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Establishment of a Quiescent Infection of HSV-1 in L929 Fibroblasts using a Mitotic Inhibitor and IFN-γ

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ESTABLISHMENT OF A QUIESCENT INFECTION OF HSV-1 IN L929 FIBROBLASTS USING A MITOTIC INHIBITOR AND IFN-γ.

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science.

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY NEELAM SHINDE ENTITLED Establishment of a quiescent infection of HSV-1 in L929 fibroblasts using a mitotic inhibitor and IFN-γ BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Wright State University, 2012. Establishment of a quiescent infection of HSV-1 in L929 fibroblasts using a mitotic inhibitor and IFN-γ.

The goal for this study was to determine if a quiescent infection of HSV-1 could be induced in murine fibroblasts L929 by treating them with the anti-mitotic agent 5-fluoro 2'deoxy uridine (FUDR) alone and with interferon-γ. Since neurons are post-mitotic and exhibit a lower metabolic rate than other cells, fibroblasts were treated with FUDR to induce a post-mitotic state. The cell cycle arrest of fibroblasts would decrease the thymidylate metabolism and impair HSV-1 replication. An evaluation of cytopathic effects of FUDR was used to determine the optimal concentration which arrests cell growth and inhibits viral replication. Image J program developed by NIH was used to analyze images of cultured L929 cells. In initial experiments cells showed protection from cytopathic effects of HSV-1 when treated with FUDR and IFN-γ. To determine whether the virus was in a quiescent state in L929 cells attempts were made to rescue viable virus from these cells. The FUDR+ IFN-γ + HSV-1 treated L929 cells were co-cultured with Vero cells or lysate from L929 cells was added to Vero cells. Viral plaques indicating viral rescue were observed after 48hrs.of incubation by staining the cells with crystal violet, indicating that HSV-1 was in a silent state in these treated L929 cells.

Hoechst staining was performed to detect the apoptosis in the cells treated with FUDR and IFN-γ. Approximately one third of the population of treated L929 cells showed protection against viral apoptosis compared to virus infected control at 12 hrs. post-infection. No difference was observed at 6 hrs. post- infection. RT-PCR analysis was conducted at 6, 10 and 16 hrs. post infection with HSV-1 at 2 multiplicity of infection to detect expression of ICP0 (infected cell protein) and LAT (Latency associated transcript) viral transcripts. LAT expression was observed at 16 hrs. in the infected control. Immuno-staining was used to detect HSV-1 ICP0 protein in treated L929 cells and virus infected control. A significant difference was observed, with higher expression of ICP0 in virus infected control than in cells treated with FUDR and IFNγ. Image J was used to merge images of actin stained and ICP0 stained cells. In these asynchronous fibroblast cultures treated with FUDR about 5- 10% cells replicate in the presence of FUDR. Four percent of the cells in the FUDR treated population showed ICP0 staining, as expected. A quiescent infection of HSV-1 was established in L929 cells treated with the mitotic inhibitor and maintained in a medium supplemented with IFN-γ. LAT was not detected in treated cells.

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LIST OF ABBREVIATIONS

CPE= Cytopathic Effects

E= Early

FUDR= 5-fluoro 2'- deoxyuridine

gB, gC, gD, gH, and gL = Glycoprotein B, C, D, H, L.

HSV-1 = Herpes simplex virus type – 1

ICP0 = Infected cell protein 0

ICP4 = Infected cell protein 4

IE = Immediate-early.

IFN = Interferon

IFN –α = interferon alpha

IFN-β = Interferon beta

IFN-γ = Interferon gamma

IFNGR1 = interferon gamma receptor 1

IFNGR2 = interferon gamma receptor 2

IRF = Interferon regulatory factor

ISRE = Interferon stimulated response elements

JAK = Janus-family tyrosine kinases

LAT= Latency Associated Transcript

L= Late

MOI= Multiplicity Of Infection

NGF β = Nerve growth factor

PFU= Plaque forming units

SOCS-1= Suppressor of cytokine signaling -1

STAT = Signal transduction and transcription

TG = Trigeminal ganglia

TS = Thymidylate synthase

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DEDICATION

I would like to dedicate this thesis to my husband Vinod and my son Rishi who have always inspired and encouraged me. Thank you for your love and support.

INTRODUCTION

Herpes Simplex Virus (HSV) is a double stranded DNA virus that belongs to Herpesviridae family. It infects its host through both lytic and latent infection. HSV-1 and -2 usually infect via the oral or genital mucosa and replicate in the stratified squamous epithelium. The virus enters into the sensory nerve fibers via the stratified squamous epithelium and then by retrograde transport to the cell body of the neuron in the dorsal root ganglion (DRG), adjacent to the trigeminal ganglion (Cunningham et al., 2006). In these sensory ganglia, HSV establishes a lifelong persistent infection (latency). However, reactivation of the virus may occur due to physical or emotional stress or UV irradiation, causing recurrent disease (Johnson et al., 2008). HSV-1 establishes latency in the sensory neurons, namely trigeminal ganglia (Perng et al., 2009). During latency the viral replicative functions are shut down and infectious virus particles are not detected (Bloom et al., 2010).

HSV-1 latency is a complex virus host interaction the molecular basis of which is not well understood. Neuron, virus and the host immune system together play a role in regulating and maintaining latency of HSV-1 (Toma et al., 2008). Type I interferons (IFN-α and IFN-β) act as the first line of defense against many viral infections. IFN-γ Type II interferon, prevents HSV-1 reactivation by inhibiting the immediate early gene ICP0 (Mossman et al., 2010). As a defense mechanism, HSV-1 exerts an anti-interferon effect by activating the protein suppressor of cytokine signalling-1(SOCS-1) as seen in

infected keratinocytes (Frey et al., 2009). In contrast, the fibroblast cell line L929 pre-treated with IFN-γ was protected against HSV-1 with a minimal increase in SOCS-1. IFN-α can induce a quiescent HSV-1 infection in porcine trigeminal ganglia neurons (Regge et al., 2010). A quiescent infection of HSV-1 in cultured cells could provide an important means of studying the molecular aspects of in vivo latency (Mcmohan et al., 2008). We proposed to develop a model system wherein we could mimic the environment of a neuronal cell and study HSV-1 infection cycle. Fibroblasts cell lines were chosen for the study based on the earlier observations by Frey et al., 2009.

The hypothesis of this study was that a quiescent infection of HSV-1 could be induced in murine fibroblasts (L929 and A.2R.1) by treating them with anti-mitotic agent FUDR and IFN-γ.

Fibroblast cell lines were treated with varying concentrations of FUDR to determine the lowest effective concentration required to induce a post-mitotic state. Lytic HSV-1 infection was demonstrated by cytopathic effects (CPE) and quiescent infections were monitored for inhibition of CPE. Nuclear staining was performed to determine if L929 cells treated with FUDR and IFN-γ were being protected from apoptosis after infection with HSV-1. As LAT is the only viral transcript produced during the latent state of HSV-1 (Bloom et al., 2010), RT-PCR was used to determine the presence of LAT in these cells under the defined experimental conditions. When the virus enters a latent state the genes

for lytic infection are not transcribed (Bloom et al., 2010). Immunofluorescence staining was used to detect HSV-1 ICP0 protein indicative of replicating virus.

LITERATURE REVIEW

HSV-1 INFECTION CYCLE:

HSV-1 can establish both productive and latent infections. Productive infection with HSV-1 leads to formation of epithelial lesions such as cold sores (Jackson et al., 2003). HSV-1 is known to infect about 60-80% people worldwide (Cunningham et al., 2006). It causes encephalitis and ocular herpes which is a major cause of blindness in the Western world (Cunningham et al., 2006). Virus attaches to the host cell surface by envelope glycoproteins gC, gB, gD, gH and gL. Glycoproteins gE and gI are involved in cell-cell dissemination (Rajcani et al., 2000) (Fig1). After entry into the cell, the nucleocapsid associates with transporter protein dynein and reaches the nuclear pore (Dodding et al., 2011). Dynein is a molecular motor associated with microtubules (cytoskeletal filaments) and is involved in the retrograde transport of HSV-1 to the cell body of neuron (Diefenbach et al., 2008). However, Kinesins are another type of motor proteins associated with microtubules involved in the anterograde transport of HSV-1 the from the neuronal cell body towards axon tips (Diefenbach et al., 2008).

Viral replication in the nucleus occurs in association with localized complexes nuclear domain 10 (ND10) and promyelocytic leukemia associated bodies (PML) (Rajcani et al., 2000). HSV-1 genes are grouped into three kinetic classes (α, β, γ) based on their time of expression in a lytic cycle. The alpha genes are the immediate early genes α 0, α 4, α 27, α 22 and α 22 (1-2 hpi and maximum protein synthesis at 2-4 hpi), β-early or delayed

early (3-7 hpi) and gamma are late genes. The major immediate early proteins necessary for lytic events are infected cell protein (ICP0, 4, and 27) (Rajcani et al., 2000). ICP0 and ICP4 are viral trans-activators for expression of early and late genes.

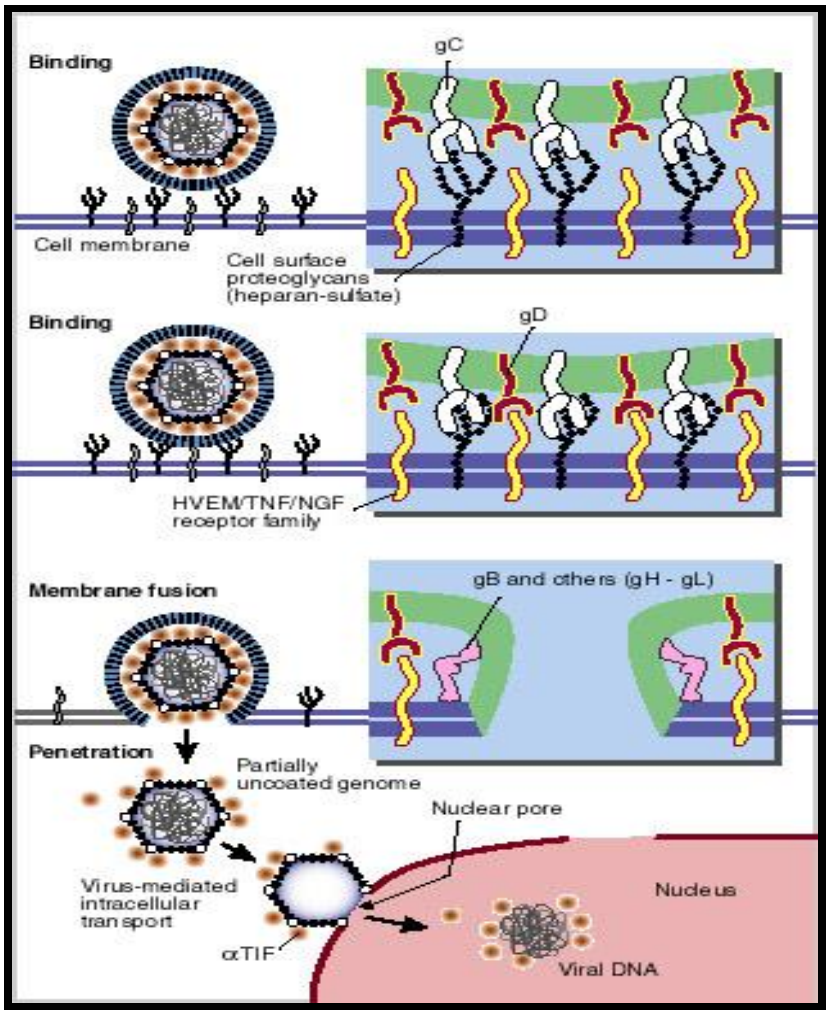


Figure 1: HSV-1 attachment and entry into host cell (Adapted from http://darwin.bio.uci.edu/~faculty/wagner/hsv4f.html).

IMMUNE RESPONSE TO HSV-1

Host immune response mediated by CD8+ and CD4+ T-cells plays a crucial role in limiting the virus at initial stages or later by preventing reactivation. Using a murine

model of genital infection, Johnson and his colleagues (2008) showed that CD4+ T cells are involved in clearance of HSV-1 from sensory ganglia and spinal cords. CD4+ T cells are also essential for priming of CD8+ T-cells during primary response to HSV-1 infection (Rajasagi et al., 2009). CD8+ T-cells are the cytotoxic effector cells in HSV-1 infection and they control infection by exocytosis of lytic granules or by secretion of cytokines IFN-γ and TNF-α (Sheridan et al., 2007). CD8+ T-cells also control the maintenance of latency and prevent reactivation of HSV-1. In ex-vivo cultures of latently infected ganglia, IFN-γ and granzyme B secreted by CD8+ T cells inhibited HSV-1 reactivation by degrading ICP4 protein (crucial for lytic events) (Knickelbein et al., 2008).

INTERFERON RESPONSE

IFN-γ exerts its effects through IFN-γ receptor (IFNGR) composed of two subunits IFNGR-1 and IFNGR-2. When a homodimer of IFN-γ binds to the receptor complex, JAK2 gets phosphorylated and it transphosphorylates JAK1. IFN-γ receptor is in turn phosphorylated promoting binding and activation of STAT1. IFN-γ/IFNGR1/STAT1 complex is internalised and translocated to nucleus where it binds to interferon response element of target gene (Eriksen S. E., 2005). SOCS-1 is an inhibitor of janus kinase activity (Vuong et al 2004) and downregulates IFN-γ expression in keratinocytes (Frey et al., 2009). IFN-α 1 plasmid transfection of mouse fibroblasts L929 lead to reduced viral load and viral gene expression (Noisakran et al., 2000). Treatment of human fibroblast (FB) cells with β and gamma interferon in combination and individually inhibits

replication of HSV-1 (Peng et al., 2007). Similar observations were made by Sainz and Halford (2002) using Vero cells pre-treated with IFN-α, β and gamma and then infected with HSV-1. Thus IFN-γ is effective in limiting HSV-1 infection. In neuronal cells CD8+ T-cells, which are a major source of cytokine IFN-γ, persist at the site and therefore prevent virus replication or reactivation from latency (Hendricks et al., 2005).

Despite effective immune response by the host against HSV-1, it survives in the host. Virus tries to evade these immune mechanisms by counteracting IFN α/β signaling. ICP27 protein of HSV-1 in detectable amounts in cells leads to secretion of heat stable, protease sensitive IFN-α antagonizing protein which negates the effects of IFN-α (Johnson et al, 2010). ICP0 also counteracts STAT 1-mediated interferon protection, thereby permitting lytic infection.

LATENCY AND REACTIVATION

HSV-1 latency is characterized by down-regulation of lytic phase genes as the genome enters a circular form and persists in a non-replicative state in the neuronal cells (Fig 2). Although sensory neurons provide a permissive environment for viral replication, HSV-1 is able to establish latency in a few neuronal cells (Thompson et al., 2001). Latent infections are characterized into three phases: establishment, maintenance and reactivation. Establishment of latency results from restricted viral gene expression which may depend upon the host cell type. As the HSV-1 genome persists in a quiescent state in neurons, low level of replication may occur to maintain the virus (Wagner and Bloom., 1997). The latent state is characterized by absence of detectable HSV-1 antigen, minimal

transcription of productive cycle genes, and high expression of latency-associated transcripts (LATs) in a subpopulation (one third) of infected neurons (Millhouse et al., 2000). Reactivation of virus occurs under conditions of stress or due to immune incompetence which results in detectable amounts of infectious virus particles in the host (Wagner and Bloom., 1997).

Latency associated transcript (LAT) is the only major transcript produced during latency, other viral products can be detected during establishment of latency. ICP4 transcripts were found in mouse ganglia latently infected with HSV-1 (Coen et al., 1995). Inman, Perng and colleagues (2001) demonstrated that LAT promotes survival of neuronal cells (N2A) transfected with plasmid expressing LAT. Similar observations were made by Thompson et al., (2001). The major reason for survival of the latently infected neurons may be due to the role of LAT in preventing apoptosis of cells. ICP0 plays a critical role in modulating the lytic-latent cycle of HSV-1. ICP0 promoter activity is repressed in the presence of IFN-γ in latently infected trigeminal ganglia neurons (Hendricks et al., 2005).

LAT prevents viral induced apoptosis in neuronal cells (Perng et al., 2000). Peng et al., (2002) showed that LAT inhibits caspase-8 and caspase-9- induced apoptosis in neuro 2A cells. Since several small regions of LAT sequence are in antisense configuration to ICP0, LAT may also influence reactivation of HSV-1 to some extent. Two types of LAT transcripts 1.5kb and 2.0kb are detected in cultures of trigeminal ganglia. 2 kb LAT transcript was detected in ex-vivo cultures of trigeminal ganglia (Hill et al., 1996, Deshmane et al., 1993).

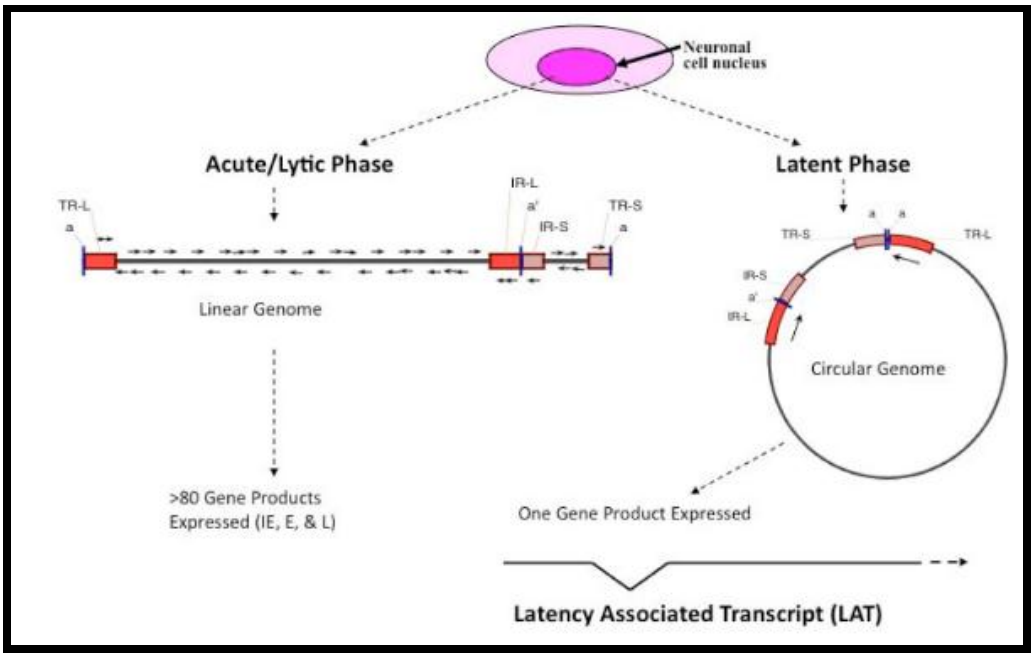


Figure 2: Molecular features of HSV-1 lytic and latent infections (Adapted from Bloom et al., 2010).

CHROMATIN STATE DURING LATENCY

Epigenetic control regulates lytic and latent events (Bloom et al., 2010). Viral genome forms an episomal structure inside neuronal cell nucleus and LAT is the only transcript produced in this state. Lytic genes remain in the transcriptionally repressed form. LAT is associated with acetylated histone H3, a euchromatin marker; while the lytic genes ICP0/ICP4 are associated with heterochromatin markers (Bloom et al., 2010). Chromatin associated with LAT is found to be facultative and thus it changes state during reactivation.

MODELS TO DEMONSTRATE LATENCY

Latent infection has been studied in animal models such as mice, rabbit and guinea pig. Latent infections were established but reactivation is not achieved successfully in these models (Wagner et al., 1997). Stringent animal safety protocols and cost of maintenance limit the use of animal models. Studying the expression of genes during latent phase and the genome structure in that state could be carried out effectively in a cell-culture model. Various cell culture models have been established to study latency of HSV-1. A long term quiescent infection of HSV-1 was established in rat pheochromocytoma (PC12) cells following differentiation with nerve growth factor (Su et al., 1999). Quiescent infection of HSV-1 was established in normal human diploid fibroblasts by McMohan and Walsh., (2008). The cells were serum starved and elevated temperature (40.5^0C) was used to maintain the virus in a dormant state.

Vierbuchen et al., (2010) converted fibroblasts to functional neurons by transfecting them with lentiviruses expressing neural-lineage specific transcription factors Ascl1, Brn2, and Myt1l. Similarly, a successful effort was made by Yoo and his colleagues (2011) using micro-RNA to convert fibroblasts to neuronal cells. Human primary fibroblasts are preferred cells over keratinocytes or lymphoid cells to study latency due to their lower metabolic state more closely resembling the neurons (Hancock et al., 2006). Murine L929 fibroblasts are also used to study HSV-1 latency as they are susceptible to the cytopathic effects of HSV-1 and show protection when transfected with an IFN-α construct (Harle et al., 2002).

Since sensory neurons are post-mitotic cells and cellular proteins required for HSV-1 replication are not synthesized viral replication is impaired in these cells. Therefore, the treatment of fibroblasts with a mitotic inhibitor (FUDR) to limit cell cycle progression was our goal in the present study. Auer et al., (1970) showed that only 10% of the population L929 cells from an asynchronous culture replicated in the presence of FUDR. Cells were arrested into G1 and early S-phase. The cell cycle time for L929 cells in culture is approximately 30 hrs. and at this time about 80-90% cells are in G1 and S-phases (Dolznig et al., 2004). Petrowsky et al., 2001 demonstrated that replication of wild type HSV-1 was impaired in the presence of 10nM of FUDR. PC-12 cells differentiated with nerve-growth factor and FUDR survived infection with HSV-1 (Moxley et al., 2002).

MODE OF ACTION OF 5-FLUORO 2-DEOXY URIDINE (FUDR):

Roobol et al., 1989 studied the metabolic pathway and target of 5-fluoro 2-deoxy uridine in L1210 leukemia cells. 5-fluoro 2-deoxy uridine is transported through the cell membrane and becomes phosphorylated to FdUMP which binds to thymidylate synthetase and inhibits DNA synthesis leading to cell cycle arrest in the S-phase. In non-replicating neurons in the central nervous system there is low level of thymidylate metabolism (Lee et al., 1991). FUDR also acts as a substrate for thymidine kinase which converts it to FdUMP which inhibits DNA synthesis (Figure 3). The wild type strains of HSV-1, including HSV-1 Syn 17+ used in the present study, are capable of producing enough thymidine kinase to replicate (Lee et al., 1991). Wild type HSV-1 strain F

showed impaired replication in the presence of FUDR (Petrowsky et al., 2001). The action of FUDR on murine L929 cells was studied by Auer et al., (1970), who found that DNA synthesis was blocked in early G1 and early S-phases. Approximately 10% of cell population replicated even in the presence of FUDR (18 hrs.) which might be the cells in late S or early G2 phase. FUDR is a drug of choice due to its reversible nature. This was demonstrated in a study by Roobol et al., (1984) who found that inhibitory effect of FUDR can be reversed by addition of thymidine. Dubbs and Kit (1964) showed that FUDR (0.01 µg/ml) mediated inhibition of L-M cells treated for 6 or 24 hrs. was reversed by an exogenous supply of thymidine.

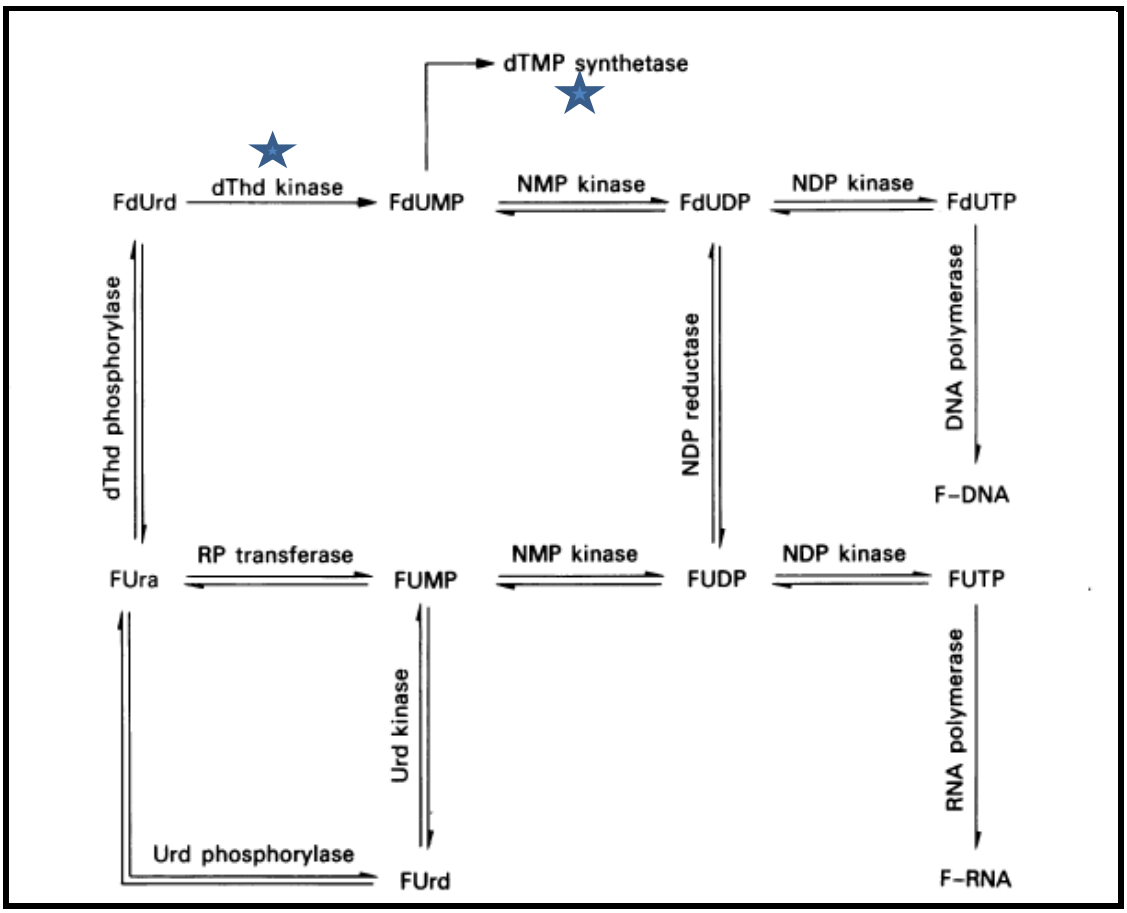


Figure 3: Metabolic pathways and targets of fluoro-pyrimidines. Stars indicate targets of 5-fluoro 2-deoxy uridine –thymidylate synthetase and thymidine kinase. (Adapted from Roobol et al., 1989).

MATERIALS AND METHODS

CELL LINES-

L929 (CCL-1, ATCC) fibroblast cells (clone of L strain derived from the connective tissue of C3H mouse).

Vero cells (CCL-81, ATCC).

The cell lines were cultured in a standard medium prepared from Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat inactivated bovine calf serum and gentamicin solution (50 µg/ml of medium). Cells were grown and maintained in 100mm² cell culture plates and incubated at 37^0C and 5% CO2 in a humidified incubator. Maintenance medium was prepared from DMEM plus 2% calf serum. Growth medium and culture plates were purchased from Fisher Scientific, Pittsburgh, PA.

Virus:

Herpes Simplex Virus-1 strain Syn 17+ (Dr.Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH) was propagated in the lab. Vero cells were grown in 75cm² flask and confluent monolayer was infected with HSV-1 at 0.1 multiplicity of infection (moi). Cells were examined and harvested along with medium when cytopathic effect (cells were rounded and detached from the bottom of the flask) was evident 3-5 days post-infection. Medium was stored as virus stock in 100 ul aliquots at -80^0C. Plaque assay was performed using Vero cells to titrate the virus.

Anti-mitotic agent:

FUDR (Fisher scientific, Pittsburgh). A stock solution was prepared in sterile distilled water at concentration of 1mg/ml and maintained at 2 to 8^0C for 2 weeks. Dilutions were freshly made prior to use.

Nerve growth factor- β (Sigma Aldrich) was stored at -20^0C in 10 ul aliquots.

Interferon-gamma- Dilutions (25 to 200 units/ml) were prepared in maintenance medium.

Cytopathic effects (CPE) assay:

An assay was performed to analyze cytopathic effects of HSV-1 on cells pre-treated with 5-fluoro 2'deoxy uridine (FUDR) and interferon gamma (IFN- γ). Two different protocols were used to perform the assay.

Protocol 1:

Cells were plated on 96, 12 or 48 well cell culture plates at a seeding density of 2.5 X 10^4 to 3.0 X 10^4 cells/ml as per experimental requirement and grown overnight in DMEM with 10% calf serum. The assay was performed using L929 cells initially. Cells at 70-80% confluence were treated with 5-fluoro 2' deoxy uridine at concentrations ranging from 160 µg/ml to 5 µg/ml of medium plus nerve growth factor-β (100 ng/ml) for 2 days. Cells were rinsed with maintenance medium (Frey et al., 2009) and treated with interferon-gamma at 100, 75, 50 and 25 units/ml for 24 hrs. Post IFN- γ treatment cells were infected with HSV-1 at multiplicity of infection of 0.1 and incubated for 2 hours

(time for virus adsorption) at 37^0C. After 2 hours the medium containing residual virus was aspirated and cells were incubated at 37^0C for 48 hrs. in DMEM with 10% calf serum. Dilutions of FUDR, IFN-γ and HSV-1 were prepared in DMEM containing 2% calf serum (maintenance medium). To examine the cytopathic effects after 48 hours, cells were rinsed with phosphate buffered saline (pH 7.4), fixed with 10% formalin for 10 mins at room temperature and stained with 0.5% crystal violet for 5 mins. Plates were rinsed with water, air dried overnight and scanned using an HP ScanJet 5300C. Scans were analyzed by Image J program provided by National Institute of Health. Experiments were performed in triplicates.

Protocol 2:

Slight modifications were made in the first protocol after analyzing the initial results. L929 cells were plated in 12 well plates in 10% DMEM and allowed to grow overnight to 70-80% confluence. They were then treated with FUDR at 5 and 10 µg/ml (concentration determined from results of first assay) for 1, 2, 3 or 6 hrs. respectively. Virus infection was carried out at 0.1 multiplicity of infection and cells were incubated for 48 and 72 hrs. A second experiment was run where cells treated with FUDR (treatment time was decided based on first experiment) were maintained in medium containing IFN-γ at 25, 50, 75 and 100 units/ml post HSV-1 infection. Virus was dispensed at 0.1 moi in all experiments and incubation period varied from 48 to 72 hours post infection. Dilutions of FUDR, IFN-γ and HSV-1 were prepared in DMEM containing 2% calf serum (Frey et al., 2009). Results were analyzed as per protocol1 (Page 14).

Rescue experiment (Plaque assay):

Based on the observations of CPE assay, about 90% of cells treated with FUDR and IFN-γ were protected from the cytopathic effects of HSV-1 as seen by absence of viral plaques. To determine if the virus was silenced in these cells, we performed a plaque assay using Vero cells. Experiment was carried out using L929 cells as per protocol 2, with a minor modification. After treating the cells with FUDR and infecting them with HSV-1 for 48 hours, the original medium was removed from cells and Vero cells in DMEM/10% calf serum were overlayed at a density of 0.1 X 10^6 cells/ml. The cells were incubated at 37^0C and 5% CO2 for 24 hours. After 24 hours medium was replaced with overlay medium containing methyl cellulose (Fisher Scientific, Pittsburgh, PA) and incubated further for 24- 48hrs to determine plaque formation. A control experiment was run alongside without overlaying Vero cells and plate was incubated till the end of the rescue experiment. Both plates were stained with 0.5% crystal violet to stain the survived cells and were scanned using HP Scan Jet 5300C. Scans were analyzed by Image J program provided by National Institute of Health.

A second rescue experiment was performed to reconfirm the results of first rescue experiment. L929 cells were lysed by two freeze thaw cycles and lysate was added on a monolayer of Vero cells. After adsorption for 2 hrs. the medium containing lysate was aspirated and fresh DMEM/10% calf serum was added to Vero cells. Plaques were observed after 48 hrs. incubation.

Hoechst staining:

To determine if cells treated with FUDR were protected from apoptotic effects of HSV-1, we performed a nuclear staining experiment. L929 cells were grown overnight on coverslips in DMEM containing 10% calf serum in a 6 well plate. The following day cells were treated with FUDR at 5 or 10 µg/ml for 3 hours. Cells were rinsed with DMEM after 3 hours and infected with HSV-1 at 10 moi. Virus was incubated with the cells for 2 hours at 37 ^{0}C. After adsorption the medium was replaced with either 10% DMEM (for control) or 2% DMEM + IFN-γ (100 units/ml) and cells were incubated for 6, 8 or 12 hours at 37 ^{0}C. After incubation cells were rinsed with 1X PBS (pH 7.4) and fixed with 1:1 methanol : acetone at -20 for 10 mins. Fixative was aspirated and cells were rinsed with 1X PBS three times five minutes per wash. Hoechst stain was added to the cells at 0.5 µg/ml concentration for 10 mins in dark. Finally cells were rinsed two times with 1X PBS. The coverslips were then mounted on glass slides using mounting medium Vectashield (Vector Labs) and then sealed with nail polish. The slides were analyzed using fluorescence microscopy.

RT-PCR:

Cells were plated into 100mm culture dishes at seeding density of 0.1X10^6 cells/ml and grown in DMEM containing 10% calf serum. At 70% confluence cells were treated with FUDR at 5 and 10 µg/ml concentration for 3 hours. The medium was removed after incubation, cells were washed with DMEM and fresh medium containing 10% calf serum (for control cells) or medium containing IFN-γ (200 units/ml) was added and cells were incubated at 37^{0}C for 6, 10 and 16 hours.

RNA extraction:

RNA was extracted from cells at 6, 10, and 16hrs. post infection. RNA was isolated by using Rneasy mini kit (Qiagen) as per manufacturer's instructions. 50µl of RNA was eluted and stored at -80 ^{0}C till further use. Integrity of isolated RNA was checked by electrophoresis on 1% agarose gel. Ethidium bromide was added to the gel. RNA samples were added in separate wells and gel was run at 95 volts. Bands were visualized under UV light and image was captured using Fuji LAS-3000 camera.

RT-PCR:

5µl of extracted RNA was amplified using Verso one step RT-PCR kit (Thermoscientific). A total reaction volume of 50 µl was used for 40 cycles and the thermal cycling was program used as per manufacturer's instructions: cDNA synthesis at 50^{0}C for 15 mins., thermostart activation at 95^{0}C 15 mins., denaturation 95^{0}C for 20 sec., annealing 55^{0}C for 30 sec., extension at 72^{0}C for 1 min and a final extension at 72^{0}C for 5 mins. The amplified product was resolved by agarose gel electrophoresis. 1% agarose gel was prepared. Ethidium bromide was added in the gel which was run at 95 volts. Bands were visualized under UV light and image was captured using Fuji LAS-3000 camera.

Immunostaining:

L929 cells were grown overnight on coverslips in DMEM containing 10% calf serum in a 6 well plate. The following day cells were treated with FUDR at 5µg/ml for 3 hours. Cells were rinsed with DMEM after 3 hours and infected with HSV-1 at 5 moi. Virus was incubated with the cells for 2 hours at 37 ^{0}C. After adsorption the medium was replaced

with either 10% DMEM (for control) or 2% DMEM with or without IFN-γ (100 units/ml) and cells were incubated for 4, 8 or 16 hours at 37 ^{0}C. After incubation cells were rinsed with 1X PBS (pH 7.4) and fixed with 4% paraformaldehyde for 15 mins. Fixative was aspirated and cells were rinsed with 1X PBS three times five minutes per wash. Cells were then permeabilized with 0.2% tritonX for 10mins and then washed with 1X PBS 3X5 mins. After permeabilization cells were blocked with 3% BSA for 1hour at room temperature. The primary mouse ICP0 antibody was prepared in the blocking buffer at a dilution of 1:25000 and then it was applied to each coverslip. The coverslips were placed in a humidified chamber cell side down and allowed to incubate at 4^{o} C overnight. Coverslips were transferred back to the dishes next day and washed 3 times with 0.5% BSA. Fluorescent-labeled (FITC) secondary antibody anti-goat IgG diluted 1:100 and Texas red conjugated phalloidin diluted 1: 100 were added to cells. Cells were allowed to incubate for 3 hours in dark at room temperature. Coverslips were transferred back to the dishes and washed 2 times with 0.5% BSA. Hoechst stain was added to the cells at 0.5 µg/ml concentration for 10 mins in dark. Finally cells were rinsed two times with 0.5% BSA. The coverslips were then mounted on glass slides using mounting medium Vectashield (Vector Labs) and then sealed with nail polish. The slides were analyzed using fluorescence microscopy.

RESULTS

Determining minimum effective dose of 5-Fluoro 2-deoxy uridine (cytopathic effects inhibition assay):

Cytopathic effects assay was performed to determine the appropriate dilution of FUDR to be used to treat cells. It was important to determine the concentration of FUDR which would only arrest the cell cycle without causing cytotoxicity to L929 cells. In figures 4, 6, and 8, higher concentrations of FUDR (160, 80, 40, 20, 10, and 5 µg/ml respectively) in addition to IFN-γ (25, 50, 75, 100 units/ml) were added to the L929 cells. Following infection with HSV-1 at 0.1moi only 20-25 % cell survival was observed. Viral plaques were seen in HSV-1 infected control, but very few cells were observed in the FUDR and IFN-γ treated wells. Pixel density as measured by Image J showed approximately 20% cell survival (figures 5 and 7) with higher concentrations and about 30- 40% (fig 9) survival with 10 or 5 µg/ml concentrations of FUDR.

Wells 1	L929 (uninfected)
Wells 2	L929 + HSV-1
Wells 3	L929 + 25 (units/ml) IFN-γ + 160 (µg/ml) FUDR + HSV-1
Wells 4	L929 + 50 (units/ml) IFN-γ + 160 (µg/ml) FUDR + HSV-1
Wells 5	L929 + 75 (units/ml) IFN-γ + 160 (µg/ml) FUDR + HSV-1
Wells 6	L929 + 100 (units/ml) IFN-γ + 160 (µg/ml) FUDR + HSV-1
Wells 7	L929 + 25 (units/ml) IFN-γ + 80 (µg/ml) FUDR + HSV-1
Wells 8	L929 + 50 (units/ml) IFN-γ + 80 (µg/ml) FUDR + HSV-1
Wells 9	L929 + 75 (units/ml) IFN-γ + 80 (µg/ml) FUDR + HSV-1
Wells 10	L929 + 100 (units/ml) IFN-γ + 80 (µg/ml) FUDR + HSV-1
Wells 11	L929 + 160 (µg/ml) FUDR
Wells 12	L929 + 80 (µg/ml) FUDR
Wells 13	L929 + 100 (units/ml) IFN-γ

Figure 4: Effects of Fudr at higher concentration (160 and 80µg/ml) on survival of L929 cells. Cell density was sparse in the center of the wells (3-10) treated with FUDR and IFN-γ and infected with HSV-1. Control wells (11-12) also showed few surviving cells. These higher concentrations of FUDR were toxic to cells and induced cell death.

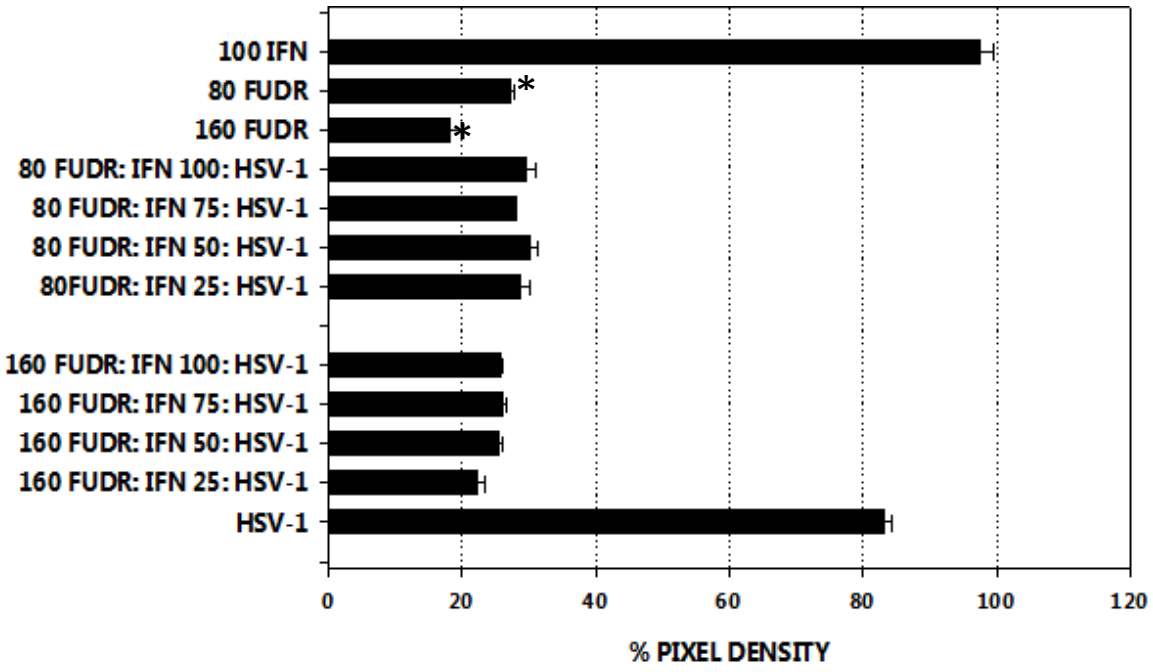
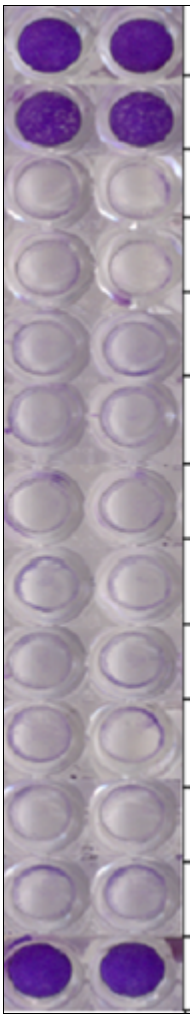


Figure 5: Effects of Fudr at higher concentration (160 and 80µg/ml) on survival of L929 cells. Higher concentrations induced cell death as seen by only 20-25% cells survival. % Pixel density was normalized against uninfected control. FUDR treated group showed significant differences in cell survival relative to HSV-1 infected control. *P<0.001 by ANOVA. % Pixel density was used as a measure of cell survival.



Wells 1	L929 (uninfected)
Wells 2	L929 + HSV-1
Wells 3	L929 + 25 (units/ml) IFN-γ + 40 (µg/ml) FUDR + HSV-1
Wells 4	L929 + 50 (units/ml) IFN-γ + 40 (µg/ml) FUDR + HSV-1
Wells 5	L929 + 75 (units/ml) IFN-γ + 40 (µg/ml) FUDR + HSV-1
Wells 6	L929 + 100 (units/ml) IFN-γ + 40 (µg/ml) FUDR + HSV-1
Wells 7	L929 + 25 (units/ml) IFN-γ + 20 (µg/ml) FUDR + HSV-1
Wells 8	L929 + 50 (units/ml) IFN-γ + 20 (µg/ml) FUDR + HSV-1
Wells 9	L929 + 75 (units/ml) IFN-γ + 20 (µg/ml) FUDR + HSV-1
Wells 10	L929 + 100 (units/ml) IFN-γ + 20 (µg/ml) FUDR + HSV-1
Wells 11	L929 + 40 (µg/ml) FUDR
Wells 12	L929 + 20 (µg/ml) FUDR
Wells 13	L929 + 100 (units/ml) IFN-γ

Figure 6: Action of FUDR at 20 and 40 µg/ml on survival of L929 cells. Viral plaques were obserevd in HSV-1 infected control (well 2). Cell density was sparse in the center of the wells (3-10) treated with FUDR and IFN-γ and infected with HSV-1. Control wells (11-12) treated with FUDR alone also showed few surviving cells. These concentrations of FUDR were also toxic to cells similar to Fig. 4.

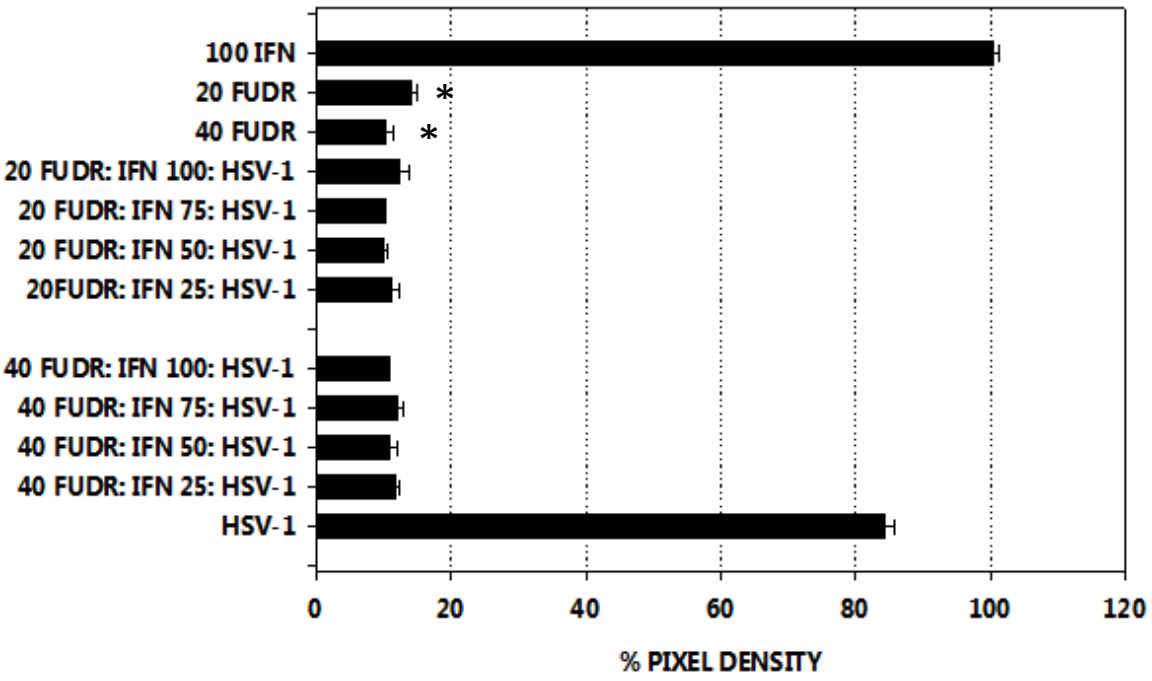
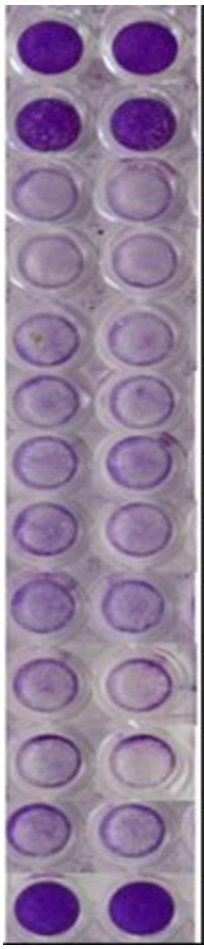


Figure 7: Effects of FUDR at 20 and 40 µg/ml. FUDR was toxic to cells at these concentrations and thus only about 10-15% cell survival was observed. % Pixel density was normalized against uninfected control. FUDR treated group showed significant differences in cell survival relative to HSV-1 infected control *P<0.001 by ANOVA. % Pixel density was used as a measure of cell survival.



Wells 1	L929 (uninfected)
Wells 2	L929 + HSV-1
Wells 3	L929 + 25 (units/ml) IFN-γ + 10 (μg/ml) FUDR + HSV-1
Wells 4	L929 + 50 (units/ml) IFN-γ + 10 (μg/ml) FUDR + HSV-1
Wells 5	L929 + 75 (units/ml) IFN-γ + 10 (μg/ml) FUDR + HSV-1
Wells 6	L929 + 100 (units/ml) IFN-γ + 10 (μg/ml) FUDR + HSV-1
Wells 7	L929 + 25 (units/ml) IFN-γ + 5 (μg/ml) FUDR + HSV-1
Wells 8	L929 + 50 (units/ml) IFN-γ + 5 (μg/ml) FUDR + HSV-1
Wells 9	L929 + 75 (units/ml) IFN-γ + 5 (μg/ml) FUDR + HSV-1
Wells 10	L929 + 100 (units/ml) IFN-γ + 5 (μg/ml) FUDR + HSV-1
Wells 11	L929 + 10 (μg/ml) FUDR
Wells 12	L929 + 5 (μg/ml) FUDR
Wells 13	L929 + 100 (units/ml) IFN-γ

Figure 8: Effect of FUDR at concentrations of 10 and 5μg/ml on survival of L929 cells. Viral plaques were observed in HSV-1 infected control (well 2). Few cells were observed in wells treated with FUDR, IFN-γ, and HSV-1 treated wells (3 to 10) similar to FUDR treated control (wells 11 and 12). The cell density of FUDR treated was increased compared to higher concentrations as seen in fig 4 and 5.

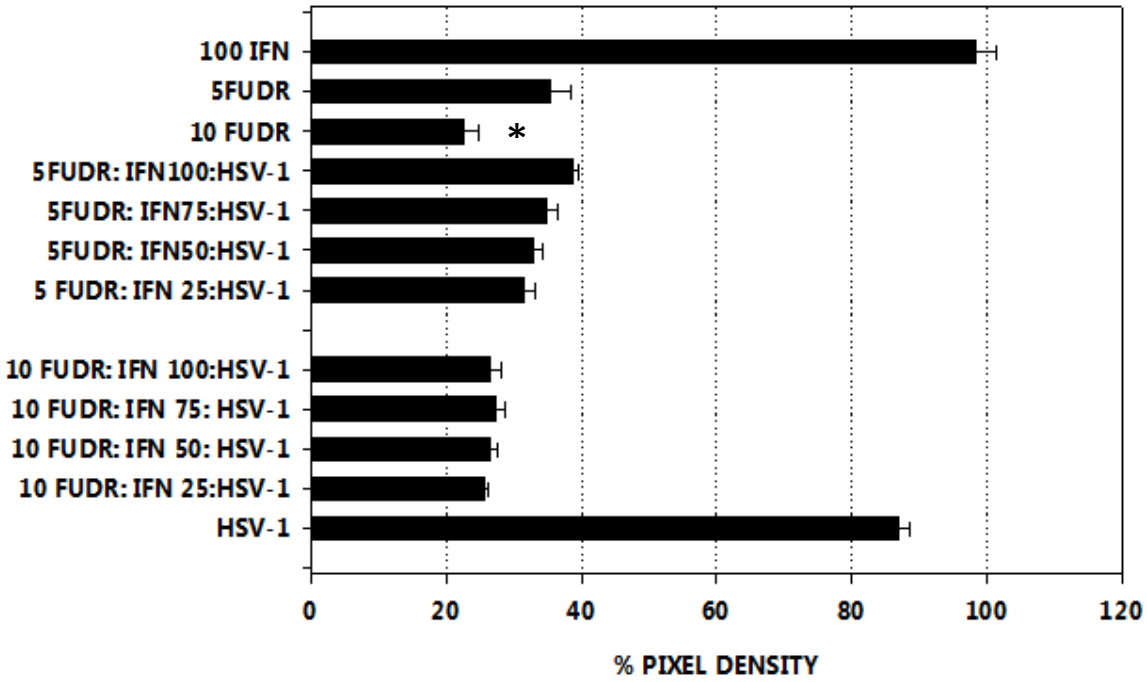


Figure 9: Effects of FUDR at 10 and 5 µg/ml. There was a little increase in cell survival about 30-40% compared to higher concentrations in fig 5 and 7. % Pixel density was normalized against uninfected control. FUDR treated groups showed significant differences in cell survival relative to HSV-1 infected control *P<0.001 by ANOVA. % Pixel density was used as a measure of cell survival.

2.5 and 1.25 µg/ml were also used, which showed improved cell survival (data not shown). These results indicated that treatment of FUDR even at lower concentrations for a prolonged period (2 days) lead to cell death. For subsequent experiments, 5 and 10 µg/ml concentrations were selected for shorter exposure times.

Comparison of varying exposure times of FUDR treatment:

Treatment of cells with FUDR for 2 days resulted in death of L929 cells, treatment time was reduced to 6, 3, 2 and 1 hr. respectively (data not shown). Cytopathic assay was performed to determine the optimum FUDR exposure time required to arrest cell growth.

After infection with 0.1 moi of HSV-1, medium was replaced with 10% DMEM (for control) and 2% DMEM for FUDR treated cells. Cell survival was analyzed at 48 and 72 hrs. post infection. Fewer viral plaques were observed in cells treated with FUDR for 2 or 3hrs. (Fig 10, 12) compared to HSV-1 infected control. A higher cell density was also seen in FUDR treated controls where treatment time was 2 or 3 hrs. (Figure 10 and 12).

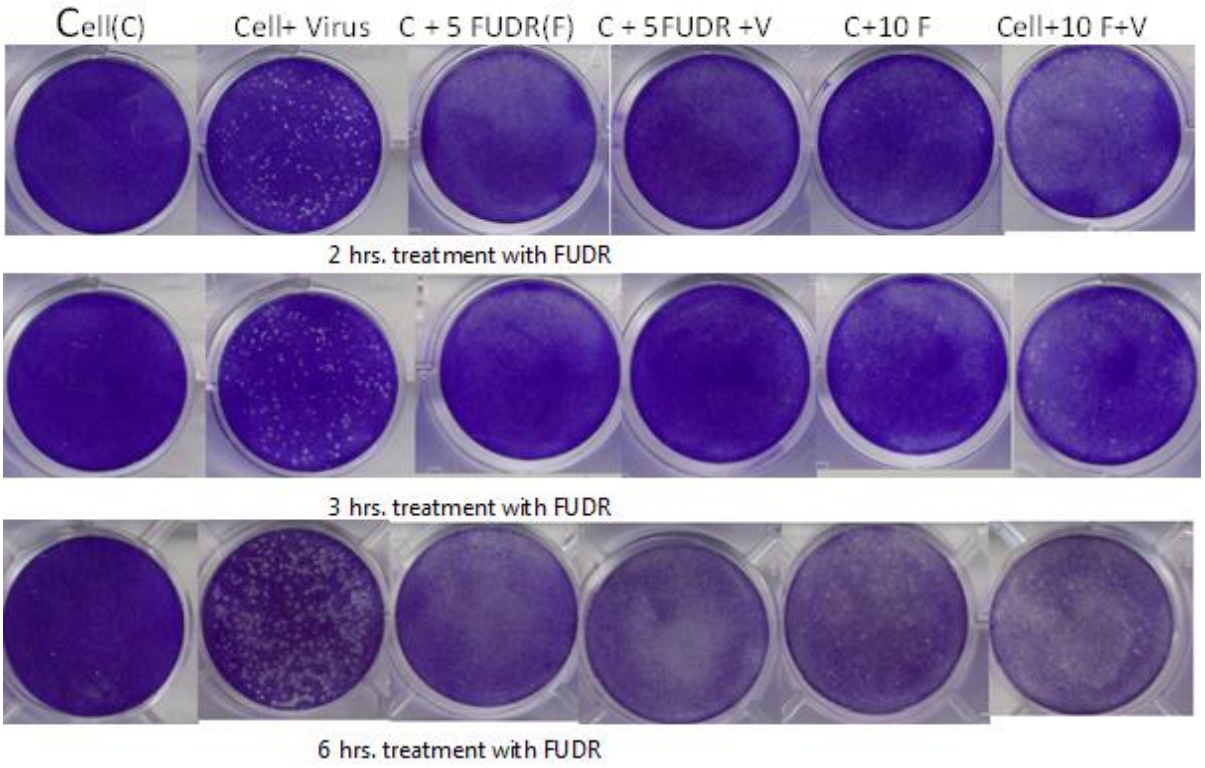


Figure 10: Comparison of FUDR treatment periods on L929 cells (48 hrs. pi.). Cells were treated with FUDR for 2, 3or 6 hrs. and then infected with HSV-1. Higher cell density and fewer plaques were observed when cells were treated with FUDR for 2 or 3 hrs. FUDR treatment for 6 hrs. reduced the cell density by about 40-50%.

An increase in the survival of L929 cells was observed when the treatment time of FUDR was reduced to 2 and 3 hrs. L929 cells treated with FUDR for 6 hrs. showed reduced cell density and lower cell survival after HSV-1 infection which was similar to FUDR treated

control cells (Fig. 10). The reason for reduced cell density for 6 hrs. treatment time may be because of the cytotoxic effects rendered by FUDR prior to HSV-1 infection. This was evident from fewer plaques in cells treated with FUDR.

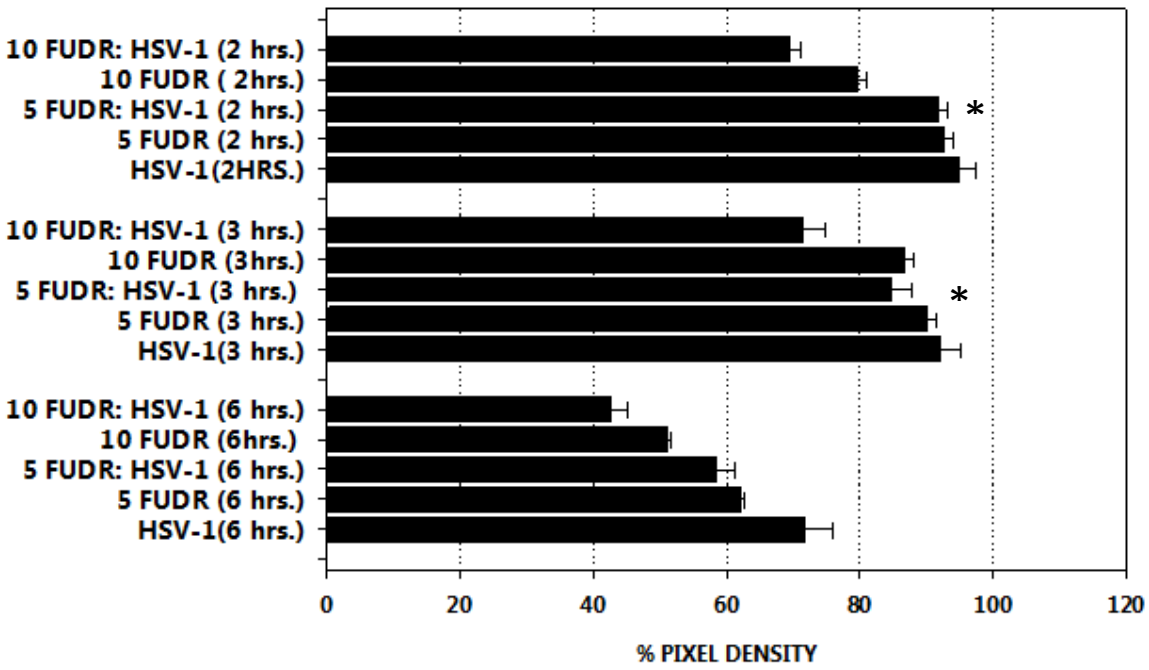


Figure 11: Comparison of FUDR treatment periods on L929 cells (48 hrs. pi.). There was a significant increase in the cell survival when time of FUDR treatment (10 and 5µg/ml) was reduced to 2 or 3 hrs. About 80-90% cell survival was observed in cells. (% Pixel density was normalized against uninfected control). *P<0.05 by ANOVA. % Pixel density was used as a measure of cell survival.

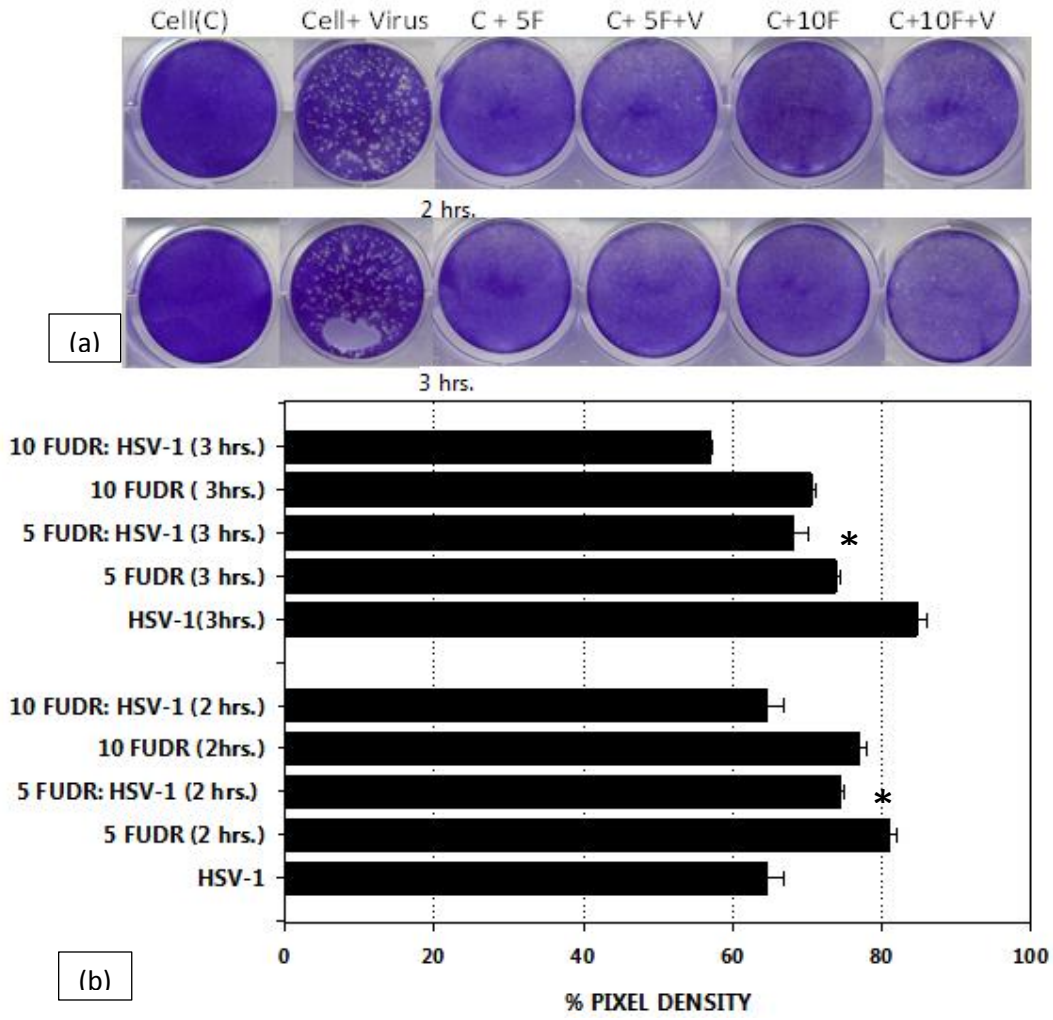
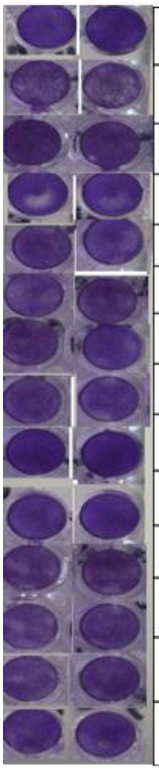


Figure 12(a, b): Effect of varying treatment periods of FUDR on L929 cells without IFN-γ (72 hrs. pi.) Treatment of L929 cells for 6 hrs. with FUDR resulted in almost 40-50% cell death. Treatment of L929 cells with FUDR for 2 or 3 hrs. showed increased protection of cells against HSV-1. (% Pixel density was normalized against uninfected control). Cells survival in treatment groups was significantly increased compared to HSV-1 control even at 72 hrs. post infection (*P<0.05 by ANOVA). % Pixel density was used as a measure of cell survival.

Combined effect of FUDR and IFN-γ on survival of L929 cells after HSV-1 infection:

In the cytopathic effects assay performed with higher concentrations of FUDR (Fig 4, 6, and 8) maximum cell death about 70% was observed. To mimic the natural infection in neurons cells were also treated with IFN-γ. After treatment with FUDR for 3 hrs (time arbitrarily selected after analyzing results in Fig. 11 and 12). Cells were infected with HSV-1 at 0.1 moi, after adsorption for 2 hrs., the medium was replaced with 2% DMEM supplemented with IFN-γ at 50, 75, 100, and 200 units/ml. Fig.13 (Frey et al., 2009).



Wells 1	L929 (uninfected)
Wells 2	L929 + HSV-1
Wells 3	L929 + 5(μg/ml) FUDR
Wells 4	L929 + 5 (μg/ml) FUDR + HSV-1
Wells 5	L929 + 10 (μg/ml) FUDR
Wells 6	L929 + 10 (μg/ml) FUDR + HSV-1
Wells 7	L929 + 5 (μg/ml) FUDR + HSV-1+ 50 (units/ml) IFN-γ
Wells 8	L929 + 5 (μg/ml) FUDR + HSV-1 + 75 (units/ml) IFN-γ
Wells 9	L929 + 5 (μg/ml) FUDR + HSV-1 + 100 (units/ml) IFN-γ
Wells 10	L929 + 5 (μg/ml) FUDR + HSV-1 + 200 (units/ml) IFN-γ
Wells 11	L929 + 10 (μg/ml) FUDR + HSV-1 + 50 (units/ml) IFN-γ
Wells 12	L929 + 10μg/ml) FUDR + HSV-1 + 75 (units/ml) IFN-γ
Wells 13	L929 + 10μg/ml) FUDR + HSV-1 + 100 (units/ml) IFN-γ
Wells 14	L929 + 10μg/ml) FUDR + HSV-1 + 200 (units/ml) IFN-γ

Figure 13: Effect of FUDR (3 hrs.) and IFN-γ on survival of L929 cells after HSV-1 infection. Few viral plaques were observed in cells treated with FUDR and IFN-γ at 100 and 200 units/ml (wells 7 to 14) as compared to HSV-1 infected control (well 2). L929 cells displayed protection from cytopathic effects of HSV-1 when treated with FUDR (5μg/ml) for 3 hrs and maintained in IFN-γ (100 or 200 units/ml) post HSV-1 infection.

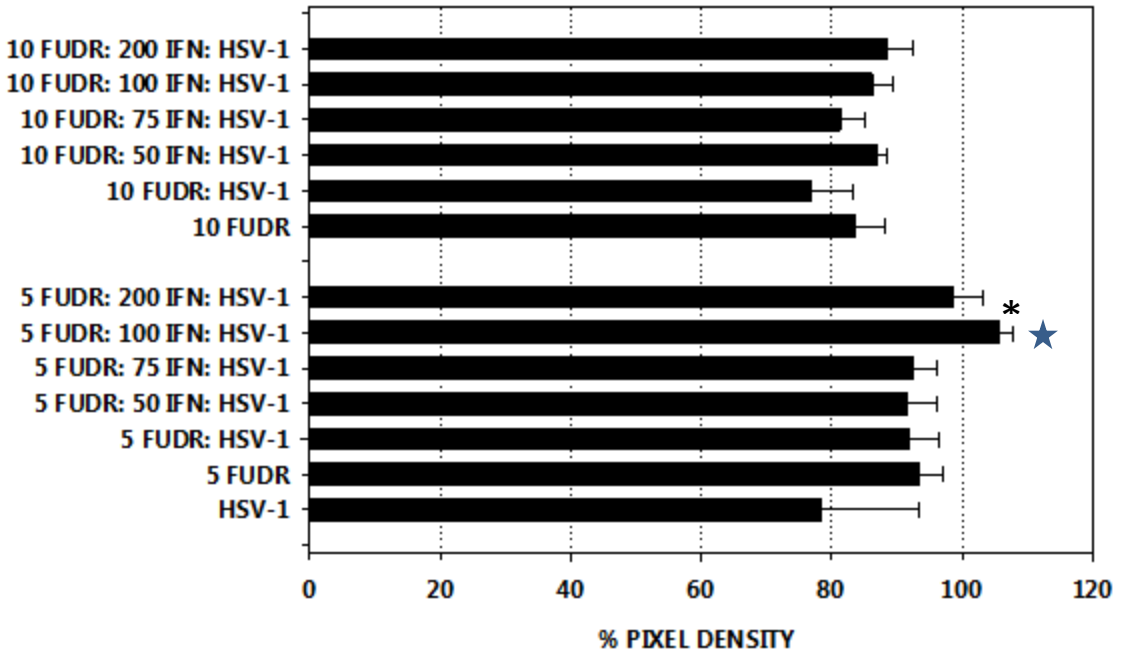


Figure 14: Effect of FUDR (3 hrs.) and IFN-γ on cell survival after HSV-1 infection. About 90% cells survival was observed after HSV-1 infection in cells treated with FUDR and IFN-γ. % Pixel density was normalized against uninfected control. FUDR and IFN-γ treatment groups showed significant increase in cell survival relative to HSV-1 infected control *P<0.001 by ANOVA. ★ Indicates cell group not significantly different from uninfected control. % Pixel density was used as a measure of cell survival.

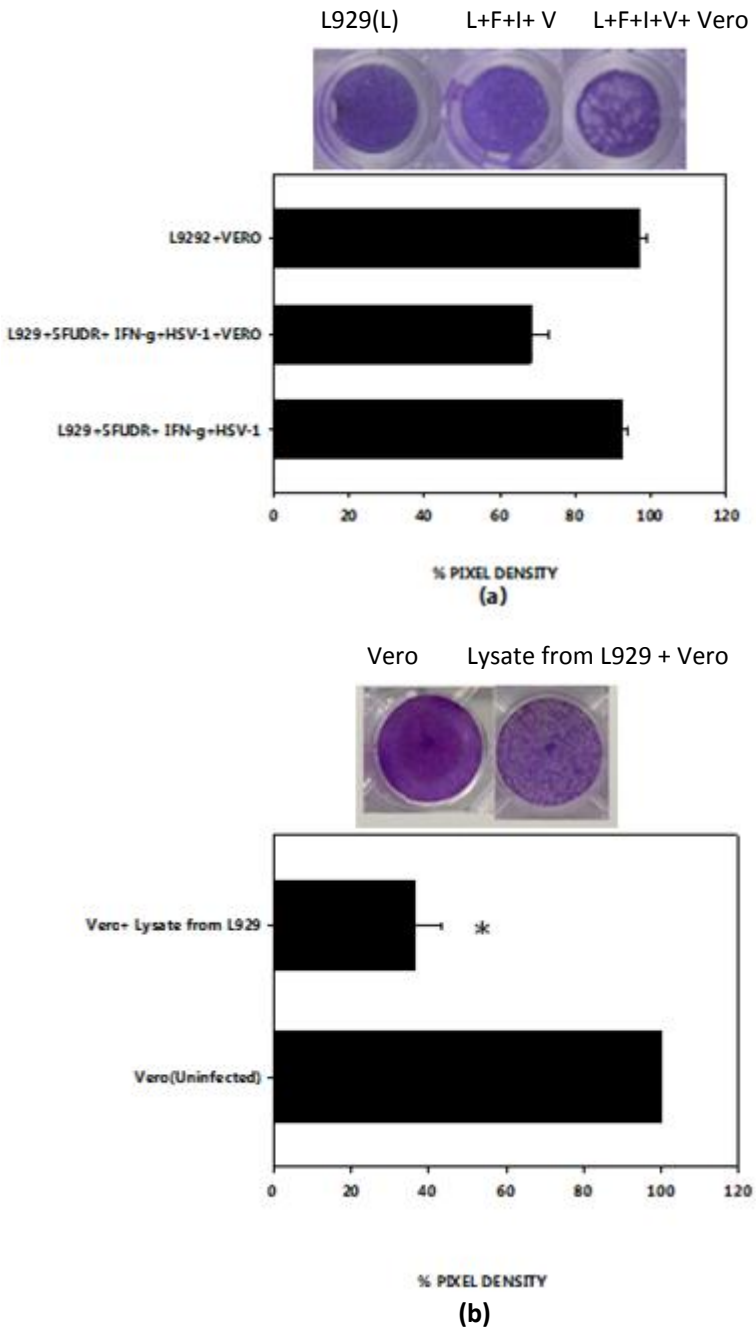


Figure 15: Virus rescue by overlaying Vero cells. Viral plaques were observed in L929 cells overlayed with Vero cells *P= 0.004 (a) and on Vero cells to which lysate from L929 cells was added *P<0.001 by ANOVA (b). % Pixel density was normalized against uninfected L929 + Vero (a) and Vero (b) control cells. Viral plaques were indicative of virus rescue from L929 cells. % Pixel density was used as a measure of cell survival.

HOECHST STAINING :

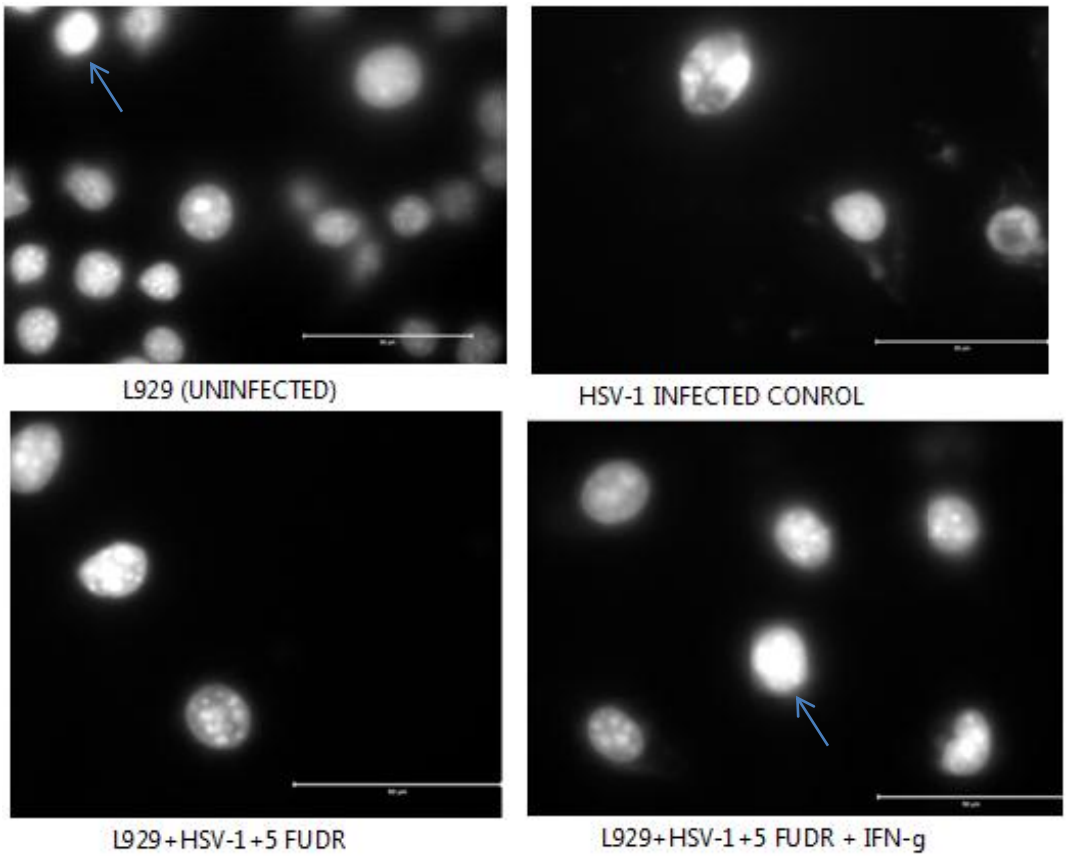


Figure 16: Hoechst staining to detect apoptosis at 6 hrs. post infection. L929 cells were grown overnight on coverslips and then treated with FUDR for 3 hrs. Cells were then infected with HSV-1 at 10 moi and then incubated in medium with IFN-γ. At 6 and 12 hrs. post infection nuclear staining with Hoechst was performed. At 6 hrs. post infection no difference in nuclear morphology was observed in L929 uninfected control and FUDR + IFN-γ treated cells. Some atypical staining was observed in both groups as indicated by arrows. Bar markers indicate 50 µm size.

No differences in nuclear morphology were evident at 6 hrs. post infection (Fig. 16).

HOECHST staining (12 HRS POST INFECTION)

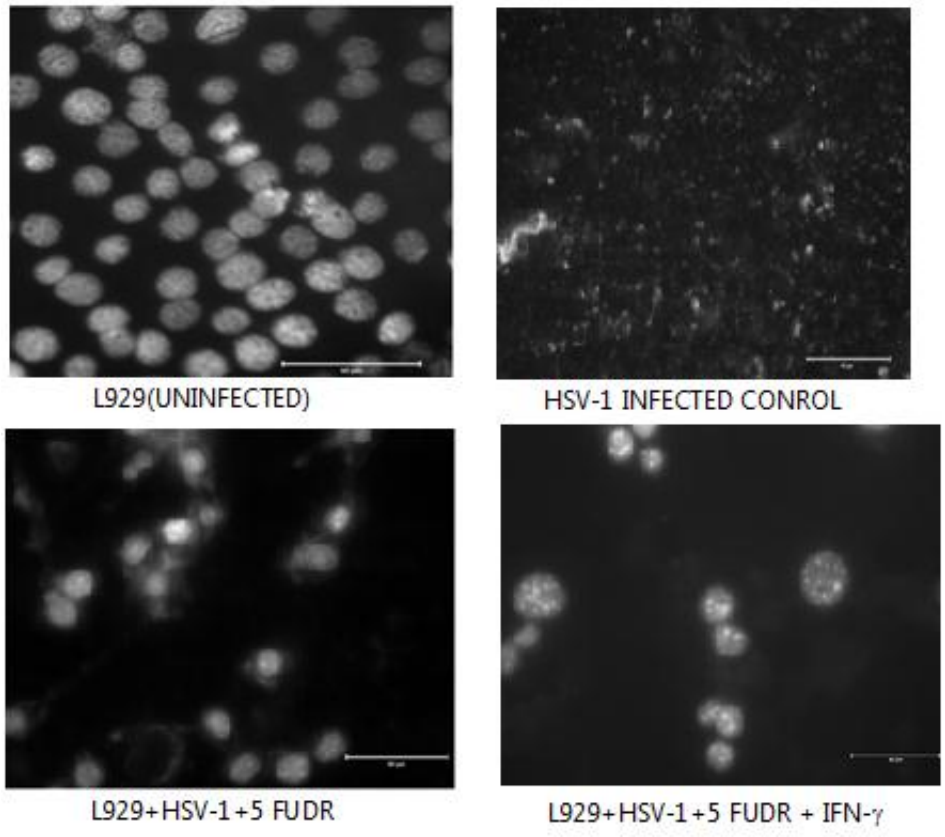


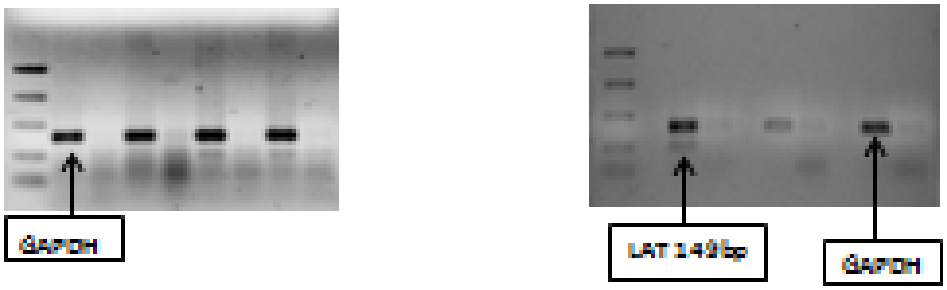
Figure 17: Hoechst staining to detect apoptosis at 12 hrs. post infection. Cells were treated in the same way as in Fig. 16. At 12 hrs. post infection about 80-90% cells were lysed in the virus infected control. About one third of the cells treated with FUDR + IFN-γ survived HSV-1 induced apoptosis. Bar markers indicate 50 µm size.

About 80% cells were lysed in the HSV-1 infected control at 12 hrs. post infection. Approximately one-third of the population of cells survived the cytopathic effects of HSV-1 in the FUDR + IFN-γ treated group (Fig. 17). In natural HSV-1 infection neurons are protected from apoptotic effects of HSV-1 because of the presence of LAT transcripts (Perng et al., 2000). These observations suggest that quiescent herpes virus infection occurred in one-third or less of the treated fibroblasts.

Detection of latency associated transcript (LAT) and ICP0 by RT-PCR:

RT-PCR analysis was conducted to analyze expression of HSV-1 LAT in L929 cells treated with FUDR and IFN-γ and infected with HSV-1 at 2 moi. Analysis was performed at 6, 10 and 16 hours post-infection. LAT expression was observed in untreated HSV-1 infected L929 cells at 16 hours post-infection but not in cells treated with FUDR and IFN-γ. No expression was seen at 6 and 10 hours (data not shown) post-infection in control or experiment.

RT-PCR (6hrs. post infection) and 16hrs. post infection:



a b

Figure 18: RT-PCR analysis of L929 cells at 6 (a) and 16 (b) hrs. post-infection. No LAT expression was observed at 6 hours post infection (a). At 16 hrs. post infection LAT expression was observed as displayed by 149 bp product (b). Arrows indicate positions of GAPDH control (a and b) and LAT (b).

Detection of ICP0 by immunofluorescence staining:

L929 cells treated with FUDR and IFN-γ were infected at 5 moi with HSV-1 and immuno-staining with antibody against HSV-1 ICP0 protein and phalloidin against Actin was performed at 4, 8 (data not shown) and 16 hrs. post infection. Cells were observed under fluorescent microscope and two images were taken per field. Two different filters were used to take images for one field because secondary antibodies used had different fluorescent labels (FITC and Texas red). Three fields were observed for each condition per experiment and 3 experiments were performed for each time point. Nuclear expression of ICP0 expression was observed as fluorescent dots. Actin expression was observed in all cells. A marked difference in the number of cells expressing ICP0 protein was observed as expected in HSV-1 infected control and other groups. Approximately 15-17% cells in HSV-1 infected control and 4% treated cells showed ICP0 protein expression. A difference in the morphology of cells was also observed: normal cell morphology was maintained in at least one-half of the cells treated with FUDR and IFN-γ compared to HSV-1 infected control. Cells in HSV-1 infected control were rounded because of the cytopathic effects of HSV-1.

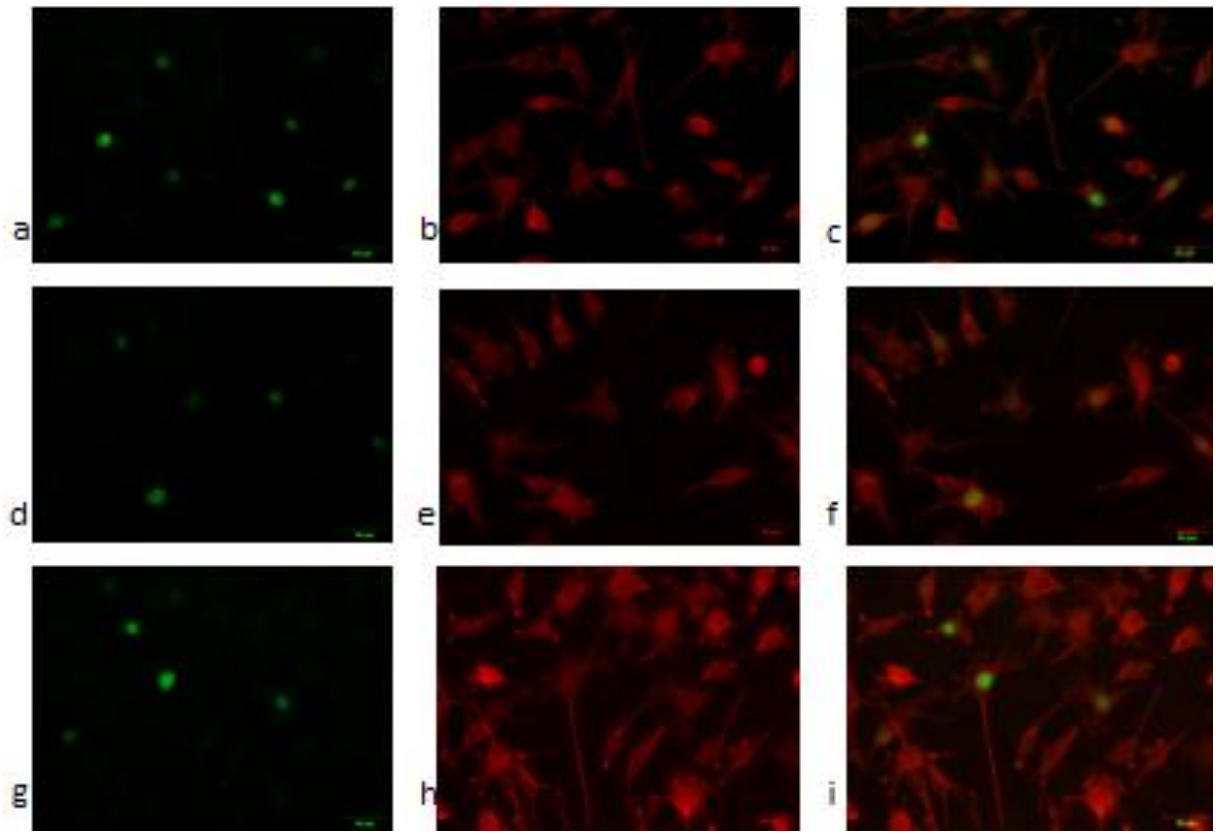


Figure 19: HSV-1 ICP0 expression at 4 hours post-infection (Green- ICP0 staining, Red- Actin) (a, b, c) HSV-1 control (d, e, f) L929+FUDR+HSV-1 (g, h, i) L929+ FUDR+ HSV-1+ IFN-γ. Nuclear expression of ICP0 was observed in HSV-1 infected control and cells treated with FUDR alone or in combination with IFN-γ. Bar markers indicate 50 µm size.

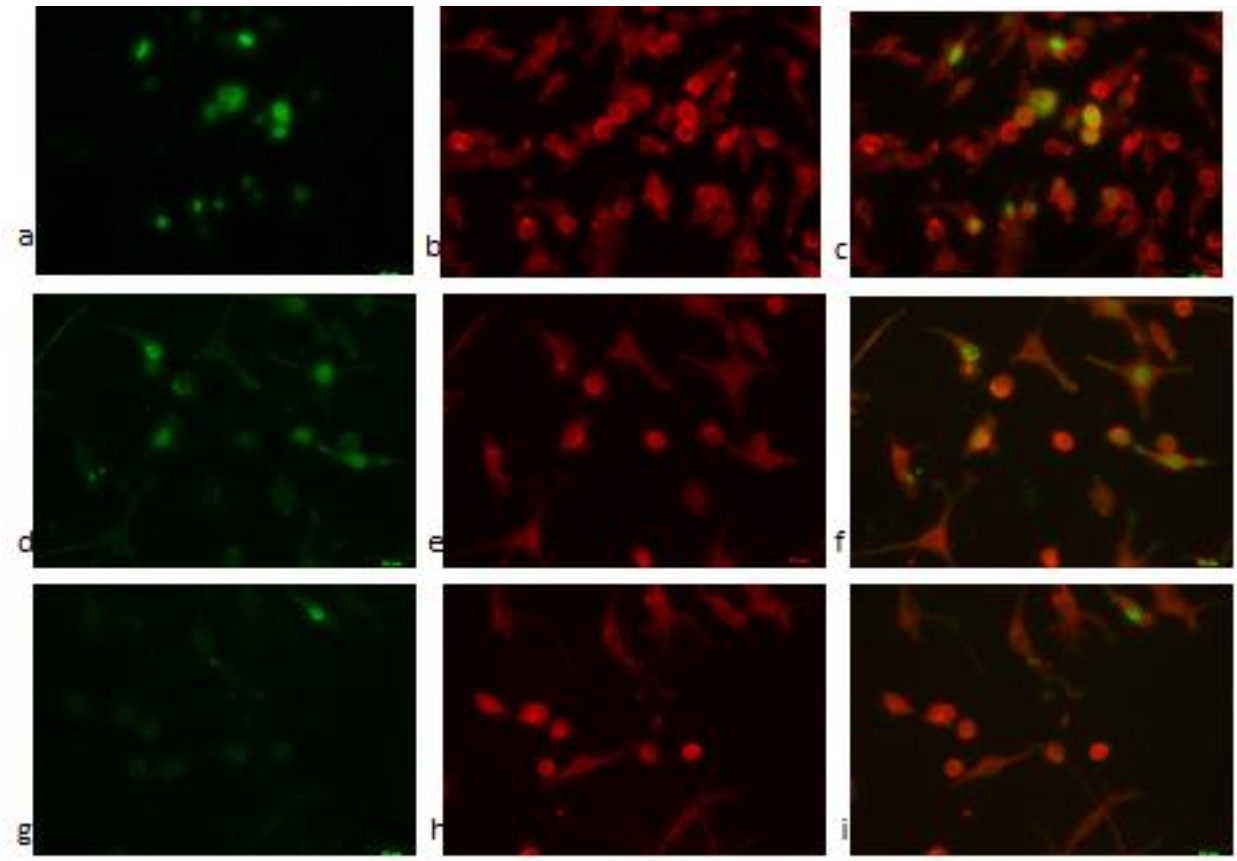
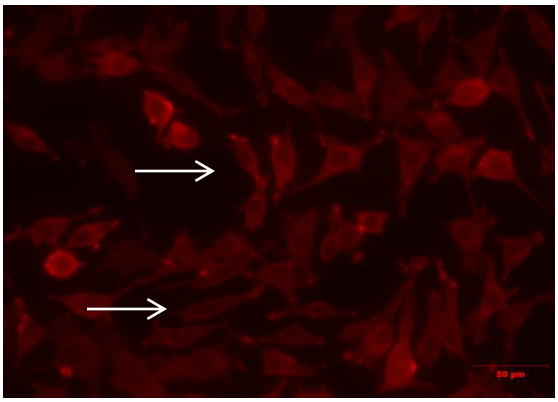
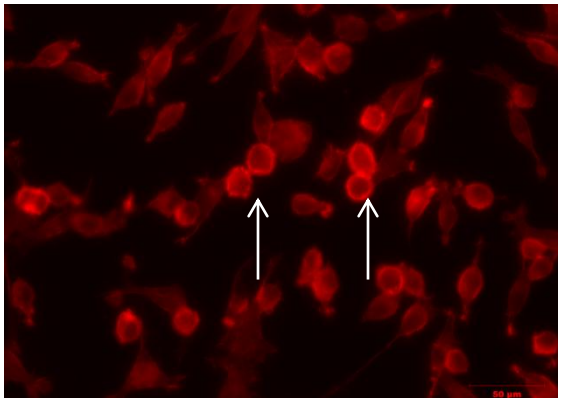


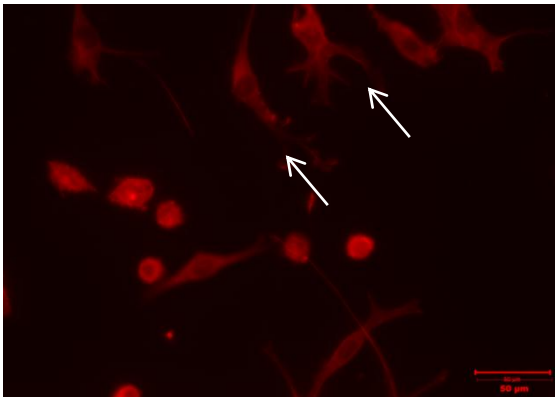
Figure 20: HSV-1 ICP0 expression at 16 hours post-infection (Green- ICP0 staining, Red- Actin) (a, b, c) HSV-1 control (d, e, f) L929+FUDR+HSV-1 (g, h, i) L929+ FUDR+ HSV-1+ IFN-γ. Nuclear expression of ICP0 was observed in infected control and in a few cells treated with FUDR and IFN-γ. Bar markers indicate 50 µm size.



a. Spindle shaped cells



b. Rounded cells



c. Long projections

Figure 21: L929 cell morphology at 16 hours post-infection (Red- Actin). (a) Uninfected control (b) HSV-1 control (c) FUDR + IFN-γ+ HSV-1 treated cells. Cells treated with FUDR and IFN-gamma retained normal cell morphology as in (a) but some cells showed longer projections (c). The cells in HSV-1 infected control (b) were rounded due to cytopathic effects of HSV-1. Arrows are showing distinct cell morphology. Bar markers indicate 50 µm size.

Since IFN-γ protected L929 cells from the viral cytopathic effects, normal cell morphology was retained in these cells treated with FUDR and IFN-γ.

DISCUSSION

HSV-1 undergoes a latent infection in a few sensory neurons (Millhouse et al. 2000). Latent infection is characterized as a non-productive state where genes for lytic cycle are repressed. Viral latency associated transcript is the only viral gene transcribed during this silent state (Millhouse et al., 2000). Schang et al., (2002) suggest that sensory neurons are post-mitotic and do not allow viral replication, in contrast to Sawtell's contention that sensory neurons have a permissive environment for HSV-1 replication. Schang et al., (2002) contend that these quiescent neurons lack cellular factors required for productive infection. Cyclin dependent kinases (cdk) 1 and 2 are not present in quiescent neurons, which limits replication of HSV-1 (Schang et al., 2002). Cdk 2 is required for cell cycle progression into S phase, while cdk1 allows cell to proceed into mitosis (Schang et al., 2002). Role of cyclin dependent kinase 5 in association with p35 in preventing HSV-1 replication was demonstrated by Haenchen et al., (2010). Cdk5 plays a role in the adhesion, migration, and differentiation of lens and corneal epithelial cells and its activity is, in part, regulated by p35 (Haenchen et al., 2010). Absence of p35 impairs ocular replication of HSV-1 in mice (Haenchen et al., 2010).

In the present study FUDR was toxic at higher concentrations (160, 80, 40 20 µg/ml respectively) concentrations as displayed by CPE. Only 20 to 30% cell survival was observed in cells treated with FUDR due to its cytotoxic effects. As FUDR leads to inhibition of DNA synthesis and repair, cells treated with the mitotic inhibitor die

eventually as they cannot replicate or repair DNA (Auer et al., 1970). L929 fibroblasts treated with lower concentrations (5 and 10 µg/ml respectively) of 5-fluoro 2'deoxy uridine (FUDR) showed protection from cytopathic effects of HSV-1 compared to untreated HSV-1 control cells. As FUDR treatment time was reduced to 3 hrs. and concentration of FUDR was low (5µg/ml) survival of cells increased to about 80 to 90% . Quiescent HSV-1 in L929 cells was rescued by two methods: cells were co-cultured with Vero cells, or lysed by freeze thaw method and lysate was added to a monolayer of Vero cells. Plaques were observed after 48 hrs. incubation. Viral plaques on Vero cells indicated that L929 cells harbored viable virus in them and was rescued from them on Vero cells. Cell survival decreased by about 30% in L929 cells overlayed with Vero cells compared to uninfected L929+ Vero cell control. The reduction in cells survival was due to cytopathic effects of rescued HSV-1 form L929 cells.

The effect of FUDR is reversible by exogenous supply of thymidine or serum in the medium (Roobol et al., 1984). For future studies, lower concentrations (nM concentrations) could be used to maintain cells in FUDR containing medium till the end of the experiment which might keep the cells in the state of arrest for a longer period.

HSV-1 induces apoptosis due to lytic events in non-neuronal cells but becomes latent in neurons. Viral production of LAT in neurons prevents apoptosis (Branco et al., 2005). Hoechst staining showed that a minor population of L929 cells was protected from apoptotic effects of HSV-1 in the presence of FUDR alone as well as FUDR and IFN-γ compared to HSV-1 infected control L929 cells at 12 hrs. post-infection. As a high

multiplicity of infection (10 moi) was used to induce apoptosis of cells, very few cells survived HSV-1 infection under these experimental conditions. Another possible reason could be that cells treated with FUDR reversed the inhibitory effects to resume the normal cell cycle leading to cell death. However, at 6 hrs. post infection, little difference was seen in the nuclear morphology except for a few cells which showed atypical staining (nucleus looked like a bright spot). A flow-cytometric analysis to detect the presence of apoptotic proteins in L929 cells treated with FUDR and HSV-1 could be conducted in future. This quantitative analysis performed using antibodies against apoptotic proteins like caspase 8 or caspase 9 would be useful in detecting if latent HSV-1 infection was established in L929 cells (Henderson et al., 2002).

These initial studies were indicative of a silent infection of HSV-1 in L929 cells. The quiescent infection established in these cells did not lead to establishment of HSV-1 latency (as no LAT transcripts were detected in experimental groups). LAT (latency associated transcript) is expressed during a latent infection in neurons (Thompson et al 2001). ICP0 is a lytic gene but due to its genomic configuration being anti-sense with LAT, it is detected during viral reactivation (Perng et al., 2010). In our experimental cells LAT or ICP0 could not be detected by RT-PCR analysis conducted at 6 and 10 hrs. post infection. At 16 hrs. post infection LAT was detected in HSV-1 infected L929 control cells but not in treated cells. Cell counts taken after termination of experiment showed that number of FUDR treated cells decreased by 20-30% by the end of the experiment. The sub-population which did not withstand treatment regime might be the one harboring latent HSV-1 and hence no transcripts were found in the remaining cells. Another

possible reason could be that there could be other neural lineage specific factors responsible to establish a latent HSV-1 infection in neurons. Therefore only FUDR and IFN- γ treatment of cells did not result in establishment of latency as hypothesized. In natural infection very few neurons (one third) express detectable LAT in latently infected cells (Su et al., 1999). It may also be possible that a low level of LAT was expressed in some of the L929 cells treated with FUDR and IFN-γ which could not be detected by RT-PCR analysis (RT-PCR kit detected 1pg -1µg of RNA). In a similar kind of study by Su et al., (1999) where a quiescent infection of HSV-1 was established in PC-12 cells, LAT transcripts were detected by southern hybridization in cells where no LAT was found by RT-PCR analysis. Use of fluorescence in-situ RNA hybridization assay or in-situ PCR performed at a single cell level would confirm the possibility of low level of LAT expression.

Fluorescence microscopy for ICP0 displayed a significant difference in the protein expression in control and treated cells. The number of cells showing ICP0 positive staining in the FUDR treated cells was in accordance with the previous studies on L929 cells. Auer et al., (1970) using L929 cells showed that only 10% of the population of cells from an asynchronous culture replicated in the presence of FUDR. Cells were arrested into G1 and early S-phase. The cell cycle time for L929 cells in culture is approximately 30 hrs. and at this time about 80-90% cells are in G1 and S-phases (Dolznig et al., 2004). Our results indicated that approximately 17% of the cells in control and 4% of the cells in treated groups expressed ICP0. In the present study the time at which cells were treated

with FUDR was the same as used by Auer et al., (1970) 5% cells were probably expected to show protein expression in presence of FUDR which was observed in our experiment.

A silent infection of HSV-1 was established in L929 cells using FUDR alone and with IFN-γ. Latency could not be established under these conditions, although primary features of a latent infection were observed. First, viable virus in L929 cells was observed in rescue experiments. Second, a notable difference was seen in the number of cells expressing ICP0 protein in treated cells compared to HSV-1 infected control. As discussed earlier, level of LAT expression might be low and thus could not be detected by RT-PCR. This important feature of latency might be confirmed by analysis of a single cell using in situ hybridization assay and thus would support further evaluation of the chromatin state of virus. A chromatin immuno-precipitation assay using L929 cells treated with FUDR and IFN-γ would permit a dissection of the virus genome association with the fibroblast chromatin. A heterochromatin state is observed with viral lytic genes and a euchromatin state associated with LAT is indicative of viral latency (Bloom et al., 2010). Chromatin modifiers could be used to treat the cells before infection with HSV-1, which would support the establishment of latency in these cells. Using the experimental approach of Vierbuchen et al., (2010), a neuronal phenotype could be induced in fibroblasts and HSV-1 latency can be studied.

REFERENCES

1. Auer, G., Zetterberg, A., Foley, G. E. (1970). The Relationship of DNA Synthesis to Protein Accumulation in the Cell Nucleus. Journal of Cell. Physiology, 76(3): 357-361.

2. Bloom, D.C., Giordani N.V., D.L. Kwiatkowski. (2010). Epigenetic regulation of latent HSV-1 gene expression. Biochim Biophys Acta, 1799(3-4): 246-56.

3. Branco F. J., Nigel W. Fraser. (2005). Herpes Simplex Virus Type 1 Latency-Associated Transcript Expression Protects Trigeminal Ganglion Neurons from Apoptosis. Journal of Virology, 79(14): 9019–9025.

4. Coen, D. M., Kramer M. F. (1995). Quantification of Transcripts from the ICP4 and Thymidine Kinase Genes in Mouse Ganglia Latently Infected with Herpes Simplex Virus. Journal of Virology, 69(3): 1389–1399.

5. Cunningham, A. L., Diefenbach, R.J., Saksena, M. M., Bosnjak, L., Kim, M., Jones, C., & Douglas, M.W. (2006). The cycle of human herpes simplex virus infection: Virus transport and immune control. The Journal of Infectious Diseases, 194, s11.

6. De Regge N., Van Opdenbosch N., Nauwynck H., Efstathiou S., Favoreel H. (2010). Interferon alpha induces establishment of alphaherpesvirus latency in sensory neurons in vitro. PLoS One, 29: 5(9).

7. Diefenbach, R. J., Miranda-Saksena, M., Douglas, M. W., Cunningham, A. L. (2008). Transport and egress of herpes simplex virus in neurons. Reviews in Medical Virology, 18: 35-51.

8. Dodding, M. P., Way, M. (2011). Coupling viruses to dynein and kinesin-1. The EMBO Journal, 30: 3527 – 3539. Review.

9. Dolznig, H., Grebien, F., Sauer T., Beug H., Müllner, E. W. (2004). Evidence for a size-sensing mechanism in animal cells. Nature Cell Biology, 6(9): 899-905.

10. Dubbs D. R., Kit, S. (1961). Effect Of Halogenated Pyrimidines And Thymidine On Growth Of L-Cells And A Subline Lacking Thymidine Kinase. Experimental Cell Research, 33: 19-28.

11. Eriksen, S. E. (2005). Macrophages and cytokines in the early defence against herpes simplex virus. Virology Journal, 2: 59.

12. Frey, K. G., Ahmed, C. M., Dabelic, R., Jager, L. D., Noon-Song, E. N., Haider, S. M., & Bigley, N. J. (2009). HSV-1-induced SOCS-1 expression in keratinocytes: Use of a SOCS-1 antagonist to block a novel mechanism of viral immune evasion. Journal of Immunology, 183(2): 1253-1262.

13. Haenchen, S. D., Utter, J. A., Bayless, A. M., Dobrowsky, R. T., Davido, D. J. (2007). Role of a cdk5-associated protein, p35, in herpes simplex virus type 1 replication in vivo. Journal of NeuroVirology, 16(5): 405–409.

14. Harle, P., Cull, V., Guo, L., Papin, J., Lawson, C., & Carr, D. J. (2002). Transient transfection of mouse fibroblasts with type I interferon transgenes provides various degrees of protection against herpes simplex virus infection. Antiviral Research, 56(1): 39-49.

15. Hancock, M. H., Corcoran, J. A., Smiley, J. R. (2006). Herpes simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression. Journal of Virology, 15; 352(1): 237-52.

16. Henderson, G., et al.,(2002)Regulation of caspase 8- and caspase 9-induced apoptosis by the herpes simplex virus type 1 latency-associated transcript. Journal of Neurovirology, 8 Suppl 2: 103-11.

17. Hendricks, R. L., Decman, V., Paul R., Kinchington., Stephen A. K., Harvey. (2005). Gamma Interferon Can Block Herpes Simplex Virus Type 1Reactivation from Latency, Even in the Presence of Late Gene Expression. Journal of Virology, 79(16): 10339–10347.

18. Hill, J. M., Bryan M., Gebhardt., Wen, R., Andree M., Bouterie., Thompson, H. W., O'callaghan, R. J., Halford, W.P., Kaufman H. E. (1996). Quantitation of Herpes Simplex Virus Type 1 DNA and Latency-Associated Transcripts in Rabbit Trigeminal Ganglia Demonstrates a Stable Reservoir of Viral Nucleic Acids during Latency. Journal of Virology, 70(5): 3137–3141.

19. Inman., Perng, G-C., Henderson, G., Ghiasi, H., Anthony B., Nesburn., Wechsler, S.L., Jones, C. (2001). Region of Herpes Simplex Virus Type 1 Latency-Associated Transcript Sufficient for Wild-Type Spontaneous Reactivation Promotes Cell Survival in Tissue Culture. Journal of Virology, 75(8): 3636–3646.

20. Jackson, S. A., DeLuca, N. A. (2003). Relationship of herpes simplex virus genome configuration to productive and persistent infections. Proceedings of the National Academy of Sciences USA, 24: 7871–7876.

21. Johnson, A.J., Chu, C.F., Milligan, C.F. (2008). Effector CD4+ T-cell involvement in clearance of infectious herpes simplex virus type 1 from sensory ganglia and spinal cords. Journal of Virology, 82(19): 9678-88.

22. Johnson, K. E., Song, B., Knipe, D. M. (2008). Role for herpes simplex virus 1 ICP27 in the inhibition of type I interferon signaling. Journal of Virology, 374(2): 487-494.

23. Johnson, K. E., Knipe, D.M. (2010). HSV-1 Infection Causes the Secretion of a Type I Interferon-antagonizing Protein and Inhibits Signaling at or before Jak-1 Activation. Journal of Virology, 396(1): 21–29.

24. Knickelbein, J., Khanna, K., Yee, M., Catherine, B., Kinchington, P., Hendricks R. (2008). Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency (supporting online material). Science, 322: 268–271.

25. Knipe, D., Cliffe, A. (2008). Chromatin control of herpes simplex virus lytic and latent infection. Nature Rev Microbiology, 6(3): 211-221.

26. Lee D. A., Shapourifar-Tehrani S., Stephenson T. R., Kitada S. (1991). The Effects of the Fluorinated Pyrimidines FUR, FUdR, FUMP, and FdUMP on Human Tenon's Fibroblasts. Investigative Ophthalmology and Visual Science, 32: 2599-2609.

27. Liu, M., Schmidt, E., Halford, W. (2010). ICP0 dismantles microtubule networks in herpes simplex virus-infected cells. PLoS One, 5(6): e10975.

28. McMahon., Walsh, D. (2008). Efficient Quiescent Infection of Normal Human Diploid Fibroblasts with Wild-Type Herpes Simplex Virus Type 1. Journal of Virology, 82(20): 10218–10230.

29. Millhouse, S., Wigdahl, B. (2000). Molecular circuitry regulating herpes simplex virus type 1 latency in neurons. Journal of NeuroVirology, 6: 6-24.

30. Mossman, K. L., Paladino, P., Collins, S. E. (2010). Cellular Localization of the Herpes Simplex Virus ICP0 Protein Dictates Its Ability to Block IRF3-Mediated Innate Immune Responses, PLoS ONE, 5(4): e10428.

31. Noisakran, S., Campbell, I. L., Carr, D.J. (2000). IFN-alpha1 plasmid construct affords protection against HSV-1 infection in transfected L929 fibroblasts. Journal of Interferon Cytokine Research, 20(1): 107-15.

32. Peng, T., Zhu, J., Hwangbo, Y., Corey, L., & Bumgarner, R. E. (2008). Independent and cooperative antiviral actions of beta interferon and gamma

interferon against herpes simplex virus replication in primary human fibroblasts. Journal of Virology, 82(4): 1934-1945.

33. Perng, G. C., Jones, C., Ciacci-Zanella, J., Stone, M., Henderson, G., Yukht, A. et al. (2000). Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science 287: 1500–1503.

34. Perng, G., Jones, C. (2010). Towards an Understanding of the Herpes Simplex Virus Type 1 Latency-Reactivation Cycle. Interdisciplinary Perspectives on Infectious Diseases, Hindawi Publishing Corporation, 2010: 262415.

35. Petrowsky, H., Gretchen, D. R., Kooby D. A., Burt B. M., Joseph J. Bennett, Delman, K. A., Stanziale, S. F., Delohery, T. M., Tong W. P., Federoff, H. J., Fong, Y. (2001). Functional Interaction between Fluorodeoxyuridine-Induced Cellular Alterations and Replication of a Ribonucleotide Reductase-Negative Herpes Simplex Virus. Journal of Virology, 75(15): 7050–7058.

36. Rajasagi, N.K., et al., (2009).CD4+ T cells are required for the priming of CD8+ T cells following infection with herpes simplex virus type 1. Journal of Virology, 83(10): 5256-68.

37. Rajčáni, J., Ďurmanová V. (2000). Early expression of herpes simplex virus (HSV) proteins and reactivation of latent infection, Folia Microbiologica Review, 45(1): 7-28.

38. Roobol,C., De Dobbeleer , G. B. E., Bernheim, J. L. (1984). 5-fluorouracil and 5-fluoro-2'-deoxyuridine follow different metabolic pathways in the induction of cell lethality in L1210 leukaemia. British Journal of Cancer, 49: 739-744.

39. Sainz. Jr., Halford W. P. (2002). Alpha/Beta Interferon and Gamma Interferon Synergize To Inhibit the Replication of Herpes Simplex Virus Type 1. Journal of Virology, 76(22): 11541–11550.

40. Sawtell, N. M. (1997). Comprehensive Quantification of Herpes Simplex Virus Latency at the Single-Cell Level. Journal of Virology, 71(7): 5423–5431.

41. Schang, L. M., Bantly A., Schaffer, P. A. (2002). Explant-Induced Reactivation of Herpes Simplex Virus Occurs in Neurons Expressing Nuclear cdk2 and cdk4. Journal of Virology, 76(15): 7724-7735.

42. Sheridan, B. S., Knickelbein, J. E., Hendricks, R. L. (2007). CD8 T cells and latent herpes simplex virus type 1: keeping the peace in sensory ganglia. Expert Opinion on Biological Therapy, 7(9): 1323-31.

43. Su, Y. H., Meegalla, R. L., Chowhan, R., Cubitt, C., Oakes, J. E., Lausch, R. N., Fraser, N., Block, T. (1999). Human Corneal Cells and Other Fibroblasts Can

Stimulate the Appearance of Herpes Simplex Virus from Quiescently Infected PC12 Cells. Journal of Virology, 73(5): 4171–4180.

44. Thompson, R. L., Sawtell, N. M. (2001). Herpes simplex virus type 1 latency-associated transcript gene promotes neuronal survival, Journal of Virology, 75 (14): 6660-75.

45. Toma, H.S., (2008). Ocular HSV-1 latency, reactivation and recurrent disease. Seminar Ophthalmology, 23(4): 249-73.

46. Vierbuchen, T., Ostermeier, A., Pang, Z., Kokubu, Y., Sudhof, T. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature, 463(7284): 1035-1041.

47. Vuong., Arenzana, T. L., Brian, M., Showalter., Losman, J., Chen, X. P., Mostecki, J., Banks, A. S., Limnander, A., Fernandez, N., Rothman, P. B. (2004). SOCS-1 Localizes to the Microtubule Organizing Complex-Associated 20S Proteasome, Molecular And Cellular Biology, 24(20): 9092–9101.

48. Wagner, E. K., Bloom, D. (1997). Experimental Investigation of Herpes Simplex Virus Latency. Clinical Microbiology Reviews, 10(3): 419–443.

49. Yoo, A. S., Sun, A. X., Li Li., Shcheglovitov, A., Portmann, T., Li, Y., Lee-Messer C., Dolmetsch, R. E., Tsien, R. W., Crabtree G. E. (2011). MicroRNA-mediated conversion of human fibroblasts to neurons, Nature, 476: 228–231.