmental cost (Bourne, Bourne,

Reising, & Stanberry, 1999; Harden et al., 2009). Curcumin demonstrated bactericidal effects against gram positive and gram negative bacteria by disrupting bacterial cell walls (Beevers & Huang, 2011). Furthermore, curcumin exhibited antiviral activity against HSV-2 in HeLa cell lines and HSV-1 infectivity, immediate-early gene expression and replication in Vero cell cultures (Bourne, Bourne, Reising, & Stanberry, 1999; Kutluay, Doroghazi, Roemer, & Triezenberg, 2008). Prior studies illustrated that curcumin slows but does not totally block HSV-1 replication, thus suggesting that viral adsorption is not completely hindered (Kutluay, Doroghazi, Roemer, & Triezenberg, 2008). Additional evidence has suggested polysaccharide extracts effectively inhibit HSV-1 binding to the host cell (Harden et al., 2009). Polyphenolic secondary metabolites have demonstrated to hinder viral adsorption to the host cell membrane and inhibit penetration of HSV-1 (Lin et al., 2011). Therefore, to further evaluate the effectiveness of curcumin on HSV-1, this study will attempt to identify the mechanism of viral inhibition in Vero cells. To determine if curcumin inhibits binding of HSV-1, penetration of HSV-1 and/or replication of HSV-1, first the cytotoxicity of curcumin on Vero cells must be established utilizing a trypan blue assay. After a safe concentration of curcumin on the Vero cell line is determined, the inhibitory effects of curcumin on HSV-1 propagation in Vero cells will be investigated through the incorporation of plaque assays, spectrophotometry, and subsequent gel electrophoresis of viral gene products.

Materials and Methods

Cells:

Cell line. Vero (African green monkey kidney cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were grown in Dulbecco modified minimum Eagle's medium (DMEM) (Mediatech Incorporated, Manassas, VA, USA) supplemented with 5% fetal bovine serum (FBS) (Biowest, Miami FL, USA) and 1% gentamicin (Lonza, Walkersville, MD, USA).

Cell cultures. Vero cells were maintained in T25 flasks with 5 mL 5% FBS-DMEM in a 37°C incubator with an atmosphere of 5% CO_2. To passage the Vero cells, the 5% FBS-DMEM was removed and the cells were washed twice with 5.0 mL 1X phosphate buffered saline (PBS) (Fisher Scientific, Fair Lawn, NJ, USA) supplemented with 1% gentamicin. Cells were trypsinized with 0.5mL of 1X trypsin-EDTA (Mediatech Incorporated, Manassas, VA, USA) at 37°C with an atmosphere of 5% CO_2 for 5 minutes to detach cells. 4.5 mL fresh 5% FBS-DMEM was added to the flask, removed and discarded. An additional 4.5mL of 5% FBS-DMEM was then added to the flask to maintain the 5.0 mL total volume. Cells were maintained at a ratio of 1:10 and subcultured one time per week.

Virus. A recombinant strain of HSV-1, GHSV-UL46, was incorporated in all experiments (ATCC, Manassas, VA, USA). The recombinant strain contains a sequence for green fluorescent protein (GFP) fused to tegument protein pUL46. To prepare HSV-1, a T25 flask with Vero cells was prepared as described in the cell culture section.

When cells were approximately 70% to 80% confluent, media was poured off the cells and 200µl of HSV-1 was added to the flask. The flask was then incubated at 37°C with an atmosphere of 5% CO_2 for one hour with intermittent rocking. Six mL of 5% FBS-DMEM was added and the flask was incubated for 48 hours at 37°C and an atmosphere of 5% CO_2. The lysate was harvested and centrifuged in a 15ml conical tube for 10 minutes at 4500 rpm to remove cellular debris. The lysate was aliquoted into cryogenic vials and stored at

-80°C.

Curcumin. Curcumin (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ USA) to prepare an initial 4% solution of curcumin. Subsequent concentrations were prepared by diluting the prior concentration by one-half, yielding concentrations of 2%, 1%, 0.5%, 0.25%, 0.125%, 0.06%, 0.03%, and 0.015% respectively.

Cytotoxicity of Curcumin:

Trypan blue assay. Vero cells were plated in 6-well plates (Corning NY, USA) with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO_2 until confluent, approximately 24-48 hours. The media was aspirated from each well and 100 µl of the curcumin concentrations was added to each respective well; as controls, 100 µL of 5% FBS-DMEM, 100 µL of methanol, and 100 µL of DMSO were each added to separate wells. Plates were incubated at 37°C with an atmosphere of 5% CO_2 for 1 hour with intermittent rocking every 15 minutes to ensure

complete coverage of cells with curcumin. After 1 hour, any remaining curcumin solution was aspirated from the cells and 3 mL of 5% FBS-DMEM was added to each well. The 6-well plates were then incubated at 37°C and an atmosphere of 5% CO_2 for 48 hours. 2 mL of 5% FBS-DMEM was then removed from each well. The cell monolayer of each well was scraped with a sterile cell scraper and 100 µL of the cell suspension for each well was transferred to a separate Eppendorf tube. 100 µL of trypan blue solution (Mediatech, Inc., Manassas, VA, USA) was then added to each separate Eppendorf tube containing the cell suspensions. After 1-2 minutes, 100 µL of each separate mixture in the Eppendorf tubes was loaded individually onto a hemacytometer (Hausser scientific, Horsham, PA, USA) and observed under a Leica ATC 200 microscope at 400X magnification. Live cells (clear) and dead cells (dark blue) were counted in the four corner squares and middle square of the grid. Total cells for each curcumin concentration were calculated, in addition to the percent live cells.

Viral Inhibition:

Cell-treated virions. Vero cells were plated in 6-well plates with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO_2 until confluent, about 24-48 hours. The media was aspirated and cells were treated with 100 µL of each of the nine concentrations of curcumin; controls included 2 wells of 100 µL of 5% FBS-DMEM. Plates were rocked and incubated at 37°C with an atmosphere of 5% CO_2 for 15 minutes. Unabsorbed solution was aspirated and 100 µL of stock virus was added to each well, excluding the media control well (mock-infected) that contained 100 µL of 5% FBS-DMEM. The plates were returned to the incubator at 37°C and 5% CO_2

for 1 hour and rocked every 15 minutes to ensure complete coverage. After 1 hour, unabsorbed virus was aspirated from each well and 2.5 mL of 5% FBS-DMEM was added to each well. The plates were incubated at 37°C with an atmosphere of 5% CO_2 for 48 hours. At that time, the lysate from each well was harvested, transferred to cryogenic vials, and stored at -80°C.

Virus-treated virions. Vero cells were plated in 6-well plates with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO_2 until confluent, about 24-48 hours. 100 µL of stock virus was mixed with 100 µL of each of the curcumin concentrations for a total of 9 Eppendorf tubes. The HSV-1 control tube contained 100 µL of the 5% FBS-DMEM in place of the curcumin plus 100 µL of the virus. An additional control tube contained no virus, only 200 µL of the 5% FBS-DMEM. The mixtures remained at room temperature (approximately 25°C) for 15 minutes. During this time, the media was aspirated from each well of the 6-well plate. 200 µL of each mixture was added to separate wells on the 6-well plate. The plates were returned to the 37°C incubator with an atmosphere of 5% CO_2 for 1 hour with rocking every 15 minutes. Unabsorbed virus/curcumin mixtures were aspirated from each well and 2.5 mL of 5% FBS-DMEM was added to each well. The plates were once again returned to the 37°C incubator with an atmosphere of 5% CO_2 for 48 hours after which the lysate was harvested, transferred to cryogenic vials, and stored at -80°C.

Cytopathic effects. Vero cells were plated in 6-well plates with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO_2 until confluent, about 24-48 hours. 200 µL of appropriate concentrations of cell-treated and

virus-treated media were added to wells of 6-well plate. The plate was incubated at 37°C and an atmosphere of 5% CO_2 for an hour with intermittent rocking. Unabsorbed media was aspirated and 3.0 ml of 5% FBS-DMEM was added to each well. The plates were returned to the 37°C incubator with an atmosphere of 5% CO_2 for 48 hours. Cell viability was observed with phase contrast microscopy. The lysates of viable cells were harvested, transferred to cryogenic vials, and stored at -80°C.

Plaque assay. Vero cells were plated in 6-well plates with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO_2 until confluent, about 24-48 hours. Viral titers were determined by performing ten-fold serial dilutions of cell-treated and virus-treated media. To start, 1% FBS-PBS was prepared by mixing 0.5 mL of FBS with 49.5 mL of PBS. 900 µL of the FBS-PBS was added to an Eppendorf tube with 100 µL of the appropriate treated media harvested from the CPE assay. 100 µL of this initial 10^{-1} dilution was then added to 900 µL of 1% FBS-PBS to yield the 10^{-2} dilution. Dilutions proceeded in this fashion until the 10^{-7} dilution was prepared. The media was aspirated from each well of the 6-well plate and 0.5 mL of each corresponding dilution was added to each well on the 6-well plate starting with the 10^{-3} dilution and concluding with the 10^{-7} dilution. One well served as the control to which 0.5 mL of 1% FBS-PBS was added. This dilution scheme was repeated for each concentration of treated media for which quantitative results were desired. The plates were returned to a 37°C incubator with an atmosphere of 5% CO_2 for 1 hour with intermittent rocking. After 1 hour any unabsorbed treated media was aspirated from each well and approximately 3 mL of nutrient agar was poured over each well. The nutrient agar was comprised of two components, solutions A and B. Solution A included 17 mL

of 3X eagle medium (Gibco Invitrogen Corporation, Grand Island, NY, USA), 1.5 mL of 5% sodium bicarbonate (Gibco Invitrogen Corporation, Carlsbad, CA, USA), 0.5 mL FBS, 0.1 mL DEAE-dextran (ICN Biomedicals Incorporated, Aurora, OH, USA), 0.1 mL penicillin/streptomycin (Cambrex, Walkersville, MD, USA), and 0.05% mL gentamicin for a total volume of 19.25 mL. Solution B consisted of an autoclaved solution of 0.2 g of bacteriological agar (Oxoid Limited, Baskingstoke, Hampshire, England) diluted with 30 mL MQ water. Solutions A and B were tempered in a 41°C water bath. After the 1 hour incubation was complete, solutions A and B were combined and added to the wells. The plates were stored in a 37°C incubator with an atmosphere of 5% CO_2 for 72 hours. The agar was then removed and the cells were stained with crystal violet. The number of plaques formed was counted in each well to calculate the viral titer in plaque forming units (PFU) per milliliter.

Virus penetration assay. Vero cells were plated in 6-well plates with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO_2 until confluent, about 24-48 hours. The media was aspirated and virus suspensions were added to each well. The plates were placed in Tupperware containers to eliminate risk of contamination and incubated at 4°C for 2 hours to permit viral attachment. Serial dilutions of 10^{-3} to 10^{-7}prepared from each treated media harvested from CPE were then added to the appropriate wells at room temperature. The plates were incubated for 10 minutes at 37°C and atmosphere of 5% CO_2 to allow penetration. Dilutions of extracts were then aspirated and the cell monolayers were briefly washed with 1% PBS. Nutrient agar comprised of Solution A and B was prepared as indicated previously. 2.5 mL of the nutrient agar was added to each well and the plates were incubated at 37°C with an

atmosphere of 5% CO_2 for 72 hours. The agar was removed and cell monolayers were stained with crystal violet.

Infectious Dose 50 (ID_{50}). Vero cells were plated in 6-well plates with 2.5 ml of cell suspension added to each well and incubated at 37°C and an atmosphere of 5% CO_2 until confluent, about 24-48 hours. The media was aspirated and serial dilutions of 10^{-2} to 10^{-6} were prepared from the 4% and 2% curcumin and HSV CPE lysates. The plates were incubated at 37°C and an atmosphere of 5% CO_2 for 1 hour with intermittent rocking. Dilutions of unabsorbed extracts were then aspirated and 3.0 ml of 5% FBS-DMEM was added to each well. The plates were returned to the 37°C incubator with an atmosphere of 5% CO_2 for 48 hours. Cell viability was observed with phase contrast microscopy. The cells were stained with crystal violet to aid in the identification of live cells.

DNA extraction. Vero cells plated in $60mm^2$ culture dishes were infected with 100 µL HSV-1 and 5% FBS-DMEM and 100 µL HSV-1, 100 µL of the virion-treated 4% curcumin lysate, and 5% FBS-DMEM respectively. The cells were incubated at 37°C with an atmosphere of 5% CO_2 for 18-24 hours. The DNA for each of the two sets of cells was extracted with the Qiagen DNeasy® Blood & Tissue Kit (Qiagen Sciences, Germantown, MD, USA), following the purification of total DNA from animal blood or cells spin column protocol provided by the manufacturer.

DNA quantification. DNA was quantified in the extracted DNA products with the NanoDrop ND-100 Spectrophotometer with the accompanying computer software

(NanoDrop Technologies Incorporated, Wilmington, DE, USA). The standard protocol as supplied by the manufacturer was followed.

Polymerase chain reaction (PCR). HSV-1 DNA was amplified by PCR as instructed by the GoTaq® PCR Core Systems quick protocol (Promega Corporation, Madison, WI, USA). The primers were sequence-specific for the HSV-1 strain incorporated in all assays. The primers coded for glycoprotein D (gD) and green fluorescent protein (GFP). Primers included:

Glycoprotein D:

gD2F: 5'-TTGTTTGTCGTCATAGTGGGCCTC-3'

gD2R: 5'-TGGATCGACGGTATGTGCCAGTTT-3'

Green Fluorescent Protein:

GFP1F: 5'-GTCAAAGCTTAAGATGGTGAGCAAGG-3'

GFP1R: 5'-CTTGAAGCTTCTTGTACAGCTCGTCC-3'

Gel electrophoresis. PCR products were visualized on a 1% agarose gel.

Results

Cytotoxicity

Trypan blue assay. Live and dead cells for each concentration of curcumin in addition to positive and negative controls were counted. Live cells exclude the trypan blue dye and therefore appear clear upon microscopic examination while dead cells uptake the dye and appear blue upon microscopic examination. For each curcumin concentration, the percentage of living cells was calculated by dividing the number of viable cells and diving by the total number of cells (Table 1). The methanol and DMSO served as negative controls whereas the 5% FBS-DMEM served as the positive media control. The Vero cells successfully tolerated all concentrations of curcumin as compared to the positive media control (Figure 1).

Table 1

Curcumin Cytotoxicity in Cultured Vero Cells: Trypan Blue Assay

Curcumin Concentration	Viable Cells
4.00%	71% ±3%
2.00%	53% ±3%
1.00%	50% ±3%
0.50%	80% ±1%
0.25%	60% ±5%
0.125%	74% ±2%
0.06%	77% ±7%
0.03%	75% ±3%
0.015%	67% ±3%
0 (Media)	71% ±4%
0 (DMSO)	0
0 (Methanol)	0

Note. For each sample, the percentage of viable Vero cells was determined microscopically by counting the number of viable (clear) and non-viable (dark blue) Vero cells in each of the four quadrants plus the middle square on the hemacytometer. The number of viable cells was then divided by the total number of Vero cells for each sample to calculate the percentages of viable Vero cells. Values represent an average of two samples ±SD.

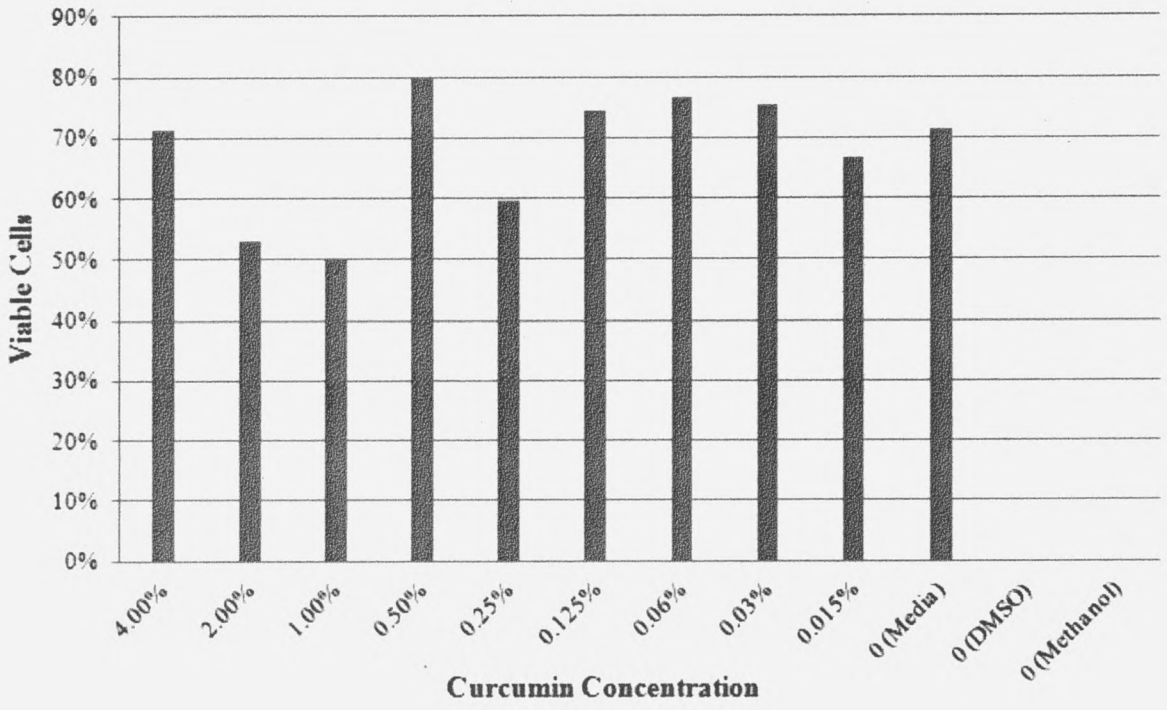

Figure 1. Percent of viable Vero cells after treatment with curcumin concentrations.

Viral Inhibition

Phase contrast microscopy. To observe the cytopathic effects of HSV-1 on Vero cells and to determine whether curcumin could inhibit HSV-1, thereby reducing or preventing observable cytopathic effects, cell-treated and virion-treated Vero cells were observed with the Motic AE31 phase contrast microscope at 400X magnification at 18-24 hours post-infection. The 4% and 2% concentrations of curcumin successfully inhibited cytopathetic effects of HSV-1, paralleling the 5% FBS-DMEM control group. The Vero cells infected with HSV-1 displayed rounding and detaching of cells, characteristic observations of cytopathetic effect.

Plaque assay. After determining that 4% and 2% curcumin could successfully inhibit cytopathic effects of HSV-1 infected Vero cells, plaque assays were performed to determine viral titer. However, the plaque assays were inconclusive as the Vero cells lifted repeatedly from the 6-well plates when the nutrient agar was removed, resulting in insufficient staining of the cells with crystal violet. However, no cytopathic effects were observed when the Vero cells were viewed under phase contrast microscopy before removal of the agar.

Virus penetration assay. The virus penetration assay was inconclusive because the Vero cells lifted repeatedly from the 6-well plates when the nutrient agar was removed, resulting in insufficient staining of the Vero cells with crystal violet. Cells were viable and no cytopathic effects were observed with both the 4% and 2% curcumin under phase contrast microcopy while the agar was still intact.

ID_{50}. The dose at which 50% or more of the cells exhibited cytopathic effects was determined to be 10^5 pfu/ml for the HSV-1 positive control, 10^2 pfu/ml for the 4% curcumin and 10^2 pfu/ml for the 2% curcumin. This data suggests that the production of new HSV-1 virions was impaired.

DNA extraction and comparison: To compare the total DNA of Vero cells infected with HSV-1 versus the total DNA of Vero cells treated with 4% curcumin and infected with HSV-1, DNA was extracted from the requisite Vero cells and measured with the NanoDrop spectrophotometer. The total DNA extracted from the untreated Vero cells measured 70.2 ng/µl and expressed a DNA/RNA A260/Protein A280 sample purity ratio of 1.98. The total DNA extracted from the 4% curcumin-treated Vero cells measured 51.7 ng/µl and expressed a DNA/RNA A260/Protein A280 sample purity ratio

of 2.04. This data suggests adequate purity of the samples and a decrease in the total amount of DNA present in the Vero cells when the HSV-1 is exposed to the 4% curcumin.

PCR and gel electrophoresis. To isolate the viral DNA from the total (cellular and viral) DNA extracted from the HSV-1 infected Vero cells and the HSV-1 infected curcumin-treated Vero cells, HSV-1 specific primers gD2 and GFP were incorporated during PCR to amplify the viral DNA. Gel electrophoresis of the PCR products displayed high intensities of bands for the HSV-1 infected cells. The bands for HSV-1 infected 4% curcumin-treated cells were lighter in intensity, suggesting that the concentration of viral DNA was reduced and that curcumin exerted an inhibitory effect on HSV-1 replication (Figure 2).

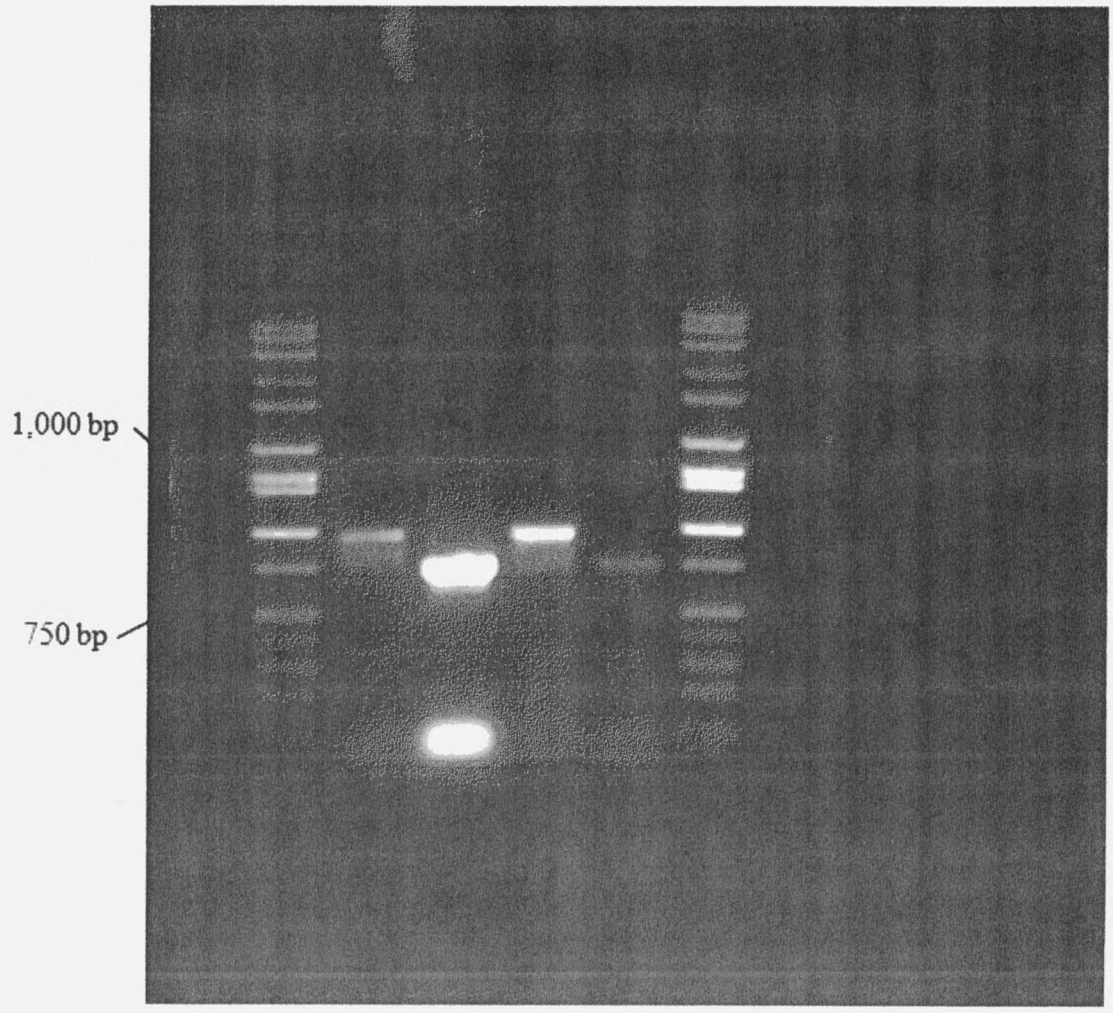

Figure 2. Gel electrophoresis of PCR products extracted from HSV-1 infected curcumin-treated and untreated cells with specific HSV-1 primers GFP and gD. Lanes 1 and 6 contain the Hi/Lo marker, lane 2 Curcumin-treated HSV-1 GFP, lane 3 HSV-1 gD, lane 4 HSV-1 GFP, lane 5 curcumin-treated HSV-1 gD..

Discussion

As herpesvirus infections remain ubiquitous world-wide, the need for safe, effective and inexpensive therapies to control the spread of the virus requires investigation and development. Although the majority of HSV infections are non-life threatening, the virus can be fatal in the neonate and immunocompromised populations. Current HSV research has focused on limiting the spread of HSV within the HIV-positive population although topical biocides and systemic antiviral therapies that eliminate the genital spread of the disease remain elusive to researchers and clinicians alike. Plant-derived products remain popular choices as microbicides, especially considering their low cost, suggested effectiveness in laboratory studies, and low toxicity (Bourne, Bourne, Reising, & Stanberry, 1999; Harden et al., 2009). Curcumin has proven promising as a bactericidal agent against different species of bacteria and fungi (Beevers & Huang, 2011). Furthermore, curcumin has demonstrated antiviral activity in HeLa cells and Vero cells (Bourne, Bourne, Reising, & Stanberry, 1999; Kutluay, Doroghazi, Roemer, & Triezenberg, 2008). However, the mechanism of curcumin's inhibition of HSV activity has not been definitively established. Plant extracts have previously been determined to interfere with HSV adsorption while curcumin has been implicated in the inhibition of viral infectivity, immediate-early gene expression and replication in Vero cell cultures (Harden et al., 2009; Kutluay, Doroghazi, Roemer, & Triezenberg, 2008; Lin et al, 2011). Therefore, this study aimed to determine the mechanism of curcumin's antiviral activity on HSV-1 in Vero cell cultures.

Viral inhibition depends upon the interruption of one or more stages of the virus's life cycle to limit and/or eliminate viral progeny. However, throughout the process of

destroying the virus, the host cells must remain viable. Therefore, the first step in determining the antiviral effectiveness of a potential therapeutic agent is to evaluate the cytotoxicity of the agent on the host cells. The curcumin was determined to be safe at the 4% concentration and all subsequent lower concentrations. Viral inhibition of HSV-1 was observed by the lack of the characteristic cytopathic effects when the Vero cells were treated with the 4% and 2% curcumin. The untreated Vero cells displayed rounding and detaching of cells when infected with HSV-1. Unfortunately, the determination of viral titer by plaque assay was inconclusive. Upon microscopic examination, the Vero cells were viable, but enumeration was prohibited by the lifting of the Vero cells when the nutrient agar was discarded. The cell monolayer was detached which made proper staining with crystal violet unsuccessful. The explanation for this disruption of the Vero cell monolayer remains elusive, but the curcumin and/or DMSO may have had an effect on the proper attachment of the Vero cells to the plates. Both substances are nonpolar and thus either alone or in combination may interfere with the monolayer's remaining fixed to the 6-well plates as the nutrient agar is scored and lifted from the individual wells. The detachment of the Vero cells also occurred during the virus penetration assay, leading to inconclusive results as well. However, the ID_{50} assay was successful in quantifying the infectious dose of HSV-1 in the Vero cells to be 10^5 pfu/ml versus the 10^2 pfu/ml for the 4% and 2% curcumin-treated Vero cells, respectively. This 1000-fold decrease in HSV-1 infectivity suggests curcumin exhibits antiviral activity against herpesvirus. Additionally, DNA extraction of the HSV-1 infected Vero cells versus the curcumin-treated Vero cells demonstrated a 26% reduction in total DNA from 70.2 ng/µl to 51.7 ng/µl. This result also suggests that HSV-1 was successful at infecting Vero cells

and that the difference in DNA concentration is due to the inhibition of HSV-1 by curcurmin. Amplification of PCR products with the HSV-1 specific primers gD and GFP resulted in decreased band intensities for the 4% curcumin-treated HSV-1 for gel electrophoresis as compared to the untreated HSV-1. This result, combined with the decrease in total DNA in the curcumin-treated cells versus the untreated cells strongly suggests that curcumin is inhibiting HSV replication in Vero cells.

The evidence from the assays conducted in this study collectively proposes that curcumin exhibits an anti-viral effect on HSV-1 in Vero cell cultures. The virus appears to adsorp and gain entrance to the cell, as demonstrated by the presence of HSV-1 in the PCR products. However, the replication of HSV-1 is inhibited by the 4% curcumin as displayed by the decrease in band intensities of the PCR products displayed by electrophoresis in the curcumin-treated cells versus the untreated cells. The specifics of the inhibition mechanism are unknown at this present time. Curcumin has been indicated as a modulator of numerous signaling pathways, including MAPKs, PI3K/PKB, nuclear factor kappa B (NF-κB),UPS, and COP9 signalosome (CSN) through inhibition of casein kinase II (CKII) and protein kinase D (PKD) (Bharti, Takada, & Aggarwal, 2004; Chaudry & Hruska, 2003; Jana, Dikshit, Goswami, & Nukina, 2004; Kim et al., 2004; Mullally & Fitzpatrick, 2002; Ehle et al., 2003). Furthermore, curcumin has been demonstrated to inhibit HSV-1 in Vero cells independent of the p300/CBP histone acetyltransferase pathway (Kutluay, Doroghazi, Roemer, & Triezenberg, 2008). Future studies should focus on the effects of curcumin on cellular signaling pathways that contribute to successful HSV-1 replication. Additionally, the lowest dose of curcumin that inhibits viral replication should be established in Vero cells. The solubility of

curcumin in DMSO and other organic solvents or in combination with organic solvents and additional emulsifiers is another factor that should be investigated, as the nonpolar nature of the curcumin and the DMSO may have had an effect on enumerating the viral titer of HSV-1. The curcumin is a difficult substance to handle successfully in the laboratory given its hydrophobic nature.

As indicated in prior research and in this study, curcumin appears to be a promising microbicide against HSV. However, extensive research is still required to validate the efficacy of curcumin as an antiviral agent, both as a systemic therapeutic and topical microbicide.

Bibliography

Aggarwal, B.B., Kumar, A., & Bharti, A.C. (2003). Anticancer potential of curcumin: Preclinical and clinical studies. *Anticancer Research,* 23, 363-398.

Akhtar, J., & Shukla, D. (2009). Viral entry mechanisms: Cellular and viral mediators of herpes simplex virus entry. *Federation of European Biochemical Societies Journal,* 276(24), 7228-7236.

Arduino, P.G., & Porter, S.R. (2006). Oral and perioral herpes simplex virus type 1 (HSV-1) infection: review of its management. *Oral Diseases,* 12(3), 254-270.

Belshe, R.B., Leone, P.A., Bernstein, D.I., Wald, A., Levin, M.J., Stapleton, J.T., Gorfinkle, I., Morrow, R.L., Ewell, M.G., Stokes-Riner, A., Dubin, G., Heineman, T.C., Schulte, J.M., & Deal, C.D. (2012). Efficacy results of a trial of a herpes simplex vaccine. *New England Journal of Medicine,* 366(1), 34-43.

Beevers, C.S. & Huang, S. (2011). Pharmacological and clinical properties of curcumin. *Botanics: targets and therapies,* 1, 5-18.

Bharti, A.C., Takada, Y., & Aggarwal, B.B. (2004). Curcumin (diferuloylmethane) inhibits receptor activity of NF-kappa B ligand-induced NF-kappa B activation in osteoclast precursors and osteoclastogenesis. *Journal of Immunology,* 172, 5940-5947.

Biswas, S.,Miguel, R.N., Sukla, S., & Field, H.J. (2009). A mutation in helicase motif IV of herpes simplex virus type 1 UL5 that results in reduced growth in vitro and

lower virulence in a murine infection model is related to the predicted helicase structure. *Journal of General Virology,* 90, 1937-1942.

Biswas, S., Swift, M., & Field, H.J. (2007). High frequency of spontaneous helicase-primase inhibitor (BAY 57-1293) drug-resistant variants in certain laboratory isolates of HSV-1. *Antiviral Chemistry and Chemotherapy*, 18, 13-23.

Bolstad, M., Abaitua, F., Crump, C.M., & O'Hare, P. (2011). Autocatalytic activity of the ubiquitin-specific protease domain of herpes simplex virus 1 VP1-2. *Journal of Virology,* 85(17), 8738-8751.

Bourne, K.Z., Bourne, N., Reising, S.F., and Stanberry, L.R. (1999). Plant products as microbicide candidates: assessment of in vitro and in vitro activity against herpes simplex virus type 2. *Antiviral Research,* 42, 219-226.

Chan, E.L., Brandt, K., & Horsman, G.B. (2001). Comparison of Chemicon SimulFluor Direct Fluorescent Antibody Staining with Cell Culture and Shell Vial Direct Immunoperoxidase Staining for Detection of Herpes Simplex Virus and with Cytospin Direct Immunofluorescence Staining for Detection of Varicella-Zoster Virus. *Clinical Vaccine Immunology,* 8(5), 900-912.

Chaudhary, L.R. & Hruska, K.A. (2003). Inhibition of cell survival signal protein kinase B/Akt by curcumin in human prostate cancer cells. *Journal of Cell Biochemistry,* 89, 1-5.

Conn, K.L., Hendzel, M.J., & Schang, L.M. (2011). Core histones H2B and H4 are mobilized during infection with herpes simplex virus 1. *Journal of Virology,* 85(24), 13234-13252.

Danve-Szatanek, C., Aymard, M., Thouvenot, D., Morfin, F., Agius, G., Bertin, I., Billaudel, S., Chanzy, B., Coste-Burel, M., Finkielsztejn, L., Fleury, H., Hadou, T., Henquell, C., Lafeuille, H., Lafon, M.E., Le Faou, A., Legrand, M.C., Maille, L., Mengelle, C., Morand, P., Morinet, F., Nicand, E., Omar, S., Picard, B., Pozzetto, B., Puel, J., Raoult, D., Scieux, C., Segondy, M., Seigneurin, J.M., Teyssou, R., & Zandotti, C. (2004). Surveillance network for herpes simplex virus resistance to antiviral drugs: 3-year follow-up. *Journal Clinical Microbiology,* 42(1), 242-249.

Everett, R.D., Parsy, M.L., & Orr, A. (2009). Analysis of the functions of herpes simplex virus type 1 regulatory protein ICP0 that are critical for lytic infection and derepression of quiescent viral genomes. *Journal of Virology,* 83(10), 4963-4977.

Farnsworth, A., Goldsmith, K., & Johnson, D.C. (2003). Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. *Journal of Virology*, 77(15), 8481-8494.

Farnsworth, A., Webb, M., Roller, R., Cohen, G., Eisenberg, R., & Johnson, D.C. 2007. Herpes simplex virus glycoproteins gB and gH function in fusion between the virion envelope and the outer nuclear membrane. *PNAS,* 104(24), 10187-10192.

Fatahzadeh, M., & Schwartz, P.A. (2007). Human herpes simplex virus infections: Epidemiology, pathogenesis, symtomatology, diagnosis and management. *Journal of the American Academy of Dermatology,* 57(5), 737-763.

Gillian, E., Hafezi, W., & Bernard, E. (2005). Deletion of the herpes simplex virus VP22-encoding gene (uL49) alters the expression, localization, and virion incorporation of ICP0. *Journal of Virology,* 79(15), 9737-9745.

Held, K., Junker, A., Dornmair, K., Meinl, E., Sinicina, I., Brandt, T., Theil, D., & Derfuss, T. *(2011).* Expression of herpes simplex virus 1-Encoded microRNAs in human trigeminal ganglia and their relation to local T-cell infiltrates. *Journal of Virology,* 85(19), 9680-9685.

Harden, E.A., Falshaw, R., Carnachan, S.M., Kern, E.R., & Prichard, M.N. (2009). Virucidal activity of polysaccharide extracts from four algal species against herpes simplex virus. *Antiviral Research,* 83(3), 282-289.

Jana, N.R., Dikshit, P., Goswami, A., & Nukina, N. (2004). Inhibition of proteosomal function by curcumin induces apoptosis through mitochondrial pathway. *Journal Biological Chemistry,* 279, 11680-11685.

Kalamvoki, M. & Roizman, B. (2011). The histone acetyltransferase CLOCK is an essential component of herpes simplex virus 1 transcriptome that includes TFIID, ICP4, ICP27, and ICP22. *Journal of Virology,* 85(18), 9472-9477.

Kim, S.M., Park, J.H., Chung, S.K., Kim, J.Y., Hwang, H.Y., Chung, K.C., Jo, I. Park, S.I., & Nam, J.H. (2004). Coxsackievirus B3 infection induces cry61 activation via JNK to mediate cell death. *Journal of Virology,* 78, 13479-13488.

Kutluay, S., Doroghazi, J., Roemer, M.E., & Triezenberg, S.J. (2008). Curcumin inhibits herpes simplex virus immediate-early gene expression by a mechanism independent of p300/CBP histone acetyltransferase activity. *Virology,* 373(2), 239-247.

Lester, J.T., & DeLuca, N.A. (2011). Herpes simplex virus 1 ICP4 forms complexes with TFIID and mediator in virus-infected cells. *Journal of Virology,* 85(12), 5733-5744.

Liptak, L.M., Uprichard, S.L., & Knipe D.M. (1996). Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures. *Journal of Virology*, 70(3), 1759-1767.

Lin, L.T., Chen, T.Y., Chung, C.Y., Noyce, R.S., Grindley, T.B., McCormick, C., Lin, T.C., Wang, G.H., Lin, C.C., & Richardson, C.D. (2011). Hydrolyzable tannins (chebulagic acid and punicalagin) target viral glycoprotein-glycosamminoglycan interactions to inhibit herpes simplex virus 1 entry and cell-to-cell spread. *Journal of Virology,* 85(9), 4386-4398.

Martin, D.W., Deb, S.P., Klaur, J.S., & Deb, S. (1991). Analysis of the herpes simplex virus type 1 OriS sequence: mapping of functional domains. *Journal of Virology,* 65(8), 4359-4369.

Maringer, K., & Elliott, G. (2010). Recruitment of herpes simplex virus type 1 immediate-early protein ICP0 to the virus particle. *Journal of Virology,* 84(9), 4682-4696.

Mettenleiter, T.C., Klupp, B.G., & Granow, H. (2009). Herpesvirus assemply: An update. *Virus Research,* 143, 222-234.

Mossman, K.L., & Ashkar, A.A. (2005). Herpesviruses and the innate immune response. *Viral Immunology,* 18(2), 267-281.

Mostafa, H.H., Thompson, T.W., Kushnir, A.S., Haenchen, S.D., Bayless, A.M., Hilliard, J.G., Link, M.A., Pitcher, L.A., Loveday, E., Schaffer, P.A., & Davido, D.J. (2011). Herpes simplex virus 1 ICP0 phosphorylation site mutants are attenuated for viral replication and impaired for explants-induced reactivation. *Journal of Virology,* 85 (23), 12631-12637.

Mullally, J.E. & Fitzpatrick, F.A. (2002). Pharmocophore model for novel inhibitors of ubiquitin isopeptidases that induce p53-independent cell death. *Molecular Pharmocology,* 62, 351-358.

Nagel, C.H., Albrecht, N., Milovic-Holm, K., Lakshnikanth, M., Keyser, B., Abel, B., Weseloh, B., Hofmann, T.G., Eibl, M.M., & Hauber, J. (2011). Herpes simplex

virus immediate early protein ICP0 is targeted by SIAH-1 for proteasomal degradation. *Journal of Virology,* 85(15), 7644-7657.

Rao, P., Pham, H.T., Kulkarni, A., Yang, Y., Liu, X., Knipe, D.M., Cresswell, P., & Yuan, W. (2011). Herpes simplex virus 1 glycoprotein B and US3 collaborate to inhibit CD1d antigen presentation and NKT cell function. *Journal of Virology,* 85(16), 8093-8104.

Rode, K., Dohner, K., Binz, A., Glass, M., Strive, T., Bauerfeind, R., & Sodeik., B. (2011). Uncoupling uncoating of herpes simplex virus genomes from their nuclear import and gene expression. *Journal of Virology,* 85(9), 4271-4283.

Roizman, B., & Baines, J. (1991). The diversity and unity of herpesviridae. *Comparative Immunology, Microbiology and Infectious Diseases,* 14 (2), 63-79

Smith, M.C., Boutell, C., & Davido, D.J. (2011). HSV-1 ICP0: paving the way for viral replication. *Future Virology,* 6(4), 421-429.

Wadsworth, S., Jacob, R.J., & Roizman, B. (1975). Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. *Journal of Virology,* 15(6), 1487-1497.

Ying-Hsiu Su, Y., Moxley, M.J., Ng, A.K., Lin, J., Jordan, R., Fraser, N.W., & Block, T.M. (2002). Stability and circularization of herpes simplex virus type 1 genomes in quiescently infected PC12 cultures. *Journal of General Virology,* 83(12), 2943-2950.

Svoboda, S. Bell, S., & Crump, C.M. (2012) Analysis of the interaction between the essential herpes simplex 1 tegument proteins VP16 and VP1/2. *Journal of Virology,* 86(1), 473-483.

Uhle, S., Medalia, O., Waldron, R., Dumdey, R., Henklein, P., Bech-Otschir, D., Huang, X., Berse, M., Sperling, J., Schade, R., & Dubiel, W. (2003). Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. *EMBO J.,* 22, 1302-1312.

Wang, Q.Y., Zhou, C., Johnson, K.E., Colgrove, R.C., Coen, D.M. & Knipe, D.M. (2005). Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. *PNAS,* 102(44), 16055-16059.